Original Article:

Isolation and diagnosis of Chikungunya virus causing outbreaks in Andhra Pradesh, India

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ABSTRACT

Background: Chikungunya fever has recently re-emerged in India with a high morbidity. However, the prevalence of chikungunya fever in India has been underreported due to non-availability of specialized kits to confirm the disease in most of the laboratories.

Methods: Nine hundred and fifty six serum samples were collected from subjects presenting with a short febrile illness from various places in Chittoor district, Andhra Pradesh, between January to October 2009 and were screened for Chikungunya Virus (CHIKV) infection. Virus isolation, reverse transcriptase - polymerase chain reaction (RT-PCR) and immunoglobulin M (IgM) rapid strip method were employed for the identification of the causative agent.

Results: Chikungunya Virus (CHIKV) infection was confirmed in 520 (68.1%) patients by RT-PCR. Seventy seven (40.1%) patients showed the presence of anti-CHIKV IgM antibodies while 12 (6.3%) patients showed the presence of both anti-CHIKV IgM and immunoglobulin G (IgG) antibodies respectively. The isolation of CHIKV was successful from five patients.

Conclusions: The re-emergence and persistence of CHIKV in Andhra Pradesh suggests the need for continuous monitoring and identification of the pathogen and thereby prevention of the spread of the virus to other parts of the country.

Key words: *Chikungunya virus, Virus isolation, RT-PCR, Surveillance, Diagnosis*

INTRODUCTION

Chikungunya fever is one of the six major vector borne diseases endemic to India and has reemerged causing severe mortality during the recent outbreak.[1] During the epidemic, clinical triad of fever, rashes and arthralgia is suggestive of Chikungunya virus (CHIKV) infection. The symptoms of CHIKV infection are most often clinically indistinguishable from those observed in dengue fever and, viruses of both diseases are transmitted by the same species of mosquitoes. Co-infection with CHIKV and dengue virus (DENV) have been reported earlier, as well as during the current outbreak.[2-6] Diagnosis of Chikungunya fever during early stages of infection is a major challenge for clinicians. Non-availability of CHIKV antigen commercial assays in many parts of India also hampers the diagnosis of Chikungunya fever. As a result of this the burden of Chikungunya fever is under estimated. The rapid diagnosis of Chikungunya fever during an outbreak is critical for instituting appropriate treatment. Any delay in diagnosis or response to an outbreak allows spread of the virus, making eradication more difficult. The present study was undertaken to assess CHIKV seroprevalence during CHIKV suspected outbreaks in Chittoor district of Andhra Pradesh, South India.

MATERIALS AND METHODS

During January 2009, the Medical Officer of Primary Health Centre (PHC) in Rangampeta, Chittoor District, Andhra Pradesh observed a large number of patients presenting with fevers and crippling arthralgias. Symptomatology of the patients suggested the causative agent to be CHIKV infection. Similar cases were also reported in different places of Chittoor district. A community based survey was carried out from January -

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October 2009 in different places of Chittoor district and blood samples of patients exhibiting symptoms suggestive of CHIKV infection were collected. As it was practically not possible to screen the blood samples for all the infections, common illness such as dengue and malaria were ruled out by the physician prior to CHIKV testing. Samples were drawn by PHC staff/physicians. Prior to sample collection "informed verbal consent" was obtained from all the patients and parents (in case of minors). The patients were also briefly informed about the various diagnostic tests proposed to be used for confirming the CHIKV infection. Details of clinical samples processed are furnished in Table 1. Samples were collected in sterile vials without anticoagulant, transported in coolant packs and stored at –80 °C until use. All samples were processed within 48 hours after the collection.

Isolation of CHIKV from 5 acute phase samples was attempted in BHK-21 cell lines following a standard protocol.[7,8] The infected and uninfected cultures were observed after 0, 24, 48, 72 and 96 hours post-infection (PI) for the presence of changes induced by the virus. The cell culture supernatants were further confirmed for the presence of CHIKV ribonucleic acid(RNA) by reverse transcription - polymerase chain reaction (RT-PCR) using primer pair DVRChk-F/ DVRChk-R.[9] Briefly, total RNA was extracted from 250 µL of cell culture supernatants using TRI Reagent® BD (Sigma-Aldrich, USA), the pellets were dissolved in nuclease free water and stored at –20 °C until use. The cDNA was synthesized in 20 µL reaction volume using Oligo $(dT)_{18}$ primer. Polymerase chain reaction (PCR) amplification was carried out in 50 µL of reaction volume containing 3 µL of cDNA, 10 pmol of forward primer (DVRChk-F:5'-ACCGGCGTCTACCCATTCATGT-3'), 10 pmol of reverse primer (DVRChk-R 5'-GGGCGGGTAGTCCATGTTGTAGA-3'), 2 mM.$MgCl_2$ (Fermentas, USA), 2.5 Units of Taq deoxyribonucleic acid (DNA) polymerase (Fermentas, USA) 0.2 mM each of dNTPs (Fermentas, USA) and nuclease free water. The

Table 1: Details of clinical samples processed during the present study

Place	Sampling Time	CHIKV suspected samples	
		Acute phase samples (No. of samples positive/ No. of samples tested)	Convalescent phase samples (No. of samples positive/ No. of samples tested)
Kalicherla	March, 2009	3/5	1/1
Madanapalle	March, 2009	11/17	5/9
Vayalpad	April, 2009	15/23	8/13
Punganuru	June, 2009	3/16	14/31
Palamner	June, 2009	10/21	15/28
Kuppam	June, 2009	1/17	7/11
Piler	July, 2009	7/28	2/13
Chinnagottigalu	April, 2009	1/7	3/9
Rangampeta	January, 2009	6/9	7/7
Pakala	February, 2009	3/13	0/7
Chittoor	February, 2009	31/57	8/15
Chandragiri	March, 2009	4/7	2/3
Tirupati	February - October, 2009	418/529	13/35
Renigunta	April, 2009	3/5	1/6
Sri Kalahasti	April, 2009	4/10	3/4
Total		520/764	89/192

CHIKV=chikungunya virus

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reaction was carried out using gradient thermal cycler (Corbett Research, Model CG1-96, Australia) and the PCR amplification conditions included an initial denaturation cycle at 94 °C for 5 min followed by 35 cycles of denaturation for 45 sec at 94 °C, annealing for 30 sec at 56 °C and extension for 1 minute at 72 °C. A final extension step of 15 min at 72 °C was carried out at the end and the PCR products along with the marker DNA (100bp DNA ladder, Fermentas, USA) were electrophoresed in 2% agarose gel. The acute phase sera samples were screened for CHIKV infection using the above RT-PCR assay and the convalescent phase serum samples were tested for CHIKV specific antibodies using SD Bioline Chikungunya IgM Rapid Tests (Standard Diagnostics, South Korea) according to manufacturer's instructions.

RESULTS

In view of the clinical features suggestive of Chikungunya fever and the ongoing epidemic in India, CHIKV infection was suspected in patients from Chittoor district in Andhra Pradesh. Isolation of CHIKV was attempted and succeeded from all the five suspected samples. CHIKV Cytopathogenic effect (CPE) was characterized by rounded, swollen, refractile and granular cells at 24 hours post infection.[8,10] These events were followed by aggregation of enlarged and vacuolated cells and, finally cell distortion and death of cells (Figure 1B) resulting in detachment of cell monolayer from the surface after 3-4 days post infection as was observed earlier.[8,10] The CPE was observed in all the five tissue culture bottles infected with CHIKV suspected clinical serum samples. The uninfected BHK-21 cells remained healthy, with no changes in the cellular morphology (Figure 1A) until 120 hours of incubation. The cell culture supernatants were further confirmed for CHIKV infection by RT-PCR assay which yielded 330 bp product (Figure 2) specific to CHIKV.[9] A total of 520 (68.1 %) serum samples were found positive for the presence of CHIKV gene specific 330bp amplicon. CHIKV positivity correlated with the early stage of the disease as all these patients
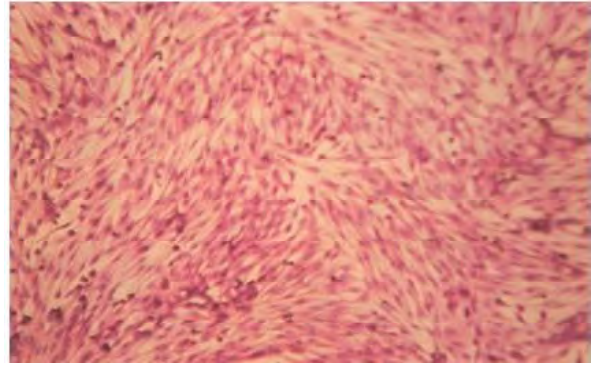

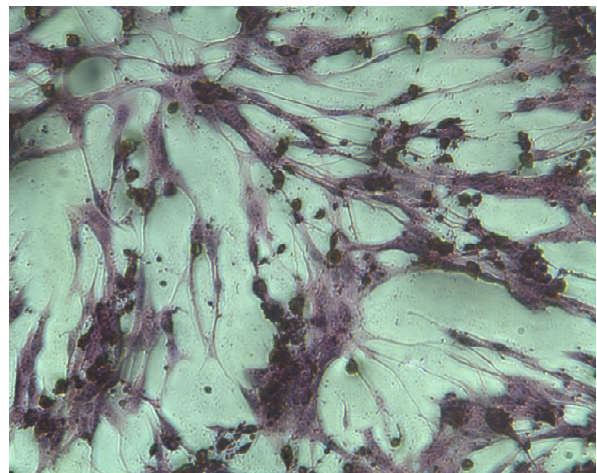**Figure 1A:** BHK-21 monolayer- uninfected control (Haemotoxylin and eosin stain × 100)

Figure 1B: BHK-21 monolayer infected with chikungunya virus showing aggregation of enlarged and vacuolated cells (Haemotoxylin and eosin, × 400)

had fever of less than 7 days duration as described earlier.[11] The serological investigation revealed the presence of anti CHIK IgM in 77 patients (40.1%) and anti-CHIK IgM and IgG in 12 patients (6.3%) respectively.

DISCUSSION

In India CHIKV outbreak was first reported in 1963 in Kolkata which accounted nearly 200 deaths.[12] CHIKV outbreaks were recorded in Chennai, Pondicherry and Vellore in 1964; Visakhapatnam, Rajahmundry, Kakinada and Nagpur in 1965; and Barsi in 1973.[13] After a gap of nearly 32 years CHIKV reemerged in India causing severe morbidity.[13] During the current outbreak, Andhra Pradesh was the first state to report CHIKV epidemic in December 2005 and was one of the worst affected.[14] After a time lapse of 40 years, Andhra Pradesh reported CHIKV
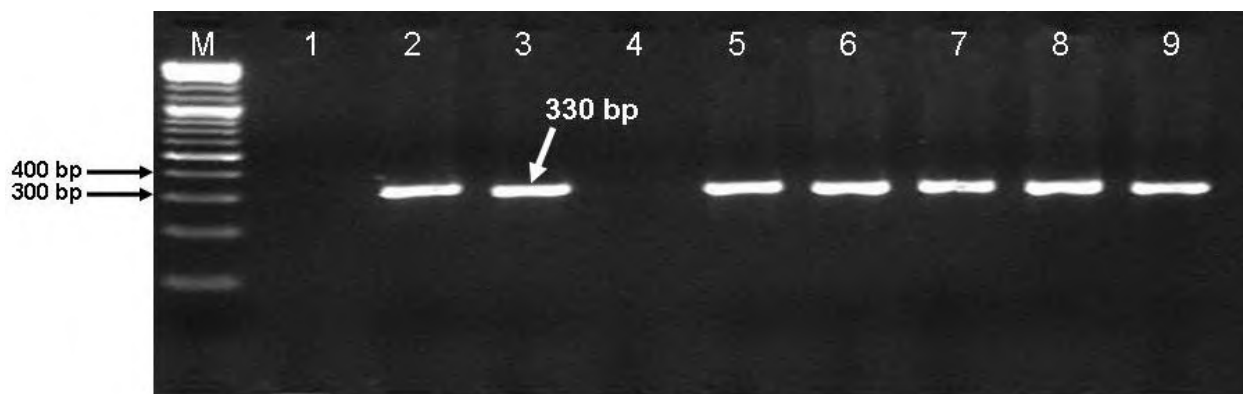
Figure 2 : Gel photograph showing 330bp fragment of E1 gene of Chikungunya virus by RT-PCR
Lane M: GeneRuler™ DNA Ladder (Fermentas);
Lane 1: Negative control
Lane 2: Positive control
Lane 3-9: CHIKV suspected samples
Lane 4: CHIKV negative sample by RT-PCR
Lanes 3, 5-9: CHIKV positive samples by RT-PCR
CHIKV= Chikungunya virus; RT-PCR = reverse transcriptase polymerase chain reaction

epidemic during 2005. Twenty three districts were affected and nearly 77,533 CHIKV cases were suspected by the end of 2006.[15]

CHIK cases have been subsequently reported from different places of Andhra Pradesh.[9,13,14,16-23] Diagnosis of CHIKV infection is mostly based on serological and PCR techniques. Serological diagnosis is reliable 5-6 days after the clinical onset of disease. The ideal tests for CHIKV detection during acute phase are virus isolation, antigen detection and PCR assays.[24,25] Virus isolation is a sensitive method but is expensive, time consuming and requires expertise.[26] The degree of success in virus isolation process is dependent on a number of complicating factors, for example, time of collection, transportation, maintenance of cold chain, storage and processing of samples.[8] Hence CHIKV isolation cannot be carried out for routine and early detection of all the samples. The diagnostic accuracy of RT-PCR assays targeting pathogen genes is clinically acceptable for the diagnosis of acute Chikungunya infection up to 7 days of illness and is superior to antibody-based technologies.[27] Thus RT-PCR appears to be a sensitive and useful technique for the early detection of CHIKV infection.[11,28] The importance of RT-PCR can be assessed by the fact that the World Health Organization (WHO) still considers RT-PCR as a standard method for detection of arboviral infections.[29] The present study revealed less seropositivity which may be attributed to the collection of samples at the very early or acute stage of illness. Therefore, based on time of sample collection, the diagnostic test used will differ. In the present study the combination of virus isolation, RT-PCR assay and one step IgM antibodies to CHIKV test served as a valuable tool for CHIKV screening during the epidemic.

In conclusion our 10 months survey revealed the circulation of CHIKV in 2009 in Andhra Pradesh and emphasizes the importance of active surveillance of the disease so as to prevent the outbreaks in the near future. The present study highlights the fact that CHIKV infection is an important but unrecognized cause of febrile illness in Andhra Pradesh and emphasizes the need of continuous surveillance for CHIKV disease using multiple diagnostic tests.

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