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# Application of real-time RT-PCR in vector surveillance and assessment of replication kinetics of an emerging novel ECSA genotype of Chikungunya virus in *Aedes aegypti*

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A B S T R A C T

Chikungunya has emerged as one of the most important arboviral infection of global significance. Expansion of Chikungunya virus endemic areas can be ascribed to naive population, increasing vector population and adaptability of virus to new vector. In this study, a SYBR Green I based quantitative RT-PCR assay was developed. The assay was found to be 10-fold more sensitive than conventional RT-PCR and no cross reactivity was observed with related alphaviruses and flaviviruses. The detection efficiency of the assay was impervious to mosquitoes of different pool sizes. Vector surveillance has resulted in detection of CHIKV RNA in *Aedes aegypti*, confirming its vectorial potential for CHIKV in northern India. The assessment of the assay was further carried out by studying the competence of Indian *Ae. aegypti* for CHIKV, which revealed 100% infection rate and dissemination rate with 60% transmission rate. The replication kinetics of CHIKV in different anatomical sites of *Ae. aegypti* revealed highest titre at day 6 post infection in midgut and at day 10 post infection in saliva, legs and wings. The implementation of the assay in detecting lower viral load makes it a remarkable tool for surveillance of virus activity in mosquitoes.



## 1. Introduction

Among the arboviruses, Chikungunya virus has emerged as a major threat to public health. Chikungunya virus belongs to genus *Alphavirus*, family *Togaviridae* and is primarily transmitted in humans *via* bite of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes (Jupp and McIntosh, 1988). In Asia, *Ae. aegypti* mosquitoes are responsible for the maintenance of urban cycle, while in Africa, CHIKV transmission involves a sylvatic cycle, primarily with *Aedes furcifer* and *Aedes africanus* mosquitoes (Powers et al., 2000). Chikungunya virus has been grouped along with Selmiki forest and Ross river as old world alphavirus (Solignat et al., 2009). CHIKV was first reported from Tanzania in 1953 and after that it was isolated from outbreaks in many other countries of Africa and Asia (Ross, 1956). The recent reemergence of CHIKV in east Africa and islands of Indian Ocean in 2005 followed by massive outbreaks throughout much of Asia has resulted in 1.2 million human cases. This epidemic is attributed to a novel clade of East Central South African genotype of CHIKV (Dash et al., 2007; Schuffenecker et al., 2006). The rapid resurgence of this arthropod borne virus in recent years is a matter of public health concern.

Chikungunya fever is a self limiting illness in humans that is often characterized by sudden onset of fever, headache, fatigue, nausea, vomiting, rash, myalgia and severe polyarthralgia, which may last for 1–10 days. However, arthralgia may persist for months to years (Lakshmi et al., 2008). In recent outbreaks, many unusual neurological signs like altered sensorium, auditory or visual hallucinations, nuchal rigidity, tense fontanelle, seizures were also documented (Robin et al., 2008).

The genome of CHIKV consists of 5′ capped positive sense single-strand RNA of ~11.8 kb that harbours a poly (A) tail in its 3′ end. The genome is composed of two open reading frames (ORFs) embedded between non-translated regions (5′ NTR and 3′ NTR). The ORF located at the 5′ end of the genome encodes a polyprotein precursor of nonstructural proteins (nsP1, nsP2, nsP3, nsP4) with replicative and proteolytic activities. The second ORF encodes the polyprotein precursor of the structural proteins (C, E1, E2) (Chevillon et al., 2008).

The laboratory diagnosis of CHIKV is achieved through a battery of assays like virus isolation, immunological assays and molecular detection. Viral isolation technique is considered as gold standard, but this is labour intensive, time consuming and requires proper maintenance of cell culture facility. The serological diagnosis is

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simple but appearance of antibody would take 6–7 days, before which the disease cannot be diagnosed. Further, this is also not applicable for mosquito surveillance studies. In contrast, PCR based molecular assays provide very fast, sensitive and specific detection of CHIKV. As compared to the real time PCR technologies, conventional RT-PCR has high risk of contamination and is less sensitive. The real time quantitative RT-PCR has the advantages of rapid detection, quantitation, and ease of standardization with high specificity and sensitivity. Real time RT-PCR is now increasingly adopted for clinical diagnosis of many infectious diseases including Chikungunya around the world (Carletti et al., 2007; Edwards et al., 2007; Santhosh et al., 2007). Apart from clinical diagnosis, it is also widely used for vector surveillance and to study virus vector interactions. The lower viral load in mosquitoes particularly mandates the application of these assays with higher sensitivity for reliable detection (Ali et al., 2010; Dash et al., 2012; Smith et al., 2009; Yang et al., 2010). Vector surveillance programme in many parts of the world now also relies on the real-time RT-PCR based assays owing to their higher sensitivity (Brault et al., 2012; Van den Hurk et al., 2012).

In the present study, a SYBR Green I based quantitative real time RT-PCR assay was developed and validated with field caught mosquitoes. This assay was further adopted to assess the replication kinetics of CHIKV at different anatomical sites in *Ae. aegypti* at different days of post infection.

## 2. Materials and methods

### 2.1. Viruses and mosquitoes

A Chikungunya virus strain isolated from a major epidemic in India in 2006 (DRDE 06 (GenBank Acc. No. EF210157) maintained in Virology Division, Defence Research and Development Establishment (DRDE), Gwalior was used in this study. The virus was grown to a titre of $1 \times 10^8$ PFU/ml in confluent monolayer of Vero cells as determined by plaque assay. The virus was aliquoted and stored in −80° C until use. For cross reactivity experiments, among the alphaviruses, Ross river virus, T48 (GQ433359) was used, while among the closely related flaviviruses *viz.*, West Nile virus, Eg101 (AF260968), Dengue virus serotypes-DEN-1, RR107 (KF289072), DEN-2, GWL18 (AY324614), DEN-3, ND143 (FJ644564), DEN-4, ND73 (HM237348), Japanese encephalitis virus, JaOArS982 (M18370) and St. Louis encephalitis virus, Parton (EF158070) were used. All these strains were propagated in C6/36 cells in Virology Division, DRDE, Gwalior. Due to non-availability of other alphaviruses, the synthetic gene constructs of O'Nyong Nyong virus, Gulu strain (M20303), Semliki Forest virus, L10 strain (AY112987) and Sindbis virus (J02363) were used for cross-reactivity study.

*Aedes (Stegomyia) aegypti* used in this study was maintained in Vector Management Division, Defence Research and Development Establishment (DRDE) at 28 ± 1 °C with 80% relative humidity and 14:10 light:dark photo period. These were collected from Gwalior district, India. Adult mosquitoes were provided with 10% sucrose solution soaked in cotton pads. They were utilized for standardization of assay and replication kinetics study.

### 2.2. Extraction of viral RNA

The viral RNA was extracted from 140 μL of infected culture supernatant, homogenates of field caught and orally infected *Ae. aegypti* mosquito using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The RNA was finally eluted in 50 μL elution buffer and stored at −80 °C until use.

### 2.3. SYBR Green-I based Real time RT-PCR

SYBR Green I based one step real time quantitative RT-PCR was performed using a primer pair: forward primer: CHIK1: 5′-ACGCAGTTGAGCGAAGCAC-3′ and reverse primer: CHIK2: 5′-CTGAAGACATTGGCCCCAC-3′ targeting to a highly conserved region of E1 gene of CHIKV (Dash et al., 2008). The quantitative RT-PCR was carried out using SS III Platinum one step qRT-PCR kit (Invitrogen, Carlsbad, CA) in Mx3000P system (Stratagene, La Jolla, CA). Samples were assayed in a 25 μL reaction volume containing 12.5 μL of 2× Master mix, 0.125 μL (0.25 μmol) each of forward and reverse primers, 0.25 μL of enzyme mix comprising of Taq DNA polymerase and reverse transcriptase, 9.5 μL of nuclease free water and 2.5 μL of RNA. The thermal profile consisted of 30 min of reverse transcription at 50 °C, 10 min of polymerase activation at 95 °C, followed by 40 cycles of PCR at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s. Following amplification, a melting curve analysis was performed with the melting curve analysis software of the Mx3000P according to the instructions of manufacturer. Positive and negative template control was also included along side in all experiments.

### 2.4. Construction of standard curve and determination of detection limit

The standard curve was constructed to ascertain the detection limit of SYBR Green I based real-time RT-PCR using *in vitro* transcribed full length RNA from an infectious clone of CHIKV (Coffey and Vignuzzi, 2011). Briefly, the purified plasmid from CHIKV infectious clone was linearized using *Not* I and *in vitro* transcribed from SP6 promoter with mMESSAGE mMACHINE kit (Ambion, Austin, TX). The *in vitro* transcription (IVT) was carried out at 37 °C for 1 h. The IVT product was then treated with DNase I and incubated at 37 °C for 15 min to remove the remaining DNA followed by inactivation of DNase I at 65 °C for 15 min. RNA was ethanol precipitated and resuspended in DEPC treated water. Purified RNA was quantified by Nanodrop spectrophotometer and 10-fold serial dilutions ($10^{11}$–$10^0$ RNA copies/μL) were prepared and subjected to real time RT-PCR. The Ct values obtained against the known concentration of serially diluted RNA were used for the construction of standard curve. The quantity of RNA as determined by Nanodrop spectrophotometer was converted to molecular copies, using the following formula,

$$Y\,(\text{molecules}/\mu\text{L}) = \left[\frac{X\,(\text{g}/\mu\text{L})}{\text{transcript length}\,(\text{nucleotides}) \times 340}\right] \times 6.023 \times 10^{23}$$

### 2.5. Conventional RT-PCR

To compare the sensitivity of conventional RT-PCR and SYBR Green-I based Real time RT-PCR, a conventional RT-PCR was carried out with same primer sets (CHIK1 and CHIK2) using Enhanced Avian HS RT-PCR kit (Sigma, St. Louis, USA). The amplification was carried out in a 25 μL of total reaction volume comprising of 5 μL of 10× Buffer, 2.5 μL of $MgCl_2$, 0.5 μL of 10 mM dNTPs, 0.25 μL (0.5 μmol) each of forward and reverse primer, 0.25 μL of Jump start AccuTaq DNA polymerase, 0.25 μL of Enhanced avian reverse transcriptase (eAMV RT), 0.25 μL of ribonuclease inhibitor, 13.25 μL of nuclease free water and 2.5 μL of RNA. The thermal profile of the PCR was cDNA synthesis at 48 °C for 45 min, initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min in a thermal cycler (ABI, Foster City, CA). PCR products were electrophoresed