Short Report: Rapid Detection and Quantification of Chikungunya Virus by a One-Step Reverse Transcription–Polymerase Chain Reaction Real-Time Assay

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Abstract. Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus. A large outbreak of CHIKV disease occurred in 2005 in the Indian Ocean Islands. Many cases have been imported in European countries. Laboratory confirmation of suspected cases is mandatory for control measures during an outbreak. We report a novel, real-time, reverse transcription–polymerase chain reaction (RT-PCR) for the nonstructural protein 1 region that can quantify a wide range of viral RNA concentrations. This assay was validated by in vitro experiments in which interferon-α, a well-known virus inhibitor, showed a dose-dependent inhibition of virus replication on Vero cells that was assessed by viral infectivity and viral RNA production. This new real-time RT-PCR was used to measure viral load in serum samples from cases recently imported to Italy, and may be a useful tool in rapid detection of CHIKV and monitoring the extent of viral replication in patients.

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus of the family Togaviridae. The clinical manifestations of infection, which include fever, rashes on limbs and trunk, severe joint pain and arthralgia, may be confused with those caused by dengue fever but, unlike dengue, CHIKV infection is not associated with hemorrhagic or shock syndromes.

This virus, which was first isolated in Tanganyika (now Tanzania) in 1952, is found in Africa, India, and southeast Asia. A large outbreak of CHIKV disease, which is now under control, began in 2005 at Comoros Islands and was associated with a fatality rate of approximately 1/1,000, although most of the deaths occurred in patients with comorbidities. Many other countries in the Indian Ocean have shown a dramatic increase in the number of cases.

As expected, cases in returning travelers have been detected in France, Germany, Belgium, Norway, Switzerland, the United Kingdom, Spain, and Italy. The increasing threat, a few specific serologic or molecular methods for its detection are available.

To date, conventional reverse transcription–polymerase chain reaction (RT-PCR) methods have been used for studying CHIKV replication in supernatants or clinical samples in epidemiologic surveys. Recently, a TaqMan RT-PCR assay and an RT–loop-mediated isothermal amplification assay have been developed that target the envelope protein 1 (E1) gene. However, this viral genome region is difficult to analyze because of variability of its nucleic acid sequence. The purpose of this study was to develop a real-time quantitative RT-PCR to detect and quantify a CHIKV gene product that is more conserved than E1. We identified the gene for nonstructural protein 1 (NSP1) (overall mean genetic distance = 0.024) as a possible region for the PCR. We also identified a region within this gene that spanned nucleotide positions 345–486 (Ross reference strain, GenBank accession no. AF490259) with a smaller overall mean genetic distance (0.014) and developed a fluorescence resonance energy transfer probe-based, quantitative, real-time RT-PCR (qRT-PCR).

Chikungunya virus isolates from the present outbreak were obtained from our laboratory and another strain linked to the Indian Ocean outbreak (R2006_OPY1) was provided by Remi Charrel (Marseille, France). Virus was grown on Vero E6 cells and C6/36 cells in the Biosafety Level 3 facilities of the Institute for Infectious Diseases L. Spallanzani (Rome, Italy). Supernatants were obtained 3–5 days after virus inoculation and stored at -80°C. Viral RNA was extracted (Qiamp viral RNA kit; Qiagen, Hilden, Germany) from a supernatant, titrated, and serial diluted (10−1–10−7).

Sequences from the GenBank database were used to design primers and probes. The primers and probes used for LightCycler probe designer version 2.0 software (Roche, Basel, Switzerland) were CHK142f (5′-CCC GAG AGA CTC GAC AAC GAC AGG ATG TTG GCG T-3′), CHK142r (5′-TGT GTA AGC AGA ATG TTA GGCC CAT CTC TGG AAA GAT CGG-3′), CHK probe1 (5′-CCT GGA CAG AAA CAT CTC TGG AAA GAT CGG-3′-fluorescein), and CHK probe2 (Red 610-5′-ACT TAC AAG CTA AAG TAA TGG CCG TGC CAG AC-3′). These primers and probes showed 100% identity with viral clusters from central Africa and central east Africa, and high identity with the sub-clusters from Asia (Figure 1).

Because the primers showed several mismatches with NSP1 sequence from strain 37997 (the only west Africa strain sequence available in GenBank), it was expected that our system would not recognize this strain, although it has not been tested. Real-time RT-PCR assays were carried out in a LightCycler instrument (version 2.0; Roche) and a One-Step LightCycler RNA Master HybProbe kit (Roche) according to the manufacturer’s instructions. The steps in the RT-PCR were reverse transcription (61°C for 20 minutes), activation (95°C for 2 minutes), amplification (50 cycles at 95°C for 10 seconds, 55°C for 12 seconds, and 72°C for 12 seconds), and cooling (40°C for 30 seconds). Final concentrations of primers and probes were 0.5 μM and 0.2 μM, respectively.

We then constructed a standard curve using as quantifica-
tion standard a 10-fold dilution series of a plasmid containing the PCR amplicon as the insert. This was obtained by cloning the PCR amplicon (using CHK142f and CHK142r primers) into the pCR 4-TOPO vector (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA). The assay showed linear results for 7 logs of CHIKV plasmid dilution, as shown in Figure 2). The analytical sensitivity (CHIKV copy number at a 95% detection rate) calculated by the Environmental Protection Agency

FIGURE 1. Multiple alignment of the nonstructural protein 1 regions of Chikungunya virus (CHIKV) with respect to the reference strain. GenBank accession numbers of representative sequences are listed on the left (Ross reference strain accession no. AF490259). The CHIKV strains used for sequence alignments belong to clusters from Central Africa, Central/East Africa, and Asia. Dots indicate sequence identity. This figure appears in color at www.ajtmh.org.

FIGURE 2. A. Amplification profile and B, standard curve of a quantitative reverse transcription–polymerase chain reaction of serial dilutions of a positive control chikungunya virus (CHIKV) plasmid with a known copy number. A linear range of 7 logs of a CHIKV plasmid dilution is shown in B. This figure appears in color at www.ajtmh.org.
Specificity of the PCR was assessed by amplifying RNA extracted from 20 uninfected human samples (urine, n = 3; swabs, n = 3; serum, n = 10; and whole blood, n = 4). In addition, viruses from patients with similar clinical symptoms (influenza and dengue) and different arboviruses were tested. No positive results were observed in uninfected clinical samples (specificity = 100%), and no cross-reactivity was observed with other viruses (influenza H5N1 and H1N1 viruses, tick-borne encephalitis virus, yellow fever virus, West Nile virus, dengue 2 and dengue 4 viruses, Venezuelan equine encephalitis virus, Japanese encephalitis virus, and Toscana virus).

To validate the use of the qRT-PCR for quantitative measurements, we performed parallel determinations by qRT-PCR and infectivity titration in experiments of inhibition of virus replication by interferon-α (IFN-α) *in vitro*. Vero cells were treated for 24 hours with recombinant IFN-α at concentrations of 1, 10, 100, 1,000 and 10,000 IU/mL and infected with CHIKV at a multiplicity of infection of 0.01. Virus yield was measured by both assays after 24 hours of infection. As shown in Figure 3A, a dose-dependent inhibition of virus yield was observed, measured as both viral infectivity and viral RNA. As shown in Figure 3B, a direct correlation (r = 0.9719) between viral titers from independent experiments, expressed as log TCID₅₀/mL and log RNA copies/mL, was observed.

The applicability of this CHIKV qRT-PCR in the clinical setting was explored using serum samples from four recently imported cases to Italy who were admitted to the National Institute for Infectious Diseases L. Spallanzani. All infections were confirmed in the laboratory by detection of CHIKV-specific IgM. IgG seroconversion, diagnostic end point RT-PCR, or virus isolation from serum samples. Acute-phase samples collected 2–6 days after the onset of symptoms, as well as convalescent samples, were tested with the qRT-PCR. The clinical and laboratory findings of these patients are shown in Table 1. Viremia was detected by the qRT-PCR in acute-phase serum samples, with viremia levels ranging from $1.3 \times 10^5$ to $6 \times 10^6$ copies/mL. The lowest CHIK viremia levels were observed in the patient who had a concomitant IgM humoral response that had been detected during the acute phase of disease.

Our method is sensitive and specific and detects a wide range of CHIKV concentrations. This quantitative method was validated by *in vitro* experiments in which IFN-α, a well-

![Figure 3](image)

**Figure 3.** Reduction of virus yield in Vero E6 cells by interferon-α (IFN-α) assessed by infectivity and viral RNA titration. A, Vero cells were treated with recombinant IFN-α (1, 10, 100, 1,000, and 10,000 IU/mL), incubated for 24 hours, and infected with chikungunya virus at a multiplicity of infection of 0.01. After 24 hours of infection, progeny virus was harvested and viral replication was measured by both quantitative reverse transcription–polymerase chain reaction and viral infectivity assay. Results are expressed as infectivity (log 50% tissue culture infectious dose [TCID₅₀/mL]) (●) and viral RNA (log copies/mL; vertical bars) titers. B, Correlation between viral titers expressed as log TCID₅₀/mL and log RNA copies/mL from the IFN-α inhibition experiments (pooled results from two independent experiments). Statistical analysis was performed with a nonparametric (Spearman’s correlation) test. Linear regression with 95% confidence intervals (dotted lines) is shown.

### Table 1

Use of qRT-PCR for establishing chikungunya virus (CHIKV) viral load in serum samples from patients with confirmed diagnosis of CHIKV infection

<table>
<thead>
<tr>
<th>Patient ID, sex, age (years)</th>
<th>Travel history, onset of symptoms</th>
<th>Sampling date</th>
<th>RT-PCR (NSP1)</th>
<th>qPCR (copies/mL)</th>
<th>Viral isolation (strain designation)</th>
<th>IFA IgM titters</th>
<th>IFA IgG titters</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGL, M, 48</td>
<td>Mauritius 3/27/06</td>
<td>3/31/06</td>
<td>Pos</td>
<td>$1.3 \times 10^5$</td>
<td>Neg</td>
<td>Neg†</td>
<td>Pos‡</td>
</tr>
<tr>
<td>TAM, M, 45</td>
<td>Mauritius 3/31/06</td>
<td>4/5/06</td>
<td>ND</td>
<td>Neg$</td>
<td>ND</td>
<td>≥ 1:320</td>
<td>Pos</td>
</tr>
<tr>
<td>RS, M, 56</td>
<td>Seychelles 5/30/06</td>
<td>5/6/06</td>
<td>Pos</td>
<td>$2.2 \times 10^5$</td>
<td>ND</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>MR, F, 35</td>
<td>India 9/7/06</td>
<td>9/9/06</td>
<td>ND</td>
<td>$5.9 \times 10^5$</td>
<td>Pos (CHIKV ITA3 MR)</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

$qRT$-PCR = quantitative reverse transcription–polymerase chain reaction; NSP1 = nonstructural protein 1; IFA = immunofluorescent antibody assay; Pos = positive; Neg = negative; ND = not done.

† r < 1.20
‡ ≥ 1:20
§ < 4 × 10²/copies/mL.
known virus inhibitor, showed a dose-dependent inhibition of virus replication, assessed by viral infectivity and viral RNA production. The wide linear range of the assay is consistent with results of in vitro studies, in which evaluation of viral replication is needed. Our results for patient samples also indicate that clinical sensitivity of this method is good, and that this method can be a useful tool for rapid detection of CHIKV during natural infection and monitoring the extent of viral replication in patients. Because of concern that CHIKV infection could become endemic in western countries where the mosquito vector (Aedes albopictus) is present, availability of methods for rapid diagnosis and patient monitoring is important in increasing global preparedness for a possible new and emerging epidemic.

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