

Chapter 2

Molecular Epidemiology of Chikungunya Virus by Sequencing

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Abstract

Molecular surveillance of Chikungunya virus (CHIKV) is important as it provides data on the circulating CHIKV genotypes in endemic countries and enabling activation of measures to be taken in the event of a pending outbreak. Molecular surveillance is carried out by first detecting CHIKV in susceptible humans or among field-caught mosquitoes. This is followed by sequencing a selected region of the virus which will provide evidence on the source of the virus and possible association of the virus to increased cases of Chikungunya infections.

Key words Molecular surveillance, RT-PCR, Sequencing, Phylogenetic tree

1 Introduction

Molecular epidemiology of Chikungunya viruses is carried out routinely to identify strains of the virus in a geographical location at a given time [1]. This is done by sequencing CHIKV isolated from patients from current infections and comparing these sequences with that of the virus from previous cases or from sequences databases such as Genbank. Molecular epidemiology of CHIKV will be effective if the exercise is done routinely as it will assist in identifying the introduction of new strains. Furthermore, the data may provide some evidence on the association of Chikungunya strains to a sudden increase in Chikungunya cases in places as this may suggest that new strains of the virus have been introduced to the population. This is due to the presence of naïve subpopulation in that area who have not been exposed to the new strain and hence may develop signs and symptoms of Chikungunya infections.

Chikungunya virus is found mostly in Africa especially in Eastern, Southern, Central, and Western Africa and Asia including parts of India, Thailand, and Malaysia. Currently there are three genotypes identified, belonging to Asian, East/Central/South African, and West African lineages [2–4]. As humans are the major reservoir of the virus for the mosquito, surveillance for the virus in both humans and mosquitoes is important to prevent outbreaks. This is done by carrying out molecular epidemiology of CHIKV.

There are many publications reporting the importance of molecular epidemiology of CHIKV [1, 5–9]. Their findings illustrate the role of Chikungunya strains causing outbreaks. In the Caribbean island of Saint Martin, two cases of Chikungunya were first reported in December 2013, indicating the start of the first documented outbreak in the Americas [8]. Since then the virus has established itself in the Americas where more than 17,000 suspected and confirmed cases were reported between December 2013 and March 2014. Probably the most significant Chikungunya outbreak of unprecedented magnitude occurred in 2005–2007, affecting countries ranging from the Reunion Island, Mauritius, Seychelles, Comoros, and Southwest India where there were reports of anecdotal deaths, encephalitis, and neonatal infections [4]. The CHIKV strain implicated was genotype East/Central/South Africa (ECSA). In India, CHIKV was implicated during the 2007 outbreak of febrile arthritis, affecting nearly 25,000 people in the Southern State of Kerala [10] while Thailand reported cases of Chikungunya associated with the A226V mutation [11, 12]. Meanwhile, molecular epidemiology of Chikungunya in Malaysia showed that the strains that were circulating in 2008–2009 also belonged to the ECSA genotype (Fig. 1; [13]). Hence, from previous investigations into the molecular epidemiology of CHIKV, the best approach is through sequencing of selected regions of the virus. The most studied region of the CHIKV is the envelope gene 1 (E1). This gene was selected as the variation noted in this region provided information on the mutations associated with environmental pressures. In addition, Sanger sequencing is a method of choice as the target fragment is small. It is more cost-effective to use this approach rather than next generation sequencing (NGS) which is usually used for full genome sequencing.

Fig. 1 Phylogenetic tree of partial glycoprotein E1 sequences of CHIKV inferred using the Neighbor-Joining method from the software MEGA 4. The evolutionary distances were computed using the Maximum Composite Likelihood method. Genotype Asian, Central/East African, and West African are indicated by *square brackets* with O'nyong-nyong virus as an outgroup. 49 CHIKV isolates from Malaysia in 2008 and 2009 are indicated in *red* and *blue* respectively. Representative strains of each genotype obtained from GenBank are labeled using the following format: "Accession number"—"isolate"—"Country of origin"—"Year isolation." Bootstrap values (>75 %) for 1000 pseudoreplicate dataset are indicated at branch nodes [13]



This chapter describes the methods used for the determination of Chikungunya strains. This involves design of primers based on the envelope gene of the virus and subsequent reverse transcriptase polymerase chain reaction of the targeted region. Subsequently, sequencing and data analysis is done to characterize the viruses according to their genotypes. This information is useful to infer molecular epidemiology of CHIKV.

2 Materials

All biological specimens including blood should be considered potentially infectious and should be handled in accordance with the appropriate national/local biosafety practices. In addition collection of blood sample from the study subjects must be approved by the Institutional Human Ethics Board. All biological and clinical waste must be decontaminated before disposal, in accordance with national and local regulations. All solutions must be prepared using ultrapure water and molecular grade reagents.

2.1 Viral RNA Extraction

1. Serum from CHIKV-infected patients or CHIKV-infected cell culture supernatant.
2. Viral RNA extraction kit (QIAGEN) or any commercially available viral RNA extraction kit.
3. RNaseZap Wipes and DNAZap.

2.2 One-Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

1. PCR primer sequences: CHIKV forward primer (Forward 5' TACCCATTTCATGTGGGGC 3'), and CHIKV reverse primer (Reverse 5' GCCTTTGTACACCACGATT 3') (*see Note 1*; [14]).
2. QIAGEN One-Step RT-PCR Kit or any commercially available RT-PCR reagents.
3. Nuclease-free water.
4. PCR Thermal Cycler.
5. Mini microcentrifuge.

2.3 Agarose Gel Electrophoresis and Purification of PCR Product

1. NuSieve® 3:1 Agarose.
2. Tris EDTA Buffer (1×): 89 mM Tris-borate, 2 mM EDTA.
3. Red Safe™ Nucleic Acid Staining Solution.
4. 100 bp DNA ladder marker.
5. 6× Loading Dye.
6. Agarose gel electrophoresis system.
7. QIAquick® Gel Extraction Kit (Qiagen).
8. QIAquick® PCR Purification Kit (Qiagen).

**2.4 Sequencing
Reaction**

1. PCR product of CHIKV.
2. Forward and reverse primers of the PCR product.
3. BigDye Terminator Ready Reaction Kit V3.1 (Applied Biosystems).
4. Nuclease-free water.
5. Thermal cycler.
6. Mini microcentrifuge.

**2.5 Purification
of Cycle Sequencing
Product**

1. Nuclease-free water.
2. 3 M sodium acetate pH 4.6.
3. 95 % Ethanol.
4. 70 % Ethanol.
5. Hi Di formamide (Applied Biosystems).
6. Centrifuge.
7. Mini microcentrifuge.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Prepare RT-PCR reaction mixture in a PCR/UV work station. Clean the work bench and pipettes with RNaseZap Wipes and DNAZap to remove all potential RNAase and DNA contamination.

**3.1 Viral RNA
Extraction**

1. All work must be carried out in a biosafety cabinet. Clean all surfaces with RNaseZap before wiping with RNase-free water.
2. Perform viral RNA extraction by using commercially available kit, QIAamp® Viral RNA Kit. Use serum from Chikungunya-infected person or Chikungunya-infected BHK cells.
3. Follow the method as described in the kit insert. Store extracted RNA at -80 °C before use (*see* **Note 2**).

**3.2 One-Step
Reverse Transcriptase
Polymerase Chain
Reaction (RT-PCR)**

1. Prepare each PCR reaction in a 25 µl reaction volume containing 5 µl of extracted RNA, 1 µl of RT enzyme, 12.5 µl of 2× One-Step RT-PCR Buffer Mix, 0.5 µl of each primers, CHIKV forward (25 µM) and CHIKV reverse primers (25 µM), and nuclease-free water to make up to 25 µl reaction mix. Follow the concentrations of buffer, dNTPs, *Taq* polymerase, and Mg²⁺ as recommended by the manufacturer.
2. Quick spin the PCR tubes in the mini microcentrifuge and load them into a PCR thermal cycler. The thermal cycling conditions consist of a 30 min reverse transcription step at 50 °C, 15 min of initial denaturation at 95 °C, followed by 40 cycles of amplification steps of denaturation at 95 °C for 30 s and

annealing/extension at 54 °C for 60 s, and a final extension at 72 °C for 10 min.

3. Upon completion of the PCR run, store the PCR amplicons at -20 °C for temporary storage or proceed to agarose gel electrophoresis (*see Note 3*) to determine the size of PCR amplicons.

3.3 Agarose Gel Electrophoresis and Purification of PCR Product

1. Prepare a 2% gel by carefully transferring 2 g of agarose powder into a clean 250 ml conical flask containing 100 ml of 1× TBE (*see Note 4*). Mix gently to ensure all powder comes in contact with TBE buffer.
2. Dissolve the agarose by heating using a microwave at a high-power setting for 2 min (*see Note 5*). Mix the flask gently to ensure that all the agarose have completely dissolved. Add 2 µl of Red safe dye into the dissolved gel and mix adequately. Pour the gel gently into suitable gel cast with comb inserted, ensuring no bubbles are formed. Leave the gel cast at room temperature for about 30 min to enable the cast to set.
3. Remove the comb from the gel cast. Mix 10 µl of PCR amplicons of all RT-PCR reactions including positive and negative controls with 1 µl of loading dye (mixing can be done on a parafilm). Load the PCR amplicons and loading dye mixture into individual wells. Also load 5 µg of 100 bp DNA ladder into one of the side wells.
4. Perform agarose gel electrophoresis at 100 V for about 45 min or until the loading dye is nearing the bottom edge of the gel. Stop the electrophoresis run. Transfer the gel into a gel documentation system and capture an image of the gel.
5. Before performing any sequencing reaction, the PCR product must undergo purification to remove excess primers and other PCR reaction mix which can inhibit the chain termination sequencing reaction. If there is only a single band of PCR product present, purify the PCR product directly using QIAquick® PCR Purification Kit. If there is more than 1 band present, then gel purification must be carried out by excising the band of interest before continuing with purification using QIAquick® Gel Extraction Kit.
6. Follow the purification method as described in the kit insert.
7. Use the purified PCR product as a template for subsequent sequencing reaction or keep at -20 °C before use.

3.4 Sequencing Reaction

1. Aliquot 4 µl of Big Dye® Ready Reaction Premix in a 200 µl sterile tube. Then add 2 µl of a single forward or reverse primer (10 pmol/µl), followed by 2 µl of BigDye sequencing buffer.
2. Subsequently, add 1 µl of template (100 ng/µl) and make up the volume to 20 µl with nuclease-free water. Quick spin the reaction tubes in a mini microcentrifuge.

3.5 Purification of Cycle Sequencing Product

3. Load the reaction tubes into a PCR Thermal cycler. The PCR conditions are as follows: Initial denaturation at 96 °C for 1 min; denaturation at 96 °C for 10 s, hybridization at 50 °C for 5 s, and elongation at 60 °C for 4 min.
1. Upon completion of the cycle sequencing, centrifuge the plate at $100\times g$ for 1 min.
2. Add 80 μ l of mixture containing 2.55 μ l 3 M Sodium Acetate pH 4.6, 54.74 μ l 95% ethanol, and 22.71 μ l nuclease-free water into each well of the plate.
3. Close the plate with a plate septa to prevent leakage. Mix gently and centrifuge at $100\times g$ for 1 min.
4. Incubate the product at room temperature for 30 min to allow precipitation. Centrifuge the plate again at $3000\times g$ for another 30 min at 4 °C.
5. Discard the supernatant. Add 150 μ l of 70% ethanol into each well and rinse the pellet. Vortex the mixture adequately. Centrifuge the plate at $3000\times g$ for 10 min at 4 °C.
6. Discard the supernatant and repeat the ethanol washing step. Briefly centrifuge the plate at $50\times g$ for a minute.
7. Discard the supernatant. Air-dry the pellet using a thermal cycler at 65 °C for 5 min.
8. In preparation for sample injection into the sequencer, add 10 μ l of Hi Di formamide into each well and resuspend the pellet. Briefly centrifuge the plate at $100\times g$ for a minute.
9. Denature the samples by heating the plate at 95 °C for 2 min. Then immediately place the plate on ice. Briefly centrifuge the plate for a minute at $200\times g$.
10. Load all samples into the designated wells in ABI 3130 genetic analyzer DNA sequencer. Proceed to run the reaction using POP 7 polymer 5 cm capillary array.
11. Analyze data using software SeqScape v 5.2.

3.6 Sequence Data Edition

The sequence data derived must be edited by using data from both primer directions of the template to ensure reliability of sequence data.

1. Download suitable sequence data edition software from the internet. For example, CHROMAS is a suitable sequence data editor and can be downloaded from the internet.
2. Open CHROMAS software. Then, open the forward direction of the sequencing reaction raw data followed by the reverse direction of the same sequencing reaction.
3. Using CHROMAS, reverse complement the reverse direction of the sequencing reaction. Now compare both sequences of

the sample, looking for any sequence data that do not match. This is to ensure reliability of the sequence data.

4. Repeat the sequencing experiment if there are mutations that do not match between both sequence data as this suggests error during the sequencing reaction.
5. Once the reliability of the raw sequence data has been established, import these edited raw sequence data into MEGA 6. Use this software to create a phylogenetic tree.

3.7 Construction of Phylogenetic Tree

1. For investigation of molecular epidemiology of viruses, phylogenetic trees must be constructed to reflect the origin of viruses. For that, MEGA 6, a free software, is highly recommended (*see* **Note 6**; [14]).
2. To obtain a reference sequence, perform a nucleotide BLAST analysis from the following website (<http://www.ncbi.nlm.nih.gov/BLAST>). For analysis, use neighbor-joining method according to the distances between all pairs of the sequences in a multiple alignment.
3. Evaluate the confidence of the sequence clustering by bootstrapping with 1000 replicates.

4 Notes

1. These primers will result in a single PCR product of 294 bp. If other bands appear, further optimization can be carried out by using a gradient PCR. Usually an increase in annealing temperature will reduce the probability of nonspecific bands. If the extra bands are still present, then the best option is to excise the 294 bp sized band and continue with PCR product purification, using gel purification protocol which is available from Qiagen. The primers are designed to be highly specific and produce a single amplicon only. This enables easier PCR amplicon purification.
2. The extracted RNA should be aliquoted into a suitable volume (12 µl suitable for two reactions) to prevent degradation due to several freeze-thawing procedures. The aliquoted RNA must be stored at -80 °C before use.
3. The PCR amplicon should be purified within 1 week after the completion of RT-PCR. This is to reduce the effect of exonuclease activity.
4. Wear heat protecting gloves when handling hot glasswares used for heating agarose. The volume of the buffer used during heating of the agarose should be 10 % less than the desired volume to ensure better solubilization of the agarose. Once the agar has been completely dissolved, add adequate volume of 1× TBE to make up to 100 ml of agarose.

5. Ensure that the agarose is completely dissolved and then have the final volume made up to the desired volume.
6. To get started, it is helpful to refer to the tutorial provided in the Mega 6 software [14].

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