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Molecular diagnosis and analysis of Chikungunya virus

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Abstract

Background: In March 2005 a Chikungunya fever outbreak began on the islands of the Indian Ocean. The number of cases of this disease dramatically rose amongst these islands before affecting over a million people in India. Travellers to these regions have returned to the UK with the disease leading to a greater than 15-fold increase in the annual number of Chikungunya virus (CHIKV) sero-positive samples in 2006.

Objectives: A real-time RT-PCR test was developed for CHIKV and designed to detect currently circulating strains of virus as well as other genotypes. Its sensitivity was compared with an existing standard RT-PCR assay and a previously published real-time assay.

Study design: A real-time RT-PCR assay was optimised and evaluated using a panel of 55 clinical serum samples and a synthetic RNA transcript as a positive control. Nucleotide sequencing of part of the E1 gene of CHIKV was used to investigate the relatedness of the samples.

Results: The real-time RT-PCR was 10-fold more sensitive than a conventional block-based RT-PCR and could detect as low as 20 copies of RNA transcript. The assay also had 10-fold improved sensitivity in detecting the outbreak strain of virus when compared to another published TaqMan assay. Analysis of sequences from patients that had travelled to India, Mauritius or the Seychelles showed high similarity with published sequences from the Indian Ocean island of Réunion.

Conclusions: A sensitive and rapid real-time RT-PCR assay has been developed for CHIKV and tested against current isolates.

Keywords: Chikungunya; RT-PCR; TaqMan; Alphavirus

1. Introduction

Chikungunya fever is a viral disease transmitted to humans by mosquitoes. The most common clinical symptoms are arthralgia, myalgia, rash, headache and fever. The symptoms usually last between 1 and 10 days although arthralgia may persist. Chikungunya virus (CHIKV) is an alphavirus of the family *Togaviridae*. It has a single strand positive sense RNA genome.

In the past, there have been outbreaks in Africa, India and South-East Asia. Sequence analysis has revealed the existence of geographically clustered lineages of the virus. Powers et al. (2000) reported that the Asian outbreak strains originated in Africa, but still formed a distinct Asian genotype separate from West African and Central/East African genotypes. More recently the virus emerged on the islands of the South-West Indian Ocean (Ligon, 2006). This outbreak is thought to have begun in Comoros Island and then circulated among the islands of Mauritius, Mayotte, Réunion and the Seychelles. The epidemic had a particularly severe impact on the island of Réunion, where over a third of the population was infected between 2005 and 2006 (Bonn, 2006). CHIKV then emerged in India, the first outbreak in 32 years (Yergolkar et al., 2006). Sequences from the recent outbreak strains have been published and those from Réunion, Mayotte, Madagascar, Mauritius and the Seychelles all align with the Central/East African genotype (Schuffenecker et

Abbreviations: CHIKV, Chikungunya virus; RT-PCR, reverse transcriptase polymerase chain reaction

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Table 1
Primers used for RT-PCR and sequencing

Primer	Sequence 5′–3′	Genome position
Standard RT-PCR		
CHIK 1	TATCCTGACCACCCAACGCTCC	9,403–9,424[a]
CHIK 2	ACATGCACATCCCACCTGCC	9,693–9,712[a]
Real-time RT-PCR		
CHIK E1 F	TCGACGCGCCCTCTTTAA	10,865–10,882[b]
CHIK E1 R	ATCGAATGCACCGCACACT	10,973–10,991[b]
CHIK E1 P	ACCAGCCTGCACCCATTCCTCAGAC	10,902–10,926[b]
Sequencing		
CHIK 10264 F	GGCGCCTACTGCTTCTG	10,264–10,280[b]
CHIK 11300R	CGACACGCATAGCACCAC	11,281–11,298[b]
CHIK 10564 F	CCCTTTGGCGCAGGAAGAC	10,564–10,582[b]
CHIK 11081 R	GACTTGTACGCGGAATTCGG	11,081–11,100[b]

[a] From CHIKV vaccine sequence GenBank accession number L37661.
[b] From CHIKV S27 prototype sequence GenBank accession number AF369024.

al., 2006). Sequences from recent isolates in India also all belonged to this Central/East African group (Yergolkar et al., 2006). These isolates were obtained from the states of Andhra Pradesh, Karnataka and Maharashtra during the early part of 2006.

This paper describes the development of a real-time RT-PCR assay to detect CHIKV using sequence information from the recent outbreak. The assay was compared with an existing in-house standard RT-PCR assay and a previously published assay that was based on Central African and West African strains (Pastorino et al., 2005). The assay described here had improved sensitivity compared to these other methods for the current Indian Ocean outbreak. Nucleotide analysis of part of the E1 gene from sequences obtained from eight patients returning to the UK provided confirmation of CHIKV designation and allowed strain identification.

2. Methods

2.1. Clinical samples

Patients positive for Chikungunya infection by IgG serological tests in 2006 had recently travelled to Mauritius, the Seychelles and India (Gujarat and Tamil Nadu). Serum samples were provided with travel history of the patient and the date of onset of illness. The clinical history of the patients presenting with the disease included headache, rash, fever and arthralgia. A panel of 55 patient samples were used to test the assay.

2.2. Standard RT-PCR

RNA was extracted using QIAamp Viral RNA mini kit according to manufacturer's instructions (Qiagen, UK). A one-step RT-PCR test was developed in-house for use on a block thermocycler (primers are listed in Table 1). The amplification product was 305 bp within the gene that codes for the viral envelope protein E2. A 25 μl volume reaction was used with Superscript III one-step RT-PCR Platinum–*Taq* kit (Invitrogen, UK), containing 12.5 μl 2× reaction mix, 10 μl water, 0.2 μM primers (Table 1), 0.5 μl RT/*Taq* and 1 μl extracted RNA. The thermocycler conditions were 50 °C for 30 min, 94 °C for 2 min, then 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 2 min 20 s with a final extension at 68 °C for 5 min.

2.3. Real-time RT-PCR

The amplification target was 127 bp of E1 gene, which codes for the structural envelope protein E1. Primers were designed using an alignment of sequences from each of the different genotypes represented in Fig. 1. The real-time

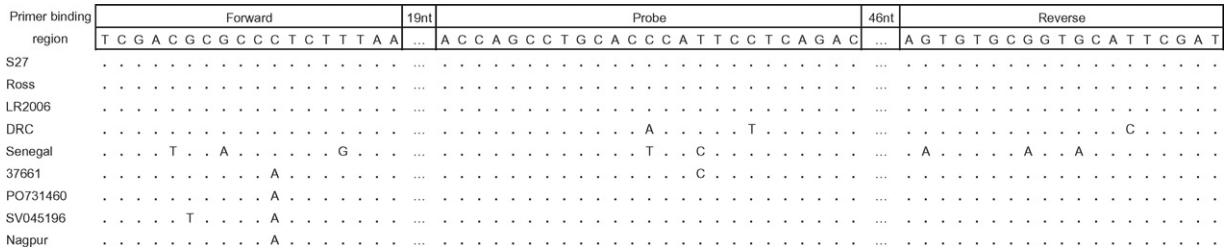

Fig. 1. Alignment of the primer/probe binding regions for the real-time RT-PCR. Sequences representing the different genotypes of CHIKV are shown and nucleotide differences are highlighted.

RT-PCR was carried out using the LightCycler 2.0 (Roche Diagnostics, UK). A 20 μl reaction volume was used with the Superscript III Platinum one-step qRT-PCR system (Invitrogen). This contained 10 μl 2× reaction mix, 0.9 μM primers (Table 1), 1.25 μM dual-labelled probe, 6.75 mM $MgS0_4$, 0.8 μl RT/*Taq* and 5 μl extracted RNA. The cycling parameters were 55 °C for 10 min, 95 °C for 1 min, 45 cycles of 95 °C for 10 s and 60 °C for 35 s, then a cooling step of 40 °C for 30 s.

2.4. Synthesis of an RNA transcript for use as a positive control

RNA extracted from CHIKV S27 prototype strain (National Collection of Pathogenic Viruses) was used to amplify 127 bp region of the E1 gene using the real-time primers (Table 1) and the conditions described in the RT-PCR method section. This product was excised from a 2% agarose gel and purified using QIAquick Gel Extraction Kit according to manufacturer's instructions (Qiagen). The product was cloned into TOPO pCR2.1 vector (Invitrogen) and transformed into TOP10 cells (Invitrogen). Recombinant plasmids were purified using QIAprep Spin Minikit (Qiagen) and the cDNA insert amplified by PCR using the CHIK E1 R primer and a T7 promoter primer. The product was gel purified as before and the RNA transcription reaction was set up using the MEGAshortscript transcription kit (Ambion Inc., UK) according to manufacturer's instructions. RNA was then purified by acidic phenol–chloroform extraction. The RNA concentration was established using a NanoDrop spectrophotometer (NanoDrop technologies).

2.5. Optimisation of the real-time RT-PCR

The assay was optimised with respect to magnesium and primer concentrations. Sensitivity was tested using a dilution series of synthetic RNA transcript, and a dilution series of S27 viral RNA was used to compare the sensitivity with that of the block-based RT-PCR. The assay was also compared to the TaqMan real-time RT-PCR method described by Pastorino et al. using CHIKV strain D5565 (an isolate from India in 2006, GenBank accession EF533650). This virus was isolated from a clinical sample and its titre in pfu/ml was determined using plaque assay on vero cells. Dilutions of this virus were then extracted using the QIAmp Viral RNA minikit and run on the assay. Specificity of the assay was tested using RNA extracts from the following viruses (National Collection of Pathogenic Viruses accessions): O'nyong nyong (accession 130), Ross River (17), Western/Eastern/Venezuelan equine encephalitis (602/598/599), Sindbis (51), Dengue 1, 2, 3 and 4 (202/151/153/569), West Nile NY99 (398), yellow fever French Neurotropic (32), Japanese encephalitis Nakayama (169), Rift Valley fever (559) and Murray Valley encephalitis (506) viruses.

2.6. Sequencing of the E1 gene for clinical samples

RT-PCR positive samples were selected from patients that had travelled to different countries and the primers CHIK 10264F and CHIK 11300R (Table 1) were used to amplify part of the E1 gene directly from the extracted RNA. The BigDye Dye Terminator Sequencing kit (Applied BioSystems, UK) and the DyeEx 2.0 Spin kit (Qiagen) were used prior to reading in the ABI 3100 sequencer (Applied BioSystems). Sequences were assembled and analysed using DNASTAR software. An overlapping 570 bp region (10,620–11,189 nucleotides from S27 reference strain numbering) of the aligned sequences was aligned using ClustalW services of the European Bioinformatics Institute website with output in PHYLIP format (Chenna et al., 2003; Felsenstein, 1989). This alignment was entered into the PUZZLE program (Institute Pasteur website: Strimmer and von Haeseler, 1996). The output data from this were assembled into a phylogram using TREEVIEW software (Eisen et al., 1998).

3. Results

3.1. Sensitivity of real-time RT-PCR assay

The real-time assay using CHIKV S27 RNA (extracted from cell supernatant) was10-fold more sensitive than the standard RT-PCR assay. The real-time method could also detect as few as 20 copies of RNA transcript. Using titrated virus stock, the detection limit of the assay was 0.1 pfu/ml of CHIKV (D5565). This was 10-fold more sensitive than the detection limit of the compared real-time assay (Pastorino et al.), which was found to be 1 pfu/ml using this strain. When testing the panel of serum samples, the real-time assay amplified three samples that were not detected by the standard RT-PCR method (Table 2). It also detected five samples that were obtained prior to patient sero-conversion, therefore, were not positive by serology. One of these was positive by virus isolation and further samples were requested from three other patients, which were proven CHIKV IgG positive. Samples that were positive by serology but negative by molecular methods were taken post-viraemia. The assay did not detect any of the other alphaviruses, flaviviruses or the phlebovirus tested in the specificity panel.

Table 2
Comparison of serological and molecular data obtained from UK patient samples

Total samples	Serology	Molecular tests
55	**18** positive	**7** positive by standard RT-PCR **9** positive by real-time RT-PCR
	37 negative	**4** positive by standard RT-PCR[a] **5** positive by real-time RT-PCR[b]

[a] One of these was positive by virus isolation and two were positive by serology in follow-up samples.

[b] One of these was positive by virus isolation and three of these were positive by serology in follow-up samples.

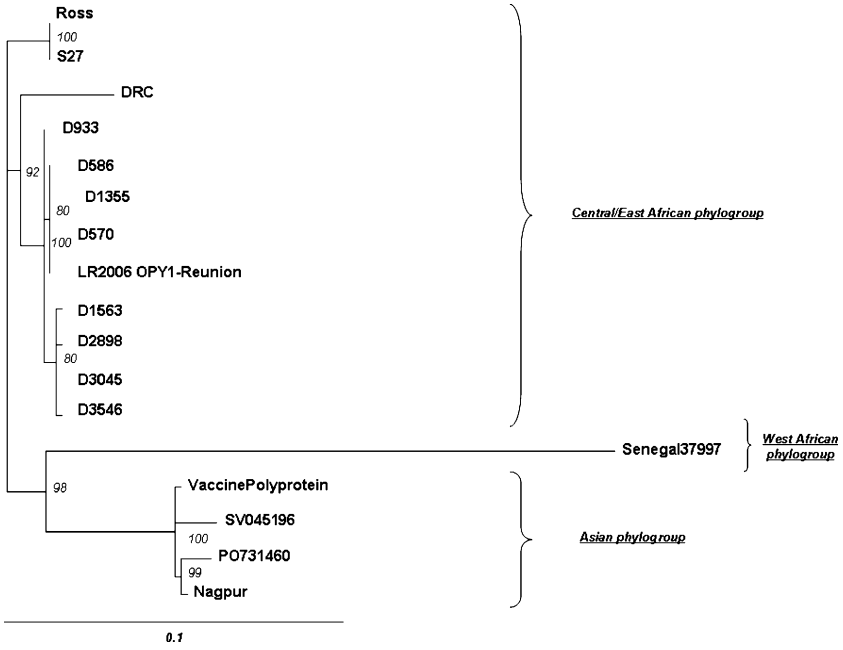

Fig. 2. Phylogenetic analysis of CHIKV sequences from this study (designated D. . .) and CHIKV samples from different regions. The tree is a phylogram from partial E1 gene sequences with an outgroup of strains S27 and Ross (isolated 1952–1953). Support values are given.

3.2. Sequence analysis

For one of the earliest virus isolates processed during this outbreak, the whole genome was sequenced (data not shown, GenBank strain D570 accession number EF012359). Nucleotide sequence was obtained for part of the E1 gene for eight samples (nucleotide position 10,620–111,89 of S27 reference strain numbering). The relatedness of the isolates examined in this study to published sequences is shown in Fig. 2. An NCBI BLAST search (Altschul et al., 1990) matched the eight sequences to the isolates from Réunion Island. The consensus sequences from the contigs showed the nucleotide difference between samples. The three sequences from Mauritius (Table 3) were identical in the 570 bp region sequenced while the sample from the Seychelles had 99.8% sequence identity (one nucleotide difference). The samples from India were identical to each other and had three nucleotide differences relative to the Mauritius strains. These were at nucleotide positions 10,743, 111,27 and 10,670 of the S27 reference strain (AF369024). The nucleotide change at position 10,670 led to an amino acid substitution from valine to alanine.

Table 3
Origin of published sequences and clinical sample sequences

Strain/sample id	Year of isolation/date received	Origin	Accession no.	Reference
S27	1952	Tanzania	AF369024	Khan et al. (2002)
Ross	1953	Tanzania	AF490259	N/A[a]
DRC1725	2000	Democratic Republic of Congo	AY549581	Pastorino et al. (2004)
LR2006_OPY1	2006	Réunion	DQ443544	Parola et al. (2006)
37997	1983	Senegal	AY726732	Powers et al. (2000)
Vaccine strain	N/A	N/A	L37661	N/A[a]
SV045196	1996	Thailand	AF192900	Powers et al. (2000)
PO731460	1973	India	AF192902	Powers et al. (2000)
Nagpur	N/A	India	AY424803	N/A[a]
D570	March 2006	Mauritius	EF012359	J. Chamberlain[b]
D586	March 2006	Mauritius	EF193853	This paper
D933	April 2006	Seychelles	EF193854	This paper
D1355	May 2006	Mauritius	EF193855	This paper
D1563	May 2006	South (India)	EF193856	This paper
D2898	August 2006	Chennai (India)	EF193857	This paper
D3045	September 2006	Chennai (India)	EF193858	This paper
D3546	October 2006	Gujarat (India)	EF193859	This paper
D5565	December 2006	India	EF533650	This paper

[a] Information not available.
[b] Sample D570 whole genome was sequenced in our laboratories by J. Chamberlain and submitted to GenBank accession number EF012359.

The sequences from samples from Mauritius clustered with the published Réunion strain LR2006_OPY1: this E1 gene region was identical between the samples. The Indian samples formed another cluster away from the earlier Indian Ocean outbreak samples but originating from the same lineage. The sequence from the Seychelles sample showed a high level of similarity to the sequence from Réunion. Support values given demonstrate the high resolution of phylogenetic relationships within each subgroup.

4. Discussion

The real-time RT-PCR assay described here was shown to detect CHIKV to a high sensitivity in clinical serum samples. Patients returning to the UK from the Indian Ocean islands and India with suspect viral illness are routinely screened for CHIKV in addition to dengue virus and Rickettsial infections. Other European countries are continuing to report the disease in travellers from the Indian Ocean and now from Africa. This demonstrates the importance of surveillance of this disease because a resurgence in cases is possible (Bonn, 2006). The risk of establishment of the imported virus in the UK is low because the climate is currently not favourable to support the vector mosquito species. The *Aedes albopictus* mosquito is responsible for transmitting the virus in the Indian Ocean islands and has been detected in many other parts of Europe (Depoortere et al., 2006). As a result, there is a need to increase awareness of the disease and of the emergence and re-emergence of other mosquito-borne virus infections throughout Europe.

Sequence analysis has provided further information about the samples tested and allowed additional insight into their ancestral background. The nucleotide substitution at position 10,670 (S27 reference strain numbering) for five of the eight sequences derived has been previously observed in Réunion isolates. Schuffenecker et al. (2006) observed that in CHIKV isolates pre-June 2005 from the Indian Ocean outbreak, there is an alanine residue at residue 226 but in isolates from these islands post-September 2005, there is a valine residue at this position. However, the sequences presented here from 2006 have a valine residue for the Mauritius strains and an alanine for the Indian and Seychelles strains. Further sequencing of clinical samples or isolates will provide more information on the progress of this disease and the specificity of the diagnostic primers.

In conclusion, we report a real-time RT-PCR assay which is highly sensitive for currently circulating strains of CHIKV. Additionally, sequence analysis provides insight into the epidemic spread of this virus.

Acknowledgements

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