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Development and evaluation of a one-step SYBR-Green I-based real-time RT-PCR assay for the detection and quantification of Chikungunya virus in human, monkey and mosquito samples

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Abstract. This paper reports the development of a one-step SYBR-Green I-based real-time RT-PCR assay for the detection and quantification of Chikungunya virus (CHIKV) in human, monkey and mosquito samples by targeting the E1 structural gene. A preliminary evaluation of this assay has been successfully completed using 71 samples, consisting of a panel of negative control sera, sera from healthy individuals, sera from patients with acute disease from which CHIKV had been isolated, as well as monkey sera and adult mosquito samples obtained during the chikungunya fever outbreak in Malaysia in 2008. The assay was found to be 100-fold more sensitive than the conventional RT-PCR with a detection limit of $4.12x10^0$ RNA copies/µl. The specificity of the assay was tested against other related viruses such as Dengue (serotypes 1-4), Japanese encephalitis, Herpes Simplex, Parainfluenza, Sindbis, Ross River, Yellow fever and West Nile viruses. The sensitivity, specificity and efficiency of this assay were 100%, 100% and 96.8% respectively. This study on early diagnostics is of importance to all endemic countries, especially Malaysia, which has been facing increasingly frequent and bigger outbreaks due to this virus since 1999.

INTRODUCTION

Chikungunya, a viral fever resembling dengue, is caused by Chikungunya virus (CHIKV), an Alphavirus of the family Togaviridae (Calisher *et al.*, 1982; USACHPPM, 2007). CHIKV has a positive, single-stranded RNA genome, a 60-70nm diameter capsid, and a phospholipid envelope. The genome is organized as follows: 5' cap-nsP1-nsP2-nsP3-nsP4-(junction region)-C-E3-E2-6K-E1-poly(A) 3' (Khan *et al.*, 2002). The virus is transmitted by bites of infected female mosquitoes, especially *Aedes albopictus* and *Aedes aegypti*, which also spread Dengue virus (DENV). Thus, both CHIKV and DENV share the same vectors, some symptoms and geographical distributions.

In recent years, chikungunya fever has become an increasing threat to Asia, and Malaysia has seen increasingly bigger outbreaks since 1999 (Lam *et al.*, 2002; Lee *et al.*, 2008; Mavalankar *et al.*, 2009;

Murtola *et al.*, 2009; Vasan & Shepard 2009). The threat posed by this virus can also be easily estimated from the experience in countries such as India and (the French) Réunion Island (Paganin *et al.*, 2006; Mavalankar *et al.*, 2009; Murtola *et al.*, 2009). The disease affected one third of the population of the French Réunion Island during the outbreak in year 2005-2006 (Paganin *et al.*, 2006), while India had 1.4 million suspected cases in 2006 alone (Shuffenecker *et al.*, 2006; Mavalankar, *et al.*, 2009; Murtola *et al.*, 2009). Besides, sporadic cases have also been reported in Burma (Thaung, 1975; Thaikruea *et al.*, 1997). CHIKV was first isolated from human serum and *Ae. aegypti* mosquitoes during an epidemic in Tanzania in 1953 (Ross, 1956). The first outbreak in Southeast Asia was reported in Bangkok, Thailand in 1958. Since then, outbreaks of this virus have been documented in Cambodia, Indonesia, Laos, Malaysia, Myanmar, The Philippines and Vietnam.

The incubation period of CHIKV infection is 2-12 days, with an average of around 3-7 days. CHIKV infection (either symptomatic or asymptomatic) is thought to confer life-long immunity. Chikungunya fever is characterized by a brief febrile episode, persistent arthralgia/ arthritis in more 80-90% of infections reported and by the absence of fatalities. In contrast, dengue fever is characterized by a sudden onset of fever, chills, headache, nausea, vomiting, arthralgia and rash. However, similarities between clinical appearances of the two diseases probably account for misclassification and some under reporting of chikungunya fever cases in areas endemic to dengue fever. Therefore, laboratory confirmation of reported cases is very important.

Currently, the diagnosis of CHIKV infection is carried out either by virus isolation or by detection of virus-specific antibodies using ELISA or by genome detection using reverse transcription-polymerase chain reaction (RT-PCR) technique (Bronzoni *et al.*, 2002; Hasebe *et al.*, 2002). PCR is a widely used technique in molecular biology and diagnostics, and is the basis for many tests and assays. Many research centres and hospitals now have their own molecular diagnostic laboratories, which make use of methods such as PCR, RT-PCR and Ligase Chain Reaction (LCR). RT-PCR reaction is useful during the early viraemic phase from 0 day to 4th day (Pfeffer *et al.*, 2002; Pastorino *et al.*, 2005), while the classic serological methods such as haemaglutination inhibition, complement binding, immuno-fluorescence and ELISA are simple and can be done from the 2nd or 3rd day until more than 15th days after the onset of fever (Thein *et al.*, 1992). The conventional RT-PCR is time consuming, with higher risk of cross contamination compared to the real-time RT-PCR (Mackay *et al.*, 2002). Recently, the TaqMan real-time RT-PCR was developed as one of the rapid diagnostic tools for detecting and quantifying African CHIKV (Pastorino *et al.*, 2005).

The real-time RT-PCR is less time consuming and has many other advantages such as high sensitivity, specificity, capability of quantitation, low risk of contamination and ease of standardization (Mackay *et al.*, 2002). Basically, all real-time RT-PCR systems depend on the detection and quantification of a fluorescent reporter, and the increasing fluorescent signals are proportional to the amount of PCR product in a reaction. The simplest and most economical format is SYBR Green I dye, which is a double-strand DNA-specific intercalating dye.

This study describes the sensitivity and specificity of a SYBR-Green I-based one step RT-PCR assay for the detection and quantification of CHIKV infection using primers targeting the *E1* gene region of the CHIKV. The specificity and sensitivity of the present assay was validated with 71 samples comprising of a panel of sera positive for other viruses (negative controls), human sera, monkey sera and adult mosquitoes obtained during the chikungunya fever outbreak in Malaysia in 2008.

MATERIALS AND METHODS

Primer design

The oligonucleotides used for the SYBR-Green I-based real-time RT-PCR assay were designed from the *E1* gene sequence of MY019IMR/06/BP CHIKV prototype strain (GenBank accession no. EU703761). The potential primers were selected by identifying highly conserved regions among CHIKV genotypes by aligning *E1* gene sequences in ClustalW 1.83 programme (Fig. 1). All primers were designed using the FastPCR software (Microsoft Office software, Biology software net). The specificity of selected primers was checked by BLAST analysis against the NCBI nucleotide database of CHIKV and related alphaviruses such as O'Nyong Nyong virus (Gulu strain, GenBank accession no. M20303.1), Semiliki Forest virus (L10 strain, GenBank accession no. AY112987), Sindbis virus (GenBank accession no. J02363.1) and Ross River virus (8961 strain, GenBank accession no. GQ433357.1). The primer sequences are as follows; sense CHIK/E1/10367/+ (CTCATA CCGCATCCGCATCAG) and anti-sense CHIK/E1/10495/- (ACATTGGCCCCACAAT GAATTTG).

Sample collection

A total of 71 samples were used to evaluate the sensitivity and specificity of the assay. These samples included (1) acute patient sera from which CHIKV was isolated and collected during 1-4 days after the onset of symptoms (n=9), (2) pools of adult mosquitoes (10 mosquitoes per pool) (n=5) and larvae (20 larvae per pool) (n=7) collected from Tangkak, Johor during a chikungunya fever outbreak in 2008, (3) monkey sera (n=4) collected from Kuala Lipis, Pahang during the chikungunya fever outbreak in peninsular Malaysia in 2008, (4) infected individual adult mosquito homogenates (from CHIKV experimental oral infections) (n=13) and (5) a panel of negative control sera that were positive for other viruses (n=32) and a panel of CHIKV negative human sera collected from healthy volunteers (n=8) (Table 1). These samples were obtained from the Institute for Medical Research (IMR) and the University Malaya Medical Centre (UMMC), Malaysia in 2008. Ethical approval of using human and monkey sera had been obtained from Malaysian Medical Research and Ethical Committee and Director General of Malaysian Wildlife and Natural Resources before sample collection began.

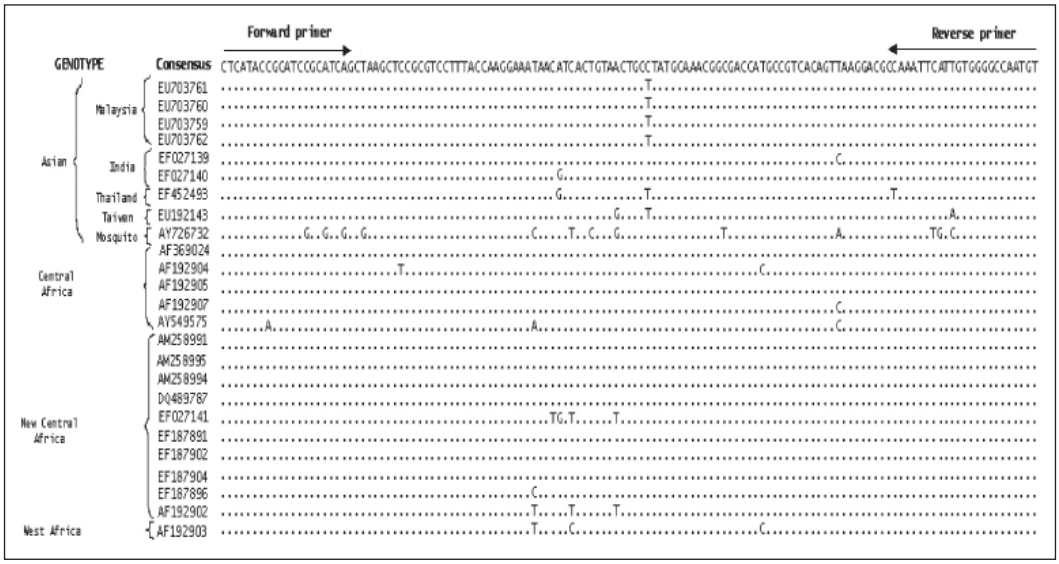

Figure 1. Multiple alignment of the *E1* gene region sequences of selected CHIKV isolates (genomic position 10367-10495). A total of 25 sequences were used to analyse the possible variation at chosen sites (.) indicates conserved nucleotides. The designed primers generate an amplicon of 129 bp

Table 1. Panel of negative controls

Other disease agents & negative sera	No. of samples
Dengue virus 1 (DENV-1)	4
Dengue virus 2 (DENV-2)	5
Dengue virus 3 (DENV-3)	4
Dengue virus 4 (DENV-4)	4
Herpes simplex virus 1 (HSV 1)	2
Herpes simplex virus 2 (HSV 2)	2
Japanese encephalitis virus (JE)	2
Parainfluenza 1	2
Parainfluenza 2	2
West Nile virus	2
Ross River virus	1
Sindbis virus	1
Yellow fever virus	1
Healthy human serum	8

Table shows the 13 groups of other disease agents and 8 healthy human sera which were used for the evaluation of the specificity of the reported assay

Preparation of positive control CHIKV

CHIKV stock (strain Bagan Panchor) was obtained from the Medical Entomology Unit, Institute for Medical Research (IMR), Malaysia. Viruses were then propagated in BHK-21 cell line and the viral RNA was extracted as described below.

RNA extraction

Total RNA was extracted using Bioneer AccuPrep Viral RNA extraction kit (Bioneer Inc., USA) according to the manufacturer's protocol. Firstly, pools of mosquitoes (10 mosquitoes per pool) and larvae (20 larvae per pool) were ground in chilled eppendorf tubes with 1.0 ml of the maintenance medium (Hank's MEM medium) supplemented with 2% fetal bovine serum (FBS), 1M Hepes, 7.5% sodium bicarbonate and antibiotics (Lee *et al.*, 1997). On the other hand, individual mosquitoes were ground in 0.3 ml of Hank's MEM maintenance medium (Vazeille *et al.*, 2007). The larvae and mosquito suspensions were centrifuged at 3,000 rpm for 15 minutes at 10°C (Lee *et al.*, 1997). The supernatant was filtered through a 0.22 µm filter unit and used for RNA extraction. In addition, all other samples (human sera, monkey sera, larvae and adult mosquito homogenates) were spun at 2000 rpm for 5 minutes. Two hundred microlitres of biological samples were placed in a 1.5 ml eppendorf microcentrifuge tube with 400 µl of binding buffer (VB) and mixed by gentle vortexing for 5 seconds. The mixture was incubated at room temperature for 10 minutes, followed by the addition of 100 µl of isopropanol. All mixtures were transferred to the binding column and centrifuged at 8000 rpm for 1 minute. RNA was washed using 500 µl of AW1 (washing buffer 1) and spun at 8000 rpm for 1 minute. The same step was repeated with AW2 (washing buffer 2). Finally, RNA was eluted in 50 µl of elution buffer. The eluted RNA was stored at -70°C.

Preparation of CHIKV RNA standard

Serial dilutions of a CHIKV stock with a known copy number were used to generate a standard curve for the quantitative real-time RT-PCR. CHIKV RNA standard was obtained by the *in vitro* synthesis of RNA transcripts from DNA templates. *In vitro* transcription of the amplified PCR product of CHIKV was done by using the MAXIscript *in vitro* transcription kit (Ambion Inc., USA). Firstly, a standard RT-PCR was carried out by using the designed forward primer modified to incorporate the T7 promoter sequence (5'- **TAATACGACTCA CTATAGGG**CTCATACCGCATCCGCATCAG-3') and the reverse primer (5'-ACATTGG CCCCACAATGAATTTG-3'). PCR product (1 µg) was subjected to *in vitro* transcription (IVT) at 37°C for 1 hour. The IVT products were treated with 1 U of DNase I and incubated at 37°C for 30 minutes to remove the remaining DNA. The DNase activity was stopped by adding 1 µl of 0.5M EDTA and heat deactivated at 95°C for 10 minutes. The excess nucleotide and pyrophosphate in the IVT products were removed by ethanol precipitation. The RNA pellet was resuspended in diethyl pyrocarbonate (DEPC) treated water. The concentration of the generated CHIKV RNA fragment was determined by spectrophotometric reading and converted to molecular copies using the formula (Krieg, 1991) as shown in Fig. 2. The RNA stock solution was 10-fold serially diluted

$$\text{Y molecules/µl} = \frac{\text{X g/µl RNA x } 6.02 \text{ x } 10^{23}}{\text{transcript length (bp) x } 340}$$

Figure 2. Calculation of the molecular copy number

using DEPC treated water containing 1 µg of RNA carrier and stored at -70°C.

One-step SYBR-Green I real time RT-PCR

The One-step SYBR-Green I-based real time RT-PCR was carried out using an iCycler Real-Time PCR machine (BioRad, Hercules, California, USA) using iScript™ One-Step RT-PCR Kit with SYBR® Green premix (Biorad, Hercules, California, USA). Samples were assayed in a 25 µl reaction containing 5 µl of extracted RNA, 0.25 µl of RNA transcriptase, 12.5 µl of iScript™ SYBR® Green premix 2X and 0.2 µM of each primer. The concentrations of buffer, dNTPs, Taq polymerase and Mg2+ were according to the recommendations of the kit's manufacturer. The thermal cycling conditions consisted of a 30 minutes reverse transcription step at 50°C, 15 minutes of initial denaturation at 95°C, followed by 40 cycles of amplification steps of denaturation at 95°C for 30 seconds, annealing at 55.8°C for 45 seconds, extension at 72°C for 60 seconds and a final extension at 72°C for 10 minutes. A RNA standard series with copy numbers ranging from 10^{10} to 10^{0} was used to construct a standard curve.

Conventional RT-PCR

In order to compare the sensitivity of the real-time SYBR-Green I-based real-time RT-PCR assay, the conventional RT-PCR assay was performed with the same set of primers to target the 129 bp region of the *E1* gene of CHIKV genome. The amplification was carried out in a 25 µl total reaction volume by using the Access Quick RT-PCR kit (Promega, USA) as suggested by the manufacturer. Reactions contained 5 µl of extracted RNA, 0.25 µl of RNA transcriptase, 12.5 µl of AccessQuick™ Master Mix 2X, and 0.2 µM of each primer. The thermal cycling conditions for conventional RT-PCR assay were same as those for the real-time assay. The RT-PCR was performed in a Mastercycler gradient machine (Eppendorf, Hamburg, Germany) and took about 2½ hours to complete.

PCR results were analysed by the gel electrophoresis. Five µl of each PCR product was loaded onto 1.5% (W/V) agarose gels (Cambrex, Rockland, ME, USA) in 1x Tris-Borate-EDTA buffer stained with ethidium bromide (EtBr), and run at 80 volts for 1 hour. A 100 bp ladder was used as the molecular weight marker. The gel was viewed under an ultra violet trans-illuminator (Ultra Lum Ins, California, USA) and the image was captured with a Polaroid camera. The method for RT-PCR and electrophoresis employed was modified from Hasebe *et al.* (2002).

Virus isolation

Isolation of viruses from suspected samples was attempted in the BHK-21 cell line (hamster kidney) based on the standard cell culture protocol (Dana *et al.*, 2005). All human sera, monkey sera and adult mosquitoes were inoculated into BHK-21 monolayer and incubated at 37°C in Hank's MEM maintenance medium supplemented with 1M Hepes, 7.5%, sodium bicarbonate, 2% heat-inactivated fetal bovine serum (FBS) and antibiotics. The infected culture fluids were harvested once the prominent cytopathic effect (CPE) was observed (4-5 days post infection) by freeze-thaw method, centrifuged and supernatants were passed through 0.22 µm filter units. Isolation of the virus was confirmed by immuno-fluorescence with monoclonal antibodies specific to CHIKV.

RESULTS

Design of CHIKV specific primers

A set of primers was designed after taking into account the mismatching and primer-dimer formation among all aligned CHIKV strains; Malaysian CHIKV strains, Indian

CHIKV strains, a mosquito CHIKV strain, a Thailand CHIKV strain, a Taiwanese CHIKV strain, Central African CHIKV strains, New Central African CHIKV strains and a West African CHIKV strain. The selected primers were specific and targeted a 129 bp region of the *E1* gene (nt 10367-10495) of the viral genome.

Amplification and melting curve analysis

The conventional PCR amplification of CHIKV *E1* gene as observed by 129bp amplicon on agarose gel analysis is shown in Figure 3A. For the Real Time PCR melting curve analysis was done by analyzing the melting temperature of the specific 129bp amplicon. The melting temperature of CHIKV was found to be 83.5°C±0.5°C. The specific amplification plot and the melting curve for CHIKV are shown in Fig. 3B.

Sensitivity and specificity of the real-time RT-PCR

The detection limit of SYBR-Green I-based real-time RT-PCR assay was $4.12x10^0$ copies of RNA/µl. In comparison, detection limit of the conventional RT-PCR was $4.12x10^2$ copies of RNA/µl (Fig. 4). Fig. 5 shows the standard curve for CHIKV constructed using the C_t values obtained against 10-fold serial dilutions of a CHIKV stock containing $4.12x10^{10}$ copies of RNA/µl. The designed primer pair was highly specific in detection of only CHIKV, having no cross-reactivity with samples from other viral infections or from the negative panels (n=40).

Real-time SYBR-Green I-based real-time RT-PCR assay was evaluated using 71 samples. The comparison of the detection rates between the present real-time assay, virus isolation and the conventional RT-PCR method is summarized in Table 2. The sensitivity of the assay was assessed in 31 human and monkey sera as well as adult mosquito samples (Table 2). Out of 31 samples, the one-step SYBR-Green I-based real-time RT-PCR assay was able to detect all samples as positive for CHIKV as compared to the conventional RT-PCR which could only detect 26 samples as positive. In comparison, by using the virus isolation method, only 19 samples were deemed as positive for CHIKV. Therefore, the SYBR-Green I-based real-time RT-PCR assay was more sensitive than the conventional RT-PCR assay and virus isolation. The sensitivity and specificity of the developed assay was 100% (Table 3) with an efficiency of 96.8% (Fig. 5). The

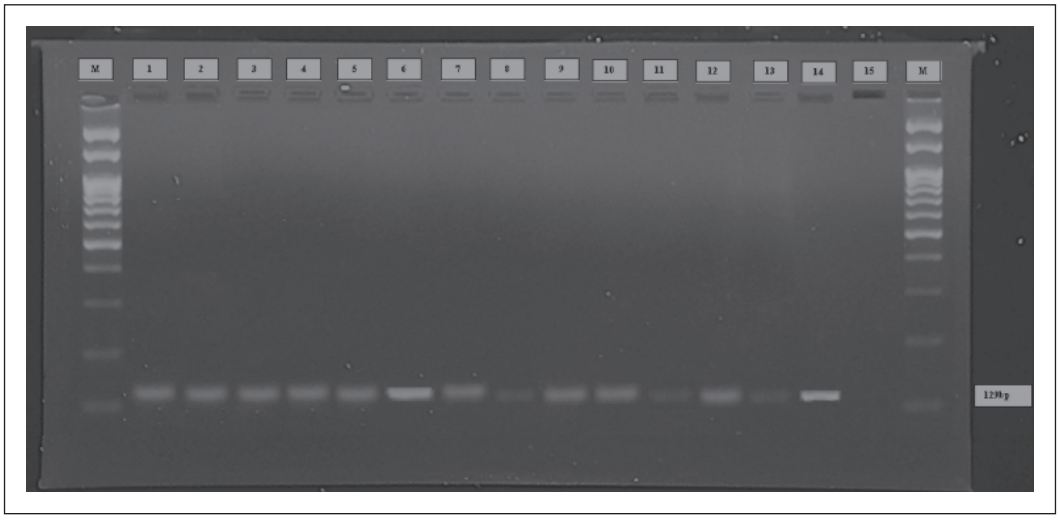

Figure 3A. The detection of CHIKV *E1* gene as observed by 129 bp amplicon on agarose gel analysis. Lane M: 100 bp DNA ladder; lanes 1-6: patient serum; lane 7-10: monkey serum; lane 11-13: infected mosquito homogenates; lane 14: CHIKV positive control; lane 15: negative control

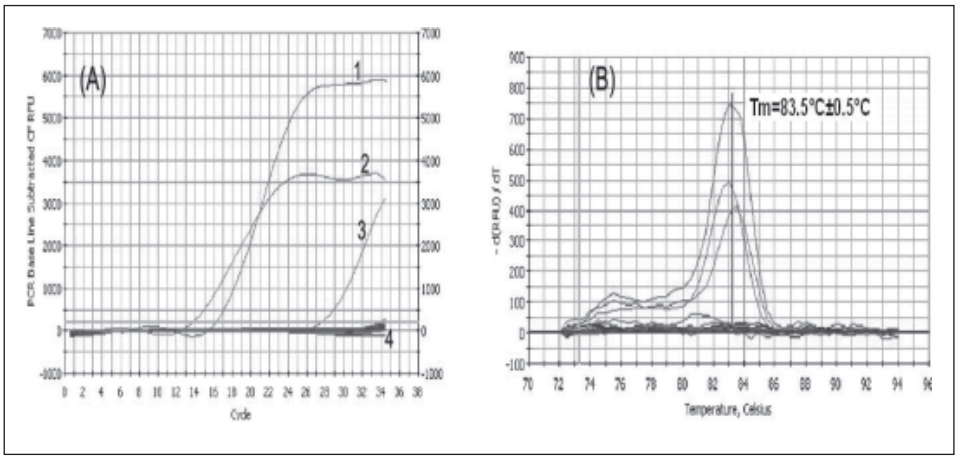

Figure 3B. Real-time kinetics of CHIKV *E1* gene-specific SYBR-Green I-based real-time RT-PCR showing the amplification and melting curve for the reference RNA from three isolates. (A) Amplification plot, (B) Melting curve analysis graph. (1-3) CHIKV positive controls, (4) Negative panels

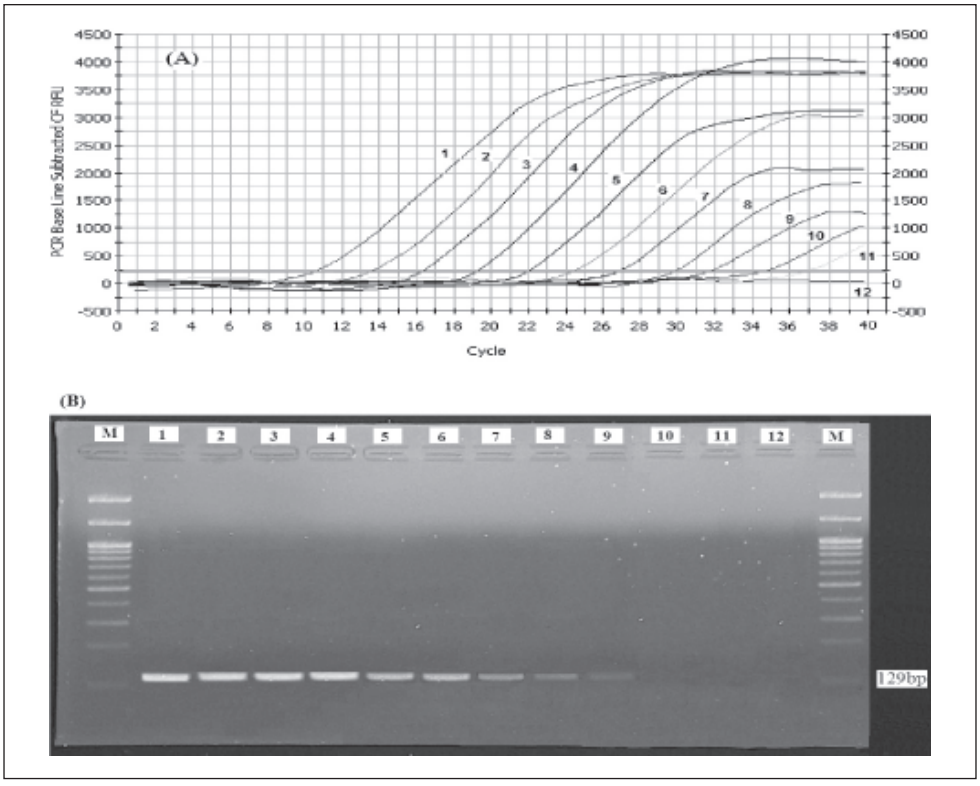

Figure 4. Comparative sensitivity of SYBR-Green I-based real-time RT-PCR assay versus conventional RT-PCR for the detection of CHIKV. (A) The amplification plots from left to right (no. 1-11 as shown in figure A) are the curves of 10-fold serial dilution of virus from $4.12x10^{10}$–$4.12x10^{0}$ copies of RNA/µl; no. 12: negative control. The detection limit for the assay was $4.12x10^{0}$ copies of RNA/µl. (B) Sensitivity of RT-PCR for the detection CHIKV *E1* gene as observed by 129bp amplicon on agarose gel analysis with a detection limit of $4.12x10^{2}$ copies of RNA/µl. Lane M: 100bp DNA ladder; lanes 1-11: 10-fold serial dilution; lane 12: negative control

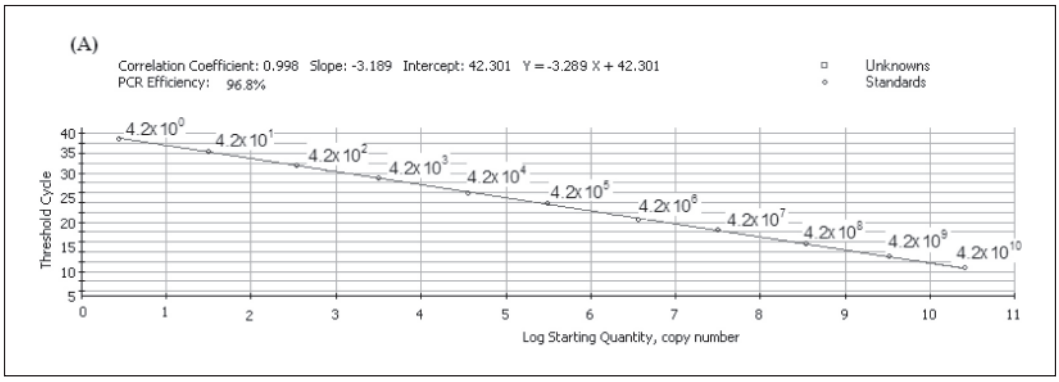

Figure 5. A representative standard curve of CHIKV RNA amplification. A total of $4.12x10^{10}$ copies of RNA/µl standard were prepared by the *in-vitro* transcription technique, which were ten-fold serially diluted and 5 µl of RNA were added into each reaction tube. The standard curve shows a correlation coefficient of 0.998 and detection limit of $4.12x10^{0}$ RNA copies/µl

Table 2. Comparison of the real-time SYBR Green RT-PCR assay, conventional RT-PCR and virus isolation for the detection of CHIKV

Category	Day of Fever Onset	Virus isolation (CPE)[c]	Result of PCR	
			Conventional RT-PCR	SYBR Green RT-PCR (copies/µl)
Patient serum[a]	1	4+	CHIKV	$3.77x10^{7}$
Patient serum[a]	1	4+	CHIKV	$4.53x10^{7}$
Patient serum[b]	1	4+	CHIKV	$8.57x10^{6}$
Patient serum[b]	3	3+	CHIKV	$2.04x10^{5}$
Patient serum[a]	2	3+	CHIKV	$8.05x10^{6}$
Patient serum[b]	4	2+	CHIKV	$4.12x10^{4}$
Patient serum[a]	2	3+	CHIKV	$1.48x10^{5}$
Patient serum[a]	1	4+	CHIKV	$6.18x10^{7}$
Patient serum[b]	4	N/A	CHIKV	$2.93x10^{3}$
Monkey serum[a]	–	3+	CHIKV	$8.55x10^{6}$
Monkey serum[a]	–	3+	CHIKV	$1.39x10^{5}$
Monkey serum[a]	–	3+	CHIKV	$3.54x10^{6}$
Monkey serum[a]	–	4+	CHIKV	$2.08x10^{7}$
Mosquito pool[a]	–	2+	CHIKV	$3.26x10^{4}$
Mosquito pool[a]	–	3+	CHIKV	$3.01x10^{6}$
Mosquito pool[a]	–	3+	CHIKV	$5.86x10^{5}$
Mosquito pool[a]	–	3+	CHIKV	$4.75x10^{5}$
Mosquito pool[a]	–	3+	CHIKV	$6.03x10^{6}$
Infected mosquito[a]	–	N/A	-ve	$1.30x10^{1}$
Infected mosquito[a]	–	2+	CHKV	$1.34x10^{4}$
Infected mosquito[a]	–	N/A	-ve	$4.80x10^{1}$
Infected mosquito[a]	–	N/A	CHIKV	$2.51x10^{2}$
Infected mosquito[a]	–	N/A	CHIKV	$2.86x10^{2}$
Infected mosquito[a]	–	N/A	CHIKV	$1.14x10^{2}$
Infected mosquito[a]	–	2+	CHIKV	$3.10x10^{4}$
Infected mosquito[a]	–	N/A	CHIKV	$2.88x10^{2}$
Infected mosquito[a]	–	N/A	-ve	$3.80x10^{1}$
Infected mosquito[a]	–	N/A	CHIKV	$4.23x10^{2}$
Infected mosquito[a]	–	N/A	CHIKV	$1.26x10^{3}$
Infected mosquito[a]	–	N/A	-ve	$6.56x10^{0}$
Infected mosquito[a]	–	N/A	-ve	$3.40x10^{1}$

[a] Samples obtained from the Institute for Medical Research, Malaysia
[b] Samples obtained from the University Malaya Medical Centre, Malaysia
[c] Prominent cytopathic effect formation (CPE); 4+ = monolayer is totally destroyed; 3+ = about 70% of cells exhibit CPE; 2+ = about 50% of cells exhibit CPE; 1+ = less than 50% of cells exhibit CPE; N/A = Not available

Table 3. Comparison of the sensitivity and specificity of the virus isolation, conventional RT-PCR and SYBR-Green I-based real-time RT-PCR assays

Category	No. of samples	No. of samples positive by		
		Virus isolation	Conventional RT-PCR	SYBR-Green I-based real-time RT-PCR
Acute patient serum from which CHIKV was isolated[a]	9	8	9	9
Monkey serum[a]	4	4	4	4
Mosquito pools[a]	5	5	5	5
Infected mosquitoes	13	2	8	13
Negative control	40	N/A	0	0
Total	71	19	26	31
Sensitivity (%)	–	61.3	83.9	100
Specificity (%)	–	–	100	100

N/A: not attempted
[a] Samples obtained during chikungunya fever outbreak in Malaysia in 2008

PCR efficiency has a major impact on the fluorescence history and the accuracy of the calculated expression result and is critically influenced by PCR reaction components. Efficiency evaluation is an essential marker in the gene quantification procedure. Constant amplification efficiency in all compared samples is one important criterion for reliable comparison between samples. This becomes crucially important when analyzing the relationship between an unknown sequence versus a standard sequence. The efficiency of PCR should ideally be 90-100%. The PCR efficiency of the assay reported in this study was 96.8% which was within the recommendation range.

Quantification of viral load in pre-collected samples

Quantification of the CHIKV load in suspected samples was determined based on their cycle threshold (C_t), by employing the standard curve generated using the C_t values obtained against the serially diluted CHIKV stock. In this study, 48% of the samples had virus concentrations in the range of 10^4–10^6 RNA copies/µl, although 16% of them had a lower amount of virus, corresponding to 10^0-10^1 RNA copies of RNA/µl.

DISCUSSION

CHIKV infection is considered a major public health concern in Asia and Africa (Kit, 2002; Shuffenecker *et al.*, 2006; Mavalankar *et al.*, 2009; Murtola *et al.*, 2009). Currently, there are a few specific serological tests or molecular diagnosis tools available despite the fact that CHIKV resurgence is associated with epidemics of unprecedented magnitude. Even though there is no commercially-available vaccine or specific treatment for this disease, rapid virus identification is essential for better patient management, control of outbreaks and epidemiological studies. Virus quantification is needed for monitoring disease progression, measuring the efficiency of antivirals and vaccine candidates, and to quantify the virus found in cell supernatants. While virus isolation has been used as the basic technique for virus detection, real-time RT-PCR and ELISA assays have also been found to be specific in detecting the virus in clinical as well as biological samples such as adult mosquitoes.

The primers that were designed in this study might be able to detect CHIKV isolates from other regions such as Central Africa, New Central Africa, West Africa,

Taiwan and India due to the high nucleotide similarity among Malaysian, Central African, New Central African, West African, Taiwanese as well as Indian strains. We feel, however, that nucleotide mismatches between strains is an advantage of this assay in detecting CHIKV from other strains because more than 80% of primer nucleotides showed similarity between Malaysian strains and other strains. So, the probability of detecting other CHIKV strains is high and to confirm further the different strains of the virus, purification and sequencing methods have to be carried out. As observed the mosquito isolate that had multiple mismatches was amplified and detected by both PCRs. However there will be limitations as any assay needs to be constantly reassessed from time to time.

The results of the reported assay showed 100% of sensitivity and specificity, and had an efficiency of 96.8%. This assay was validated using 71 samples, consisting of a panel of negative control sera, human sera, monkey sera and adult mosquito samples from which CHIKV has been isolated. The detection limit of this assay was $4.12x10^0$ RNA copies/µl and was 100-fold more sensitive than the conventional RT-PCR. This observation is supported by the previous results obtained by Santosh *et al.* (2007). Further analysis showed that there were five positive samples that had low viral loads which were not within the detection limit of conventional RT-PCR.

The standard curve-based quantification of viral load in a majority of biological samples showed a viral load between 10^4–10^6 RNA copies/µl. This observation reflects the high viral load during the viraemic phase of CHIKV infections. Besides that, the real-time assay also showed a high degree of specificity for CHIKV, because there was no cross-reactivity between other members of *Alphavirus* (Ross River virus and Sindbis virus), healthy human sera and other human pathogens such as Yellow fever virus, Dengue (serotypes 1-4), Herpes simplex, Japanese encephalitis virus, Parainfluenza and West Nile virus.

Real-time RT-PCR assay has many advantages for the diagnosis of acute CHIKV infections as compared to the conventional methods such as virus isolation, ELISA and conventional RT-PCR (Hasebe *et al.*, 2002; Pfeffer *et al.*, 2002). The advantages of using SYBR-Green real-time RT-PCR assay in this study are its sensitivity, rapidity (takes less than 3 hours to complete run) and accuracy. Moreover, the assay is quantitative, can be easily standardized with very low detection limit of $4.12x10^0$ RNA copies/µl and carries a lower risk of contamination. On the other hand, virus isolation, which is the gold standard, is laborious and time consuming, as the results are available after 3-7 days, and hence not useful for the management of patients or for immediate disease/ vector control measures (Hundekar *et al.*, 2002).

Real-time PCR assays have been used in many laboratories and employ either DNA binding fluorogenic molecules such as SYBR Green I or 5'-3' nuclease oligoprobe (TaqMan) in assay formats (Morrison *et al.*, 1999). A TaqMan probe-based quantitative RT-PCR has been reported by Pastorino *et al.* (2005). In that study, a sensitive and specific CHIKV one-step TaqMan RT-PCR assay was developed by targeting the *E1* region and was evaluated using clinical samples and cell supernatants. The assay was developed as a tool for the diagnosis of CHIKV as well as a rapid indicator of active infection by quantifying viral load (Pastorino *et al.*, 2005). Carletti *et al.* (2007) also reported a TaqMan RT-PCR assay for the non-structural protein 1 (ns*P1*) region that can quantify a wide range of viral RNA product and was used to measure the viral load in serum samples from CHIKV cases recently imported to Italy. In the present study, SYBR-Green I was chosen over TaqMan probe as SYBR-Green I is cost effective and relatively simple to manipulate, although it has a slightly lower sensitivity and specificity as compared to the TaqMan probe. The TaqMan probe real-time RT-PCR uses an extra probe which is highly specific for the detection of the chosen region.

In conclusion, the one-step SYBR-Green I-based real-time RT-PCR assay developed in this study can be used as an excellent epidemiological tool for the detection and quantification of CHIKV. This method is rapid, sensitive, specific, carries low risk of contamination and has greater ability to quantify the virus load in biological samples. In addition, it can also be used to study the association between the viral load and the disease severity. The early and precise diagnosis of infection will be important to clinicians for early patient management, while the early detection of virus in mosquitoes or larvae enables implementing control measures promptly to prevent further spread of the disease.

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