



## RESEARCH ARTICLE

# A TaqMan minor groove binder probe-based quantitative reverse transcription polymerase chain reaction for detection and quantification of chikungunya virus

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### ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus with widespread distribution across the globe. Since 2016, CHIKV re-emerged in several countries including Indian subcontinent and Southeast Asia. A proper diagnostic tool for early diagnosis of CHIKV infection is crucial to facilitate patient management and control virus transmission at the earliest stage of outbreak. Therefore, a TaqMan minor groove binder (MGB) probe-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay was developed to detect and quantify the CHIKV. The primers and probe were designed based on a conserved genomic region of 730 global CHIKV sequences that is located between *nsP1* and *nsP2* genes. The nucleotide mismatches of primers and probe with 730 global CHIKV sequences and 13 alphaviruses were then analysed *in silico*. In this study, the last 5 nucleotides at 3' end of primers and 5' end of probe were considered to be the critical regions for priming. *In silico* analysis revealed that the critical regions of primers and probe were at least 99.6% matched with the 730 global CHIKV sequences. Besides, the primers and probe showed at least 5/20 (25.0%) and 4/17 (23.5%) nucleotide mismatches with 13 alphaviruses respectively. The amplification efficiency of qRT-PCR assay was 100.59% (95% CI= 93.06, 109.33) with a R<sup>2</sup> score of 0.957. Its limit of detection (LOD) at 95% probability level was 16.6 CHIKV RNA copies (95% CI= 12.9, 28.9). The qRT-PCR assay was specific to CHIKV without cross-reacting with all dengue virus serotypes, Getah virus, Tembusu virus and Zika virus. The diagnostic results of qRT-PCR assay were perfectly agreed ( $\kappa=1.000$ ,  $p=0.003$ ) with a commercial triplex assay, with sensitivity of 100% (95% CI= 61, 100) and specificity of 100% (95% CI= 44, 100). Overall, the developed qRT-PCR assay is ideal for rapid, sensitive and specific detection as well as quantification of CHIKV.

**Keywords:** CHIKV; qRT-PCR; infectious disease; diagnosis; mosquito.

### INTRODUCTION

Chikungunya virus (CHIKV) is an alphavirus of the *Togaviridae* family that is predominantly transmitted between humans by *Aedes aegypti* and *Aedes albopictus* (Sobieszczyk & Hay, 2004; Simon *et al.*, 2008). The CHIKV genome is a single-stranded, positive-sense RNA with a length of about 11.8 kilobases (Khan *et al.*, 2002). It comprises two open reading frames (ORFs), one at its 5' region and another at its 3' region. The 5' ORF encodes for four non-structural proteins (*nsP1*, *nsP2*, *nsP3*, and *nsP4*), while the 3' ORF encodes for five structural proteins, such as capsid protein (C), envelope proteins (E1, E2 and E3) and accessory protein (6K) (Khan *et al.*, 2002; Solignat *et al.*, 2009). The CHIKV genome is surrounded by C proteins and enveloped by a lipid bilayer which is embedded with trimeric spikes made up of E1-E2 heterodimers (Vu *et al.*, 2017). The CHIKV phylogeny comprises three genotypes such as West African, Asian and East/Central/South African (ECSA) genotypes (Presti *et al.*, 2014). CHIKV is classified under the Old World virus group as it

causes fever, joint pain and rash (Kumar *et al.*, 2020). Some patients may experience erratic, recurring, and incapacitating joint pain for several months (Pialoux *et al.*, 2007).

Since 2016, CHIKV first emerged in India and subsequently spread to Pakistan and Kenya in the same year, as well as Bangladesh and Italy in 2017 (Aamir *et al.*, 2017; Venturi *et al.*, 2017; Maljkovic Berry *et al.*, 2019; Rahman *et al.*, 2019; Jain *et al.*, 2020). The CHIKV was also imported into Zhejiang Province, China in 2017 by a traveller who returned from Bangladesh (Pan *et al.*, 2019). Besides, Thailand reported a CHIKV outbreak from 2018 to 2019 due to importation of CHIKV from Bangladesh (Khongwichit *et al.*, 2021). Thereafter, a CHIKV outbreak was reported in Mandalay city, Myanmar in 2019 and the viral strain was closely similar to that in Thailand (Kyaw *et al.*, 2020). The CHIKV was then imported from Myanmar into its adjacent country, Yunnan Province, China in 2019 (Yin *et al.*, 2021). In Malaysia, CHIKV re-emerged in 2019 after a 10-year gap following the previous outbreak from 2008 to 2009 (Sam *et al.*, 2009; Ministry of Health Malaysia, 2022; Khor *et al.*, 2023).

The clinical manifestations of CHIKV infection are identical to dengue virus (DENV) and Zika virus (ZIKV) infections, especially during early febrile phase (Waggoner *et al.*, 2016b). Therefore, a proper diagnostic test is required to identify the CHIKV infection. Virus isolation through cell cultures is the gold standard for laboratory diagnosis but it is expensive for maintenance of cell culture, time-consuming in incubation for the virus to induce a cytopathic effect and requires technical professional for microscopical assessment of cell culture monolayer (Leland & Ginocchio, 2007). Consequently, it is impracticable for rapid diagnosis and usually reserved for research only. CHIKV infection is usually diagnosed using real-time reverse transcription polymerase chain reaction (RT-PCR) at early stage of illness and serology at late stage of illness (Reddy *et al.*, 2012). Center for Disease Control and Prevention (CDC) diagnostic testing algorithm for CHIKV infection recommends the diagnosis of patients before and after day 6 of illness by real-time RT-PCR and IgM enzyme-linked immunosorbent assay (ELISA), respectively (Johnson *et al.*, 2016). Hence, real-time RT-PCR is the method of choice for early diagnosis of CHIKV infection.

This study aims to develop a TaqMan minor groove binder (MGB)-based real-time quantitative RT-PCR (qRT-PCR) assay for rapid, sensitive and specific detection as well as quantification of CHIKV. Considering the re-emergence of CHIKV in Indian subcontinent and Southeast Asia, it is essential to have a proper diagnostic tool to detect the current circulating CHIKV strains in Malaysia. A qRT-PCR assay with up-to-date primers and probe sequences can ensure a good coverage of different CHIKV strains. Early diagnosis of CHIKV infection by qRT-PCR assay is beneficial in facilitating patient management and controlling transmission of CHIKV at the earliest stage of outbreak.

## MATERIALS AND METHODS

### Design of CHIKV-specific primers and probe

A total of 730 global CHIKV complete genome sequences were retrieved from GenBank. These CHIKV sequences were then aligned manually by using GeneDoc software (version 2.7) to identify the conserved genomic region of 730 global CHIKV sequences. Following that, the forward primer, reverse primer and TaqMan MGB probe were designed based on the conserved genomic region. The characteristics of primers and probe including the GC content, melting temperature, formation of hairpin and dimers were analysed by using web IDT OligoAnalyser Tool (<https://sg.idtdna.com/calc/analyser>). In this study, the last 5 nucleotides at 3' end of primers and 5' end of probe were considered to be the critical regions for priming (Teoh *et al.*, 2020; Chin *et al.*, 2022). The nucleotide mismatches of primers and probe with 730 global CHIKV sequences were analysed *in silico*. Besides, the reference sequences of alphaviruses including the Barmah Forest virus (BFV), eastern equine encephalitis virus (EEEV), Getah virus (GETV), Mayaro virus (MAYV), Middleburg virus (MIDV), Ndumu virus (NDUV), o'nyong-nyong virus (ONNV), Ross River virus (RRV), Semliki Forest virus (SFV), Sindbis virus (SINV), Una virus (UNAV), Venezuelan equine encephalitis virus (VEEV) and western equine encephalitis virus (WEEV) were from retrieved GenBank. These reference sequences of alphaviruses were then aligned by using ClustalX software (version 2.1). The nucleotide mismatches of CHIKV-specific primers and probe with 13 alphaviruses were analysed *in silico*. Lastly, the designed primers and probe were commercially synthesized by Integrated DNA Technologies Pte. Ltd. (Singapore).

### Preparation of CHIKV RNA standards for qRT-PCR assay

A recombinant plasmid that consisted of a pUC57-mini vector, target CHIKV sequence, T7 promoter and BamH1 restriction site was designed by using GeneDoc software (version 2.7). The restriction

sites of recombinant plasmid were then analysed by using web NEB cutter V2.0 (<https://nc2.neb.com/NEBcutter2/>). The designed recombinant plasmid was subsequently commercially synthesized by GenScript Biotech Corporation (Singapore). The recombinant plasmids were introduced into *E. coli* TOP10F' through calcium chloride heat shock transformation. Following that, the transformed *E. coli* TOP10F's colonies were selected through ampicillin selection on Luria Broth (LB) agar plates. The transformed *E. coli* TOP10F' colony was then cultured in LB broth for bacterial growth. The recombinant plasmids were extracted from the bacteria culture by utilizing FavorPrep Plasmid Extraction Mini Kit (Favorgen, Taiwan) according to the instruction manual. The extracted recombinant plasmids were then linearized by BamH1 restriction enzymes (Promega, USA) following the instruction manual. The linearized recombinant plasmids were analysed by 1.2% agarose gel electrophoresis, followed by purification using NucleoSpin PCR and Gel Clean-up Kit (Macherey-Nagel, Germany) in accordance with the instruction manual. Subsequently, the linearized recombinant plasmids were *in vitro* transcribed, followed by purification through DNase treatment and lithium chloride precipitation by using MEGAScript Kit (Life Technologies, USA) in accordance with the instruction manual. The copy number of *in vitro* transcribed synthetic CHIKV RNA was then calculated by the web EndMemo Copy Number Calculator (<http://endmemo.com/bio/dnacopynum.php>). Lastly, CHIKV RNA standards with known copy numbers were prepared and then stored at -80°C until further use.

### One-step real-time qRT-PCR reaction and thermocycling conditions

The SensiFAST Probe Hi-ROX One-Step Kit (Meridian, USA) was utilised for one-step real-time qRT-PCR reaction. The reaction mixture with a final volume of 15 µl comprised the following reagents: 7.5 µl of 2×SensiFAST Probe Hi-ROX One-Step Mix, 0.9 µl of 10 µM forward primer, 0.9 µl of 10 µM reverse primer, 0.225 µl of 10 µM probe, 0.15 µl of reverse transcriptase, 0.3 µl of RiboSafe RNase inhibitor, 3.025 µl of nuclease-free water and 2 µl of RNA template. The thermocycling conditions were adjusted as follow: reverse transcription (10 mins at 45°C), polymerase activation (2 mins at 95°C), followed by 40 cycles of amplification (5 s at 95°C, 10 s at 55°C and 20 s at 60°C). All reactions were performed using StepOnePlus Real-time PCR system (Applied Biosystems, USA). The raw data was then analysed by using StepOne software (version 2.2.1) to determine the threshold cycles (Ct). The Ct value below 40 was considered as positive detection.

### Characteristics of qRT-PCR assay

The qRT-PCR assay was performed by using ten-fold serially diluted CHIKV RNA standard ( $10^6$  to  $10^0$  copies/reaction), in duplicate. It was repeated for five times consecutively using the same batch of reagents by the same operator. The equation, slope and coefficient of determination ( $R^2$ ) of linear regression graph were then determined through simple linear regression analysis by using GraphPad Prism software (version 9.0.0). Besides, the amplification efficiency (E) was calculated through the formula:  $E = (10^{-1/\text{slope}} - 1) \times 100$ . A linear regression graph of Ct value against  $\log_{10}$  (copies) was plotted by using GraphPad Prism software (version 9.0.0).

### Limit of detection (LOD) of qRT-PCR assay at 95% probability level

The qRT-PCR assay was performed by using ten-fold serially diluted CHIKV RNA standard ( $10^3$  to  $10^0$  copies/reaction), in quadruplicate. It was repeated for five times consecutively using the same batch of reagents by the same operator. The 95% LOD was then determined through probit regression analysis by using IBM SPSS Statistics software (version 26). The probit regression graph of percentage of positive detection against  $\log_{10}$  (copies) was plotted by using GraphPad Prism software (version 9.0.0).

**Cross reactivity of qRT-PCR assay**

The cross-reactivity of qRT-PCR assay was evaluated by testing on different arboviruses such as CHIKV, dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4), Getah virus (GETV), Tembusu virus (TMUV) and Zika virus (ZIKV). All the arboviruses were acquired from the TIDREC's viral repository (Teoh et al., 2013; Sam et al., 2018; Chin et al., 2022). The viral RNAs were extracted by using QIAamp Viral RNA Mini Kit (Qiagen, USA) in accordance with the instruction manual.

**Comparison between developed qRT-PCR assay and commercial triplex assay**

This study had obtained ethical approval from the UMMC Medical Ethics Committee (MRECID No.: 202259-11224). The viral RNAs were extracted from the archived clinically-suspected chikungunya serum samples (n=9) by using QIAamp Viral RNA Mini Kit (Qiagen, USA) in accordance with the instruction manual. The extracted RNA samples were then tested by both developed qRT-PCR assay and GenoAmp Triplex Real-time RT-PCR Zika/Den/Chiku assay (Mediven, Malaysia). The commercial triplex assay acted as a comparative assay and was performed according to the instruction manual. The kappa measure of agreement between the diagnostic results of both assays were determined by using IBM SPSS Statistics software (version 26). The sensitivity, specificity, positive and negative predictive values of qRT-PCR assay were calculated by web EBM Diagnostic Test Calculator (<https://ebm-tools.knowledgetranslation.net/calculator/diagnostic>).

**RESULTS**

**In silico analysis of CHIKV-specific primers and probe**

The primers and probe targeted a conserved genomic region of 730 global CHIKV sequences that is located between *nsP1* and *nsP2* genes (Table 1). The characteristics of primers and probe such as GC content, melting temperature, formation of self-dimer, hetero-dimer and hairpin were analysed (Supplementary Table S1). Besides, the information on recombinant plasmid such as size, vector, promoter, target sequence and restriction site were shown (Supplementary Table S2). The nucleotide mismatches of primers and probe with 730 global CHIKV sequences and 13 alphaviruses were analysed *in silico*. In this study, the last five nucleotides at 3' end of primers and 5' end of probe was considered to be the critical regions for priming. *In silico* analysis revealed that only the critical regions of reverse primer and probe were perfectly matched (100%, 730/730) with 730 global CHIKV sequences. For forward primer, there was one nucleotide mismatch found between its critical region and three CHIKV sequences (0.4%, 3/730) (Figure 1). In addition, forward primer showed 5/20 (25.0%) to 11/20 (55.0%) nucleotide mismatches with 13 alphaviruses, while there were 5/20 (25.0%) to 12/20 (60.0%) nucleotide mismatches found between reverse primer and 13 alphaviruses. For probe, the number of its nucleotide mismatches with 13 alphaviruses ranged from 4/17 (23.5%) to 10/17 (58.8%) (Figure 2).

**Evaluations of qRT-PCR assay**

The amplification efficiency of qRT-PCR assay was 100.59% (95% CI= 93.06, 109.33) with R<sup>2</sup> score of 0.957 (Figure 3). Besides, the percentage of positive detection for 10<sup>3</sup>, 10<sup>2</sup>, 10<sup>1</sup> and 10<sup>0</sup> copies/reaction were 100% (20/20), 100% (20/20), 60% (12/20) and 5% (1/20) respectively. The 95% LOD of qRT-PCR assay was 16.6 CHIKV RNA copies (95% CI= 12.9, 28.9) (Figure 4). Moreover, the qRT-PCR assay could detect CHIKV and there was no amplification signal observed for other tested arboviruses such as DENV-1, DENV-2,

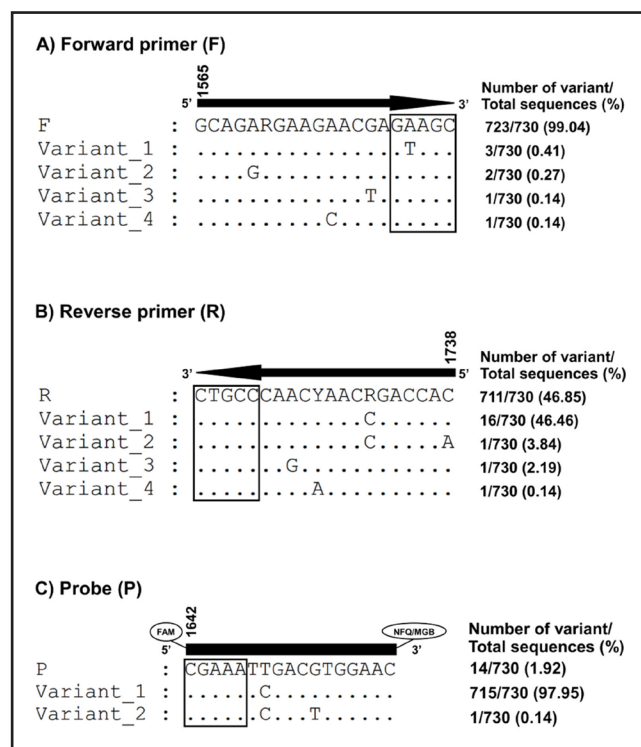
DENV-3, DENV-4, ZIKV, TMUV and GETV (Figure 5). Furthermore, the kappa measure of agreement between the diagnostic results of developed qRT-PCR assay and commercial triplex assay was perfect (κ=1.000, p=0.003) (Supplementary Table S3). The sensitivity and positive predictive value of qRT-PCR assay were 100% (95% CI= 61, 100), while the specificity and negative predictive value were 100% (95% CI= 44, 100) (Table 2).

**Table 1.** CHIKV-specific primers and probe designed in this study

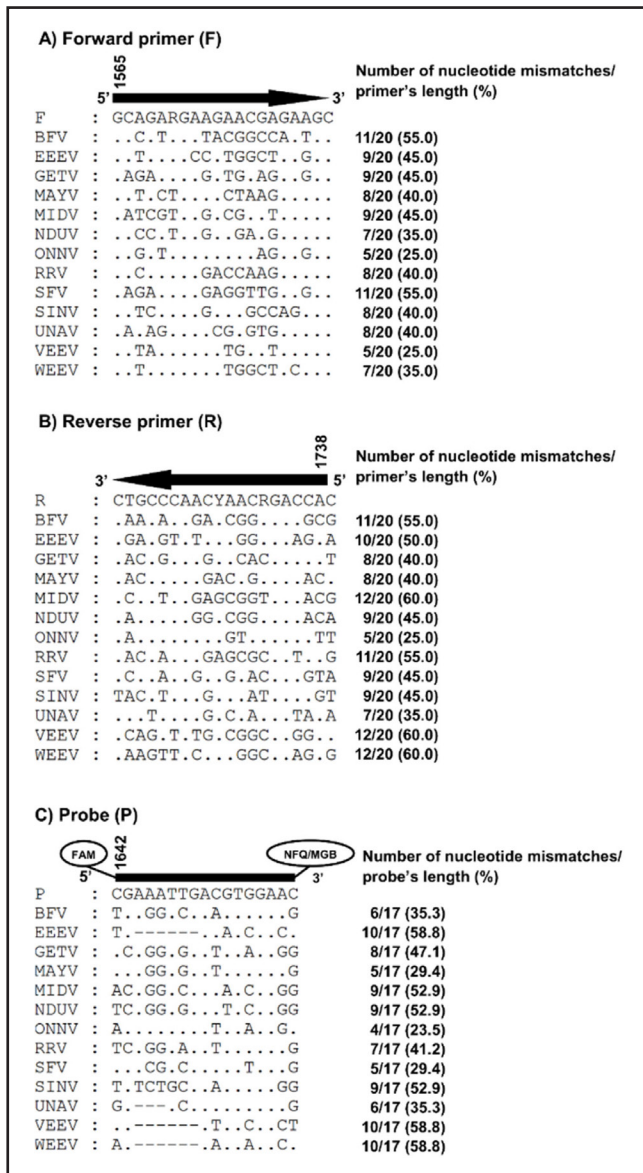
	Nucleotide sequences (5' 3')	Length (bp)	Position (bp)
F	GCAGARGAAGAACGAGAAGC	20	1565-1584
R	GTGGTCYGTTRGTTGGGCAG	20	1719-1738
P	FAM-CGAAATTGACGTGGAAC-NFQ/MGB	17	1642-1658

F=forward primer; R=reverse primer; P=probe; FAM=TaQMan fluorescent dye 6-carboxyfluorescein; NFQ/MGB=non-fluorescent quencher/minor groove binder.

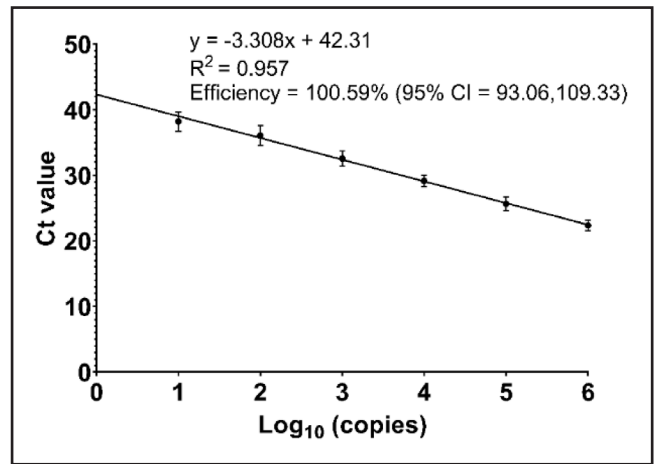
The nucleotide positions refer to the reference sequence of CHIKV S27-African prototype (GenBank accession number: NC\_004162.2).



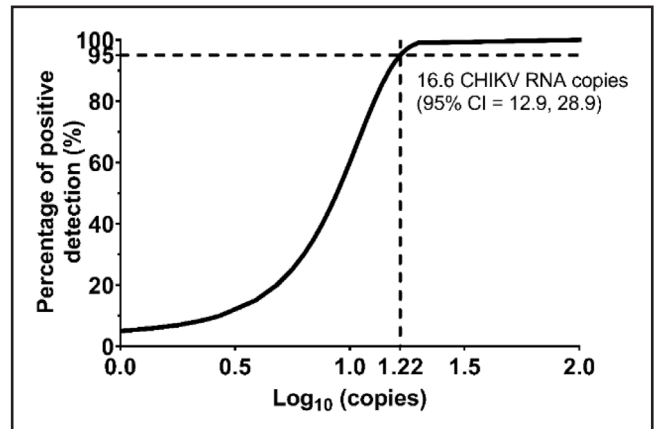
**Figure 1.** Nucleotide mismatches of the qRT-PCR primers and probe against 730 CHIKV genome sequences. The top sequences are primers (F and R) and probe (P) sequences. Total numbers of CHIKV genomes with 100% sequence similarity to the primers and probe are shown at the end of top sequences. Only the CHIKV genomes with nucleotide mismatches to the primers and probe are listed. The dots indicate the same nucleotides as the top sequence. The boxes indicate the nucleotide positions which are critical for priming and amplification.



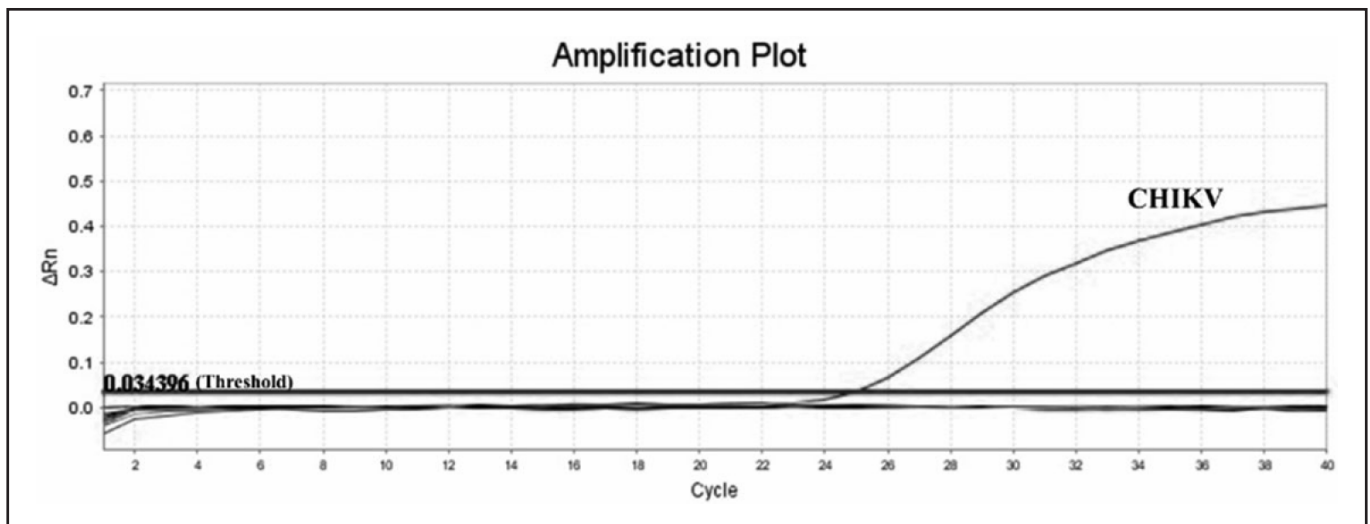
**Figure 2.** Nucleotide mismatches of the qRT-PCR primers and probe against 13 alphaviruses' genome sequences. The top sequences are primer (F and R) and probe (P) sequences. The dots indicate the same nucleotides as the top sequence. The hyphens indicate the gaps in the sequence.



**Figure 3.** Characteristics of qRT-PCR assay. Ten-fold serially diluted CHIKV RNA standard ( $10^6$  to  $10^0$  copies/reaction) was tested in duplicates and repeated for five times over consecutive days.



**Figure 4.** Limit of detection of qRT-PCR assay at 95% probability level. Ten-fold serially diluted CHIKV RNA standard ( $10^3$  to  $10^0$  copies/reaction) was tested in quadruplicates and repeated for five times over consecutive days.



**Figure 5.** Amplification plot of qRT-PCR assay for different arboviruses. The cross reactivity of qRT-PCR assay was evaluated by testing on CHIKV, dengue viruses (DENV-1, DENV-2, DENV-3 and DENV-4), Getah virus (GETV), Tembusu virus (TMUV) and Zika virus (ZIKV). Only CHIKV showed an amplification of signal with Ct value of 24.91.

**Table 2.** Diagnostic performance of the developed qRT-PCR assay in comparison to a commercial triplex assay

		Triplex assay		
		Positive test	Negative test	
qRT-PCR assay	Positive test	6	0	PPV=100% (95% CI= 61, 100)
	Negative test	0	3	NPV=100% (95% CI= 44, 100)
		Sensitivity=100% (95% CI= 61, 100)	Specificity=100% (95% CI= 44, 100)	

PPV=positive predictive value; NPV=negative predictive value.

## DISCUSSION

In this study, a TaqMan MGB probe-based real-time qRT-PCR assay capable of identifying CHIKV RNA in clinical serum samples was developed. The qRT-PCR assay successfully detected the CHIKV strain utilized in the study, while demonstrating no cross-reactivity with various closely related arboviruses, such as DENV-1, DENV-2, DENV-3, DENV-4, GETV, TMUV and ZIKV. In general, the TaqMan-based qRT-PCR assay has advantages over SYBR Green 1-based for instances more specific in detection, probes can be attached with different dyes for multiplex assay and post-PCR processing is not required (Wang & Yang, 2013). Besides, the TaqMan probe was modified by attaching a minor groove binder (MGB) to the 3' end quencher. The MGB moiety can stabilize the hybridization between the probe and target DNA. This hyper-stabilized duplex results in a significant increase in the melting temperature ( $T_m$ ) of probe by approximately 10 to 20°C. The increased  $T_m$  allows a shorter probe to be used. As the fluorescent dye is closer to the quencher in a shorter probe, this enhances the quenching effect on the dye. Consequently, the background noise is reduced, resulting in a higher signal to background noise ratio (Kutyavin *et al.*, 2000; Yao *et al.*, 2006; Garson *et al.*, 2012). Not only that, the MGB probe can improve single-nucleotide polymorphism (SNP) or mismatch discrimination. This is because the nucleotide mismatches between MGB probe and target DNA will have a stronger destabilizing effect on the probe-template duplex. (Kutyavin *et al.*, 2000; Yao *et al.*, 2006). Hence, the MGB-modified probe is advantageous over an unmodified probe.

At the present, most of the CHIKV-specific real-time RT-PCR assays targeted the *E1* gene but were limited for non-structural genes (Edwards *et al.*, 2007, 2017; Wang *et al.*, 2016). The qRT-PCR assay developed in this study targeted a conserved genomic region that is located between *nsP1* and *nsP2* genes. The primers and probe were up-to-date as they were designed based on 730 global CHIKV sequences that isolated from different geographical regions and temporal periods, including the recent epidemic strains. This is important to ensure that the qRT-PCR assay is able to detect a diverse variety of CHIKV strains. Ideally, the primers and probe should be perfectly matched with the target sequence because every single nucleotide mismatch will destabilize the primer or probe-template duplex. However, it is difficult to accomplish in practice. There are studies showed that the nucleotide mismatches at 3' end region of primers and 5' end region of probe have greater destabilizing effect (Klungthong *et al.*, 2010; Stadhouders *et al.*, 2010). Therefore, the last five nucleotides at 3' end of primer and 5' end of probe were considered to be the critical regions for priming in this study. The critical regions of reverse primer and probe were perfectly matched with 730 global CHIKV sequences but there was one nucleotide mismatch found between critical region of forward primer and three CHIKV sequences (0.41%, 3/730). This nucleotide mismatch was located at the fourth position from the 3' end of forward primer. As it was further away from 3' end, it had lesser impact on priming efficiency (Klungthong *et al.*, 2010). The good match of primers and

probe with a diverse variety of CHIKV strains ( $\geq 99.6\%$  of 730 global CHIKV sequences) can reduce the risk of false negative result in diagnosing CHIKV-infected patients. Moreover, the CHIKV-specific primers and probe varied significantly with 13 closely related alphaviruses ( $\geq 4$  nucleotide mismatches). This prevents the cross reactivity of CHIKV-specific qRT-PCR assay with other alphaviruses.

The qRT-PCR assay showed a nearly perfect amplification efficiency of 100.59% (95% CI= 93.06, 109.33) with high  $R^2$  value of 0.957. This allows the qRT-PCR assay to quantify the CHIKV accurately. Virus quantification is useful in determining the disease severity, effectiveness of potential vaccines and antiviral drugs (Aykac *et al.*, 2021). Besides, the 95% LOD of qRT-PCR assay (16.6 CHIKV RNA copies per reaction) was comparable to those of previously reported RT-PCR assays (3.8 – 60.0 CHIKV RNA copies per reaction) (Panning *et al.*, 2009; Chen *et al.*, 2015; Simmons *et al.*, 2016; Waggoner *et al.*, 2016a; Edwards *et al.*, 2017). A study reported that the median viral load in CHIKV-infected patients during the first four days of illness was  $1.3 \times 10^8$  copies/ml (Boddu *et al.*, 2019). Hence, the low 95% LOD of the developed qRT-PCR assay (16.6 copies per reaction was equivalent to  $3.56 \times 10^3$  copies/ml) allows it to detect CHIKV in patients during the viremia phase. CHIKV is transmitted from an infected human with high-titer viremia to other naive humans through mosquitoes' bites (Morrison, 2014). Early diagnosis of CHIKV infection during the viremia phase can help in facilitating patient management by advising the confirmed CHIKV-infected patients to wear protective clothing, apply mosquito repellents, use bed nets while sleeping and avoid going to forested areas for three weeks in order to prevent mosquito bites (Chang *et al.*, 2018). This can mitigate the transmission of CHIKV and eventually preventing an outbreak from occurring.

The diagnostic performance of developed qRT-PCR assay was perfectly agreed with the commercial triplex assay ( $\kappa=1.000$ ,  $p=0.003$ ), with high sensitivity and positive predictive value of 100% (95% CI= 61, 100) as well as high specificity and negative predictive value of 100% (95% CI= 44, 100). These statistical measures for diagnostic test, however should be interpreted with caution as only small sample size of clinical samples was tested in this study. Further evaluation of the qRT-PCR assay with a larger clinical sample size is desirable.

## CONCLUSION

The TaqMan MGB probe-based real-time qRT-PCR assay developed in this study is ideal for rapid, sensitive and specific detection as well as quantification of CHIKV. It is beneficial in disease surveillance and molecular epidemiology. Early diagnosis of CHIKV infection was crucial in facilitating the patient management and preventing further transmission of CHIKV at the earliest stage of outbreak.

### Conflict of interest statement

The authors declared that they have no competing interests.

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