RESEARCH ARTICLE

Development of a TaqMan minor groove binding probe-based quantitative reverse transcription polymerase chain reaction for the detection and quantification of Zika virus

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ARTICLE HISTORY

ABSTRACT

Zika virus (ZIKV) infection has emerged as a global health concern following epidemic outbreaks of severe neurological disorders reported in Pacific and Americas since 2016. Therefore, a rapid, sensitive and specific diagnostic test for ZIKV infection is critical for the appropriate patient management and the control of disease spread. A TaqMan minor groove binding (MGB) probe-based quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was developed based on the conserved sequence regions of 463 ZIKV NS2B genes. The designed ZIKV qRT-PCR assay was evaluated for its detection limit, strain coverage and cross-reactivity. We further assessed the clinical applicability of qRT-PCR assay for ZIKV RNA detection using a total 18 simulated clinical specimens. The detection limit of the qRT-PCR assay was 11.276 ZIKV RNA copies at the 95% probability level (probit analysis, $p \leq 0.05$). Both Asian and African ZIKV strains were detected by the qRT-PCR assay without cross-reacting with DENV-1, DENV-2, DENV-3, DENV-4, CHIKV, JEV, LGTV, GETV and SINV. The qRT-PCR assay demonstrated a perfect agreement (κ = 1.000, P < 0.001) with the reference assay; the sensitivity and specificity of the qRT-PCR assay were 100% (95% CI= 79.6-100) and 100% (95% CI= 43.9-100) respectively. The qRT-PCR assay developed in this study is a useful diagnostic tool for the broad coverage detection and quantification of both the Asian and African ZIKV strains.

Keywords: Infectious disease; vector-borne; mosquito; MGB probe; diagnostics.

INTRODUCTION

Zika virus (ZIKV) is an arthropod-borne virus (arbovirus) that belongs to the family *Flaviviridae,* genus *Flavivirus.* ZIKV is transmitted to humans mainly by the bites of infected female *Aedes* spp. mosquitoes including *Ae. aegypti*, *Ae. africanus* and *Ae. furcifer*. Zika virus of African lineage was first isolated in 1947 from a sentinel rhesus macaque and was later detected in *Ae. africanus* mosquitoes in 1948 in Forest of Uganda, east Africa (Dick *et al*., 1952). On the other hand, the ZIKV of Asian lineage was first identified from *Ae. aegypti* in Bentong, Pahang in 1966 (Marchette *et al*., 1969). In 2007, Asian lineage ZIKV caused the first massive outbreak in Yap Island of the Federated States of Micronesia (Ioos *et al*., 2014), followed by a French Polynesia outbreak in 2013 with over 30,000 symptomatic cases (Musso *et al*., 2014). In 2015, another large Asian lineage ZIKV outbreak occurred in Brazil with approximately 440,000 – 1,300,000 suspected cases of ZIKV infection (Schuler-Faccini *et al*., 2016). The recent ZIKV infections have been documented across Asian countries including China, Cambodia, India, Indonesia, Philippines, Thailand, Singapore and Malaysia (Lim *et al*., 2017). Due to its rapid spread

and the serious consequences of infection, Zika was declared as Public Health Emergency of International Concern (PHEIC) in 2016 (Sikka *et al*., 2016).

Like all flaviviruses, ZIKV is a positive-sense single-stranded RNA virus consisting of a roughly 11 kb long RNA genome. The open reading frame (ORF) which is framed by 5' and 3' untranslated regions (UTRs), encodes three structural proteins [the capsid (C), membrane (M), envelope (E)] and seven non-structural proteins (NS; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) essential for viral replication (Galán-Huerta *et al*., 2016). Previous studies reported that the significant NS1 codon usage adaptation to human housekeeping genes could facilitate viral dissemination, increase viral titers and inhibit interferon-β induction (de Melo Freire *et al*., 2015; Xia *et al*., 2018).

Clinical symptoms of ZIKV infection in human range from asymptomatic to mild, self-limiting and non-specific symptoms (Ioos *et al*., 2014; Plourde & Bloch, 2016). Fever, rash, arthralgia, myalgia and conjunctivitis are among the most commonly reported symptoms, which usually recover within two weeks and mortality are rare. Nevertheless, it is indistinguishable from the symptoms of other

arbovirus infections [e.g., dengue virus (DENV) and Chikungunya virus (CHIKV) infection] which may confound and increase the rate of misdiagnosis for ZIKV infections (Haddow *et al.*, 2012). Severe neurological disorders including microcephaly in newborns and Guillain-Barré syndrome (GBS) in adults have been associated with the ZIKV infection (Fagbami, 1979; Dominguez-Moreno *et al.*, 2014).

With no specific antiviral or vaccine available, accurate diagnosis of ZIKV infection is of paramount importance for the appropriate patient management and the control of disease spread. Conventional serological assays including enzyme-linked immunosorbent assay (ELISA) and plaque reduction neutralization test (PRNT) are commonly used for diagnosis of ZIKV infection (Fagbami, 1979; Huzly *et al.*, 2016; Landry & St. George, 2017; Shan *et al.*, 2017). Nevertheless, ELISA method demonstrates possible cross-reactivity among flavivirus infections and the PRNT is time demanding (Waggoner & Pinsky, 2016). On the other hand, nucleic acid amplification technique enables the accurate diagnosis during the acute phase of ZIKV infection (up to 7 days post onset of symptoms) (Plourde & Bloch, 2016). To date, numerous RT-PCR (Balm *et al.*, 2012; Waggoner *et al.*, 2013) and real-time RT-PCR assays (Faye *et al.*, 2013; Pyke *et al.*, 2014; Tappe *et al.*, 2014, 2015; Corman *et al.*, 2016; Waggoner & Pinsky, 2016; Chan *et al.*, 2017) have been developed. In this study, we developed a TaqMan minor groove binding (MGB) probe-based real-time quantitative RT-PCR (qRT-PCR) that utilized primers and probe designed based on 463 ZIKV genomes from GenBank.

MATERIALS AND METHODS

Viruses
Four ZIKV strains including Asian strains of the ZIKV isolates: P6-740 (Malaysia, 1966), PRVABC59 (Puerto Rico, 2015), H/FP/2013 (French Polynesia, 2013) and African strain of the ZIKV isolates: MR766 (Uganda, 1947) were used in this study. The viruses were propagated in Vero cell lines that maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, NY, USA) enriched with 2% heat-inactivated fetal bovine serum (FBS; Bovogen, Australia), L-glutamine and non-essential amino acids (Gibco, NY, USA). After 7 days of incubation at 37°C in the presence of 5% CO_2, the infected cell culture supernatants were harvested, titrated using plaque assay and kept in -80°C for further use. All the viruses were archived in Tropical Infectious Diseases Research & Education Centre (TIDREC) at Universiti Malaya, Kuala Lumpur, Malaysia.

Simulated clinical specimens
The study obtained approval from the UM Institutional Biosafety and Biosecurity Committee (Approval Number: UMIBBC/NOI/R/TNCPNI/TIDREC-007/22072020) and the UMMC Medical Ethics Committee (Ethics Committee/IRB Reference Number: 908.11). In order to produce ZIKV-positive simulated clinical specimens, Asian ZIKV strains P6-740 virus with known plaque-forming-unit (PFU) titer was spiked into human serum, saliva and urine at final concentrations between 10^3 to 10^{-1} PFU/ml. Specimens spiked with serum-free media were served as negative simulated clinical specimens. A number of 18 simulated clinical specimens were prepared, including 6 serum, 6 saliva and 6 urine specimens. All serum, saliva and urine specimens were obtained with the informed consent of the healthy donors.

Plaque assay
A monolayer of Vero cells (1×10^5 cells/well) were grown in a 24-well plate overnight until more than 80% confluency. The cells were then inoculated with 200 µl of the serial 10-fold dilutions of virus stock with serum-free media and the plate was placed on a rocker for virus absorption. After 1 hour, the inoculum was removed from each well and overlaid with 500 µl plaque media consisting DMEM supplemented with 2% FBS, L-glutamine, non-essential amino acids

and 0.8% high viscosity carboxymethylcellulose (HV-CMC; Sigma-Aldrich, USA). The plate was then incubated for 5 days at 37°C with 5% CO_2. After 5 days, the overlaying media was removed from each well and the wells were washed with phosphate-buffer saline (PBS). After washing, the cells were fixed with 4% paraformaldehyde (SigmaAldrich, USA) in PBS for 30 minutes at room temperature. The cells were then rinsed three times with PBS. Afterwards, the cells were stained by adding 0.5% crystal violet in 20% alcohol (Sigma-Aldrich, USA) for 15 minutes. A Nikon SMZ 1000 stereomicroscope (Tokyo, Japan) was used to count the virus plaques and the viral infectious titer was quantified in plaque forming units per millilitre (PFU/ml).

Design of ZIKV specific qRT-PCR assay primers and TaqMan probe set
Complete genome sequences of Asian and African lineages of ZIKV strains were retrieved from GenBank and aligned with the genome sequences of other flaviviruses using the Clustal X 2.0 software (Thompson *et al.*, 1997). Manual comparative analyses were performed between the genome sequences of ZIKV and other flaviviruses to eliminate cross-reactivity. The conserved ZIKV-specific sequence regions of NS2B were then identified as the suitable site for the primer and probe design. Primer Express 3.0.1 was used to design forward primer, reverse primer as well as TaqMan MGB probe. The melting temperature, GC content, hairpin formation and presence of self- and hetero-dimers were further determined for the primers and probe using IDT's OligoAnalyzer Tool (Supplementary Table S1). The primers and probe were synthesized by Applied Biosystems Inc. (Foster City, CA, USA). In addition, the primers and probe designed were exhaustically compared with an alignment of 463 ZIKV genomes. The five nucleotides at the 3' end of both forward and reverse primers as well as the five nucleotides at 5' end of probe were considered as the critical sites for priming and amplification.

Generation of RNA standard for qRT-PCR
The recombinant plasmid vector containing specific promoter, restriction sites and the target sequence for ZIKV was designed using GeneDoc. *In silico* analysis of restriction enzyme cleavage was carried out with NEBcutter at http://tools.neb.com/NEBcutter. In this study, the recombinant plasmid designed was 3540 bp in length after inserting 1454 bp of target sequence of ZIKV (pIDTSmart, Ampicilin resistance). The plasmid was purchased from the Integrated DNA Technology (Coralville, IA). The recombinant plasmid was used to transform competent state of tetracyclin resistant *E.coli* TOP10F' by heat shock. After antibiotic selection on the LB-Amp agar, the cloned plasmid was extracted by using the FavorPrep Plasmid Extraction Mini Kit (Favorgen Biotech Corporation, Taiwan) following manufacturer's recommendations. The extracted plasmid DNA was cleaved by *Bam*H1 restriction endonuclease (Promega, USA) and was further analyzed by 1.2% agarose gel electrophoresis. The linearized plasmid DNA in the gel was then purified by QIAquick Gel Extraction Kit (Qiagen, USA). The *in vitro* transcribed RNA was generated using MEGAscript Kit (Ambion, Austin, TX) and was then quantified using the NanoPhotometer™ UV/Vis Spectrophotometer (Implen, Germany and *Qubit* 2.0 *Fluorometer (Invitrogen,* Life Technologies, CA, USA). The RNA copy number (copies/µl) was calculated using the ENDMEMO software at http://www.endmemo.com/bio/dnacopynum.php. Seven serial 10-fold dilutions of *in vitro* RNA transcripts with known copies number between 1×10^6 to 1×10^0 copies/µl, were used as assay standards to establish the standard curve.

RNA extraction and real-time qRT-PCR assay
The viral RNA from infected cell culture supernatants or simulated clinical specimens were extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany) following manufacturer's protocol. The eluted viral RNA was then amplified and quantified using qRT-PCR assay,

which was performed using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA). The reagent mixture was used as follows: 3.75µl of 4X TaqMan Fast Virus One-Step Master Mix, 0.75 µl of 20X TaqMan Gene Expression Assay mix, 8.5 µl of nuclease free water and 2 µl of RNA template or nuclease free water as negative control (total 15 µl/ reaction). All the samples were analyzed in duplicate. The reverse transcription was carried out at 50°C for 5 minutes, 95°C for 20 seconds and 40 cycles of 95°C for 3 seconds, 60°C for 30 seconds. Viral copy numbers in each sample were analyzed by StepOne Software v2.2.1 depending on the threshold cycles (Ct). The standard curve slope was used to determine the assay's efficiency.

Detection limit of qRT-PCR assay
The qRT-PCR assay's detection limit was evaluated using serial 10-fold diluted *in vitro* RNA transcripts (1×10^3 to 1 copy numbers). The qRT-PCR detection limit test was performed with ten independent runs.

Strain coverage and cross-reactivity of qRT-PCR assay
The strain coverage of the qRT-PCR assay was evaluated using viral RNA extracted from four strains of ZIKV (P6-740, PRVABC59, FP/2013 and MR766). The qRT-PCR assay's cross-reactivity was validated against various arboviruses including all four DENV serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), CHIKV, Japanese encephalitis virus (JEV), Langat virus (LGTV), Getah virus (GETV) as well as Sindbis virus (SINV).

Evaluation of qRT-PCR assay using simulated clinical specimens
The diagnostic performance of the qRT-PCR assay to detect ZIKV RNA was evaluated using the simulated clinical specimens. The performance of the designed qRT-PCR assay was then compared to the reference assay, Genesig Real-Time qRT-PCR ZIKV Detection Kit (Primerdesign Ltd, United Kingdom). The Genesig Real-Time qRT-PCR ZIKV Detection Kit assay was performed as previously described (Teoh *et al.*, 2020).

Statistical analysis
Data analysis was performed using IBM SPSS Statistics, V21 (IBM Corp., Armonk, NY, USA). A probit analysis was performed to analyze the detection limit of the qRT-PCR assay at a probability level of 95% and subsequently plotted using Graph Pad Prism, version 7 (Graph Pad Software Inc., San Diego, CA, USA). The degrees of agreement [kappa value (κ)] between the results of the qRT-PCR and reference assay were assessed. The Evidence-based Medicine (EBM) Diagnostic Test Calculator (https://ebm-tools.knowledgetranslation.net/calculator/diagnostic) was used to calculate the sensitivity, specificity, positive predictive value and negative predictive value of the qRT-PCR assay in comparison to the reference assay.

RESULTS

Design of ZIKV specific primers-probe set
A set of primers and probe was designed by targeting the conserved region of NS2B: ZIKV-F (5'-GGCACTAGATGAGAGTGGTGA-3'), ZIKV-R (5'-CTATTGGGTTCATGCCACAGA-3') and ZIKV-P (5'-AGAGAGATCATACTCAAG-3') which hybridized in the ZIKV genome from GenBank accession number (HQ234499) (Table 1 and Supplementary Figure S1). Our *in silico* coverage analysis was performed by comparing the primers and probe sequences with the alignment of 463 ZIKV genomes retrieved from GenBank (Supplementary Figure S2). In this comparison, ZIKV-F (forward primer) and ZIKV-R (reverse primer) respectively showed 0.22% (1/463) and 0.65% (3/463) of critical nucleotide mismatches with the ZIKV genomes, whereas ZIKV-P (TaqMan probe) showed no critical nucleotide mismatch with all ZIKV genomes. In addition, the

in silico cross reactivity analysis revealed that the primers-probe set was highly divergent from other arboviruses including Usutu virus, Murray Valley encephalitis virus, JEV, West Nile virus, St. Louis encephalitis virus, DENV and yellow fever virus (Supplementary Figure S3).

Detection limit, cross-reactivity and strain coverage of qRT-PCR assay
The designed qRT-PCR assay's detection limit was evaluated by ten independent runs on the previously generated *in vitro* RNA transcripts with known copies number ranging from 1×10^3 to 1 copy numbers. The positive detection rates by qRT-PCR assay (n = 10) for 1000, 100, 10, and 1 ZIKV RNA copy were 100% (10 of 10), 100% (10 of 10), 100% (10 of 10), and 10% (1 of 10), respectively. The detection limit of the qRT-PCR assay was 11.276 ZIKV RNA copies at the 95% probability level (probit analysis, p≤ 0.05) (Figure 1). In cross-reactivity testing, a total of nine closely related arboviruses common in the region including DENV-1, DENV-2, DENV-3, DENV-4, CHIKV, JEV, LGTV, GETV and SINV were tested. None of the arboviruses showed amplification, indicating that the qRT-PCR assay did not cross-react with other arboviruses (Supplementary Figure S4). Moreover, all four reference ZIKV strains were detected by the qRT-PCR assay, suggesting the designed qRT-PCR assay was capable of targeting both Asian and African ZIKV strains (Supplementary Figure S5).

Evaluation of qRT-PCR assay using simulated clinical specimens
The qRT-PCR assay for ZIKV RNA detection was tested on a total of 18 simulated clinical specimens: 15 positive specimens with ZIKV

Table 1. ZIKV qRT-PCR primers and TaqMan probe designed in this study

Name	Sequence (5' to 3')	Position[a]
Forward primer	GGCACTAGATGAGAGTGGTGA	4344-4364
Reverse primer	CTATTGGGTTCATGCCACAGA	4433-4453
TaqMan probe	FAM- AGAGAGATCATACTCAAGG -MGB/NFQ	4398-4417

[a] All nucleotide positions refer to the published Zika virus isolate P6-740 gene sequence (GenBank accession number: HQ234499). FAM, TaqMan fluorescent dye 6-carboxyfluorescein; MGB/NFQ, minor groove binder/non-fluorescent quencher.
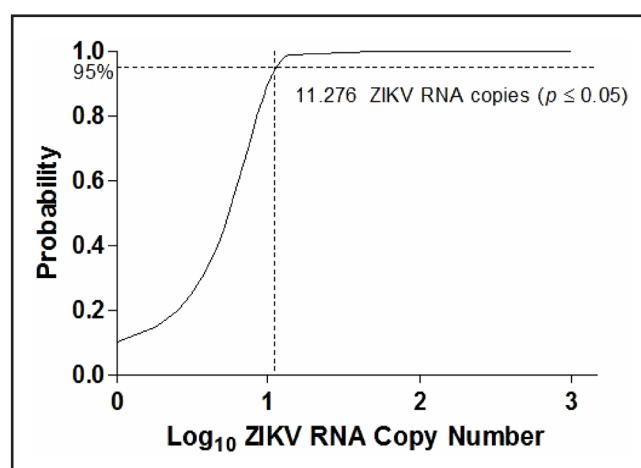

Figure 1. Detection limit of the designed qRT-PCR assay. The probit regression curve was obtained from ten replicates of ZIKV RNA in four serial 10-fold dilutions (1000, 100, 10, 1 copy numbers).

Table 2. Diagnostic performance of the qRT-PCR assay against that of the reference assay (Genesig Real-Time qRT-PCR ZIKV Detection Kit) using the simulated clinical samples (n = 18)

	Results[a,b]	Simulated clinical sample (Reference assay)		Sensitivity [% (95% CI[c])]	Specificity [% (95% CI)]	PPV[d] [% (95% CI)]	NPV[e] [% (95% CI)]
		Pos (n)	Neg (n)				
qRT-PCR	Pos	15	0	100% (79.6-100)	100% (43.9-100.0)	100% (79.6-100.0)	100% (43.9-100)
	Neg	0	3				

[a] Agreement of results between qRT-PCR assay and reference assay: κ = 1.000, P < 0.001.
[b] Pos, positive; Neg, negative.
[c] CI, confidence interval.
[d] PPV, positive predictive value.
[e] NPV, negative predictive value.

titers ranging from 10^3 to 10^{-1} PFU/ml and three negative specimens. The performance of the designed Zika qRT-PCR assay was compared against that of the reference assay (Genesig Real-Time qRT-PCR ZIKV Detection Kit). Concordance analysis showed that there was a perfect agreement (κ = 1.000, *P* < 0.001) between the designed Zika qRT-PCR assay and the reference assay. In this study, the sensitivity and specificity of designed qRT-PCR assay were 100% (95% CI= 79.6-100) and 100% (95% CI= 43.9-100) respectively (Table 2). The designed ZIKV qRT-PCR showed an amplification Ct value of 23.08-35.96, whereas the reference assay showed an amplification Ct value of 23.07-36.43. Both the designed qRT-PCR and reference assays detected as low as 0.1 PFU/ml in ZIKV-positive simulated specimen. No amplification was observed for the ZIKV negative specimens in both assays (Supplementary Table S2).

DISCUSSION

In the present study, a TaqMan MGB probe-based real-time qRT-PCR assay was developed for the detection of ZIKV RNA in simulated clinical specimens. The qRT-PCR assay detected both the Asian and African strains used in this study without cross-reacting with several other closely related arboviruses including DENV-1, DENV-2, DENV-3, DENV-4, CHIKV, JEV, LGTV, GETV and SINV.

In comparison to the previous SYBR green-based real-time RT-PCR assay for detecting ZIKV (Xu *et al.*, 2016), the probe-based approach improves the specificity of the signal generated and omits the requirement of melt curve analysis. Recently, several probe-based real-time RT-PCR assays have been developed for the detection of ZIKV by targeting the different viral genome regions including E (Lanciotti *et al.*, 2008; Pyke *et al.*, 2014; Corman *et al.*, 2016), prM (Lanciotti *et al.*, 2008), NS1 (Pyke *et al.*, 2014; Corman *et al.*, 2016), NS2B (Waggoner & Pinsky, 2016), NS3 (Tappe *et al.*, 2014, 2015), NS5 (Faye *et al.*, 2013) and 5′UTR (Chan *et al.*, 2017). In contrast to previous studies that used conventional TaqMan probes, here we designed a TaqMan MGB probe which would help to improve the specificity and reduce the background signal noise of the real-time RT-PCR assay (Kutyavin *et al.*, 2000; Yao *et al.*, 2006). In addition, the synthetic RNA target with known copy number was used to enable the designed real-time RT-PCR assay for quantification purposes.

Similar to the real-time RT-PCR assay developed by the Pan American Health Organization (PAHO) (Waggoner & Pinsky, 2016), we also designed the primers and probe targeting the highly conserved NS2B sequences of ZIKV. Our *in silico* coverage and cross reactivity studies revealed that the designed primers-probe set was specific to both Asian and African ZIKV lineages, but highly divergent from other arboviruses. In addition, the primers-probe set designed showed no critical mismatches with at least 99% of the 463 ZIKV genome sequences, indicating its usefulness for broad coverage detection of both the Asian and African ZIKV lineages. In

consistence with the *in silico* analyses, the designed qRT-PCR assay detected both Asian and African ZIKV strains used in this study without cross-reacting with other arboviruses.

The detection limit of developed qRT-PCR assay (~11 ZIKV RNA copies) was comparable to those of previously reported (Lanciotti *et al.*, 2008; Faye *et al.*, 2013; Pyke *et al.*, 2014; Tappe *et al.*, 2014; Corman *et al.*, 2016; Waggoner & Pinsky, 2016; Chan *et al.*, 2017); the detection limits ranged from 2 to 25 copies of ZIKV RNA. Our qRT-PCR assay also demonstrated a similar performance as the reference assay (Genesig Real-Time qRT-PCR ZIKV Detection Kit); both assays detected as few as 0.1 PFU/ml of viral load from all types of simulated clinical specimens. This assay is more sensitive than the previously reported assay which detected up to 50 PFU/ml from the simulated human plasma (Faye *et al.*, 2013). In general, the ZIKV titers in viremic patients ranged from 10^3 to 10^8 PFU/ml, with asymptomatic patients having 2.5×10^3 to 8×10^6 RNA copies/μl and symptomatic patients having 7.28×10^6 to 9.3×10^8 RNA copies/μl (Lanciotti *et al.*, 2008; Musso & Gubler, 2016). Thus, our qRT-PCR assay is useful for the detection of viremic patients who are potentially infectious. Early detection of viremic patients would aid physicians in providing appropriate supporting therapy and counselling. Early diagnosis would also allow immediate execution of proper vector control measures to mitigate the disease spread.

Previous studies reported the presence of ZIKV RNA in a variety of specimen types, including serum (Macnamara, 1954; Fagbami, 1979; Marchi *et al.*, 2020), urine (Gourinat *et al.*, 2015; de M Campos *et al.*, 2016; Rozé *et al.*, 2016), saliva (Musso *et al.*, 2015; Barzon *et al.*, 2016; Khurshid *et al.*, 2019), semen (Atkinson *et al.*, 2016, 2017; Mansuy *et al.*, 2016) and breast milk (Dupont-Rouzeyrol *et al.*, 2016, Blohm *et al.*, 2018). In this study, the designed qRT-PCR assay was evaluated using only a small number (n = 18) of the simulated clinical specimens due to the unavailability of actual clinical specimens.

CONCLUSIONS

In summary, a rapid, sensitive and specific qRT-PCR assay was developed for the detection and quantification of ZIKV. The qRT-PCR assay could detect both Asian and African ZIKV strains without cross-reacting with other arboviruses. In addition, the qRT-PCR assay utilized updated primers and probe sequences to ensure an excellent ZIKV strain coverage.

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SUPPLEMENTARY DATA

https://msptm.org/files/Vol39No4/tb-39-4-005-Chin-K-L-supplementary-data.pdf