RESEARCH ARTICLE

Development of a reverse transcription quantitative polymerase chain reaction-based assay for broad coverage detection of African and Asian Zika virus lineages

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The Zika virus (ZIKV) is an arbovirus that has spread rapidly worldwide within recent times. There is accumulating evidence that associates ZIKV infections with Guillain-Barré Syndrome (GBS) and microcephaly in humans. The ZIKV is genetically diverse and can be separated into Asian and African lineages. A rapid, sensitive, and specific assay is needed for the detection of ZIKV across various pandemic regions. So far, the available primers and probes do not cover the genetic diversity and geographic distribution of all ZIKV strains. To this end, we have developed a one-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay based on conserved sequences in the ZIKV envelope (E) gene. The detection limit of the assay was determined to be five RNA transcript copies and 2.94×10^{-3} 50% tissue culture infectious doses ($TCID_{50}$) of live ZIKV per reaction. The assay was highly specific and able to detect five different ZIKV strains covering the Asian and African lineages without nonspecific amplification, when tested against other flaviviruses. The assay was also successful in testing for ZIKV in clinical samples. Our assay represents an improvement over the current methods available for the detection ZIKV and would be valuable as a diagnostic tool in various pandemic regions.

INTRODUCTION

The Zika virus (ZIKV) was first identified in 1947 from a sentinel rhesus monkey during surveillance of yellow fever in Uganda (Dick et al., 1952). The virus was subsequently isolated in humans in Uganda and Tanzania in 1952 (Macnamara, 1954). The ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Similar to other flaviviruses, ZIKV is a single-stranded positive RNA virus with a genome of approximately 10.8 kb, containing a single open reading frame (ORF), flanked by two untranslated regions (UTR) located at the 5′ and 3′ ends of the genome (Kuno and Chang, 2007; Saiz et al., 2016). The single ORF encodes the viral polyprotein that is cleaved by cellular and viral proteases into three structural proteins: the capsid (C), premembrane/membrane (prM/M), and envelope (E); and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Kuno and Chang, 2007; Saiz et al., 2016). Phylogenetic studies have shown that ZIKV can be separated into two major lineages, Asian and African, and that it shows the highest genome similarity to other mosquito-borne flaviviruses, such as dengue virus (DENV), West Nile virus (WNV) and Japanese encephalitis virus (JEV) (Lanciotti et al., 2008; Wang L. et al., 2016a). The reservoir for ZIKV is not clearly defined, but it is speculated that the virus is likely maintained in a primate-mosquito-primate sylvatic cycle that includes nonhuman primates and/or humans, and a broad range of mosquitoes that mainly belong to the *Aedes* genus (Wolfe et al., 2001; Grard et al., 2010; Musso and Gubler, 2016).

For half a century after the discovery of ZIKV, only sporadic infections in humans were documented, mainly in Africa and Southeast Asia (Faye et al., 2014; Saiz et al., 2016). This might be partially due to the high frequency of asymptomatic infections (up to 80%), as well as the mild, self-limiting nature of ZIKV fever, with clinical manifestations that could be mistaken for other infections (Duffy et al., 2009; Saiz et al., 2016). Clinical manifestations in symptomatic cases include fever, rash, arthralgia, headache, etc. that are similar to those of other arboviral infections, such as DENV and chikungunya virus (CHIKV) (Dick et al., 1952; Hamel et al., 2016). In 2015, an outbreak of ZIKV fever originated in Brazil (Faria et al., 2016), and as of January 5, 2017, over 70 countries and territories have reported continued mosquito-borne transmission of ZIKV within their borders (World Health Organization [WHO], 2017). Accumulating evidence suggests that ZIKV infection is associated with microcephaly of the fetus in pregnant women, as well as an increased incidence of Guillain-Barré Syndrome (GBS); moreover, the infection can be sexually transmitted and poses a potential risk of testicular damage (Govero et al., 2016; Lucchese and Kanduc,

2016; Ma et al., 2016; Malkki, 2016; Mlakar et al., 2016; Parra et al., 2016; WHO, 2016b; Wong et al., 2016). These findings suggest that ZIKV is more dangerous than previously thought. As such, the WHO declared ZIKV a public health emergency of international concern (WHO, 2016a).

Due to the general nature of disease symptoms, in addition to the co-circulation with DENV and CHIKV in many ZIKV-affected areas, accurate diagnosis of ZIKV fever is difficult (Dick et al., 1952; Hamel et al., 2016; Saiz et al., 2016). Virus isolation and serological methods are still commonly used as diagnostic tools, despite their shortcomings. For instance, ZIKV isolation is time consuming, as it requires days to grow the virus on permissible cell lines; whereas serological methods could have limited cross-reactivity with related flaviviruses (Hamel et al., 2016; Saiz et al., 2016) and are not indicative of current (active) ZIKV infection. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)-based assays are known for their ability to provide rapid, sensitive, and specific pathogen detection. Currently, the available primers and probes do not cover the genetic diversity and geographic distribution of all ZIKV strains (Lanciotti et al., 2008; Faye et al., 2013; Musso and Gubler, 2016). Furthermore, ZIKV displays high genetic diversity even within the same lineage and region (Shi et al., 2016; Zhang et al., 2016). To address this problem, we describe a novel qRT-PCR assay with broad coverage of ZIKV strains, including new circulating isolates.

MATERIALS AND METHODS

Viruses and RNA extraction

The DENV 1–4 strains were provided by Prof. Chengfeng Qin. Strains of the ZIKV isolates MR_766 and PRVABC59 were provided by Prof. Mifang Liang, and the PLCal_ZV strain was provided by Prof. Gary Kobinger (University of Laval and Public Health Agency of Canada). The yellow fever virus (YFV) BJ01 strain and ZIKV SZ_SMGC-1 and CAS01 strains were isolated by our group from previously imported cases to China. Viral stocks were prepared using Vero or C6/36 cell lines. The RNA was extracted from viral stocks using the QIAamp RNA Viral Kit (Qiagen, Heiden, Germany) and MagaBio plus Virus RNA Purification Kit (Automatic Nucleic Acid Purification System NPA-32+, BIOER, China) according to the manufacturer instructions. The RNA was washed with buffers AW1 and AW2, and eluted in 50 μL of AVE buffer and stored at –80 °C for subsequent use.

Viral stock titration by TCID$_{50}$

Vero cells in 96-well plates were grown to 90% confluence and infected with 10-fold serial dilutions of the cell supernatant for 1 h at 37 °C. The inoculum was then

removed, and cells were overlaid with fresh DMEM plus 2% FBS. At 6 days post infection (dpi), plates were assessed for the lowest dilution at which 50% of the wells exhibited cytopathology. The $TCID_{50}$ values were calculated according to the Reed-Muench method (Reed and Muench, 1938).

Primer and probe design

The *E* gene of ZIKV was chosen as the target for the primer design because of its unique characteristic that facilitates its differentiation from those of other flaviviruses. All sequences of the 81 ZIKV strains used in the present study were downloaded from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/genome/viruses/variation/Zika/), and aligned using the Clustal X program (Thompson et al., 1997). Conserved ZIKV-specific sequences that were divergent from other flaviviruses were identified, and the primers (ZIKV-F/ZIKV-R) were designed using the Primer Premier 5 software. The probe (ZIKV-P) was labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5′-end and the fluorescent quencher dye 6-carboxytetramethylrhodamin (TAMRA) at the 3′-end. The sequences and genome positions of the primer and probe set are shown in Figure 1.

Generation of RNA standards

A section of the *E* gene, 98 base pairs in size, was amplified with the primers ZIKV-F/ZIKV-R, and the product was purified using the Gene JET PCR Purification Kit (Thermo, MA, USA) and ligated to the pGEM-T vector

(Promega, Madison, USA). *In vitro* transcription was performed using the MEGAscript T7 Transcription Kit (Thermo, MA, USA) and quantified using the Nanodrop 2000 Spectrophotometer (Thermo, MA, USA). The RNA copy number (molecules/µL) was calculated using the following equation: $[C \times A / 330 \times L]$, where C represents the concentration of RNA (g/mL) assessed by the optical density measurement; A is the Avogadro number (6.023×10^{23}); L is the length of the synthetic RNA (number of nucleotides); and 330 is an approximation of the molecular weight of a nucleotide (g/mol).

Clinical samples

Urine and serum samples were obtained from two confirmed cases of ZIKV infection in China (Deng C. et al., 2016a; Deng YQ. et al., 2016b; Liu et al., 2016; Wang Q. et al., 2016b). The RNA was extracted using the QIAamp RNA Viral Kit (Qiagen, Heiden, Germany) according to manufacturer recommendations.

Quantitative reverse transcription polymerase chain reaction

The RNA samples were tested by qRT-PCR in an ABI QuantStudio 7 Real-Time cycler (Applied Biosystems, Foster City, USA). The One Step PrimeScript™ RT-PCR Kit (Takara, Dalian, China) was used as follows: 0.8 µL enzyme mixture (including reverse transcriptase [RT] and Taq polymerase), 10 µL 2 × One Step RT-PCR buffer III, 0.4 µL of each primer and probe (20 µmol/L), 0.4 µL ROX Reference Dye II, 2.6 µL RNase free water, and 5 µL RNA (total 20 µL/reaction mixture). Each
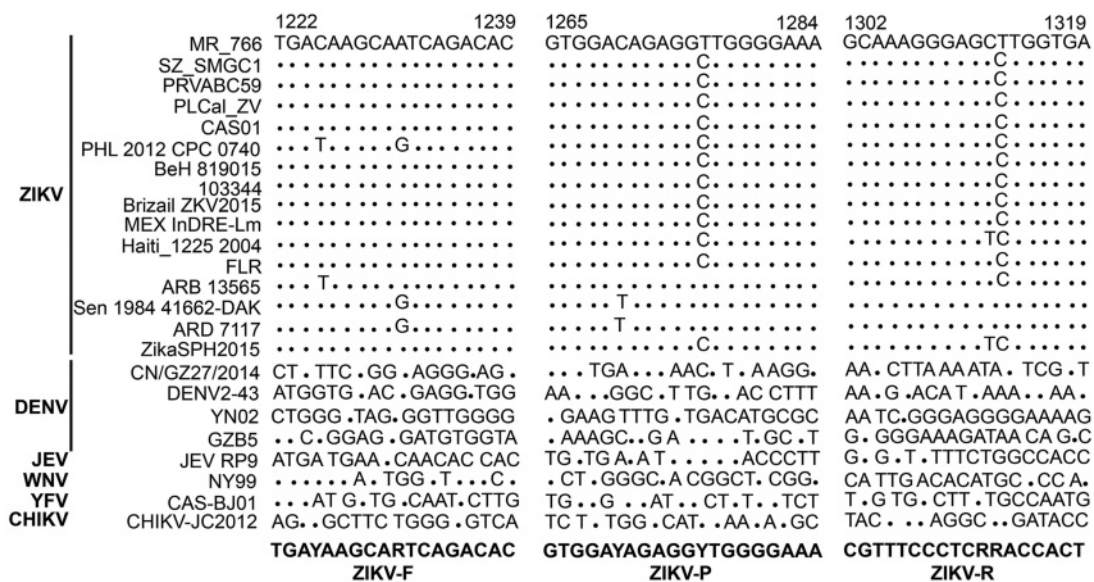

Figure 1. Conserved sequence selection for primer and probe design. The *E* genes of 81 ZIKVs were downloaded for alignment; the most conserved regions that were divergent from other flaviviruses were selected for the primer and probe design. Alignment of the regions of designed primers and probe with reference strains of ZIKVs and other flaviviruses are shown. Dots indicate identity with the consensus sequence on top of the alignment.

qRT-PCR run contained one negative and one positive control. The negative control consisted of water in place of the RNA sample. The positive control was ZIKV nucleic acid extracted from viral stocks, as described above. The qRT-PCR assay conditions were as follows: reverse transcription for 5 min at 42 °C; 10 s at 95 °C for reverse transcriptase inactivation and DNA polymerase activation followed by 40 cycles of 5 s at 95 °C; and 30 s at 55 °C (annealing-extension step). The data were analyzed using the QuantStudio™ Real-Time PCR Software (Applied Biosystems, Foster City, USA). Commercial qRT-PCR kits were also used for the detection of DENV, YFV, and ZIKV (Da An Gene Co., Ltd., Guangdong, China) following manufacturer instructions. All samples were analyzed in triplicate with three independent runs.

RESULTS

Design of the primer-probe set

The genomes of all ZIKVs were downloaded from the NCBI database for alignment. After systematic analysis, we identified a highly conserved region of 98 nucleotides (nt) in length on the *E* gene that was specific to ZIKV, but divergent from other flaviviruses. Based on the analysis, we designed the following primer-probe set for this conserved region: ZIKV-F (5′-TGAYAAGCAR-TCAGACAC-3′), ZIKV-R (5′-TCACCARRCTCCCT-TTGC-3′) and ZIKV-P (5′-FAM-GTGGAYAGAGG-YTGGGGAAA-TAMRA-3′), which hybridized to positions 1222–1239, 1302–1319, and 1265–1284, respectively, in the ZIKV genome (Figure 1, as calculated from GenBank accession number AY632535). The primer-probe set was then used for ZIKV detection by the one-step qRT-PCR method, as described in the Materials and Methods section.

Specificity of the qRT-PCR assay

To test the specificity of our qRT-PCR method, different flaviviruses including DENV 1–4, YFV, and several ZIKV strains were used. All five ZIKVs, including both African and Asian lineages (Figure 2) could have been
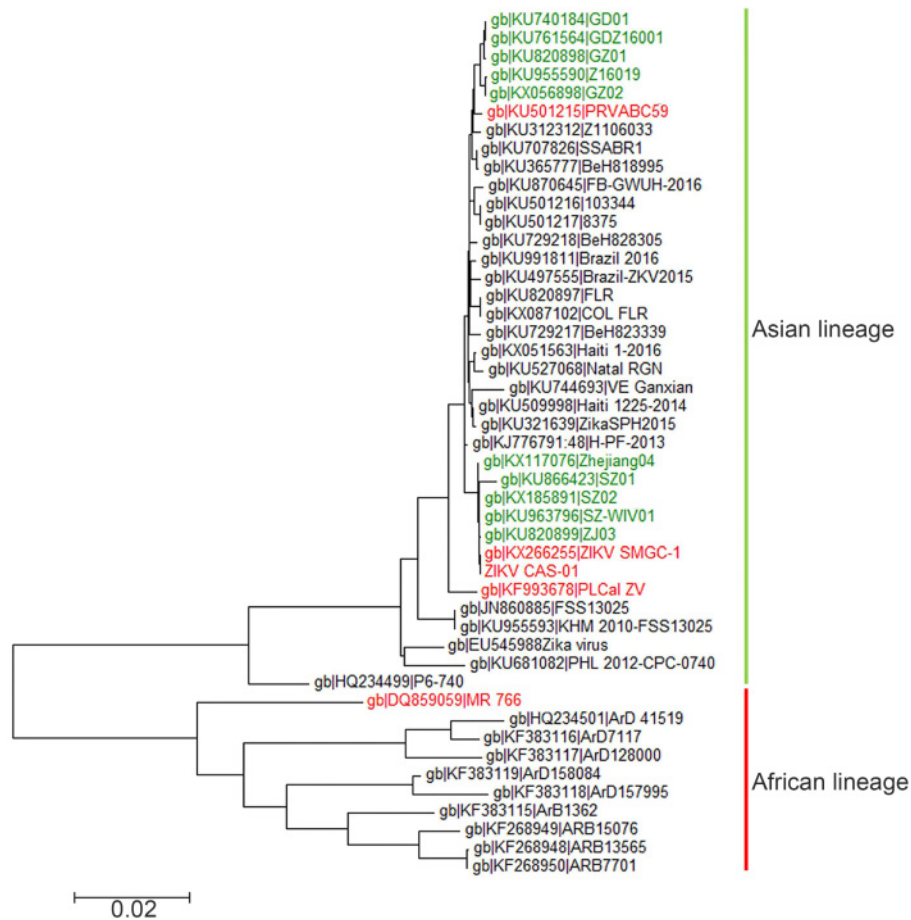

Figure 2. Phylogenetic analysis of ZIKVs. Genomes of ZIKVs were analyzed using the Mega 7 software. Strains of ZIKV imported into China and reference strains used in the present study are colored in green and red, respectively.

detected by our primer-probe set (Table 1). In addition, with our primer-probe set, amplification was not observed in any of the RNA preparations from the DENV and YFV strains, or gene fragments of WNV. The novel qRT-PCR with the new primer-probe set displayed high specificity for ZIKVs without any amplification of other flaviviruses.

Sensitivity of the qRT-PCR assay

The detection limit of the novel qRT-PCR assay was evaluated using the quantitative RNA standards from a pGEM-T vector expressing the target sequence of the *E* gene, and viral RNAs prepared from serial ten-fold dilutions of the five ZIKV stocks. Assays were performed in triplicate for both methods. The qRT-PCR method using the RNA standards as a template showed that cycle threshold (Ct) values were linear between 1 (mean Ct value = 39.14) and 1×10^8 molecules (mean Ct value = 14.92). The regression coefficient ($R^2 = 0.999$) indicated that over this range, the assay was both accurate and precise (Figure 3A). The detection limit was determined to be five RNA transcript copies per reaction, based on the standard curve (Figure 3A) and specific amplification curves (Figure 3B). Moreover, RNA samples were extracted from ten-fold serial dilutions of stock ZIKV ranging from 3.2×10^5 to 2.1×10^{-2} TCID$_{50}$/mL, and were used to test the detection limit. Results showed that the detection limit of the qRT-PCR assay was similar among the five ZIKV strains under evaluation, that is, between 2.94×10^{-3} and 4.48×10^{-3} TCID$_{50}$ per reaction (2.94×10^{-3} TCID$_{50}$ for MR_766; 3.78×10^{-3}

TCID$_{50}$ for CAS01; and 4.48×10^{-3} TCID$_{50}$ for the other three strains). Viral titers were shown to correlate well with the obtained Ct values, ranging from 17.71 to 38.74 (Figure 4A-4E, Table 2). The Ct values across the quantitative range showed a standard deviation ranging from 0.01 to 0.58 (Table 2). According to our results, the specimen was considered positive if the Ct value was less than 38, and negative if it was undetermined. Any Ct values between 38 and 40 with typical amplification curves (Figure 3B) were considered indeterminate and positive if the repeat results were similar to the previous results, and this was confirmed by sequencing the amplicon.

Evaluation of the qRT-PCR assay in clinical samples

To assess the performance of the qRT-PCR assay in a clinical setting, tests were conducted on urine and serum samples from two confirmed ZIKV-infected cases imported to China, as mentioned in the Materials and Methods section. As expected, all samples tested positive for ZIKV and displayed comparatively lower Ct values with the qRT-PCR assay than those obtained with the commercial detection kit for ZIKV. However, no statistically significant differences were observed between the two results (Table 3).

DISCUSSION

In the present study, the detection limit was determined to be as low as five RNA transcript copies from a pGEM-T

Table 1. ZIKV and other flavivirus strains used in the present study

Flavivirus species	Reference	Lineage	GenBank no.	Virus titer (TCID$_{50}$/mL)	RT-qPCR assays This publication
ZIKV	MR_766	African	KX377335	2.1×10^6	14.75 ± 0.22
ZIKV	PRVABC59	Asian	KX377337	3.2×10^4	20.22 ± 0.16
ZIKV	PLCal_ZV	Asian	KF993678	3.2×10^5	17.59 ± 0.22
ZIKV	SZ_SMGC-1	Asian	KX266255	3.2×10^5	17.96 ± 0.44
ZIKV	CAS01	Asian	NA	2.7×10^5	18.59 ± 0.23
DENV	CN/GZ27/2014	Serotype 1	KP723473	NA[c]	U[d]
DENV	DENV2-43	Serotype 2	AF204178	NA	U
DENV	YN02	Serotype 3	KF824903	NA	U
DENV	GZB5	Serotype 4	AF289029	NA	U
YFV	BJ01/2016	—[b]	KY495641	NA	U
WNV[a]	NY99	—[b]	NC_009942	1×10^6(copies/μL)	NA

Note: ZIKV, Zika virus; DENV, dengue virus; YFV, yellow fever virus; WNV, West Nile virus. [a] The synthetic envelope gene was used instead of the live virus; [b] Not applicable; [c] Not available; [d] Undetected. The Ct values of the commercial kits for DENVs (CN/GZ27/2014, DENV2-43, YN02, and GZB5) and YFV were 17.22, 12.18, 15.23, 16.42, and 15.59, respectively.
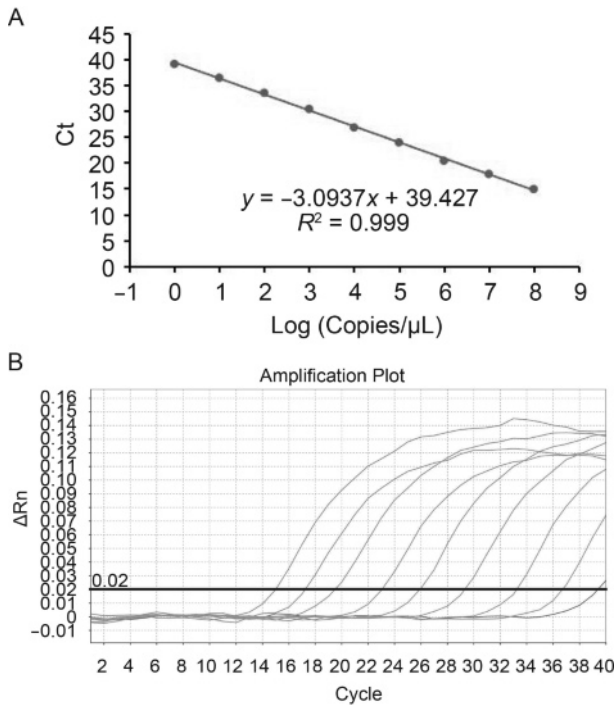
Figure 3. Sensitivity of the qRT- PCR assay using synthetic ZIKV RNA. (A) Standard curve for ten-fold serial dilution of synthetic ZIKV RNA. The log number of ZIKV RNA transcripts (Copies/µL) is expressed linearly on the x-axis, whereas Ct values obtained from qRT-PCR are expressed linearly on the y-axis. (B) Representative amplification curves of the different concentrations of synthetic ZIKV RNA.

vector expressing the target sequence, and 2.94×10^{-3} $TCID_{50}$ of live ZIKV per reaction, with high accuracy and precision ($R^2 = 0.999$). These findings reflect greater sensitivity than the previously developed qRT-PCR targeting NS5 gene and other traditional RT-PCR assays that target the *E* gene (Lanciotti et al., 2008; Faye et al., 2013; Basarab et al., 2016). The difference in abundance of the *E* and *NS5* genes in the ZIKV virion might influence the sensitivity of qRT-PCR detection. Currently, diagnosis of ZIKV infection is mainly based on the detection of ZIKV RNA during the first few days after the onset of symptoms (Musso and Gubler, 2016). According to previous studies, ZIKV RNA can be detected in several types of bodily fluids, including blood, urine, saliva, breast milk, and semen (Musso and Gubler, 2016). In symptomatic patients, viremia ranges from 7.28×10^6 to 9.3×10^8 copies/mL, and in asymptomatic patients, from 2.5×10^3 to 8×10^6 copies/mL (Besnard et al., 2014; Waehre et al., 2014; Aubry et al., 2016; Musso and Gubler, 2016). In urine, viral loads range from 3.8×10^3 to 2.2×10^8 copies/mL with a greater persistence than in serum (10 to > 20 days, and > 7 days, once it becomes undetectable in serum) (Besnard et al., 2014; Gourinat et al., 2015; Musso et al., 2015). Accordingly, our assay qualifies for the surveillance of ZIKV from various types of clinical samples without nonspecific amplification of other flaviviruses. In comparison to qRT-PCR, ELISAs are limited by cross-reactivity with other flaviviruses, due to the close relatedness to, and co-circulation of other flaviviruses in ZIKV endemic regions. Furthermore, detection of ZIKV is best achieved during the acute-phase; however, it is difficult to
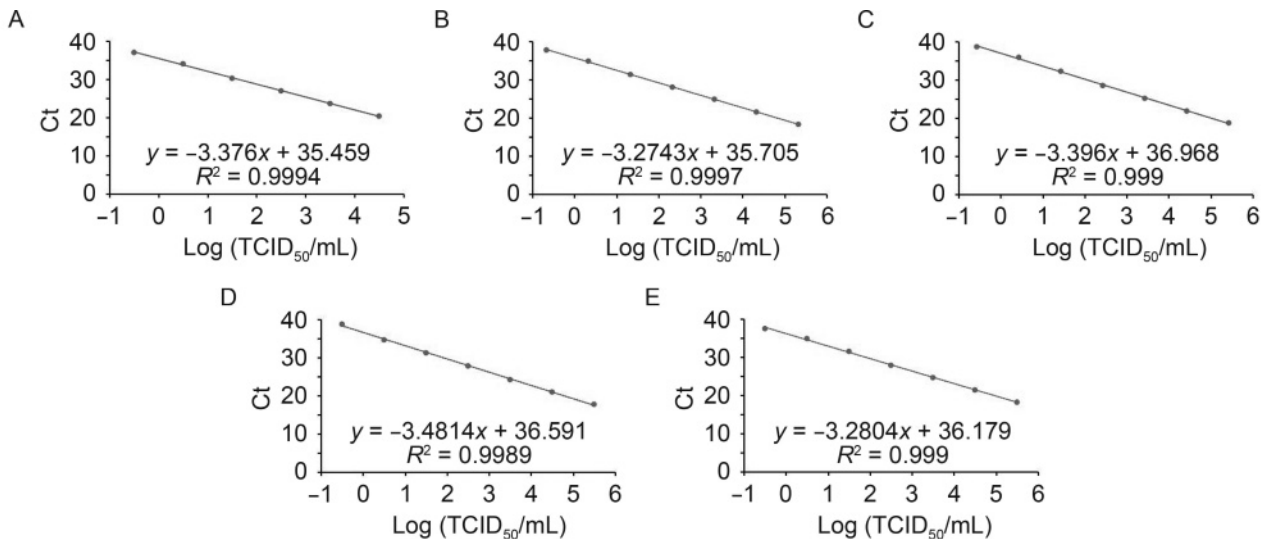

Figure 4. Sensitivity of the qRT- PCR assay using ZIKV viral RNAs. (A–F) Standard curves for ten-fold serial dilutions of stock ZIKV strains PRVABC59, MR_766, CAS01, PLCal_ZV, and SZ_SMGC-1, respectively. The log number of live ZIKV ($TCID_{50}$/mL) is expressed linearly on the x-axis, whereas Ct values are expressed linearly on the y-axis.

Table 2. Sensitivity of the qRT-PCR assay for ZIKV RNA detection

Serial dilution[a]	Ct Values				
	MR_766	CAS01	PLCal_ZV	SZ_SMGC-1	PRVABC59
Viral stock	17.92 ± 0.04	18.69 ± 0.23	17.71 ± 0.40	18.16 ± 0.39	20.32 ± 0.06
10^{-1}	21.13 ± 0.18	21.80 ± 0.25	20.95 ± 0.01	21.36 ± 0.42	23.62 ± 0.18
10^{-2}	24.49 ± 0.06	25.15 ± 0.05	24.19 ± 0.13	24.62 ± 0.48	26.96 ± 0.02
10^{-3}	27.71 ± 0.11	28.51 ± 0.09	27.80 ± 0.10	27.86 ± 0.45	30.26 ± 0.04
10^{-4}	31.01 ± 0.18	32.25 ± 0.10	31.21 ± 0.05	31.51 ± 0.61	34.06 ± 0.10
10^{-5}	34.52 ± 0.01	35.89 ± 0.58	34.64 ± 0.10	34.86 ± 0.36	37.03 ± 0.31
10^{-6}	37.75 ± 0.22	38.66 ± 0.54	38.74 ± 0.25	37.48 ± 0.13	Neg
10^{-7}	Neg[b]	Neg	Neg	Neg	Neg

Note: [a]The titers of stock ZIKV were 2.1×10^5 $TCID_{50}$/mL (MR_766), 2.7×10^5 $TCID_{50}$/mL (CAS01), 3.2×10^5 $TCID_{50}$/mL (SZ_SMGC-1), 3.2×10^5 $TCID_{50}$/mL (PLCal_ZV) and 3.2×10^4 $TCID_{50}$/mL (PRVABC59), respectively; [b] Negative.

Table 3. Detection of ZIKV in clinical samples

Samples		qRT-PCR assays		P^{a}
		Our method	Commercial kit	
Patient 1	Serum	29.67 ± 0.82	30.63 ± 0.13	0.1146
	Urine	29.54 ± 0.61	30.47 ± 0.12	0.0606
Patient 2	Serum	26.71 ± 0.58	27.66 ± 0.84	0.1823

Note: [a]Data were analyzed using the Student's *t*-test, and P-values represent a comparison of our method with that of the commercial kit.

determine the period of the onset of symptoms, as the majority of cases are asymptomatic (Hamel et al., 2016; Saiz et al., 2016).

In conclusion, we have developed a rapid, specific qRT-PCR assay with high sensitivity and broad coverage of circulating ZIKV strains (both African and Asian lineages). This assay would be of value in surveillance efforts across various regions of the outbreak.

ACKNOWLEDGMENTS

COMPLIANCE WITH ETHICS GUIDELINES

The authors declared that they have no conflict of interest. The studies have been approved by our institutional research ethics committee, and written informed consent was obtained from all patients.

AUTHOR CONTRIBUTIONS

YHB and YY designed the experiments. YY, GW, BGY, YHB, SHL, SQL, HXZ and QW carried out the experiments. YY, YHB and GW analyzed the data. YXL, LL, MFL and GFG acquired the clinical samples and provided scientific input. YY, GW and YHB wrote the paper. All authors read and approved the final manuscript.

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