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Development of a direct reverse-transcription quantitative PCR (dirRT-qPCR) assay for clinical Zika diagnosis

Lang Li[a,b,1], Jian-an He[b,1], Wei Wang[c,1], Yun Xia[b], Li Song[a,b], Ze-han Chen[a], Hang-zhi Zuo[a], Xuan-Ping Tan[d], Aaron Ho-Pui Ho[e], Siu-Kai Kong[f], Jacky Fong-Chuen Loo[e,f,*], Hua-wen Li[a,**], Gu Dayong[b,c,***]

[a] School of Public Health, The Second School of Clinical Medicine, Guangdong Medical University, Dongguan, 523808, PR China
[b] Shenzhen International Travel Health Care Center and Shenzhen Academy of Inspection and Quarantine, Shenzhen Customs District, Shenzhen, 518033, PR China
[c] Department of Laboratory Medicine, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Health Science Center, Shenzhen, 518035, PR China
[d] Shenzhen gene-one Biotechnology Co., Ltd., 518000, PR China
[e] Department of Biomedical Engineering, The Chinese University of Hong Kong, Hong Kong SAR, PR China
[f] Biochemistry Programme, School of Life Sciences, The Chinese University of Hong Kong, Hong Kong SAR, PR China

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ABSTRACT

Objective: The nucleic acid-based polymerase chain reaction (PCR) assay is commonly applied to detect infection with Zika virus (ZIKV). However, the time- and labor-intensive sample pretreatment required to remove inhibitors that cause false-negative results in clinical samples is impractical for use in resource-limited areas. The aim was to develop a direct reverse-transcription quantitative PCR (dirRT-qPCR) assay for ZIKV diagnosis directly from clinical samples.
Methods: The combination of inhibitor-tolerant polymerases, polymerase enhancers, and dirRT-qPCR conditions was optimized for various clinical samples including blood and serum. Sensitivity was evaluated with standard DNA spiked in simulated samples. Specificity was evaluated using clinical specimens of other infections such as dengue virus and chikungunya virus.
Results: High specificity and sensitivity were achieved, and the limit of detection (LOD) of the assay was 9.5×10^1 ZIKV RNA copies/reaction. The on-site clinical diagnosis of ZIKV required a 5 μl sample and the diagnosis could be completed within 2 h.
Conclusions: This robust dirRT-qPCR assay shows a high potential for point-of-care diagnosis, and the primer–probe combinations can also be extended for other viral detection. It realizes the goal of large-scale on-site screening for viral infections and could be used for early diagnosis and the prevention and control of viral outbreaks.

Introduction

Zika virus (ZIKV), a mosquito-borne *Flavivirus*, is a positive single-stranded RNA virus with a nucleic acid length of about 10.7 kb. This virus has been spreading rapidly and there have been large-scale outbreaks worldwide in recent years (Haddow et al., 2012; Harris et al., 2006; Calvet et al., 2016; Dick et al., 1952). ZIKV has drawn attention as it may cause severe symptoms while no specific medical treatment or vaccine is currently available (Campos et al., 2015; Hancock et al., 2014; Brasil et al., 2016; Cao-Lormeau et al., 2016). Rapid diagnosis is the only option to monitor and control the spread of ZIKV, and nucleic acid-based diagnostic assays, i.e. polymerase chain reaction (PCR), have shown high efficiency due to their sensitivity and specificity (Bonaldo et al., 2016; Huzly et al., 2016; Wang et al., 2016; Zanluca et al., 2015). However, clinical samples contain innate constituents that are regarded as PCR inhibitors, which inactivate DNA polymerases or degrade target nucleic acids or primers, such as

* Corresponding author at: Department of Biomedical Engineering, The Chinese University of Hong Kong, Hong Kong SAR, PR China.
** Corresponding author.
*** Corresponding author at: Department of Laboratory Medicine, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Health Science Center, Shenzhen, 518035, PR China.
E-mail addresses: jackyfcloo@cuhk.edu.hk (J.F.-C. Loo), chineseli@163.com (H.-w. Li), wanhood@163.com (D. Gu).
[1] Lang Li, Jian-an He, and Wei Wang contributed to the work equally and should be regarded as co-first authors.

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immunoglobulin G and hemoglobin in blood, and these have resulted in false-negative results in tests on these samples (Schrader et al., 2012; Al-Soud et al., 2000; Abu Al-Soud and Radstrom, 2001; Wang et al., 1992; Khan et al., 1991; Abu Al-Soud and Radstrom, 2000; Abu Al-Soud and Radstrom, 1998). The required purification of the nucleic acid from infected specimens prior to detection is not only time- and labor-intensive, but also increases the risk of human error and accidental exposure (Abu Al-Soud and Radstrom, 2001). The incomplete removal of qPCR inhibitors during nucleic acid extraction could still lead to inaccurate detection. In this context, the detection of target nucleic acid directly from samples without purification is desirable.

The direct reverse-transcription quantitative PCR (dirRT-qPCR) amplification method simplifies the operating process, reduces the time from sample-to-answer detection, reduces the risk of exposure to infectious substances, and reduces the sample volume required from the milliliter level to as low as the sub-microliter level (Kang et al., 2015). These advantages support dirRT-qPCR as a practical point-of-care diagnostic method for on-site disease screening. Several methods for sample pretreatment such as preheating and freezing–thawing have been reported for direct sample detection without purification (Watkins-Riedel et al., 2004; Jadaon et al., 2009; Panaccio et al., 1993; Victor et al., 2009). The use of various PCR enhancers such as dimethyl sulfoxide (DMSO), non-ionic detergents, bovine serum albumin (BSA), and PCR enhancer cocktail has also been reported in direct PCR detection (Zhang et al., 2010; Watkins-Riedel et al., 2004; Nishimura et al., 2000; Yang et al., 2007; Bu et al., 2008). Yet, the fragility of RNA and the existence of high levels of RNases, which can cause RNA degradation and compromise RNA integrity, are the challenge in direct RNA detection from clinical samples (Dongyang et al., 2018).

We have therefore developed a dirRT-qPCR assay to detect ZIKV directly from multiple crude clinical samples, including saliva, serum, throat swab, whole blood, and urine, without the need for any sample pretreatment. With the optimized combination of Taq DNA polymerase variant, PCR and enzyme stabilizers, this dirRT-qPCR assay was found to have high sensitivity and high specificity for ZIKV detection in the presence of other interference substances and other arbovirus infections, such as dengue virus (DENV) and chikungunya virus (CHIKV). Since this robust assay can be completed in 2 h with as little as 5 μl of sample, dirRT-qPCR reaction mixture, and a thermocycler only, and the primer–probe combination can be extended for the detection of other viruses, it has a high potential for application in rapid on-site screening, such as at entry–exit ports and hospitals. This assay realizes the goal of large-scale preliminary screening for viral infections and could be used for early diagnosis and the prevention and control of viral outbreaks.

Materials and methods

Collection and processing of clinical samples

Nine clinical samples of ZIKV infection, i.e. three saliva, two serum, one throat swab, two urine, and one whole blood, were provided by Zhejiang Inspection and Quarantine Bureau. Information on the patients infected with ZIKV is summarized in Table 1. All normal clinical specimens, 54 DENV-positive samples, and eight CHIKV-positive samples were obtained from the Health Quarantine Laboratory of Shenzhen International Travel Health Care Center and were collected according to the standard clinical sample collection protocol, with ethical approval and written consent. Clinical whole blood samples and the throat swabs were additionally treated with dipotassium ethylenediaminetetraacetic acid (EDTA-K2) anticoagulation and Minimal Essential Medium Eagles with Earle's Balanced Saltsa (MEM/EBSS) medium, respectively.

Table 1
Basic information for the clinical samples collected from the Zika virus-infected patients.

Patient	Sex	Age (years)	Symptoms	Types of samples collected
1	Male	38	Conjunctivitis, fever, skin rash	Serum, saliva
2	Male	8	Conjunctivitis, fever, skin rash	Serum, saliva, urine
3	Female	42	Conjunctivitis, fever, skin rash	Saliva, throat swab, whole blood, urine

ZIKV lentivirus, ZIKV RNA, bacterial genomic DNA, arbovirus and bacterial plasmids, and influenza RNA

The ZIKV lentivirus at a stock concentration of 2×10^8 Transducing Units/mL TU/ml was obtained from Hanbio Biotechnology Co., Ltd.. Extracted ZIKV RNA and one yellow fever virus (YFV) in cell culture supernatant were provided courtesy of the Health Quarantine Laboratory of Guangdong Inspection and Quarantine Technology Center; the concentration was approximately 1.9×10^{10} copies/μl. Influenza RNA was obtained from the Health Quarantine Laboratory of Shenzhen International Travel Health Care Center. Three types of bacterial genomic DNA were provided by Shenzhen Disease Prevention and Control Center (SZCDC). Five types of arbovirus plasmid and bacterial plasmid were synthesized by Sangon. Information on these nucleic acids is show in Table 2. To evaluate the specificity of this assay, five different arbovirus plasmids, eight different bacterial plasmids, and three types of influenza virus (H1N1, H3N1, and B) were used. Meanwhile, these arbovirus plasmids were added to the reaction together with RNA to further identify the specificity of the RT-qPCR assay. Specifically, 5 μl of these plasmids (10^5 copies/μl) were respectively added last to the reaction mixture or 2 μl of plasmid (10^5 copies/μl) and 3 μl ZIKV RNA (10^5 copies/μl) were added together to the reaction mixture.

Design of the primer pair and probe

The design of the primer pair and probe was based on the alignment of the NS5 non-structural protein gene published in GenBank. The primers and the probe were designed with Primer Premier 5.0 and Primer Express 3.0.1, which generated an amplicon size of 113 bp. The sequence of the primer pair and the hydrolysis probe, along with the sizes of the expected amplicons, are summarized in Table 3. The primer pair and probe synthesized by Sangon were purified by ultra-polyacrylamide gel electrophoresis (ULTRAPAGE).

dirRT-qPCR assay

Four DNA polymerases, namely OmniTaq, AlphaTaq (VitaNavi), Tth, and TTX (TOYOBO), showing a certain degree of tolerance to inhibitors from samples, and one conventional HotTaq DNA polymerase (Sangon) were screened in the optimization step. The corresponding buffer for DNA polymerase was selected for the reaction system and the initial PCR conditions were established according to the melting temperature (Tm) value of the primer and probe and the recommended reaction temperature of DNA polymerase, where the reaction mixture contained optimized concentrations of $MgCl_2$, dNTPs, KCl, Triton X-100, OmniTaq/

Table 2
Information on influenza virus RNA, viral plasmids, bacterial plasmids, and genomic DNA used in this study.

Name	Accession number	Genome region	Type	Name	Accession number	Genome region	Type
DENV	**M29095.1**	3′-UTR	Plasmid	*Bacillus anthracis*	**AF205319.1**	rpoB	Plasmid
CHIKV	**KC488650.1**	E1	Plasmid	*Brucella*	**JX081250.1**	IS711	Plasmid
YFV	**FJ654700.1**	E	Plasmid	*Burkholderia mallei*	**AM087433.1**	Flip	Plasmid
WNV	**AY646354.1**	E	Plasmid	*Burkholderia pseudomallei*	**AF074878.2**	TTS	Plasmid
JEV	**L47349.1**	E	plasmid	*Francisella tularensis*	**M32059.1**	Tul4	plasmid
H1N1	-	-	RNA	*Salmonella*	-	-	Genomic DNA
H3N1	-	-	RNA	Typhoid bacillus *Salmonella typhi*	-	-	Genomic DNA
B	-	-	RNA	*Shellogell Shigella*	-	-	Genomic DNA

DENV, dengue virus; CHIKV, chikungunya virus; YFV, yellow fever virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; H1N1, influenza virus H1N1; H3N1, influenza virus H3N1; B, influenza B virus; UTR, untranslated region.

Table 3
Sequences of the primer pair and hydrolysis probe used in this study.

Name	Sequence (5′→3′)	Length (bp)
Forward primer	TTCGGAATATGGAGGCTGAG	20
Reverse primer	TCGTTTGAGCCTATCCCATC	20
Probe	FAM-AGAAAGTGACCAACTGG TTGCAGAGCA-BHQ1	27

AlphaTaq/ReverAce, RNase inhibitor, primer pair, probe, and sample. Each experiment was repeated in triplicate. A positive control (ZIKV RNA) and a no template control (NTC) were also included.

To optimize the dirRT-qPCR assay, KCl, Triton X-100, and PCR enhancer cocktail (PEC-2; composed of 0.2% NP-40, 0.15 mol/l D-(+)-trehalose, and 0.12 mol/l L-carnitine) were employed. On the basis of the previous reaction system corresponding to OmniTaq DNA polymerase, KCl and Triton X-100 were added to the reaction system separately or in combination. dNTPs act with a chelating effect to interact with magnesium ion (Mg^{2+}), an important co-factor for the DNA polymerase, subsequently affecting the activity of DNA polymerase. Therefore, the concentrations of dNTPs (0.2–0.8 mM) and $MgCl_2$ (2.5–6.5 mM) were optimized by orthogonal design. Several PCR enhancers were optimized by their supplementation, individually or in combination, in the reaction mixture of the dirRT-qPCR to further improve the amplification efficiency. Since different PCR enhancers show different capacity to enhance the efficiency of amplification in different samples, eight common PCR enhancers were screened: sodium dodecyl sulfate (SDS) (0.05%), Tween-2 (0.1%), glycerol (1%), BSA (0.4 mg/ml), gelatin (1/10 volume), dithiothreitol (DTT) (1/10 volume), DMSO (10%), and deionized formamide (10%). These enhancers can be divided roughly into three categories, i.e. contribute to the cleavage of virus capsid and the release of nucleic acid to provide sufficient templates (SDS, Tween-20); help in stabilizing the polymerase and protecting its activity (BSA, DTT, gelatin, glycerol); help in the interaction between the primers and templates (DMSO, formamide). The dirRT-qPCR reactions were performed in a quantitative PCR instrument (7500; Applied Biosystems) with a volume of 25 μl. The dirRT-qPCR reactions for detecting ZIKV infection in the clinical samples were performed in a Mini8 Plus RT-PCR thermocycler (Coyote). The effectiveness of the dirRT-qPCR assay in terms of sensitivity, specificity, and repeatability was evaluated in simulated sample, and clinical samples were used to validate this practical performance of the assay.

Statistical analysis

Statistical analyses were conducted using OriginPro2018, GraphPad Prism 5, and Microsoft Excel. The correlation coefficient (R^2) was calculated by linear regression analysis. The repeatability of the dirRT-qPCR assay was determined by analyzing the mean cycle threshold (Ct) values of parallel reactions and standard deviations (SD) of Ct values. The mean Ct value was used as the ordinate, and the logarithm of the concentration (copies/μl) value was used as the abscissa to construct the standard curve.

Results

Optimization of the dirRT-qPCR assay

Optimization of DNA polymerase is the most important procedure to validate the best choice of polymerase that can tolerate inhibitors in multiple clinical samples with good amplification efficiency. Figure 1 shows the amplification curves using five DNA polymerases, namely OmniTaq (0.25 μl), AlphaTaq (0.25 μl), Tth (0.5 μl), TTX (0.25 μl), and HotTaq (0.5 μl), with various clinical samples, i.e. saliva (Figure 1A), serum (Figure 1B), throat swab (Figure 1C), urine (Figure 1D), and whole blood (Figure 1E). The amplification efficiency of OmniTaq DNA polymerase was higher than that of the other four polymerases for all sample types. Although the amplification efficiency of AlphaTaq DNA polymerase was similar to that of OmniTaq DNA polymerase for saliva, serum, and throat swab samples (Figure 1A–C), OmniTaq DNA polymerase was the only available polymerase that could amplify the target from the whole blood samples (Figure 1E). Meanwhile, HotTaq DNA polymerase did not work with any sample at all. As a result, OmniTaq DNA polymerase was selected for the downstream dirRT-qPCR assay and the corresponding reaction procedure was as follows: reverse transcription was performed for 5 min at 50 °C, then 94 °C for 5 s and 50 °C for 5 s for 15 cycles, followed by a PCR cycle of 94 °C for 1 min for 1 cycle, 95 °C for 5 min and 50 °C for 30 s for 40 cycles.

After investigating the choice of polymerase, optimization of the reaction mixture with conventional direct PCR enhancers, i.e., KCl (40 mM), Triton X-100 (0.1%), and PEC-2 (0.2% NP-40, 0.15 mol/L D-(+)-trehalose, and 0.12 mol/L L-carnitine), on dirRT-qPCR was performed. The Ct values and end-point fluorescence value for these experiments are summarized in Figure 2. The dirRT-qPCR assay worked better for different specimens with PEC-2, shown by the higher fluorescence intensity of the reaction in the presence of PEC-2 (about 6.3×10^5) than in the absence of PEC-2 (about 2.4×10^5) (Figure 2). It was not dependent on KCl or Triton X-100 addition, as shown by the insignificant difference in Ct values. Therefore, PEC-2 was employed in the dirRT-qPCR reaction mixture. Nonetheless, the improved amplification efficiency, determined from end-point fluorescence ΔRn intensity or end-point Ct value, using the combination of KCl and Triton X-100 could be used as an alternative to PEC-2 alone.

Optimization of the concentration of dNTPs (0.2–0.8 mM) and $MgCl_2$ (2.5–6.5 mM) could also improve the amplification

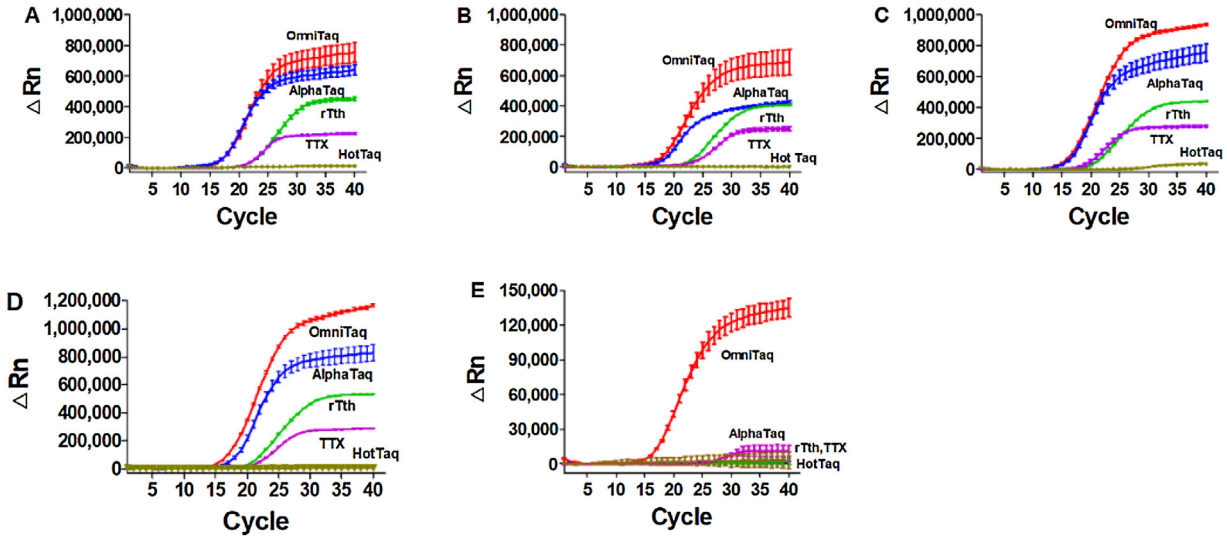

Figure 1. Amplification curves showing the amplification efficiency of five DNA polymerases, namely OmniTaq, AlphaTaq, rTth, TTX, and HotTaq, for the saliva (A), serum (B), throat swab (C), urine (D), and whole blood (E) samples in dirRT-qPCR assay.

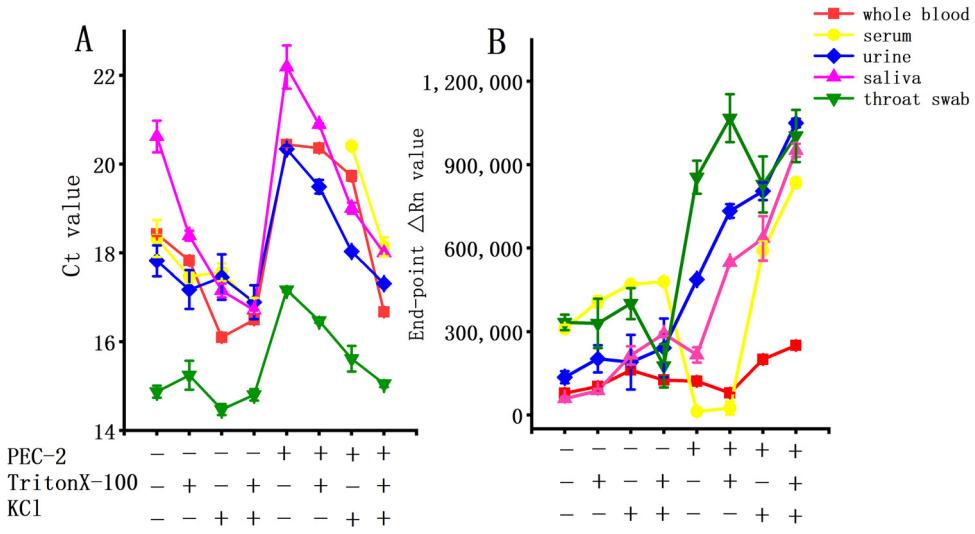

Figure 2. The Ct values (A) and end-point ΔRn value (B) of the dirRT-qPCR assay on saliva (pink), serum (yellow), throat swab (green), urine (blue), and whole blood (red) samples in the reaction mixture, with and without KCl, Triton X-100, and PEC-2 addition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

efficiency of the dirRT-qPCR assay. The Ct values and end-point fluorescence ΔRn value are summarized in three-dimensional bar graphs in Figure 3. The optimal concentrations of dNTPs and $MgCl_2$ were determined by the lowest Ct value as well as the relatively high end-point ΔRn value. The increase in concentration of Mg^{2+} showed an insignificant improvement in amplification efficiency, while a high concentration of dNTPs inhibited the amplification at a low concentration of Mg^{2+}. In this context, the optimal concentration of Mg^{2+} and dNTPs for the different specimens were 5.5 mM and 0.4 mM, respectively, and these concentrations were used in the dirRT-qPCR assay.

To further optimize the reaction mixture for dirRT-qPCR, supplementation with additional PCR enhancers was evaluated. The amplification curves for these experiments are shown in Figure 4A–E and the Ct values are summarized in Figure 4F. The addition of DTT or DMSO enhanced the amplification efficiency for all types of sample, while the addition of other additives could only enhance the amplification efficiency in some samples; e.g., the addition of gelatin could enhance amplification efficiency in urine but not in whole blood. By considering the ability to improve amplification efficiency in terms of both the Ct value (which determined the positive/negative ZIKV infection) and end-point fluorescence value (which evaluated the removal of inhibition), DTT was selected as the additional PCR enhancer for this dirRT-qPCR assay.

Validation of the detection sensitivity of the dirRT-qPCR

The sensitivity, including detection range and limit of detection (LOD), of this dirRT-qPCR assay for ZIKV RNA detection was first evaluated using simulated clinical samples with standard concentrations of ZIKV RNA ranging from 1.9×10^0 to 1.9×10^6 copies/μl of spiked, as well as standard concentrations of RNA from 1.9×10^0 to 1.9×10^6 copies/μl as positive control. The correlation between the copies of ZIKV RNA and the Ct value was analyzed. The real-time amplification curves and the standard curve are provided in the Supplementary Material (Figure S1A–F and G, respectively). The detection limits of ZIKV RNA from mimic saliva, serum, throat

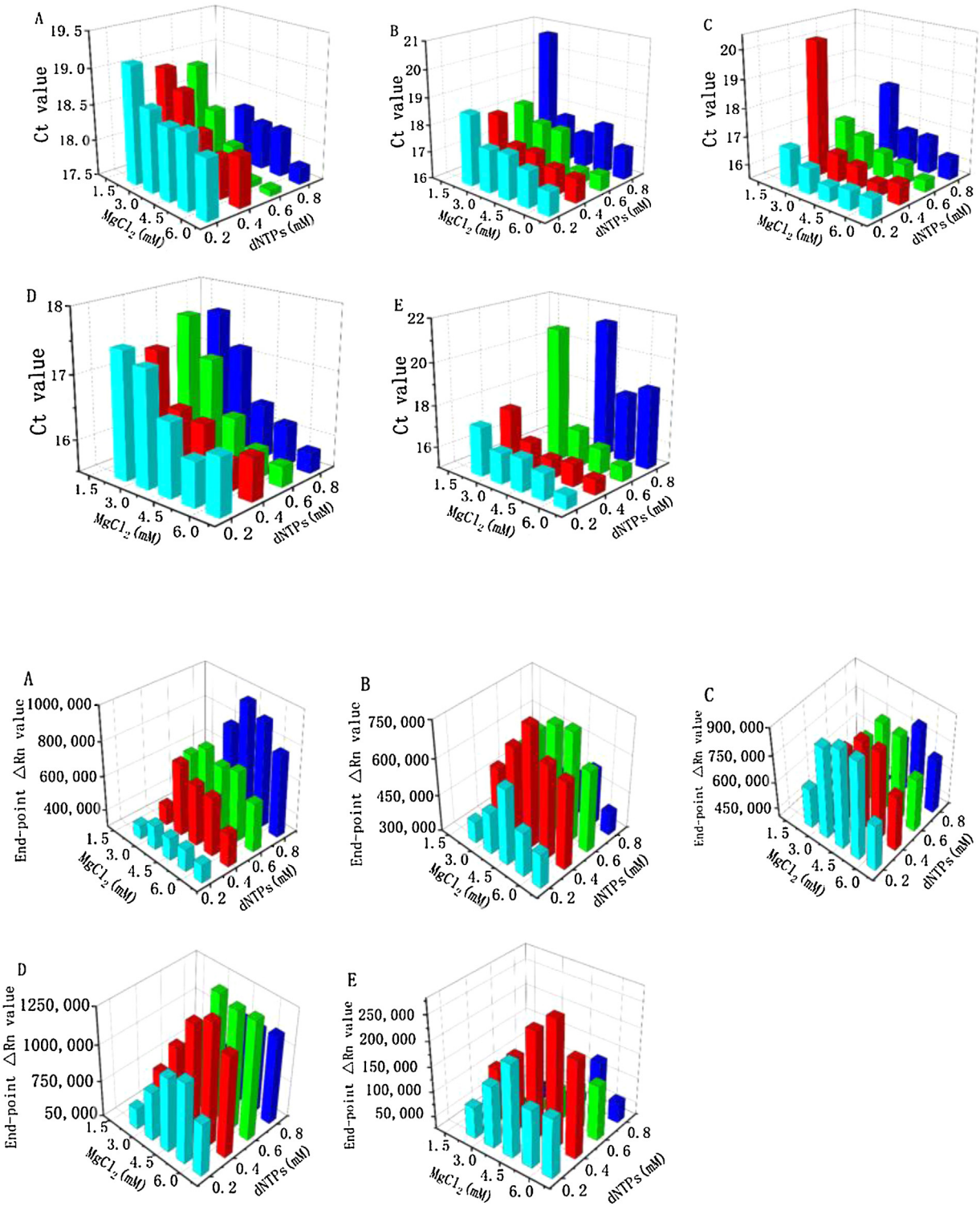

Figure 3. The (I) Ct values and (II) end-point ΔRn value showing the optimization of the concentration of $MgCl_2$ and dNTPs used in the dirRT-qPCR assay on the saliva (A), serum (B), throat swab (C), urine (D), and whole blood (E) samples.

swab, urine, and RNA were 19 copies/μl, while that from simulated whole blood was 1.9×10^2 copies/μl. The mean and standard deviation Ct values of the lowest LOD and the LODs of the different samples are summarized in Table 4. The slope of −3.398 with R^2 of 0.998, indicating an amplification efficiency of 99.673%, in the dynamic range from 1.9×10^2 to 1.9×10^6 copies/μl measured in the standard curve of the positive control (Supplementary Material Figure S1G), supported the effective quantification, with LOD of 9.5 $\times 10^1$ copies/reaction in ZIKV RNA detection. Also, a similar mean Ct on standard RNA addition between water and simulated samples showed that this dirRT-qPCR could be performed with direct sample addition without sample pretreatment.

The reliability of this dirRT-qPCR assay was determined according to its reproducibility with the standards of 1.9×10^1 to 1.9×10^6 copies/μl ZIKV RNA. The mean and SD Ct values for each concentration were obtained to measure the coefficient of

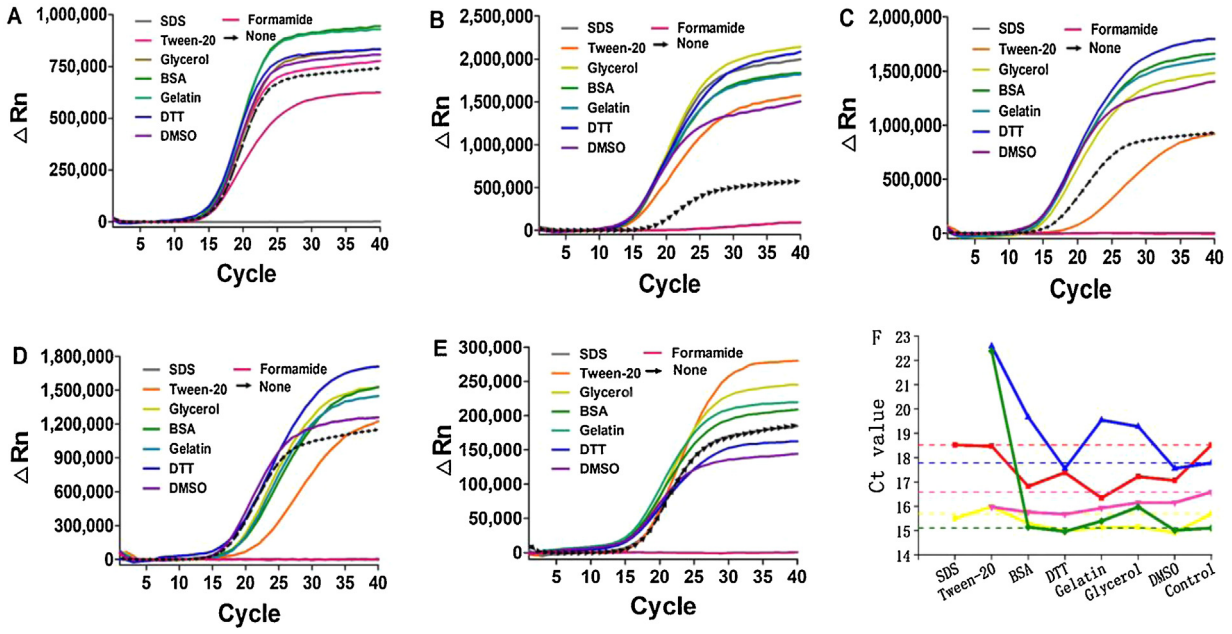

Figure 4. Amplification curves showing the additional use of eight PCR enhancers on the saliva (A), serum (B), throat swab (C), urine (D), and whole blood (E) samples, with the line graph (F) summarizing the Ct values of the saliva (pink), serum (yellow), throat swab (green), urine (blue), and whole blood (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Table 4
Summary of sensitivity and standard curve equations for the dirRT-qPCR assay.

Samples	Concentration range	Standard curve	Ct mean ± SD	LOD (copies/reaction)
RNA	1×10^2 to 1×10^6	$y=-3.398x+27.80$	25.55 ± 0.10	9.5×10^1
Saliva	1×10^2 to 1×10^6	$y=-3.203x+29.157$	24.46 ± 0.66	9.5×10^1
Serum	1×10^2 to 1×10^6	$y=-3.612x+30.145$	26.52 ± 0.36	9.5×10^1
Throat swab	1×10^2 to 1×10^6	$y=-3.438x+32.732$	24.42 ± 0.06	9.5×10^1
Urine	1×10^2 to 1×10^6	$y=-3.751x+31.706$	25.16 ± 0.65	9.5×10^1
Whole blood	1×10^2 to 1×106	$y=-3.477x+29.989$	24.42 ± 0.10	9.5×10^2

Ct, cycle threshold; SD, standard deviation; LOD, limit of detection.

variation (CV) in the three parallel samples set. The results showed a CV% lower than 3%, supporting the sensitive quantification as well as qualification of ZIKV.

Validation of the detection specificity of the dirRT-qPCR

The specificity of the dirRT-qPCR assay was evaluated with different types of virus and bacteria. Influenza, a common viral infection, may occur along with ZIKV infection. DENV is often confused with ZIKV infection since both are mosquito-borne diseases with similar symptoms and arise in similar regions of the world. Bacterial infections such as Salmonella and ShelloShigella show similar symptoms and may also lead to false-positive ZIKV detection. Therefore, the dirRT-qPCR assay was performed on other viruses and bacteria to demonstrate the high specificity of the assay for ZIKV detection. ZIKV RNA was detected among influenza viral RNA, viral plasmid DNA, and bacterial genomic and plasmid DNA, which remained steady below the threshold (Supplementary Material Figure S2A). This suggested that there was no cross-reaction to yield false-positive results with the other eight strains of viruses and eight strains of bacteria, as listed in Table 2. In addition, the specificity of the assay in the presence of interfering materials was evaluated. Reactions containing ZIKV RNA with or without spiking into various types of viral and bacterial plasmid and other nucleic acids as interfering materials were performed. ZIKV RNA could be amplified to the same extent with and without the presence of interfering materials (Supplementary Material Figure S2B). This suggested that the specificity of the assay was not affected by the co-presence of other infections, such as viral or bacterial infections, along with the ZIKV infection.

Validation of the detection accuracy of the dirRT-qPCR

The amplification curve and repeatability parameters of the dirRT-qPCR assay on ZIKV detection in simulated saliva, urine, and whole blood samples are shown in Supplementary Material Figure S3 and Table 5, respectively. The CV% of the Ct values, with repeated measurement six times at two different concentrations in these three samples, were 1.33% and 1.54%, 1.03% and 1.80%, and 1.07% and 1.63%, respectively. Since good repeatability was shown, with all CV% below 5%, this suggested that the ZIKV dirRT-qPCR detection method established and optimized in this study could ensure the reliability and accuracy of test results using different biological samples.

Evaluation of the dirRT-qPCR assay on clinical samples

Clinical validation of the specificity of the established dirRT-qPCR assay was performed using a total of 83 clinical samples (nine ZIKV-positive samples, four CHIKV-positive whole blood samples and four CHIKV-positive serum samples, 27 DENV-positive serum samples (15 DENV-1, nine DENV-2, one DENV-3, and two DENV-4),

Table 5
Repeatability parameters of the Zika virus dirRT-qPCR assay.

Sample	Concentration	Repeats	Mean	SD	CV (%)
Saliva	10^6	6	18.53	0.2	1.07%
	10^4	6	24.14	0.39	1.07%
Urine	10^5	6	20.27	0.3	1.33%
	10^2	6	30.11	0.51	1.54%
Whole blood	10^6	6	18.88	0.2	1.54%
	10^4	6	25.78	0.46	1.80%

SD, standard deviation; CV, coefficient of variation.

27 DENV-positive whole blood samples (15 DENV-1, nine DENV-2, one DENV-3, and two DENV-4), and 12 normal samples. Five microliters of each sample was added directly to the dirRT-qPCR assay. All experiments included a negative control (NTC).

Clinical validation of the established dirRT-qPCR assay was performed with a total of 83 clinical samples. To support a high specificity of detection using clinical samples as well as simulated samples prior to detecting ZIKV clinical infection, the detection of clinical specimens of non-ZIKV infection, including different arbovirus infections and non-arbovirus infection samples, and 12 normal clinical specimens from healthy individuals were first evaluated. The absence of fluorescence signal in the total 40 cycles for the other arbovirus infection samples (DENV and CHIKV), YFV cell culture supernatant (Supplementary Material Figure S4A), and normal clinical specimens (Supplementary Material Figure S4B) when compared to the positive control suggested a low probability of false-positive results.

Clinical validation of the capacity of the dirRT-qPCR for ZIKV detection in multiple clinical ZIKV-infected specimens was performed (Table 1; Supplementary Material Figure S5). All ZIKV infection samples were detected as expected, with Ct values ranging between 15 and 30. There were slight differences in the Ct for the different samples from the same individual, suggesting that the viral concentration differs in different regions of the body. Table 6 shows a summary of viral concentrations measured in multiple clinical samples, as shown in Supplementary Material Figure S5A–C, with the corresponding standard curves shown in Supplementary Material Figure S1G. The similar viral concentrations measured in serum with and without nucleic acid validate the same efficiency of the dirRT-qPCR for clinical ZIKV diagnosis in comparison to the reference standard RT-PCR.

Table 6
Summary of the s, sensitivity, Ct and the corresponding viral concentrations of the dirRT-qPCR assay in different samples and patients.

Patient	Sample	Ct	Log(viral concentration)
1	Saliva	23.59	3.3
	Serum	18.75	2.9
	Serum (RNA extract)	17.38	3.1
	Urine	27.09	0.9
2	Saliva	23.51	3.3
	Serum	19.19	2.8
	Serum (RNA extract)	17.29	3.1
3	Saliva	24.75	3.0
	Urine	26.47	1.1
	Throat swab	26.14	3.1
	Whole blood	25.62	1.5

Ct, cycle threshold.

The clinical positive and negative predictive values, sensitivity, and specificity were calculated using a real-time RT-PCR (the current standard ZIKV detection assay for sample evaluation) as the reference test. Table 7 shows a summary of the dirRT-qPCR assay for ZIKV detection. In reference to the reference RT-PCR assay, the reported assay has 100% positive and negative predictive values, sensitivity, and specificity for ZIKV diagnosis.

Discussion

The dirRT-qPCR assay developed here offers a simple operating process to improve the time and efficiency of detecting clinical ZIKV infection. Serum and blood are currently the most common samples acquired from patients. However, whole blood reduced the sensitivity of dirRT-qPCR in all optimized conditions due to its intrinsic high concentration of complex inhibitors in comparison to other samples. The highest viral concentration was found in saliva or throat swab, then serum, while the lowest was found in urine. In this context, non-invasive saliva collection could be an alternative to replace invasive venipuncture for ZIKV diagnosis. In fact, it was observed that positive detection of viral genetic markers in the blood was only possible within 2 days after the onset of the disease, while detection in saliva was possible for at least 4 days on average. On the other hand, the calculated viral concentrations in saliva and serum samples from patients 1 and 2, which were similar to one another, matches with the fact that the samples were obtained from members of the same family. The range of the RNA viral load in blood (7.28×10^3 to 9.3×10^5 copies/μl) and urine (2.5×10^1 to 8×10^3 copies/μl) in patients in another study supports the measured viral concentrations in this study (Atif et al., 2016).

To eliminate the RT-PCR inhibition in clinical samples, the dirRT-qPCR assay was first optimized with the mutant of Taq polymerase, where its variants have also been reported with a higher tolerance in as high as 20% blood (Kermekchiev et al., 2009). Thermally activated DNA polymerase is favorable, as reverse transcription is necessary prior to PCR for RNA detection, while non-specific amplification during reverse transcription at a relatively low temperature could be prevented with thermally activated DNA polymerase. Efficient release of viral RNA in this dirRT-qPCR assay is another factor in effective diagnosis. With conventional RT-PCR, lysis reagents such as TRIzol and a high temperature (85–95 °C) can promote the release of viral RNA (Bachofen et al., 2013; Nishimura et al., 2010). For example, HP-PRRSV RNA was effectively released at 55 °C in 30 min 22. However, pretreatment at high temperatures will lead to the degradation of RNA. A non-ionic detergent, such as Triton X-100, and ingredients such as NP-40 in the PEC-2 additionally facilitate the denaturation of viral capsid protein and release of the RNA 20.

The dirRT-qPCR for clinical samples simplifies the operation, supporting point-of-care diagnosis for on-site screening. Indeed, the uncontrolled spread of ZIKV in high population areas is an emerging

Table 7
Summary of the positive and negative predictive values, sensitivity, and specificity of the dirRT-qPCR assay.

dirRT-qPCR	Standard qPCR		
	Positive	Negative	
Positive	9	0	Positive predictive value = 9/9 = 1.00
Negative	0	74	Negative predictive value = 74/74 = 1.00
	Sensitivity = 9/9 = 1.00	Specificity = 74/74 = 1.00	

issue, as the transmission of ZIKV by *Aedes aegypti* and *Aedes albopictus* vectors in heavy traffic environments such as borders has already been observed in Asia (Dasti, 2016; Wu et al., 2011). This dirRT-qPCR method supports the high-throughput detection of ZIKV and other viral infectious diseases, as the primer–probe combination could also be extended. It ultimately realizes the goal of large-scale on-site screening of viral infection for early diagnosis and the prevention and control of epidemic viral outbreaks.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.06.007.

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