Establishment of micro droplet digital polymerase chain reaction and real-time fluorescence quantitative polymerase chain reaction technologies for detecting Zika virus

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Abstract: Establishment of diagnostic methods with low detection limits plays a critical role in the maintenance of early diagnosis, prevention of serious neurological complications, and control of the spread of ZIKA. In this study, we established the micro-droplet digital polymerase chain reaction (ddPCR) and real-time fluorescent quantification PCR (qPCR) protocols for the detection of Zika virus based on the NS5 gene. For the Zika standard
plasmid, the standard curve of $R^2$ was 0.999, and the amplification efficiency was 92.203%, as determined by qPCR. Both ddPCR and qPCR were positive for cell culture of Zika nucleic acid. The minimum detection limit of ddPCR is 1–2 times lower than qPCR. Moreover, all tests of Dengue virus (1–4 serotypes) were negative in cell culture. Overall, these results suggested that ddPCR may have a lower limit of detection than qPCR.

**Keywords:** Zika virus; nucleic acid detection; micro-droplet digital polymerase chain reaction; real-time fluorescence quantitative polymerase chain reaction

1. Introduction

Zika virus (ZIKA) is a single-stranded RNA virus belonging to the family *Flaviviride* genus *Flavivirus*. It was first isolated from a forest in Uganda in 1947 [1] and encodes three structural proteins (capsid protein C, precursor protein pr M, and envelope protein E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [2].

The NS5 gene is the largest gene in the ZIKA genome and encodes NS5 protein, which has the highest relative molecular weight of all proteins encoded by ZIKA. NS5 contains an RNA-dependent RNA polymerase active site at the N terminal and harbors a methylation site, which is associated with the viral cap reaction of the viral RNA. The ZIKA genome has only one open reading frame (ORF). NS5 is located at the 3' end of the ORF and is a nucleic acid detection target commonly used by the flavivirus [3]. Currently, several laboratories have established nucleic acid detection technologies for ZIKA; the main targets of detection are the E and NS5 genes [4]. The E gene has undergone several adaptive changes, whereas the NS5 gene is relatively stable. Therefore, the conserved sequence of NS5 gene may be more reliable as a detection target.

ZIKA is transmitted through mosquito bites, particularly those from *Aedes aegypti* and *A.*
and can also spread between independent mosquitoes [6]. More importantly, the virus can be transmitted from mother to fetus via amniotic fluid and fetal tissues [7] and can be transmitted through sexual activity [8]. In humans, ZIKA infection is characterized by mild fever (37.8–38.5°C), joint pain (particularly pain in the hands and feet), myalgia, headache, orbital pain, conjunctivitis, and skin rash and can also cause microcephaly and Guillain-Barré syndrome [9]. However, only 20% of infected individuals exhibit symptoms [10], and ZIKA infection can be a benign, self-limiting disease [11]. As a result, the incidence of ZIKA infection may be underestimated in epidemics or returning travelers. For the detection of ZIKA, Giada Rossini et al. [12] showed that the blood test period is longer after onset of the disease than urine or whole blood tests. ZIKA can be detected in whole blood in about 3–26 days and in the plasma in 3–10 days [13].

Accordingly, in this study, we aimed to establish micro-droplet digital polymerase chain reaction (ddPCR) and real-time fluorescent quantification PCR (qPCR) protocols for the detection of Zika virus based on the NS5 gene.

2. Materials and methods

2.1 Sample processing

Asian Zika virus Z16006 strain was provided by the Institute of Microbiology in the Center for Disease Control and Prevention of Guangdong Province, China (GenBank no. KU955589.1). Four serotypes of Dengue virus (DV1 Hawaii strain, DV2 NGC strain, DV3 H87 strain, and DV4 H241 strain) were preserved in our laboratory. The above viruses were all amplified and cultured in C6/36 cells. After observing the cytopathic effect, the cells were
repeatedly frozen and thawed three times, centrifuged and frozen at -80℃. Clinically positive
ZIKA blood samples were obtained from the Center for Disease Control and Prevention of
Jiangmen, Guangdong Province, China.

2.2 Instruments and reagents

The micro-ddPCR instrument, amplification premixed reagent, Droplet PCR Supermix, and
micro-drop reagent were all from Bio-Rad (Hercules, CA, USA). Reverse transcription was
performed using a TAKARA Prime Script RT reagent Kit (Perfect Real Time kit; TaKaRa,
Shiga, Japan), Bestar qPCR Master Mix (TaqMan Probe) (DBI Bioscience, Germany), and a
QuantStudio 6 Flex Real-Time PCR System (ThermoFisher, USA).

2.3 Design of primers and probes

The sequences of 30 strains of ZIKA were downloaded from GenBank and compared. After
identifying conserved sequences, primers and probes were designed using Primer 5.0 with the
ZI6006 strain as the reference strain. The primers and probes were designed using Oligo 7.

2.4 RNA extraction and reverse transcription

Samples were brought to room temperature before use. Viral RNA was extracted using the
QIAamp® (Qiagen, Hilden, Germany), with a final RNA volume of 50 μL. The RNA was
separated into aliquots and preserved at -80℃.

For reverse transcription, the components were prepared on ice, as follows: 2 μL
PrimeScript RT Enzyme Mix I, 0.5 μL Oligo dT Primer, 2 μL Random 6-mers, 4 μL total RNA,
and 1 μL RNase-free dH2O. The reaction conditions were as follows: reverse transcription at
37°C for 15 min, reverse transcriptase inactivation at 85°C for 5 s, and a final hold at 4°C.

2.5 ddPCR

For ddPCR, the 20μL reaction system contained 10 μL ddPCR Supermix (no dUTP), 6 μL primer probe premix (initial concentration of 10 μM upstream primer and 0.4 μL downstream primer, probe 0.2 μL, deionized water 5 μL), and 4 μL nucleic acid extract. After mixing, 20 μL of the sample reaction system was added to the middle of a DGB cartridge. Next, 70 μL oil was added to the bottom row of each lane to avoid the formation of bubbles, and the wells were covered. The reaction system and droplet-forming cartridge were placed in a droplet generator and subjected to micro-droplet treatment.

Droplets were generated in the top row of the wells, and the suction volume was adjusted to 40 μL. Samples were then slowly transferred to 96-well plates, and a preheated PX1 heating sealing device was used as a sealing film (with the red line up) at 180°C for 5 s. The PCR conditions were as follows: predenaturation at 95°C for 10 min, temperature change rate of 2°C/s; and 40 cycles of 94°C for 30 s and 55°C for 1 min, at 2°C/1°C/s. The 96-well plate containing the PCR-amplified products was then placed on a QX200 micro-drop reader and analyzed using Quanta Soft software.

2.6 Real-time fluorescent qPCR

For qPCR, reactions contained Bestar qPCR Master Mix (10 μL), upstream and downstream primers (0.4 μL each), probe (0.2 μL), template DNA (4 μL), and ddH2O (5 μL). The amplification conditions for qPCR were as follows: 95°C denaturation for 2 min, follows by 40 cycles of 95°C for 10 s and 60°C for 30 s.
3. Results

3.1 Design and evaluation of primers and probes

We downloaded 30 popular ZIKA strains, compared them, and found conserved sequences. Primers were designed according to the NS5 gene, keeping the GC content and Tm values as similar as possible. The length of the target fragment was within 100 bp without hairpin structures to avoid the formation of stable dimers and mismatches at the C-terminus. The designed primers are shown in Table 1.

Table 1. Primers and probe for ZIKA detection

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>ZK-F</td>
<td>5’T-GCCTTGCATCAGTCG-3’</td>
</tr>
<tr>
<td>ZK-R</td>
<td>5’T-ATGGAGCATCCGAGACT-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>5’T-FAM-TGGCAAGCTYCTTTATTTCCACARAAG-BHQ1-3’</td>
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3.2 Evaluation of the sensitivity of detection

Ten-fold dilutions of ZIKA plasmid standards (3.2 x 10^{10} copies/μL) were performed. Detection by qPCR showed that the standard curve R² value was 0.999, and the amplification efficiency was 92.203%. The relationship between the Ct value and the copy number was as follows: Y = -3.524X + 38.045, based on the standard curve (Figure 1).
Figure 1. The standard curve for fluorescent quantitative PCR. The concentration of each point on the standard curve increased 10-fold from left to right.

The initial concentration of $7.8 \times 10^6$ copies/μL was gradient diluted 5-fold from the cell culture ZIKA solution, and qPCR was used to test each sample in order to evaluate the detection method. The results are shown in Figure 2; a detection limit of about 100 copies/μL was obtained.

Figure 2. Results of 5-fold dilutions of ZIKA cell culture by qPCR.
Next, we used ddPCR to detect ZIKA from cell cultures, as described above for qPCR. As shown in Figure 3A, qPCR detected ZIKA with clear differences among sample concentrations. In Figure 3B, the threshold was set to 1000, with positive nucleic acid droplets showing values above 1000 and negative nucleic acid droplets showing values below 1000. As indicated in Figure 3C, almost all samples generated more than 10,000 droplets, suggesting that ddPCR may be more sensitive, with a detection limit of 1 copy/μL.

Figure 3A. Nucleic acid concentration.
Figure 3B. Scatter plot showing event numbers.

Figure 3C. Histogram showing event numbers.

Figure 3. Results of ddPCR for 5-fold dilutions of cell culture.

3.3 Evaluation of the accuracy of qPCR and ddPCR

Next, we evaluated the accuracy of the assays using four Dengue virus serotypes. All of qPCR results were negative. Figure 4 shows the results of ddPCR. As shown in Figure 4A,
nucleic acid concentrations detected by the absolute quantification method were all zero. Moreover, only negative micro-droplets were observed (Figure 4B), and histogram analysis showed that the total number of droplets was more than 10,000, of which none were positive (Figure 4C).

Figure 4A. Absolute quantitative detection.

Figure 4B. Scatter plot of the total number of droplets (from left to right: DV4, DV3, DV2,
DV1, N; N is the negative control).

**Figure 4C.** Total number of micro-droplets, as shown in a histogram (positive on left and total on right).

3.4 qPCR detection of Dengue virus samples in clinically positive blood samples

The lowest Ct value of clinically positive blood samples in qPCR analysis was 38.868 after 5-fold dilutions; this exceeded the detection range of qPCR (Ct values of 15–35; Figure 5).

**Figure 5.** Standard curve of qPCR. $R^2 = 0.998$, amplification efficiency = 91.226%, $Y = -3.552X + 39.06$. The red dots are plasmid standards, and the blue dots are positive blood samples.

DdPCR analysis showed that the sample concentration was 14.2 copies/μL (Figure 6).

**Figure 6A** shows the scatter plot of the events, and **Figure 6B** shows the concentrations based on absolute quantification. Finally, the histogram of the event number showed that the total
event number was 10923, with 131 positive events (Figure 6C).

**Figure 6A.** Scatter plot showing the event numbers.

**Figure 6B.** Concentrations determined by absolute quantification.

**Figure 6C.** Histogram showing event numbers.
4. Discussion

Approximately 20% of individuals infected with ZIKA may develop symptoms similar to other flaviviruses [11], presenting a great challenge to clinical diagnosis. There are two types of clinical diagnostic tests for ZIKA, serological and molecular detection. IgM specific neutralizing antibodies and antibody capture enzyme-linked immunosorbent assays can be used for qualitative detection of ZIKA IgM antibodies, but may crossreact with other flaviviruses, making the results difficult to explain. Therefore, molecular biology diagnosis has become a commonly used means of laboratory testing.

In molecular biology, traditional PCR can only roughly detect the amount of amplification after the reaction has ended and cannot be used to quantitatively detect nucleic acids in the sample. Real-time fluorescence qPCR technology utilizes changes in the fluorescence intensity of chemical substances in the reaction system to realize quantitative detection of nucleic acids. Xu et al. [14] used one-step SYBR Green real-time PCR for the detection of ZIKA with a detection limit of at least 1.0 PFU/mL (1 PFU is approximately equal to $2 \times 10^5$ RNA genome copies). However, this method allows the simultaneous detection of both specific and nonspecific PCR products and therefore produces false positives. Calvert et al. [15] used reverse transcriptase loop-mediated isothermal amplification to detect RNA from ZIKA as low as 1.2 copies/μL; however, they observed a very high false-negative rate. Our laboratory uses a probe-based real-time PCR method with a detection limit of approximately 100 copies/μL.
Micro-ddPCR detects nucleic acid molecules without relying on external standard curves based on the Poisson distribution principle. With the advantage of absolute quantitative, this method permits better accuracy at low concentrations without the need for preparing standard curves [16].

In this study, we designed specific primers and probes based on the NS5 gene of ZIKA and established a method for ZIKA nucleic acid detection using ddPCR and qPCR. Our results showed that good specificity could be obtained by designing primers and probes based on the NS5 gene and that these primers/probes could be used for detection the differential ZIKA and four serotypes of Dengue virus. Moreover, our findings demonstrated that ddPCR had good sensitivity but was inaccurate for samples with high concentrations. Thus, ddPCR may be more suitable for low viral loads. Thus, ZIKA, as well as four serotypes of Dengue virus, can be detected by analyzing the NS5 gene at concentrations of about 1–10^5 copies/μL.

In summary, in clinical samples with low concentrations of ZIKA, such as those at 1–3 days after infection, micro-droplet digital ddPCR may show the best diagnostic accuracy and sensitivity, whereas in routine analyses laboratory of viral nucleic acid detection, including analysis of clinical samples at more than 3 days after infection, fluorescence qPCR can be used.

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**Author Contributions:** Yuan Hui, Bao Zhang, Weiwei Xiao and Qinghua Wu conceived and designed the experiments; Wei Zhao and Junhe Liang contributed reagents/materials/analysis tools; Zhiming Wu, Zhiran Qin and Li Zhu performed the experiments, designed the experiments; Xujuan Li, Hanmin Han and Shiyu Feng performed part of the experiments, analyzed the data and wrote the paper.

**Competing interests:** The authors declare no conflicts of interest.

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