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Rapid and sensitive detection of Zika virus by reverse transcription loop-mediated isothermal amplification

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Background: Zika virus (ZIKV) is an arbovirus that recently emerged and has expanded worldwide, causing a global threat and raising international concerns. Current molecular diagnostics, *e.g.*, real-time PCR and reverse transcription PCR (RT-PCR), are time consuming, expensive, and can only be deployed in a laboratory instead of for field diagnostics.
Objectives: This study aimed to develop a one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) platform showing sensitivity, specificity, and more convenience than previous methods, being easily distributed and implemented.
Methods: Specific primers were designed and screened to target the entire ZIKV genome. The analytical sensitivity and specificity of the assay were evaluated and compared with traditional PCR and quantitative real-time PCR. Three different simulated clinical sample quick preparation protocols were evaluated to establish a rapid and straightforward treatment procedure for clinical specimens in open field detection.
Results: The RT-LAMP assay for detection of ZIKV demonstrated superior specificity and sensitivity compared to traditional PCR at the optimum reaction temperature. For the ZIKV RNA standard, the limit of detection was 20 copies/test. For the simulated ZIKV clinical samples, the limit of detection was 0.02 pfu/test, which was one order of magnitude higher than RT-PCR and similar to real-time PCR. The detection limit of simulated ZIKV specimens prepared using a protease quick processing method was consistent with that of samples prepared using commercial nucleic acid extraction kits, indicating that our ZIKV detection method could be used in point-of-care testing.
Conclusions: The RT-LAMP assay had excellent sensitivity and specificity for detecting ZIKV and can be deployed together with a rapid specimen processing method, offering the possibility for ZIKV diagnosis outside of the laboratory.

1. Introduction

Zika virus (ZIKV) is an arbovirus member of the *Flaviviridae family*, genus *Flavivirus*, which is related to Dengue fever, Yellow fever, West Nile fever, Japanese encephalitis, and Chikungunya viruses (Petersen et al., 2016), and it has two lineages (Asian and African) (Haddow et al., 2012). ZIKV is a single-stranded, positive-sense, RNA virus (Fleming et al., 2016) that causes common symptoms (*e.g.*, fever, rash, and joint pain) similar to those of many other diseases. Thus, it has not received much attention, and most ZIKV infections in humans are actually asymptomatic (Duffy, 2009). Recently, however, the emerging ZIKV outbreak in the Americas has attracted global attention.

ZIKV is primarily transmitted to people through the bite of infected *Aedes* species mosquitoes, but it can also be transmitted through blood, perinatally, and sexually (Haddow et al., 2012; Ibrahim, 2016). ZIKV was first identified and isolated from a

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rhesus monkey in Uganda in 1947 (Haddow et al., 2012), and the first isolation of ZIKV from humans was in Nigeria in 1954. Subsequently, sporadic human ZIKV infections occurred until a major epidemic on Yap Island, Micronesia in 2007 (Duffy, 2009; Hayes, 2009; Lanciotti et al., 2008). In 2015, ZIKV was identified in Brazil, and then spread rapidly to the Neotropics and other continents around the world, causing characteristic birth defects associated with microcephaly and Guillain-Barré syndrome related to neurological disorders (Calvet et al., 2016; Johansson et al., 2016; Rasmussen et al., 2016; Smith and Mackenzie, 2016; Victora et al., 2016). As of April 2016, a total of 4800 laboratory-confirmed, natural ZIKV infections have been reported in over 46 countries, and 13 ZIKV cases are known in China since the first patient was reported on February 5, 2016 (Zhang et al., 2016b). The genetic diversity of ZIKV may have gradually increased since late 2013 with its geographic expansion (Shi et al., 2016). On Feb 1, 2016, the World Health Organization (WHO) declared ZIKV as a public health emergency of international concern (Heymann et al., 2016; WHO, 2016).

Considering the above, ZIKV displays the potential to be introduced into new areas and cause an epidemic in the future. Thus, there is a critical need for a rapid, specific, and reliable diagnostic method to detect the virus. However, current diagnostic tests for ZIKV are limited (Pardee et al., 2016). The traditional diagnosis of ZIKV is based on serology, which has low specificity due to cross-reaction with other flaviviruses circulating in the region (Zammarchi et al., 2015). Viruses can also be directly isolated from humans, animals, and mosquitoes, but this is time consuming (Ibrahim, 2016). RT-PCR and real-time PCR are more sensitive and specific molecular diagnosis assays that have also been applied to confirm ZIKV infection (Faye et al., 2013, 2008; Lanciotti et al., 2008), but they must be deployed in a laboratory, not in the resource-limited field. Recently, a paper-based ZIKV molecular detection platform combining isothermal RNA amplification was developed that can be used in low-resource locations (Pardee et al., 2016). This detection platform resolves practical limitations to the deployment of diagnostics in the field, but the detection time is still too lengthy. In view of this, rapid and accurate molecular diagnostics outside of research laboratories are critically needed to monitor the ongoing ZIKV outbreak.

Loop-mediated isothermal amplification (LAMP) is a promising tool that is simple, efficient, and robust. The assay is generally conducted under isothermal conditions (60–65 °C) within 60 min, and its results can be analyzed by the naked eye through a color change of the reaction mixture (Nemoto et al., 2010). Further, LAMP assays can be performed with impure templates, decreasing sample processing times. Such assays have been widely used for the detection of various pathogens due to their low cost, simplicity, convenience, speed, sensitivity, and specificity (Liu et al., 2014, 2012; Livingstone et al., 2016).

In this study, we developed a one-step reverse-transcription LAMP (RT-LAMP) platform capable of detecting ZIKV specimens in the field and coupled it with a rapid and simple method for pretreatment of clinical samples. The detection platform can perform all sample processing and testing steps, utilizing a portable battery-powered metal bath within 1 h, and the results can be analyzed by the naked eye. This detection platform makes the ZIKV test procedure easier, faster, and more convenient for sample processing, the actual reaction step, and interpretation of the results. The test specifically detected ZIKV with no cross-reactivity to other arboviruses or influenza virus. These advantages suggest that our diagnostic platform is useful for identifying ZIKV cases in the laboratory or for point-of-care testing in endemic rural areas. The objective of this study was to develop a novel, portable, rapid, low-cost, and accurate ZIKV RT-LAMP detection platform, including a rapid nucleic acid release technique for clinical specimens.

2. Materials and methods

2.1. Viruses and simulated clinical specimens

The ZIKV strain (SZ_SMGC-1) used in this work was isolated from a serum sample of an imported case returning to China from Fiji and Samoa at the Shenzhen Port (Wang et al., 2016). The entire viral genome was sequenced (GenBank: KX266255). The plaque forming units (pfu) of the ZIKV stock solutions were titrated on Vero cells (African Green Monkey Kidney). The simulated clinical specimens were prepared by spiking ZIKV stocks into normal human saliva, urine, or serum (1%) at a final concentration of 2×10^4 pfu/mL. The saliva, urine, and serum samples were collected from healthy donors. Four different serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4) and influenza A virus strain PR8 were propagated in C6/36 and MDCK cells, respectively, and used to identify the specificity of the RT-LAMP assay because they cause similar clinical symptoms to ZIKV (Ibrahim, 2016). Viral RNAs were extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol.

2.2. Preparation of the reference RNA template

To prepare the ZIKV RNA standard, the NS1 gene fragment of ZIKV was synthesized (GenScript, Nanjing, China) and cloned into the *Nco*I and *Bam*HI sites of the pET15a vector. Then, the recombinant plasmid was linearized with *Eco*RI (New England Biolabs). RNA transcripts were generated using T7 RNA polymerase (Promega) according to the manufacturer's instructions. The transcripts were further treated with RNase-free DNase I (Promega) to remove any template plasmid DNA. After purification using an RNeasy clean up kit (Qiagen), the copy numbers of RNA transcripts were calculated based on the concentrations determined by a Nano-Drop 2000 spectrophotometer (Thermo Fisher Scientific). Serial dilutions of RNA were prepared and frozen at −80 °C.

2.3. Primer deign

Nucleic acid sequence alignments of ZIKV and other Flavivirus strains were performed using ClustalX. According to the sequence of ZIKV in GenBank (accession number: KU740184.2), primers specific to a highly conserved ZIKV region were designed using the online open source Primer ExploerV.4 software (Eiken Chemical, Japan, http://primerexploer.Jp/e/). They include an outer pair (F3, B3), an inner pair (FIP, BIP), and an addition loop pair (LF, LB).

2.4. Specimen processing

To determine the most effective technique for quick sample processing, we tested three different strategies: boiling lysis, proteolysis, and alkaline lysis. For thermal lysis processing, simulated clinical specimens were directly heated to 95 °C for 5 min. For proteolytic processing, proteinase K was added to the specimens to a final concentration of 20 μg/mL, incubated at 65 °C for 10 min, and inactivated at 95 °C for 2 min. The simulated serum specimens needed to be pre-diluted 10 times in water, but the other two types did not. For alkaline lysis processing, 180 μL of 50 mM NaOH was added to 20 μL of simulated specimen, mixed by vortexing, incubated at 95 °C for 10 min, and then 20 μL 1 M Tris-HCl (pH 8.0) was added to the mixture. The resulting lysates were directly used to initiate RT-LAMP reactions.

2.5. RT-LAMP

The RT-LAMP reactions were performed using a Loopamp RNA Amplification Kit (Eiken Chemical Co., Ltd., Japan) with 1.6 μM each