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Short Communications: Development of a universal and lineage-specific primer sets for Zika virus (ZIKV) rapid detection in blood and urine samples by using one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP)

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Short Communications: Development of a universal and lineage-specific primer sets for Zika virus (ZIKV) rapid detection in blood and urine samples by using one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP)

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**Summary**

Zika is a mosquito-borne disease that is causing significant public health threats in recent years. Zika virus (ZIKV), the causative agent of this disease, is classified into two distinct genetic lineages: Asian and African lineages. While molecular nucleic acid methods have been proved useful for the diagnosis of ZIKV infection, development of assays based on one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) offers advantages including shorter incubation times, ease of handling and rapid detection. In this study, a universal LAMP primer set was developed to target conserved sequence of known ZIKV lineages. Additionally, Af7462 and As1788 primer sets were designed based on LAMP-based SNPs typing for the specific detection of African and Asian lineages. The RT-LAMP assays detected specifically African and Asian lineages, with the limit of detection range from 0.17 FFU/ml – 2.3x10^2 FFU/ml. As ZIKV viremia ranges between 10^2 to 10^6 PFU/ml or 10^3–10^6 copies/mL, the data indicate that the viremia range of clinical samples is within our detection range. Because of the high specificity and sensitivity and ease of use, the results suggest the utility of the assay in early clinical diagnosis applications.
Main Text

The emerging of Zika, an arthropod-born disease caused by Zika virus (ZIKV) recently has been raising a global healthcare concern (1). ZIKV infection may be asymptomatic or cause fever, rash, and conjunctivitis. Between two distinct genetic lineages of ZIKV: Asian and African, the infection with Asian lineage was associated with Guillain-Barré syndrome and microcephaly in newborns (2-4).

The Loop-mediated Isothermal Amplification (LAMP) is a technique for the amplification of a target DNA at a constant temperature between 60°C – 65°C, using four primers recognizing six distinct regions: outer pair (F3, B3) and inner pair (FIP, BIP). As a result, abundant of cauliflower-like amplicons were produced and visualized by the naked eye or under UV light due to the presence of magnesium pyrophosphate. To accelerate the amplification, loop primers LB and LF are added to the reaction. In this regard, the development of LAMP assays provided advantages of short time incubation, simple handling, and detection (5). Sharing the same principle of LAMP but with a modification at 5’ end of inner primers, the LAMP-based SNPs (single-nucleotide polymorphism) typing allows it discriminate two sequences with one different nucleotide (6). Notably, the RT-LAMP was developed to amplify the RNA template and was applied successfully to detect a variety of viral RNA such as dengue virus, Ebola virus (7, 8). In this study, we aimed to develop a one-step RT-LAMP assay to detect all two ZIKV lineages and RT-LAMP-based SNPs typing to distinguish each of them.

Asian lineage ZIKV strains PRVABC59 (KX601168), MRS_OPY_Martinique_Pari_2015 (KU647676) and H/PF/2013 (KJ776791) and African lineage strain MR766 (LC002520) were propagated in baby hamster kidney cell line-21 (BHK-21) and then were titrated by focus forming assay. Pooled sera (Innova Inc., Michigan USA) or
urine samples were spiked with ZIKV culture fluid at determined virus titre (FFU/ml). The viral RNA was extracted by QIAmp viral RNA mini kit (Qiagen, Germany) and was titrated by real-time PCR (9). Twelve and 118 ZIKV nucleic acid sequences of Asian and African lineages, respectively from Genbank was aligned by CLC Sequence viewer (Qiagen). The conserved regions were subjected to Primer Explorer V.5 software (Eiken, Japan) to design three LAMP primer sets (Universal set for all lineages, As1788 and Af7462 set for Asian and African lineage, respectively) (Table 1). A single step RT-LAMP reaction was performed using 5µl of RNA template and Loopamp RNA Amplification Kit (Eiken, Japan) with 1.6 µM each of the FIP and BIP, 0.2 µM each of F3 and B3, 0.8 µM each of LF and LB. However, Af7642LB and Af7642LF were not included in the tube due to non-specific reactions. The temperature was maintained at 61°C for 60 minutes. Real-time turbidity changes in reaction tube were recorded by a Loopamp Realtime Turbidimeter LA-200 (Eiken, Japan). The sample was interpreted as positive when the OD value was ≥0.06.

Using the RNA template prepared from culture fluids, the universal primer set was able to amplify ZIKV RNA of both Asian and African lineage (Fig. 1a). Notably, the primer set Af7462 was able to amplify the MR766 template but not Asian lineage strains (Fig 1b). In contrast, increased turbidity was observed only in the reaction tubes with primer set As1788 and Asian lineage RNA templates and but not with African lineage RNA template (Fig. 1c). No amplification was seen in the reaction of three primer sets with other Flavivirus (dengue virus 1, 2, 3, 4 strain VN/2013/Hue265, VN/2013/Hue552, VN/2013/Hue400, VN/2013/Hue1221, respectively, yellow fever virus vaccine strain 17D, Japanese encephalitis virus strain JaOArS982), or arbovirus (chikungunya virus S27-African prototype), suggesting that these
primer sets specifically detect all Asian and African lineages of ZIKV or discriminate the two of them (Fig 1).

Next, we evaluated the sensitivity of the assay using a series of 10-fold dilution of ZIKV RNA strains MRS_OPY_Martinique_Pari_2015 and MR766 extracted from spiked serum/urine sample. The lowest point was continuously diluted at 2-fold dilution to examine the limit of detection (LOD). Results in Table 2 show that the Universal primers detect as low as 30-100 and 40-69 genome copies/test for Asian and African lineage, respectively. The LOD for the As1788 and Af7462 was $2.6 \times 10^3$ and $1 \times 10^4$ genome copies/test, respectively. The differences in the sensitivity between universal and lineage-specific primer set could be explained by the more strict-amplification condition of the primers BIP and FIP in As1788 and Af7462 set. Next, we compared the sensitivity of the RT-LAMP assay with nested RT-PCR (10) and real-time RT-PCR (9). In our system, the LOD of the Universal primer set is comparable to that of real-time RT-PCR while the LOD of a lineage-specific primer set was comparable to that of nested RT-PCR (Table 2). The RT-LAMP in this study detected ZIKV RNA down to $0.17-2.3 \times 10^2$ FFU/ml depending on primer set and type of sample (Table 2) and is comparable to those reported by other investigators (11). The ZIKV viremia during acute infection range from $10^2-10^6$ PFU/ml (12, 13) or $10^3-10^6$ copies/mL (14), indicating that the viremia range of clinical samples is within our detection range. Thus, the results suggest the utility of the assay in the detection of ZIKV RNA in clinical samples. While further studies are needed to define the viral determinants that influence disease severity in human, a recent study has demonstrated that a single amino acid substitution in the pre-membrane region of ZIKV Asian lineage may correlate with disease severity and fetal microencephaly in mice (15). Further manipulations of the RT-LAMP-based
SNPs typing method developed this study would allow discrimination of SNPs and detection of ZIKV mutations.

In conclusion, in this study, we developed three RT-LAMP assays that could detect specifically either both African and Asian lineages of ZIKV or each of them. Because of the high specificity and sensitivity as well as ease of use, the results suggest the utility of the assay in early diagnosis applications.
Conflict of interest

The authors declare that they have no competing interests.

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Ethical considerations

This study was approved by the Institutional Review Board of NEKKEN, Nagasaki University (EAN: 08061924–7).
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Figure legend

**Figure 1.** The implementation of RT-LAMP primers. (a) Universal primers specifically detect ZIKV RNA of African and Asian lineage. (b-c) Af7642 and As1788 primers exclusively detect ZIKV African lineage and Asian lineage, respectively. OD value of ≥0.06 is interpreted as positive.
Table 1. RT-LAMP Primer sets used in the study.

| Primer Set                  | Sequence                                      |
|----------------------------|-----------------------------------------------|
| **Universal primer set**   |                                               |
| UF3                        | TTGGGGAAAGCTGTGCAG                             |
| UB3                        | AAAGGGTGGAAGGCTGTCG                             |
| UFIP                       | GGCTTYTTCCGTGCGCATGGCAGAGAAGCTGGGAAACC         |
| UBPF                       | CTGCCGTGAGCCCTCGATCTTCTCCATCTGCGC              |
| ULF                        | TTMTCGCGCTGACTAT                               |
| ULB                        | AAAACCCCAYGCCTTGGGA                           |
| **Af7462 primer set**      |                                               |
| 7462F3                     | CGTTGTGGGATGGAAATAGTGG                        |
| 7462B3                     | CAAGGGGAGGGTYGCTGCGW                         |
| 7462FIP                    | AGCACTGGAGAYRGCTACTARGTGAGGAAGAGATGGGAC       |
| 7462BIP                    | TGTGCTRCTGCRACYGCPCCAGCTCCCCCCT              |
| **As 1788 primer set**     |                                               |
| 1788F3                     | ACACGGGCCCTTGTGGAG                             |
| 1788B3                     | TTRTGTAATGTGAACGCTGC                         |
| 1788FIP                    | ACATTTCAAGTGCCAGAGGAGCTGAGATGGAGATGGGwGC     |
| 1788BIP                    | TCGCCTGAAAATGGATAAACTCAAGGAGGTGACACGCCCC     |
| 1788LF                     | ACAGCCTTCCCTT                                 |
| 1788LB                     | TAGATTGAA                                    |

* Base on the conserved sequence at 3’-UTR (Untranslated region) of ZIKV, the universal primer set (Universal set) detecting all lineages was designed using Primer Explorer V.5 software (Eiken, Japan).

** To discriminate the two ZIKV lineages, a SNPs at position 1788 in Envelope region and 7462 in NS4A region that were conserved and specific for ZIKV Asian and African lineage, respectively, were allocated to design the 5’ end of inner pair primers (FIP and BIP).
Table 2. Limit of detection of RT-LAMP primer sets developed in this study as compared to that of nested PCR and quantitative real-time PCR*.

| Virus RNA          | Lineage | Assay          | FFU*/test | FFU/ml  | RNA copies/ test |
|-------------------|---------|----------------|-----------|---------|------------------|
| 1. Universal primer set |         |                |           |         |                  |
| Spiked serum       | Asian   | RT-LAMP        | 0.002     | 0.2     | 3.0 x 10^1       |
|                    | African | RT-LAMP        | 0.01      | 0.9     | 4.0 x 10^1       |
| Spiked urine       | Asian   | RT-LAMP        | 0.006     | 0.6     | 1.0 x 10^2       |
|                    | African | RT-LAMP        | 0.017     | 1.5     | 6.9 x 10^1       |
| 2. Asian lineage-specific primer set SNP1788 |         |                |           |         |                  |
| Spiked serum       | Asian   | RT-LAMP        | 0.2       | 17.0    | 3.0 x 10^3       |
| Spiked urine       | Asian   | RT-LAMP        | 0.2       | 12.7    | 2.6 x 10^3       |
| 3. African lineage-specific primer set SNP7462 |         |                |           |         |                  |
| Spiked serum       | African | RT-LAMP        | 2.7       | 2.3 x 10^2 | 1.1 x 10^4   |
| Spiked urine       | African | RT-LAMP        | 2.3       | 2.0 x 10^2 | 1.0 x 10^4   |
| 4. Comparison of with other molecular assays |         |                |           |         |                  |
| Spiked serum       | Asian   | Nested PCR     | 0.4       | 36.8    | 6.5 x 10^3       |
|                    | African | Nested PCR     | 0.05      | 4.7     | 2.2 x 10^3       |
| Spiked urine       | Asian   | Nested PCR     | 0.8       | 67.9    | 1.2 x 10^4       |
|                    | African | Nested PCR     | 0.05      | 4.7     | 2.2 x 10^3       |
| Spiked serum       | Asian   | qReal-time PCR  | 0.06      | 5.3     | 9.5 x 10^2       |
|                    | African | qReal-time PCR  | 0.1       | 10.7    | 5.0 x 10^2       |
| Spiked urine       | Asian   | qReal-time PCR  | 0.02      | 2.3     | 4.2 x 10^2       |
|                    | African | qReal-time PCR  | 0.2       | 20.2    | 9.4 x 10^2       |

*The LOD experiment was conducted using the RNA template of MRS.OPY_Martinique_Pari_2015 strain as ZIKV Asian lineage and MR766 strain as ZIKV African lineage.

*FFU indicates focus forming units.
Figure 1.

a) Universal primer set

b) Af7462 primer set

c) As1788 primer set

- PRVABC59
- Martinique
- H/PF/2013
- MR 766
- DENV-1
- DENV-2
- DENV-3
- DENV-4
- YFV
- JEV
- CHIKV
- DW