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Short Communication

Development of Universal and Lineage-Specific Primer Sets for Rapid Detection of the Zika Virus (ZIKV) in Blood and Urine Samples Using One-Step Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)

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SUMMARY: Zika is a mosquito-borne disease that has been posing a significant threat to public health in recent years. The Zika virus (ZIKV), the causative agent of this disease, is classified into 2 distinct genetic lineages, namely Asian and African. While molecular nucleic acid analysis methods have been shown to be useful for the diagnosis of ZIKV infection, the development of assays based on one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) offers several advantages, such as shorter incubation times, ease of handling, and rapid detection. In this study, a universal LAMP primer set was developed to target conserved sequences of known ZIKV lineages. Additionally, the Af7462 and As1788 primer sets were designed based on LAMP-based single-nucleotide polymorphism (SNPs) typing for the specific detection of the African and Asian lineages. The developed RT-LAMP assays could specifically detect the African and Asian lineages of ZIKV, with a detection limit ranging from 0.17 FFU/mL to 2.3×10^2 FFU/mL. As ZIKV viremia ranges between 10^2 to 10^6 PFU/mL or 10^3 to 10^6 copies/mL, the data indicate that the viremia range of clinical samples is within the detection range of our assay. Due to the high specificity and sensitivity, as well as the ease of use of our assay, it could potentially be used for early clinical diagnosis applications.

The emerging of Zika, an arthropod-born disease caused by the Zika virus (ZIKV), has recently become a rising global healthcare concern (1). ZIKV infection may be asymptomatic or cause fever, rashes, and conjunctivitis. ZIKV has to distinct lineages, namely Asian and African. However, between the two, infections with the Asian lineage was associated with Guillain-Barré syndrome and microcephaly in newborns (2–4).

The Loop-mediated Isothermal Amplification (LAMP) is a technique used for the amplification of a target DNA sequence at a constant temperature between 60˚C– 65˚C, using 4 primers: an outer pair (F3, B3) and inner pair (FIP, BIP), which recognizing 6 distinct regions. As a result, abundant cauliflower-like amplicons are produced, which can be visualized with 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. As such, the development of LAMP assays provided advantages such as short incubation times, as well as simple handling and detection (5). Based on the same principle as LAMP, but with a modification at 5′ end of inner primers, the LAMP-based SNPs typing permits discrimination between 2 sequences which differ by 1 nucleotide (6). Notably, the RT-LAMP assay was developed to amplify RNA templates and was successfully applied for the detection of a variety of viral RNAs, such as the Dengue and Ebola virus (7,8). In this study, we aimed to develop a one-step RT-LAMP assay to detect both ZIKV lineages and RT-LAMP-based SNPs typing to distinguish between the 2 lineages.

Asian lineage ZIKV strains PRVABC59 (KX601168), RS_OPY_Martinique_Pari_2015 (KU647676), and H/PF/2013 (KJ776791) and African lineage strain MR766 (LC002520) were propagated in the baby hamster kidney cell line-21 (BHK-21) and were then titrated via a focus forming assay. Pooled sera (Innova Inc., West Bloomfield, MI, USA) or urine samples were spiked with ZIKV culture fluid at a determined virus titer (FFU/mL). The viral RNA was extracted using the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) and was titrated via real-time PCR (9). Twelve and 118 ZIKV nucleic acid sequences of the Asian and African lineages, respectively, were obtained from GenBank and aligned using the CLC Sequence viewer (Qiagen). The conserved regions were entered into the Primer Explorer V.5 software (Eiken, Tokyo, Japan) to design 3 LAMP primer sets (Universal set for all lineages, and the As1788 and Af7462 set for the Asian and African lineages, respectively) (Table 1). A single step RT-LAMP reaction was performed using 5μL of RNA template and the Loopamp RNA Amplification Kit (Eiken) with 1.6 μM each of the FIP and BIP primers, 0.2 μM each of F3 and B3 primers, and 0.8 μM each of LF and LB primers. However, Af7642LB and Af7642LF were not included

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Table 1. RT-LAMP Primer sets used in the study

Universal primer set[1)]	
UF3	TTGGGGAAAGCTGTGCAG
UB3	AAAGGGTGGGGAAGGTCG
UFIP	GGCTTYTTCCGTGCCATGGCAGGAGAAGCTGGGAAACC
UBIP	CTGCCTGTGAGCCCCTCAGATCTTTTCCCATCCTGCGC
ULF	TTMTCGGCCTGACTAT
ULB	AAAACCCCAYGCGCTTGGA
Af7462 primer set[2)]	
7462F3	CGTTGTGGATGGAATAGTGG
7462B3	CAAGGTGGAGGTYGCTGCW
7462FIP	AGCACTGGAGAYRGCTACTARGTGGAGAAGAAGATGGGAC
7462BIP	TGTGCTRCTGCGRACYGCYCCAGCCTCCCCCCAT
As 1788 primer set[2)]	
1788F3	ACACGGCCCTTGCTGGAG
1788B3	TTRGTGAATGTGAACGCTGC
1788FIP	ACATTTCAAGTGGCCAGAGGAGGCTGAGATGGATGGwGC
1788BIP	TCGCCTGAAAATGGATAAACTCAAGGAGTATGACACGCCC
1788LF	ACAGCCTTCCCTT
1788LB	TAGATTGAA

[1)]: Based 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, Tokyo, Japan).
[2)]: To discriminate the 2 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).

in the tube due to non-specific reactions. The reaction was then incubated at a constant temperature of 61°C for 60 min. Real-time turbidity changes in the reaction tube were recorded via the Loopamp Realtime Turbidimeter LA-200 (Eiken). The sample was considered positive when the OD value was ≥ 0.06.

This study was approved by the Institutional Review Board of NEKKEN, Nagasaki University (EAN: 08061924–7). Using the RNA template prepared from culture fluids, the universal primer set was able to amplify the ZIKV RNA of both the Asian and African lineages (Fig. 1A). Notably, the Af7462 primer set was able to amplify the MR766 template but not the Asian lineage strains (Fig 1B). In contrast, increased turbidity was observed only in the reaction tubes with the As1788 primer set and Asian lineage RNA templates and but not in the tubes with the African lineage RNA template (Fig. 1C). No amplification was detected when analyzing the reaction between the 3 primer sets and other *Flavivirus* (dengue virus 1, 2, 3, 4 strain VN/2013/Hue265, VN/2013/Hue552, VN/2013/Hue400, and VN/2013/Hue1221, respectively, yellow fever virus vaccine strain 17D, and Japanese encephalitis virus strain JaOArS982) or arbovirus (chikungunya virus S27-African prototype), suggesting that these primer sets specifically detect both the Asian and African lineages of the ZIKV and can discriminate between them (Fig 1).

Next, we evaluated the sensitivity of the assay using a series of 10-fold dilutions of ZIKV RNA strains MRS_OPY_ Martinique_Pari_2015 and MR766 extracted from spiked serum/urine samples. The lowest point was continuously diluted at a 2-fold dilution to examine the limit of detection (LOD). As shown in Table 2, our results revealed that the Universal primers have a detection limit of 30–100 and 40–69 genome copies/test for Asian and African lineages, respectively. The LOD for the As1788 and Af7462 primers was 2.6×10^3 and 1×10^4 genome copies/test, respectively. The sensitivity differences between the universal and lineage-specific primer sets could be explained by the more strict-amplification conditions of the BIP and FIP primers in the As1788 and Af7462 set. Next, we analyzed the sensitivity of the RT-LAMP assay compared to nested RT- PCR (10) and real-time RT-PCR (9). When using the developed assay, the LOD of the Universal primer set was comparable to that of real-time RT-PCR, whereas the LOD of a lineage-specific primer set was comparable to that of nested RT- PCR (Table 2). The RT-LAMP assay used in this study could detect ZIKV RNA at values as low as $0.17–2.3 \times 10^2$ FFU/mL depending on the primer set and type of sample (Table 2), which is comparable to results reported by other studies (11). The ZIKV viremia during acute infections ranges 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 the detection range of our assay. Thus, our results suggest that the assay could be used for the detection of ZIKV RNA in clinical samples. While further

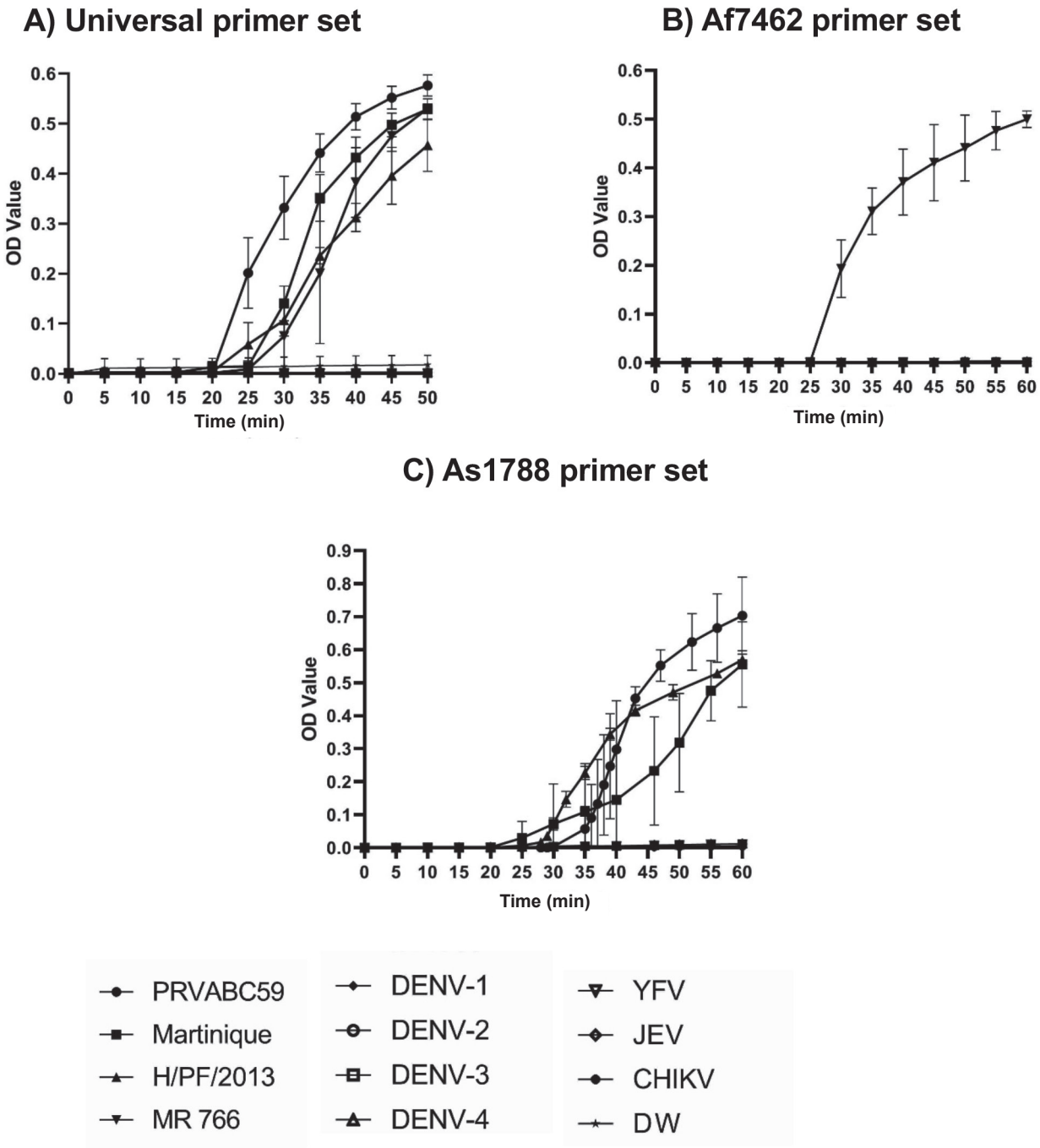

Fig. 1. The implementation of RT-LAMP primers. (A) Universal primers specifically detect ZIKV RNA of African and Asian lineage. (B,C) Af7642 and As1788 primers exclusively detect ZIKV African lineage and Asian lineage, respectively. OD value of ≥ 0.06 is interpreted as positive.

studies are needed to define the viral determinants that influence disease severity in humans, a recent study has demonstrated that a single amino acid substitution in the pre-membrane region of the Asian lineage of ZIKV may be correlated with disease severity and fetal microencephaly in mice (15). Further manipulations of the RT-LAMP-based SNPs typing method developed in this study would allow identification of unique SNPs and detection of ZIKV mutations.

In conclusion, we developed three RT-LAMP assays that could specifically detect both the African and Asian lineages of ZIKV, as well as discriminate between the two. Because of the high specificity and sensitivity, as well as ease of use, our results indicate that this assay could be used for early diagnosis applications.

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[1)]

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

[1)]: 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.
[2)]: FFU indicates focus forming units.

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Conflict of interest None to declare.

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