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LAMP Detection of Virus-Derived DNA of Zika Virus in Vector Mosquito

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Detection of infectious viruses in mosquitoes is one of the prerequisite measures to monitor the prevalence of vector-borne viral diseases. Determining which mosquitoes are currently infected with arboviruses such as Zika, dengue, and chikungunya virus is not yet practical in endemic areas due to multiple causes including the difficulty of dealing with the virus' unstable RNA. In this study, instead of handling viral RNA, virus-derived DNA (vDNA) was introduced as a target template for nucleic acid amplification. In combination with loop-mediated isothermal amplification (LAMP), we examined a LAMP-based vDNA detection assay (vDNA-LAMP) targeting Zika virus (ZIKV). The vDNA-LAMP reaction amplifying part of the NS3 region of ZIKV successfully detected its vDNA from crude DNA purified from artificially infected cultured cells and *Aedes* mosquitoes. This rapid, simple, and versatile method may provide a promising field-surveillance method for arbovirus circulation *via* vector mosquitoes.

Keywords: Zika virus, vDNA, RNA, mosquito, loop-mediated isothermal amplification, arbovirus

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INTRODUCTION

Zika virus (ZIKV) is one of the family Flaviviridae, genus *flavivirus*, which is closely related to dengue virus. Infection with ZIKV in humans passes with an asymptomatic course in most cases. However, ZIKV infection during pregnancy causes microcephaly and adverse fetal outcomes (1). Unlike the dengue virus, for which the natural vertebrate host is humans, ZIKV outbreaks are often a result of spillover from sylvatic reservoirs (2). ZIKV transmission is expected to be induced primarily by blood feeding of *Aedes* mosquitoes including *Aedes aegypti* and *Ae. albopictus*. Vertical transmission of ZIKV in *Aedes* mosquitoes, like other flaviviruses including dengue, has been demonstrated by laboratory studies (3–5). Moreover, it has been reported that wild *Ae. aegypti* larvae carried ZIKV, presenting natural vertical transmission (6). The emergence of ZIKV-infected larvae was also demonstrated by artificial contamination of water-inhabiting larvae with ZIKV, suggesting that infection of mosquitoes occurred without blood feeding (7). Thus, hindering the authentic and alternative ZIKV transmission cycles *via* mosquitoes may mitigate this increasing health burden.

Routine surveillance of potential vector populations is one of the priorities for prevention strategies and monitoring virus circulation to manage endemic situations. However, detection or surveillance of ZIKV in field-caught mosquitoes is not yet routine in endemic areas, including those with high transmission. Several studies reported the detection of ZIKV viral RNA in mosquitoes using

conventional RT-PCR or real-time RT-PCR (8–13). Possible explanations for the lack of ZIKV detection from wild mosquitoes were proposed: delay between identifying illness in people and conducting field investigations, difficulty in approaching households with patients, inefficient sampling methods, and the prompt spraying of houses once cases were identified (14). In addition, because RNA is more unstable and prone to degradation than DNA, we also expect difficulty in storing and handling RNA in endemic areas. The genome of ZIKV is RNA, and prospective monitoring of ZIKV in wild mosquitoes may require trained people, appropriate tools to store RNA, and special equipment for RNA handling. These requirements likely prevent on-site mosquito monitoring in endemic area, and the establishment of a faster and easier measure will be required for fruitful surveillance.

Virus-derived DNA (vDNA), a newly identified form in the life cycle of RNA virus infecting arthropods, was first discovered in *Drosophila melanogaster* infected with Flock House Virus (FHV) (15). Evidence of the generation of vDNA during other arbovirus infections in mosquitoes has also been reported from studies using conventional PCR (16, 17). Given that the presence of vDNA may indicate the presence of an RNA virus, vDNA may be a suitable candidate target for molecular surveillance because of its greater stability.

In this study, we applied the loop-mediated isothermal amplification (LAMP) method to detect vDNA instead of viral RNA in arbovirus-infected mosquitoes. LAMP is a simple and rapid DNA detection method employing *Bst* DNA polymerase that enables DNA amplification in isothermal conditions with high sensitivity and specificity (18). It has been applied for detecting malaria parasite in artificially infected mosquitoes and filaria in both artificially infected and wild vector mosquitoes (19, 20). We demonstrated that LAMP detected vDNA of ZIKV from both cultured cells and vector mosquitoes, making LAMP targeting vDNA (named vDNA-LAMP) a candidate method applicable for on-site surveillance of mosquitoes carrying arboviruses.

METHODS

Virus and Cell Culture

Two standard ZIKV strains, MR-766-NIID (African lineage) and PRVABC59 (Asian lineage), were used in this study. ZIKV were passaged in *Ae. albopictus* C6/36 cells twice following in African green monkey kidney Vero cells (ATCC) once to generate viral stocks. C6/36 cells were cultured in D-MEM, high glucose (Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Vero cells were cultured in E-MEM supplemented with 10% FBS, MEM Non-Essential Amino Acids Solution (Thermo Fisher Scientific Inc.), and penicillin-streptomycin.

Mosquito Rearing

The Higgs strain of *Ae. aegypti* was used in this study. Mosquito larvae were reared at 28°C and fed with a mixture of yeast powder (Taiwan Sugar Corporation) and goose liver powder (#7573, NTN) in a 1:1 ratio. Adults were maintained in a temperature and humidity controlled room (28°C and ~70% relative humidity) with a 12-hour light/dark cycle and provided with a constant 10% sucrose solution.

Viral RNA Extraction and cDNA Synthesis

Viral RNAs were extracted from viral stocks using High Pure Viral Nucleic Acid Kit (Roche Ltd.), following the manufacturer's instructions but without Proteinase K treatment. cDNA was synthesized from ZIKV RNA as follows: 1 μg of extracted RNA dissolved in 8.5 μl of RNase-free water was incubated with a reaction mixture [3 μl of 40 μM Random Primers (Thermo Fisher Scientific Inc.), 6 μl of 5× First-Strand Buffer (Thermo Fisher Scientific Inc.), 1.9 μl of 0.1 M DTT (Thermo Fisher Scientific Inc.), and 7.5 μl of dNTP Mixture (Takara Bio Inc.)] at 65°C for 5 min. Then, 0.6 μl of RNase inhibitor (Promega Co.) and 0.5 μl of M-MLV reverse transcriptase (Thermo Fisher Scientific Inc.) were added to the reaction mixture and incubated at 37°C for 90 min.

Virus Infection

C6/36 cells were seeded in the culture flask and incubated at 28°C. Infection with the MR strain was performed at multiplicities of infection (MOI) of 0.1 or 1. For the PR strain, accurate MOI of the viral stock was not determined, and a series of 10-fold dilution of the viral stock was used for infection. Infected cells were kept at 28°C for 5 days.

A natural vector species transmitting ZIKV, *Ae. aegypti*, was used in this study. For oral infection, 1×10^7 PFU/mL virus stock (PR strain) was 1:1 mixed with mouse blood and fed to female mosquitoes at 37°C for 30 min *via* a membrane-covered metal plate. Blood-engorged mosquitoes were kept at 28°C for 10 days post infection by feeding with sugar solution. These mosquitoes were killed, dried for 12 hours at room temperature, and stored at -20°C until DNA extraction.

DNA Extraction

DNA was extracted from ZIKV-infected cultured cells and mosquitoes as follows. Briefly, cells or mosquitoes were homogenized with a plastic homogenizer in 100 μl of Buffer A (0.1 M Tris (pH 9.0), 0.1 M EDTA, 1% SDS, and 0.5% DEPC), and incubated for 30 min at 70°C. Next, 22.4 μl of 5 M KOAc was added to the mixture, followed by incubation of the mixture for 30 min on ice. Supernatant was collected by centrifugation at 20,400 × g for 15 min at 4°C, and then mixed with 45 μl of isopropanol. Precipitated DNA was collected after centrifugation at 20,400 × g for 20 min at 4°C, rinsed with 70% ethanol, and dried. Each DNA pellet was dissolved and diluted with TE to adjust the concentration so that 1 μl of solution contained DNA from 1.2×10^4 infected cultured cells or from one-fiftieth of each mosquito pool. One microliter of each DNA solution was used as a template for the LAMP reaction.

LAMP Reaction

Primers for the LAMP reaction were designed using Primer Explorer V5 (Fujitsu Ltd.). LAMP reactions were performed as described in the manufacturer's instructions (Eiken Chemical

Co., Ltd.) with a half volume. Briefly, each reaction was performed in a total volume of 12.5 µl of reaction mixture containing 20 pmol of each FIP and BIP primers, 2.5 pmol of each F3 and B3 primers, 10 pmol of each Loop-F and Loop-B primers, and 6.25 µl of 2× reaction mixture (**Table 1**). For DNA amplification, the reaction mixture also contained 1.0 µl of extracted DNA solution and 0.5 µl of *Bst* DNA polymerase. For LAMP with reverse transcription step, the reaction mixture contained 2.5 µl of extracted RNA solution and 0.5 µl of enzyme mix (*Bst* DNA polymerase and AMV reverse transcriptase). Each reaction mixture was incubated at 62°C for 60 min or 90 min and terminated by incubation at 80°C for 5 min using Loopamp Realtime Turbidimeter (LoopampEXIA; Eiken Chemical Co., Ltd.). Amplified products of the reaction were examined by electrophoresis in 2% agarose gels. The gels were stained with ethidium bromide and visualized under UV light.

RESULTS

Detection of Viral cDNA Using LAMP

To develop vDNA-LAMP method for detecting various ZIKV strains, we used a primer set we reported previously (21). Full genomic RNA sequences of ZIKV MR-766-NIID strain (GenBank: LC002520.1) and PRVABC59 (GenBank: KU501215.1) strain were aligned to find high consensus sequences for making LAMP primers (**Supplementary Figure 1**). Several candidate primer sets were made based on the sequence of the MR strain, and eventually one of the primer sets targeting the NS3 region was selected due to its adequate sensitivity (21) (**Figure 1** and **Table 1**).

Prior to detecting vDNA, viral cDNA of ZIKV was prepared and provided for the LAMP reaction. Serially diluted RNA (3×10^2 ng, 3×10^1 ng, and 3×10^0 ng) of each strain was transcribed to form cDNA using reverse transcriptase, followed by the LAMP reaction to detect these cDNAs under optimized conditions (60 min at 62°C) in one-step. The target region of ZIKV was amplified from all cDNA samples, suggesting that the primer set can be applicable to vDNA-LAMP (**Supplementary Figure 1B**).

LAMP Detection of Virus-Derived DNA in ZIKV-Infected Culture Cells

To examine whether LAMP can amplify vDNA of ZIKV, we first attempted to detect vDNA in cultured mosquito cells that were spiked with the virus. DNA was extracted from cultured *Aedes* C6/36 cells at 5 days post infection with ZIKV MR or PR strain and provided as a template for the LAMP reaction (**Figure 2A**). LAMP reaction was performed in conditions that was optimized for the detection of ZIKV cDNA. Amplified products were examined by electrophoresis, showing the presence of DNA fragments derived from both ZIKV MR and PR strains (**Figure 2B**). These data suggested that vDNA-LAMP is sufficient to detect the vDNA form of ZIKV, which is generated in mosquito cells.

TABLE 1 | Sequences of primers for LAMP designed in accordance with the nucleotide sequence of the ZIKV MR strain.

Primer	Sequence (5'-3')
F3	TTA TGA CTG CCA CAC CAC C
B3	GCT GAG CTG TAT GAC CCG
FIP (F1c-F2)	GGC TCT CTC TGG GAC TTC CAC TGA ACC CGT GAT GCG TTT CC
BIP (B1c-B2)	TTG GTT CGT TCC AAG CGT GAG ATT TCC AGC CTT TGT CAG ACA
Loop-F	GTC CAT GAT TGG TGA GTT AGA GTC A
Loop-B	AAC GGA AAT GAA ATC GCA GCC

Application of vDNA-LAMP to ZIKV-Infected Mosquitoes

To evaluate the usability of vDNA-LAMP in virus-transmitting mosquitoes, we attempted to detect ZIKV vDNA from experimentally infected mosquitoes. *Ae. aegypti* female mosquitoes were orally exposed to ZIKV PR strain by artificial membrane feeding using infected blood. At 10 days post infection, mosquitoes were killed and pooled in 12 groups (5 mosquitoes per group). These mosquitoes were dried at room temperature for 12 hours and then kept in the freezer. DNA was extracted from each group and then subjected to the LAMP reaction (**Figure 3A**). As a result, out of 12 groups of infected blood-fed mosquitoes, 5 groups were identified as positive by vDNA-LAMP reaction and following electrophoresis, suggesting that vDNA transcribed from ZIKV RNA in infected mosquitoes can be detected using the LAMP reaction (**Figure 3B**).

DISCUSSION

Infection with arboviruses such as Zika, dengue, chikungunya, and Japanese encephalitis virus, remains an important issue for global public health. In particular, Zika virus disease, which caused large outbreaks mainly in the Americas, has suddenly gained attention due to reports of fetal microcephaly. Both Zika virus and dengue virus are transmitted by *Aedes* mosquitoes, continuously spreading worldwide, including *Ae. aegypti* and *Ae. albopictus*. Rapid and accurate surveillance is still strongly in demand because it is needed to strengthen and integrate with health information systems to guide proper vector control.

The vDNA-LAMP, newly developed in this study, demonstrated that it can successfully detect ZIKV vDNA in infected cultured mosquito cells and mosquitoes artificially exposed to ZIKV. The vDNA-LAMP primer set, which was designed to detect two ZIKV strains (MR and PR), actually corresponded to the sequence of the MR strain. It detected vDNA of both MR and PR strains in cultured cells and vDNA of PR strain in *Aedes* mosquitoes. These findings suggested that several mismatches in primer regions may not affect the sensitivity of the amplifying reaction, although longer reaction time was needed to detect the PR strain, as we reported previously (21).

In the experiment using mosquitoes, vDNA-LAMP identified half of the pooled groups (each containing 5 mosquitoes) as

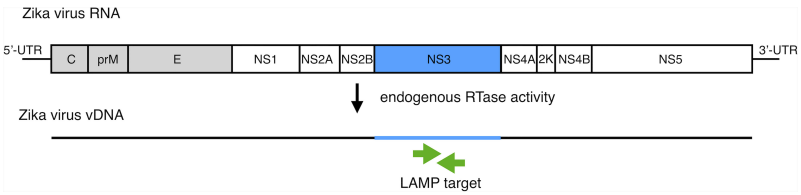

FIGURE 1 | Schematic diagram of the ZIKV RNA genome with LAMP primer positions for amplifying vDNA, which is generated from ZIKV genomic RNA using endogenous RTase activity in mosquito cells. The vDNA region between a set of primers (green arrows) is the target for the LAMP reaction.

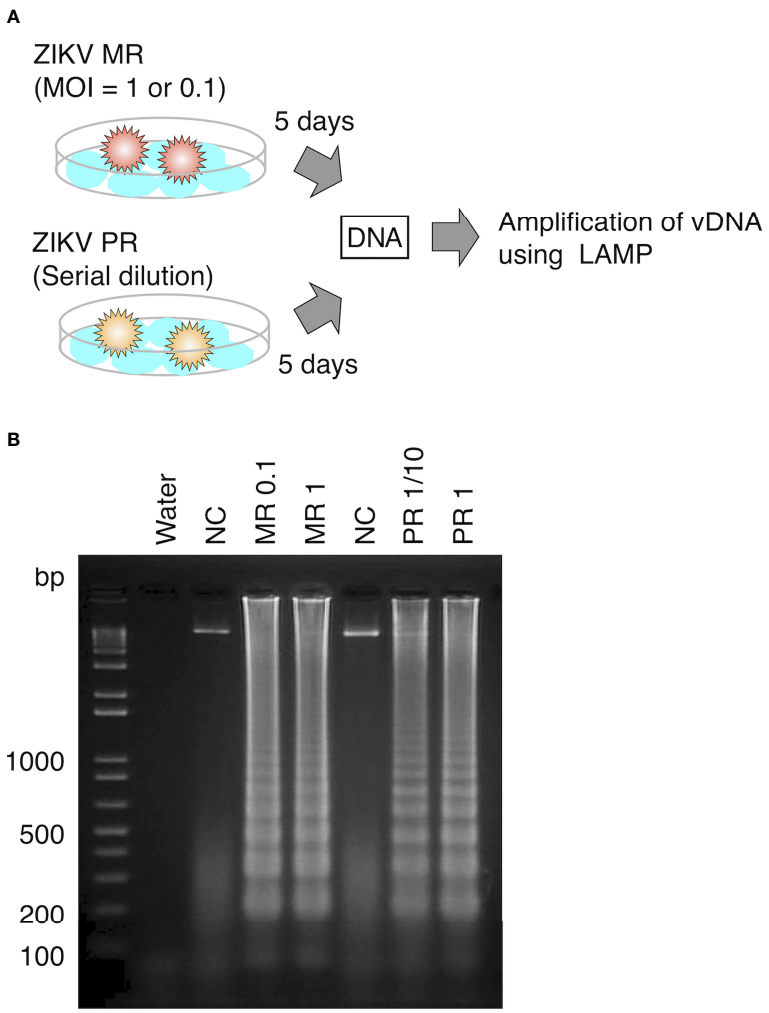

FIGURE 2 | Detection of vDNA using LAMP in ZIKV-infected mosquito cells. **(A)** Scheme of preparing ZIKV-infected cultured cells for vDNA detection. DNA was purified from ZIKV-infected cells and uninfected cells at 5 days post infection and utilized for LAMP reaction. **(B)** vDNA was amplified using LAMP from mosquito C6/36 cells infected with ZIKV MR or PR strain. Amplified products were separated on 2% agarose gels. Water served as a negative control. MR: cells infected with MR strain at an MOI = 1 or 0.1. PR: cells infected with PR strain (PR 1, infection without dilution of virus stock; PR 1/10, 10-fold dilution of virus stock). NC, negative control (uninfected cells).

positive, whereas the other 6 groups did not reach the amplification of the ZIKV target sequence. What can be assumed is the possibility that not all mosquitoes that were orally exposed to the virus established an infection, possibly due to the host defense system in the midgut (22). In cases where exposed mosquitoes did not develop an infection or presented with a low virus titer, few or no copies of vDNA could be isolated from those mosquitoes.

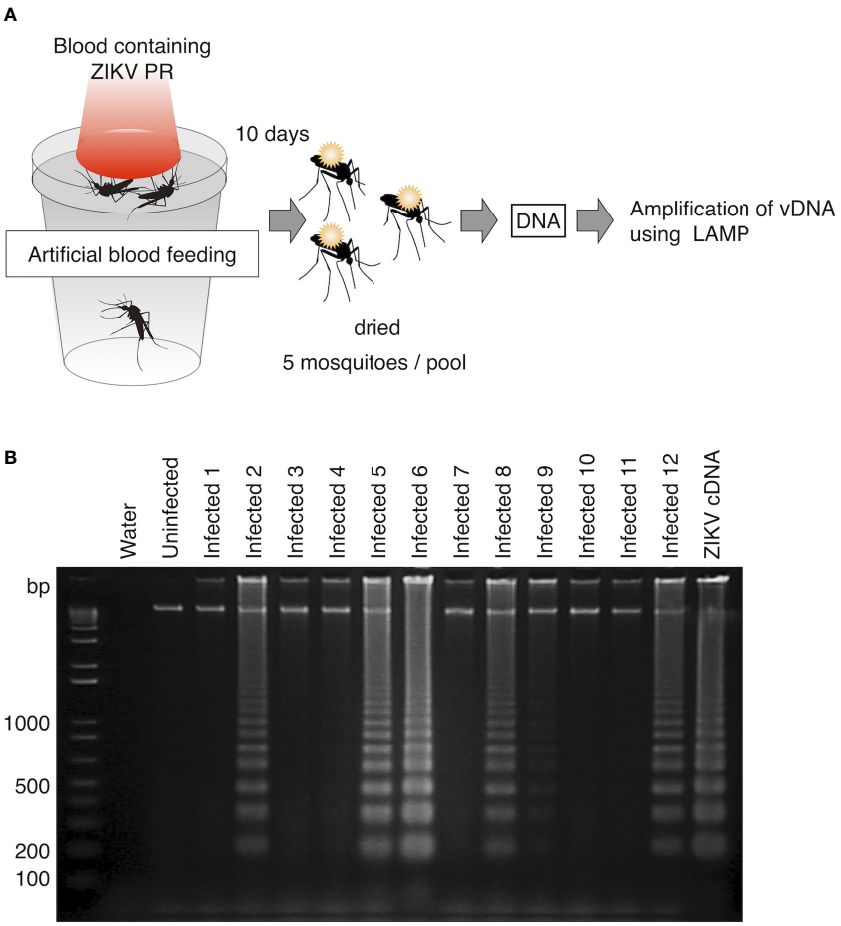

FIGURE 3 | Detection of vDNA using LAMP in ZIKV-infected mosquitoes. **(A)** Scheme for preparing ZIKV (PR strain)-infected mosquitoes for vDNA detection. Five infected mosquitoes were pooled in each group and subjected to DNA extraction. One-fiftieth of the extracted DNA per group was used as template for the LAMP reaction. **(B)** vDNA of ZIKV in infected mosquitoes was detected using LAMP in 6 groups (Infected 2, Infected 5, Infected 6, Infected 8, Infected 9, and Infected 12) out of 12. Amplified products were electrophoresed on 2% agarose gels. ZIKV cDNA was used as positive control. Water served as a negative control. Numbers of the left indicated migration of molecular weight marker (bp).

It has been argued that molecular monitoring of mosquitoes that carry viruses is challenging in particular in developing countries where arbovirus-borne diseases are often endemic; detection of viral RNA in mosquitoes needs freezing storage of mosquito samples, trained personnel to handle RNA, and expensive equipment such as PCR machines. It is notable that vDNA-LAMP, being based on isothermal amplification, is a simple and rapid virus-detecting method targeting stable DNA, not RNA, in isothermal conditions. DNA is more stable than RNA because it is double stranded and does not contain reactive OH groups as in RNA. Indeed, vDNA was detected even from dried mosquito samples in which RNA is expected to be easily degradable. These features may provide a great advantage to molecular surveillance of disease-vector mosquitoes which is carried out outside or in conventional laboratories. Further experiments comparing conventional qPCR with RNA and vDNA-LAMP will clarify the assessment of the applicability of vDNA-LAMP in routine surveillance.

CONCLUSION

The present study developed a new method for detecting arbovirus, vDNA-LAMP, employing vDNA as a target of amplification using the LAMP reaction, and we demonstrated the detection of vDNA of ZIKV in infected cells and mosquitoes. vDNA-LAMP is easily applicable for field surveys of virus infection in wild mosquitoes without RNA storage and handling. To confirm whether vDNA-LAMP will be a realistic alternative to conventional surveillance methods, further experiments using wild mosquito populations need to be performed. Implementing vDNA-LAMP in endemic areas may offer a promising strategy in monitoring virus circulation *via* vector mosquitoes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

HA and HK conceived the study and wrote the manuscript. HA performed the experiments and analyzed the data. II prepared cultured cells, standard strain of viruses, and virus RNA. MO prepared infected cultured cells. J-CL reared and prepared artificially infected mosquitoes. ST and MS provided viruses and assisted with virus infection of cultured cells. C-HC conceived and supervised virus infection of mosquitoes. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2022.759375/full#supplementary-material

Supplementary Figure 1 | (A) LAMP primers for amplifying vDNA of ZIKV. Partial sequences of the NS3 region according to the nucleotide sequences of the MR (upper) and PR (lower) strains. Nucleotide mismatches between MR and PR strains are indicated as Y, R, M, and K (Y: C or T, R: A or G, M: A or C, K: G or T). Location and direction of primers, FIP (F1c-F2), BIP (B1c-B2), F3, B3, Loop-F, and Loop-B, are indicated by arrows. **(B)** LAMP detection of cDNA of ZIKV. Serially diluted RNA (3×10^2, 3×10^1, and 3×10^0 ng) of the MR or PR strain was used as template for reverse transcription to obtain cDNA, followed by the LAMP reaction in one-step. Amplified products were electrophoresed on 2% agarose gels. Water served as a negative control. Numbers of the left indicated migration of molecular weight marker (bp).

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