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Short communication

Detection of *Kudoa amamiensis* Using Loop-Mediated Isothermal Amplification (LAMP)

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(Received March 13, 2015)

ABSTRACT—In this study, we have developed a method for detecting *Kudoa amamiensis* using loop-mediated isothermal amplification (LAMP). After initial cloning and sequencing of the internal transcribed spacer (ITS) region between 18S and 28S rRNA genes of *K. amamiensis*, a set of four primers consisting of two inner and two outer was designed based on ITS1 and 5.8S rRNA sequence for use in the LAMP reaction. Reaction time and temperature were optimized for 60 min at 60°C, respectively. No cross-reaction of the LAMP method was observed with *K. iwatai*. Therefore, this LAMP technique was considered to be a rapid and simple detection method of *K. amamiensis.*

Key words: *Kudoa amamiensis*, ITS region, molecular diagnosis, loop-mediated isothermal amplification, LAMP

Kudoosis amami caused by *Kudoa amamiensis* was first reported from yellowtail farmed in Okinawa Ocean Expo in 1975 (Egusa and Nakajima, 1978). In subsequent investigations, the infection was reported from Amami and Okinawa Districts only and not from the mainland of Japan (Sugiyama *et al*., 1999). Subsequently, *K. amamiensis* was also reported from Australian waters (Whipps *et al*., 2003; Burger *et al*., 2008). Although, in past kudoosis amami was limited to Okinawa District in Japanese waters, there is a possibility that it may occur in Kyushu in future due to increasing water temperature as a result of global warming in recent years.

As nucleic acid based molecular diagnosis techniques are faster, sensitive and highly accurate, they are efficiently used to diagnose pathogens from diseased and latent fish as well as aquatic environment. Although these techniques can amplify a short nucleic acid sequence with a high specificity, they require an expensive equipment to perform the reaction process. Due to this disadvantage, field level application of these techniques is not popular. This drawback can be overcome using a simpler technique, a loop-mediated isothermal amplification (LAMP) of a target nucleic acid (Notomi *et al*., 2000). In LAMP assay, the whole reaction is completed under an isothermal condition in a short time. This technique has been successful in detection of several clinical as well as fish and shellfish pathogens (Biswas and Sakai, 2014). In this study, we established a new diagnosis method of *K. amamiensis* using the LAMP.

Materials and Methods

Parasite samples and DNA extraction

K. amamiensis from the yellowtail *Seriola quinqueradiata* cultured in Okinawa Prefecture and *Kudoa iwatai* from the blackhead seabream *Acanthopagrus schlegelii* captured in Shizuoka Prefecture were used as parasite samples. Several strains of a species should be examined to establish the diagnostic method for any *Kudoa* species, however only one strain of each was used in this study because of difficulty in obtaining more strains. Genomic DNA was extracted from 10^5 spores of *K. amamiensis* and *K. iwatai* using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions.

Cloning and sequencing of ITS regions from K. amamiensis

Based on sequences of the 18S and 28S rRNA genes (GenBank accession numbers: AF034638 and AB693049), a set of primers, forward (Ka-ITS-Fw) and reverse (Ka-ITS-Rv) was designed (Table 1) to amplify the region containing ITS region. PCR was performed in a 50-μL reaction volume containing 4 μL dNTP mixture (2.5 mM) and 10 μL 5 × PrimeSTAR GXL buffer, 1 μL PrimeSTAR GXL DNA polymerase (0.025 U/μL) (Takara Bio Inc.), 3 μL of each primer (10 μM) (Ka-ITS-Fw and Ka-ITS-Rv), 2 μL of template DNA (3 ng/μL) and 27 μL nuclease free water. A pre-denaturation step at 98°C for 1 min was followed by thirty amplification cycles consisting of denaturation at 94°C for 45 s, annealing at 60°C for 60 s and extension at 72°C for 45 s. A final extension at 68°C for 5 min was also included in the PCR profile. The amplified products

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Table 1. Oligonucleotide primers used in this study

Primer	Type	Sequence (5´→3´)	Length (nt)	Application
Ka-ITS-Fw	Forward	GCCGATTTTCAAACATTGGCA	21	Cloning
Ka-ITS-Rv	Reverse	CTCCGCTTACTGATATGCTTA	21	Cloning
Ka-FIP	Forward inner (F1c-TTTT-F2)	CGCGTTCTTCATCGATACACTTTT- -ATAAAATAAAACAACCGTTAGC	46	LAMP
Ka-BIP	Backward inner (B1c-TTTT-B2)	CCAACTGCGATAATTGGTGCTTTT- -GTAATCTAACATGCCATTTGCA	46	LAMP
Ka-F3	Forward outer	CTTTTGGAATATTTTGTGTGATC	23	LAMP
Ka-B3	Backward outer	ACCAGACATACGTTAGACTT	20	LAMP

were electrophoresed in 1.5% agarose gels and purified using NucleoSpin Gel and PCR Clean-up kit (MACHERY-NAGEL). The gel purified PCR products were ligated into pGEM-T Easy vector (Promega) following the manufacturer's guidelines and transformed into RapidTrans TAM1 Competent E. coli (Active Motif). The recombinants were identified through red-white color selection when grown on MacConkey agar (Sigma-Aldrich). Plasmid DNA from the clones was extracted using the High Pure Plasmid Isolation Kit (Roche) and subjected to sequencing at Eurofins Genomics sequencing services. The sequences were compared with those in the database using the BLAST2 algorithm (Altschul *et al*., 1990) and deposited to GenBank database (Accession number: LC060248).

Design of primers for LAMP

Specific LAMP primers were designed based on sequence of *K. amamiensis* ITS determined in this study (GenBank Acc. No. LC060248). A set of four primers composed of two outer primers (Ka-F3 and Ka-B3) and two inner primers (Ka-FIP and Ka-BIP) was designed (Fig. 1) using the PrimerExplorer v4 software (https://primerexplorer.jp/elamp4.0.0/index.html, Eiken Chemical Co., Ltd.). Ka-FIP consisted of two distinct sequences corresponding to the complementary sequence of F1 (F1c) and sense F2 sequence of the target, with a TTTT spacer in between. Similarly, Ka-BIP contained the complementary sequence of B1 (B1c), a TTTT spacer and the sense B2 sequence. The primer sequences and their position in the ITS region are shown in Table 1 and Fig. 1, respectively.

Determination of LAMP conditions

LAMP was carried out in a 12.5-μL reaction volume containing 1 μL (20 pmol) each of Ka-FIP and Ka-BIP, 0.5 μL (2.5 pmol) of Ka-F3 and Ka-B3, 6.25 μL of 2 × reaction mixture [40 mM Tris-HCl, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betaine, and 2.8 mM dNTPs each], 0.5 μL (8U) of *Bst* DNA polymerase, 1 μL of target DNA, 57.5°C, 60°C, 62.5°C and 65°C for 60 min, and the reaction was terminated by heating at 80°C for 2 min. The reaction time (15, 30, 45, 60, 75 and 90 min) was optimized and LAMP was carried out at a predetermined temperature (60°C). LAMP products (2 μL) were electrophoresed

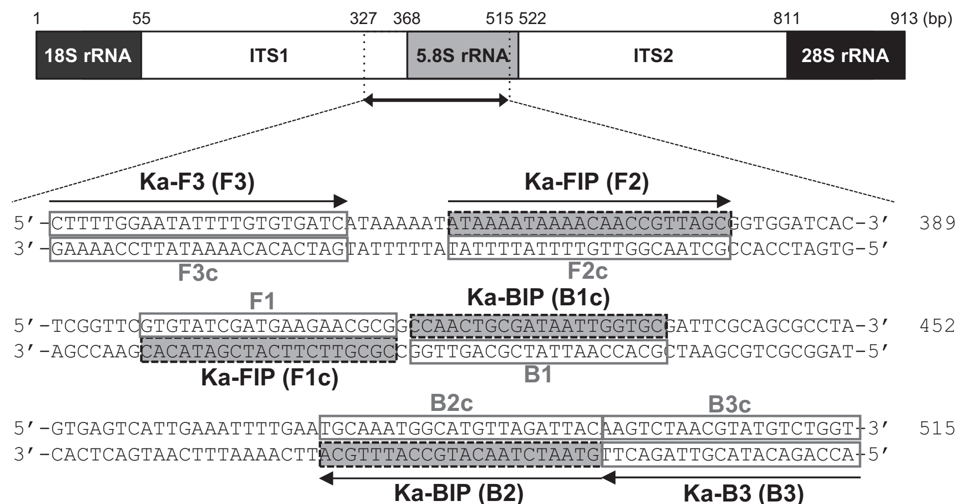

Fig. 1. Schematic representation of the 913-bp *Kudoa amamiensis* genomic sequence (GenBank Acc. No.: LC060248) that contains partial 18S and 28S rRNA genes with ITS1, 5.8S rRNA gene and ITS2 lying in between. A part of the ITS1 and 5.8S regions was used for LAMP primers design. The positions of designed primers in ITS1 and 5.8S regions were indicated with boxes and arrows.

on 1.5% agarose gels stained with ethidium bromide to determine the optimal conditions.

Sensitivity of LAMP assay

To determine the sensitivity, a 10-fold serial dilution of an initial *K. amamiensis* DNA sample (5.5 ng/μL, 10^0–10^{-4}) was prepared and LAMP was conducted following the predetermined conditions (60°C and 60 min) as described above.

Specificity of LAMP assay

To check the specificity of detection, LAMP was carried out with the genomic DNA templates of *K. amamiensis* and *K. iwatai* following the predetermined conditions (60°C and 60 min).

Results and Discussion

Determination of LAMP conditions for K. amamiensis *detection*

The LAMP was carried out using *K. amamiensis* DNA as template to determine the optimal temperature and time of reaction. Formation of LAMP products was found at 60°C and 62.5°C with no amplified products at 57.5°C and 65°C (Fig. 2A). However, 60°C was considered the optimal temperature due to clarity and deepness of bands. With regard to the reaction time, no amplification of the LAMP products was seen at 15, 30 and 45 min; however, the amplification of target gene was observed at 60, 75 and 90 min, resulting in formation of many bands of different sizes (Fig. 2B). Moreover, bands were more clear and prominent at 60 min. Therefore, the reaction conditions were optimized for 60 min at 60°C.

Sensitivity of LAMP

Based on initial *K. amamiensis* genomic DNA, a 10-fold serial dilution was used in the subsequent LAMP. LAMP displayed amplification of products up to 10^{-2} diluted template DNA (Fig. 3).

Specificity of LAMP

The specificity of LAMP was examined using genomic DNA templates extracted from spores of *K. amamiensis* and *K. iwatai*. After incubation at 60°C for 60 min, only *K. amamiensis* gave a positive reaction, whereas no amplification was observed for *K. iwatai* (Fig. 4). These results indicate that the LAMP technique developed in this study is highly specific for *K. amamiensis* detection. These findings were consistent with other studies related to specificities of LAMP assays in parasitic detection (El-Matbouli and Soliman, 2005; Sakai *et al.*, 2009).

In the present study, we have established a

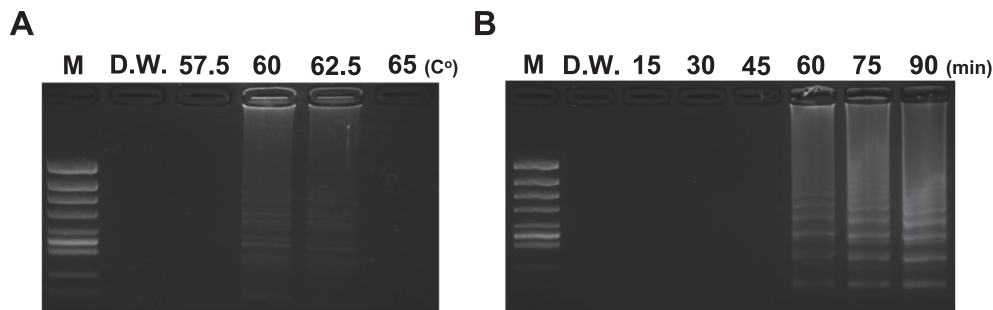

Fig. 2. Determination of LAMP conditions: effect of temperature and time on amount of LAMP product. (**A**) Temperature: lane M, 100 bp DNA marker; D.W., negative control (distilled water); the other lanes, LAMP carried out at 57.5°C, 60°C, 62.5°C and 65°C, respectively. (**B**) Time: lane M, 100 bp DNA marker; D.W., negative control (distilled water); the other lanes, LAMP carried out for 15, 30, 45, 60, 75 and 90 min, respectively. All the products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

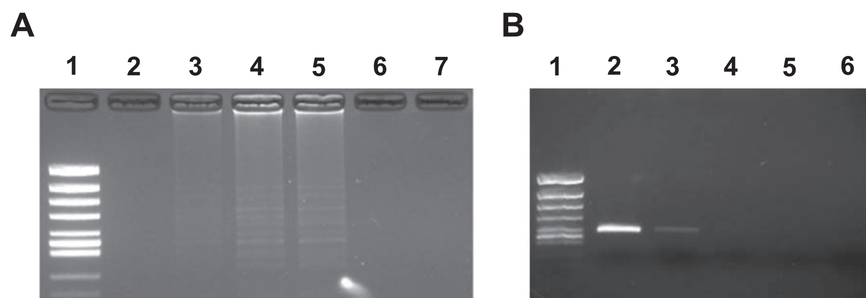

Fig. 3. Sensitivity of *Kudoa amamiensis* detection by LAMP. Lane 1, 100 bp DNA marker; lane 2, negative control (distilled water); lanes 3–7, LAMP carried out using a 10-fold serial dilution (10^0–10^4) of *K. amamiensis* DNA (5.5 ng/μL). All the products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

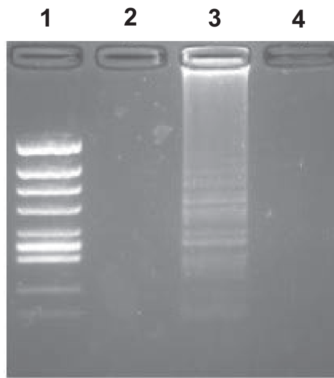

Fig. 4. Comparison of specificity for detection of *Kudoa amamiensis* DNA by LAMP. Lane 1, 100 bp DNA marker; lane 2, negative control (distilled water); lanes 3 and 4, *K. amamiensis* and *K. iwatai* (5.5 ng spore DNA of *K. amamiensis* and *K. iwatai* was used for one LAMP reaction as a template, respectively). All the products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

method for detecting *K. amamiensis* using the LAMP method. Yokoyama *et al.* (2000) already established a nested PCR and indirect fluorescent antibody technique (IFAT) for the diagnosis of *K. amamiensis*, and concluded that the nested PCR is more sensitive than IFAT. In general, nested PCR has been known to be sensitive analysis for detection of target DNA. Although we did not examine comparative sensitivity between LAMP method and nested PCR, LAMP method has an advantage of less time consumption without using a thermal cycler. However, to establish the complete diagnostic method using LAMP, several strains need to be examined to confirm the universality. Furthermore, for practical application of this method at aquaculture farm, detection using the tissue of infected fish should be demonstrated in further study.

Although *K. amamiensis* can infect important aquaculture species like yellowtail and amberjack (Sugiyama *et al*., 1999), this disease is limited only to Okinawa region in high water temperature. However, in future, global warming may cause an increase in water temperature of the Japanese mainland and likely occurrence of this disease cannot be ruled out. Therefore, the LAMP technique developed in this study seems to be a useful method for rapid diagnosis of this disease.

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