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RESEARCH PAPER

Detection of *Kudoa septempunctata* in the Japanese Flounder by Loop-Mediated Isothermal Amplification (LAMP) Targeting the Internal Transcribed Spacer (ITS) Regions

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Abstract

Recent food poisoning outbreaks from consumption of the Japanese flounder, *Paralichthys olivaceus* infested with a myxozoan parasite, *Kudoa septempunctata* have become a serious human health concern. In this study, we sequenced the internal transcribed spacer (ITS) regions between the 18S and 28S rRNA genes of *K. septempunctata* from cultured flounder in Japan and imported flounder from Korea. Sequence analysis indicates that the ITS1 regions are highly conserved in Japanese and Korean isolates of the parasite. A loop-mediated isothermal amplification (LAMP) assay targeting the ITS1 region was developed for rapid, sensitive and specific detection of either isolate of the parasite.

Keywords: *Kudoa septempunctata*, ITS region, Japanese flounder (*Paralichthys olivaceus*), LAMP.

Introduction

Recently, ingestion of raw Japanese flounder, *Paralichthys olivaceus* meat caused serious health complications in humans including transient, but strong diarrhea and vomiting (Kawai *et al.*, 2012). The etiological cause of this food-borne illness was identified as *Kudoa septempunctata* (Kawai *et al.*, 2012), a myxozoan parasite that infects the trunk muscle of *P. olivaceus* by forming pseudocysts in myofibers (Matsukane *et al.*, 2010). This parasitic manifestation and its' consequence have become a real concern for flounder breeders, culturists and post-harvest traders. Early detection of *K. septempunctata* can prevent transmission of this parasite and the subsequent human health risks, locate exactly the source of infection and ensure food safety.

DNA-based molecular techniques, such as polymerase chain reaction (PCR) and real-time PCR have been used in the detection of fish infected with *K. septempunctata* (Grabner *et al.*, 2012; Harada *et al.*, 2012). A loop-mediated isothermal amplification (LAMP) technique has recently been successful in the detection of several fish and shellfish pathogens (Biswas & Sakai, 2014), including *K. septempunctata* (Jeon *et al.*, 2014). Recently, a LAMP method was devised by Jeon *et al.* (2014), targeting the 28S rRNA gene of *K. septempunctata* and this technique was rapid, sensitive and specific when only Korean isolates were tested.

K. septempunctata was first described in the cultured *P. olivaceus* in Japan using the seed that was imported from Korea (Matsukane *et al.*, 2010) with the transmission route of the parasite remained unconfirmed. In this work, we have targeted the internal transcribed spacer (ITS) regions of the rRNA that are typically much more variable than the small subunit (SSU) rRNA to reliably examine the intra-species specific comparisons (van Herwerden *et al.*, 2000). Our aim was to check the distinction among geographically different *K. septempunctata* strains of Japan and Korea by analyzing the ITS regions. Thus, this approach is different from the method described by Jeon *et al.* (2014) who screened only the Korean isolates of *K. septempunctata*. Currently, information on the nucleotide sequence of the ITS regions within *K. septempunctata* is not available. Therefore, in this study, we performed PCR-amplification, cloning and sequencing of the ITS1 and ITS2 regions, including the 5.8S rRNA gene located between 18S and 28S

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rRNA genes, of the Japanese and Korean isolates of *K. septempunctata*. A LAMP protocol targeting the ITS1 region was developed for rapid, sensitive and specific detection of the parasite.

Materials and Methods

K. septempunctata Samples and DNA Extraction

Two Japanese isolates of *K. septempunctata* were collected from randomly sampled specimens of *P. olivaceus* from an aquaculture farm with previous incidences of *K. septempunctata* infection. Two Korean isolates of the parasite were harvested from imported *P. olivaceus*, which caused food poisoning cases in Japan. Additionally, four parasite isolates were obtained from *P. olivaceus* of unknown origin. Genomic DNA was extracted from pooled muscle blocks of individual fish using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Cloning and Sequencing of ITS Regions from *K. septempunctata*

Based on the sequences of the 18S and 28S rRNA genes (GenBank accession numbers: AB553293 and AB693040, respectively), a set of primers, forward (Ks-Fw) and reverse (Ks-Rv) were designed (Table 1) to amplify the ITS regions from *K. septempunctata* using PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Shiga, Japan). A pre-denaturation step at 98ºC for 3 min was followed by thirty amplification cycles consisting of denaturation at 98ºC for 10 s, annealing at 60ºC for 15 s and extension at 68ºC for 2 min. A final extension at 68ºC for 5 min was also included in the PCR program. The amplified products were purified using the NucleoSpin Gel and PCR Clean-up kit (MACHERY-NAGEL, Düren, Germany). The gel purified PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's guidelines. Plasmid DNA from the clones was sequenced using Eurofins Genomics' sequencing services (http://eurofinsgenomics.jp/jp/home.aspx). The sequences were compared with those in the database using the BLAST2 algorithm (Altschul *et al*., 1990).

LAMP Primer Design

Four specific LAMP primers composed of two outer primers (Ks-F3 and Ks-B3) and two inner primers (Ks-FIP and Ks-BIP) were designed based on the conserved sequence (among different strains, Figure 2) of the ITS1 region of *K. septempunctata* using the PrimerExplorer V4 software (https://primerexplorer.jp/elamp4.0.0/index.html). Ks-FIP consisted of two distinct sequences corresponding to the sense (F1) and antisense (F2c) sequences of the target, with a TTTT spacer in between. Similarly, Ks-BIP contained the complementary sequence of B1 (B1c), with a TTTT spacer and the sense B2 sequence. The primer sequences and their positions in the ITS1 region are shown in Table 1 and Figure 1. The LAMP reaction was performed using the Loopamp DNA Amplification Kit (Eiken Chemical Co., Ltd., Tokyo, Japan) in a 12.5-µL reaction volume containing 1 µL (20 pmol) each of Ks-FIP and Ks-BIP, 0.5 µL (2.5 pmol) of Ks-F3 and Ks-B3, and 1 µL of target DNA.

Determination of LAMP Reaction Conditions

LAMP was carried out in a 12.5-µL reaction volume containing 1 µL (20 pmol) each of Ks-FIP and Ks-BIP, 0.5 µL (2.5 pmol) of Ks-F3 and Ks-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, and 1.75 µL of distilled water (Eiken Chemical Co., Ltd.). To determine the optimal temperature, several incubations were carried out at 60ºC, 63ºC, 65ºC and 68ºC for 60 min, and the reaction was terminated by heating to 80ºC for 2 min. The reaction time (30, 45 and 60 min) was varied and it was determined that 60 min was the optimal time for the LAMP reaction. LAMP products (2 µL) were electrophoresed on 1.5% agarose gels stained with ethidium bromide to determine the optimal conditions.

Table 1. PCR and LAMP primers used in this study

Primer	Type	Sequence (5′→3′)	Length (nt)	Application
Ks-Fw	Forward	CAGGTCTGTGATGCCCTTCGATGTTCTG	28	ITS cloning
Ks-Rv	Reverse	ACAGTACTTGTTCGCTATCGGTTTCGTGT	29	
Ks-FIP	Forward inner (F1c-TTTT-F2)	CCTTGTTACGACTTTTACTTCCTCC -TTTT-AATGTTAGGACTTGCGGC	43 (F1c: 25; F2: 18	LAMP
Ks-BIP	Backward inner (B1c-TTTT-B2)	CCTGCGGAAGGATCATCAATGA -TTTT-CACTTCACTTCAACACTACAC	43 (B1c: 22; B2: 21)	LAMP
Ks-F3	Forward outer	CGACTGGATGTTGCTTCG	18	LAMP
Ks-B3	Backward outer	CGACACTTTACCATCCACTT	20	LAMP
Fw	Forward	TCATTCATTTCAAGTTTGTGTG	22	PCR
Rv	Reverse	ATTACATTTCCACCAACATCA	21	PCR

Sensitivity of the LAMP Assay and Comparison to PCR

To determine the sensitivity, a 10-fold serial dilution of an initial *K. septempunctata* DNA sample (3 ng/ µL, 10^{0}~10^{-9}) was prepared and LAMP was conducted following the predetermined conditions as described above. To compare the detection sensitivity of LAMP to standard PCR, the serially diluted DNA was subjected to thermal cycling using Fw (as forward) and Rv (as reverse) primers from the ITS1 region (Table 1) to amplify a 206-bp product. PCR was carried out in a 20-µL reaction volume containing 10 µL GoTaq® Green Master Mix (Promega), 2 µL (10 µM) of each primer (Fw and Rv), 5 µL distilled water and 1 µL DNA. A total of 30 amplification cycles were performed, each cycle consisting of a denaturation step at 95ºC for 30 s, annealing at 55ºC for 40 s and extension at 72ºC for 1 min. A final extension at 72ºC for 5 min was also included in the PCR program. The products were electrophoresed on 1.5% agarose gels stained with ethidium bromide and documented using a gel doc system (AlphaImager Mini, Alpha Innotech, Santa Clare, CA, USA).

Specificity of the LAMP assay and comparison to PCR

To check the specificity of detection, LAMP was carried out with the genomic DNA templates of *K. septempunctata*, *K. thyrsites*, *K. lateolabracis*, *K. amamiensis* and *K. iwatai* following the predetermined conditions (at 65ºC for 60 min). The specificity of PCR amplification with conditions described above was also determined and compared to the LAMP results.

Results

Sequence analysis of *K. septempunctata* ITS regions

A 2147-bp *K. septempunctata* genomic sequence was amplified, cloned, sequenced and submitted to the GenBank database (Accession number: LC028894). Sequence analysis revealed that there are two ITS regions between the 18S and 28S rRNA genes and they are 476 (ITS1) and 794 (ITS2) bp long. Moreover, there is a short 5.8S rRNA segment (158 bp) lying between the ITS1 and ITS2 regions (Figure 1A). Examination of the ITS1 sequences from the obtained isolates revealed 3 variable nucleotide positions and 2 deletions (Figure 2), representing a maximum sequence variability of only 1.05% among the isolates.

Determination of Conditions for *K. Septempunctata* Detection Using LAMP

Detection of LAMP products was observed at 63ºC and 65ºC (Figure 3A). However, 65ºC was considered the optimal temperature due to clarity and depth of bands. With regard to reaction time, the amplification of the target gene occurred at 45 and 60 min, resulting in the formation of several different sized bands (Figure 3B). Moreover, the bands were clearer and more prominent at the 60 min time point. Therefore, the optimized reaction conditions were

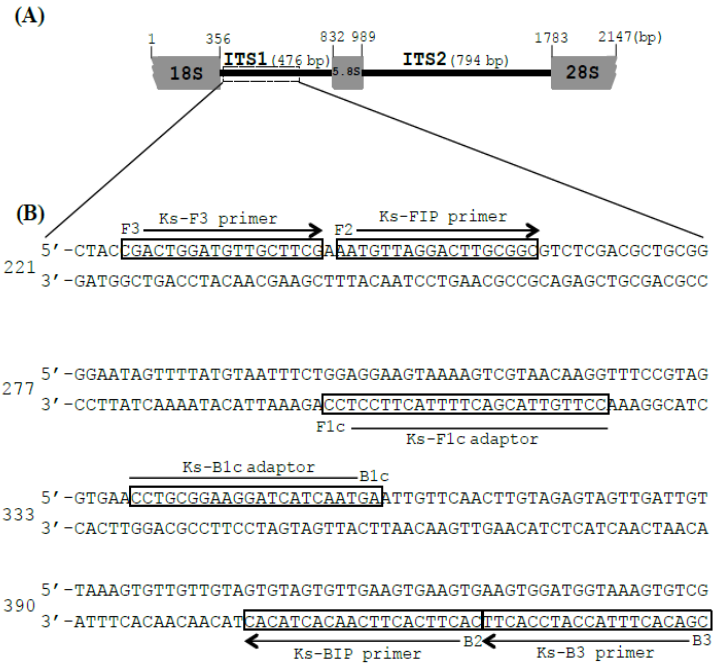

Figure 1. (A) Schematic representation of the 2,147-bp *Kudoa septempunctata* genomic sequence (GenBank Accession Number: LC028894) that contains partial sequence for the 18S and 28S rRNA genes with the ITS1 region (476 bp), 5.8S rRNA gene (158 bp) and the ITS2 region (794 bp) lying in between. A part of the ITS1 region was used for LAMP primer design. (B) Primer positions in the ITS1 nucleotide sequence. The DNA sequences used for primer design are marked with boxes and arrows.

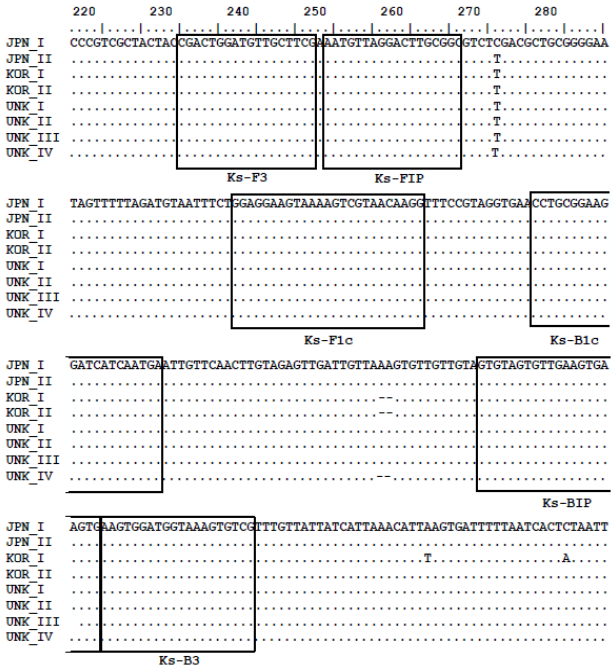

Figure 2. Multiple sequence alignment of DNA sequences from the ITS regions of different *Kudoa septempunctata* strains isolated from different geographical locations. The position of the LAMP primers on the conserved area is denoted by boxes. The alignment was made from the following sequences: JPN_I (Japanese isolate; GenBank Acc. No. LC028894), JPN_II (Japanese isolate; LC037195), KOR_I (Korean isolate; LC037199), KOR_II (Korean isolate; LC037202), UNK_I (unknown isolate; LC037196), UNK_II (unknown isolate; LC037197), UNK_III (unknown isolate; LC037198), and UNK_IV (unknown isolate; LC037200). The regions selected for the LAMP primers and analysis were 100% conserved in all isolate sequences.

determined to be 60 min at 65ºC.

Sensitivity of LAMP

Based on the initial *K. septempunctata* DNA sample (3 ng/ µL), a 10-fold serial dilution (10^0~10^-9) was made and the respective DNA was used for subsequent LAMP and PCR reactions following the predetermined conditions described above. The LAMP reaction was able to detect *K. septempunctata* up to 10^{-2} dilution (Figure 4A). However, PCR could detect this parasite up to 10^{-1} dilution of template DNA (Figure 4B).

Specificity of LAMP

The specificity of LAMP was examined using DNA templates of five *Kudoa* species, namely *K. septempunctata*, *K. thyrsites*, *K. lateolabracis*, *K. amamiensis* and *K. iwatai*. After incubation at 65ºC for 60 min, a positive reaction was observed only for *K. septempunctata*, no amplification was observed for the other four kudoid species tested (Figure 5A, B).

Discussion

In this study, we have developed a highly specific LAMP method targeting the ITS1 region of *K. septempunctata*. For this purpose, we have cloned and sequenced a 2147-bp genomic fragment containing ITS1 and ITS2 regions between the 18S and 28S rRNA genes. We compared the ITS1 sequences from various *K. septempunctata* strains to check any variability. A high variation (65.6%) in *K. thyrsites* ITS1 sequences among geographically different isolates suggested that there were some genetically distinct populations of the parasite around the world (Whipps & Kent, 2006). In contrast, the ITS1 sequences of *Myxobolus cerebralis* from European and American isolates were almost identical, supporting a hypothesis that the current global distribution of the parasite was a result of recent dispersal via anthropogenic movement of fish (Whipps *et al*., 2004a). With results similar to those for *M. cerebralis*, minor variations in the ITS1 sequences from *K. septempunctata* isolates suggest the recent introduction of the parasite from Korea to Japan, or vice versa. Jung *et al.* (2011) reported the ITS regions of the scuticociliate *Miamiensis avidus* were identical among multiple strains isolated from *P. olivaceus* in Korea and Japan. The results of these two studies imply a possible mixing of parasite populations between the two countries through transportation of juvenile or adult *P. olivaceus*.

The optimal condition for detecting *K. septempunctata* was found to be as 65ºC for 60 min. Although, a positive reaction was observed at 65ºC for 45 min, the bands were inconspicuous. It indicates

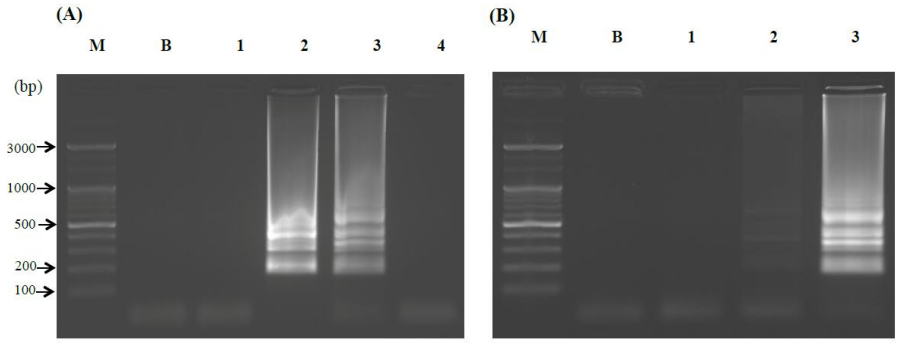

Figure 3. Determination of optimal LAMP conditions: the effect of temperature and time on the amount of LAMP product. (A) Temperature: lane M, 100 bp DNA marker; B, negative control (distilled water, DW); lanes 1-4, LAMP carried out at 60, 63, 65 and 68ºC, respectively. (B) Time: lane M, 100 bp DNA marker; B, negative control (DW); lanes 1-3, LAMP carried out for 30, 45 and 60 min, respectively. All of the products (2 µL) were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

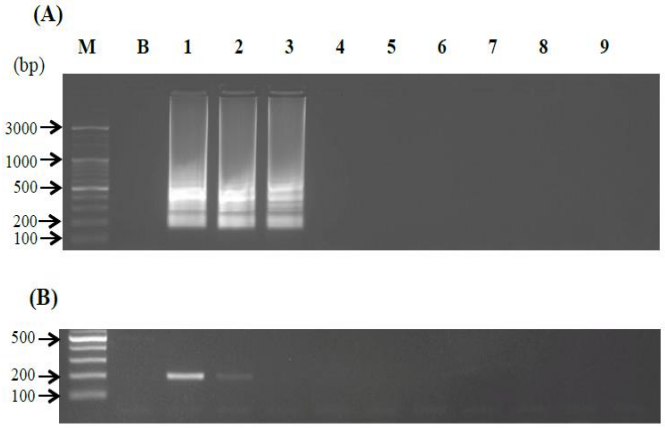

Figure 4. Sensitivity of *Kudoa septempunctata* detection by (A) LAMP and (B) PCR. M, 100 bp DNA marker; B, negative control (DW); lanes 1~10, LAMP carried out using different concentrations of *K. septempunctata* DNA (3 ng µL^{-1}) extracted from the Japanese flounder muscle: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}, respectively. All of the products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

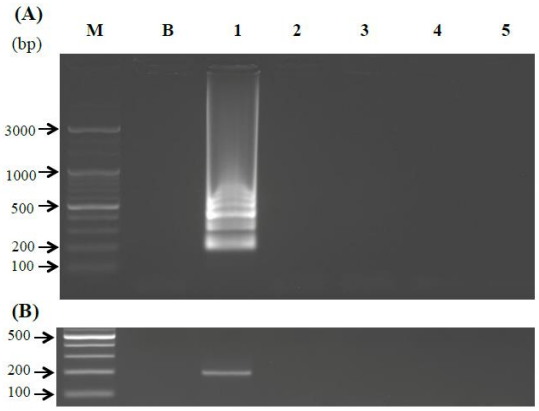

Figure 5. Comparison of the specificity of detection of *Kudoa septempunctata* DNA by (A) LAMP and (B) PCR. M, 100 bp DNA marker; B, negative control; lane 1, *K. septempunctata*; lane 2, *K. thyrsites*; lane 3, *K. lateolabracis*; lane 4, *K. amamiensis* and lane 5, *K. iwatai*. All of the products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

that detection of *K. septempunctata* is possible in less than an hour. Our results are comparable to those (for 45 min at 63ºC) observed by Jeon *et al*. (2014).

The LAMP method used for *K. septempunctata* detection was found to be highly sensitive, as it could detect this parasite up to 10^{-2} dilution of the template

DNA, whereas by PCR the detection was possible up to 10^{-1} dilution. It indicated that LAMP is ten times more sensitive than PCR. Moreover, the LAMP method is much faster when compared to the 2-3 hours it takes to complete a standard PCR reaction.

When examining the specificity of the LAMP assay for *K. septempunctata* detection, the primers amplified only this parasitic species and no other *Kudoa* species. These results suggested that the LAMP technique developed in this study is highly specific for the detection of *K. septempunctata*.

The LAMP method described in this report provides a sensitive, specific and rapid diagnostic protocol for detecting the existence of the parasite, *K. septempunctata* during routine examination as well as for surveillance/quarantine procedures. Although, our method targets amplification of ITS1 region, which generally shows intra-species specific variability, the primers were designed based on the conserved sequence of the ITS1 regions among various strains including the Japanese and Korean isolates of *K. septempunctata*. Therefore, this LAMP technique can be applied uniformly in the detection of either isolate of the parasite.

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