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Development of a loop-mediated isothermal amplification method for detection of *Perkinsus* spp. in mollusks

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ABSTRACT: *Perkinsus* is a genus of unicellular protozoan parasite responsible for mass mortality of several commercially valuable mollusks. Surveillance and inspection of its epidemiology in the field calls for convenient and rapid detection methods. Here, a loop-mediated isothermal amplification (LAMP) assay was developed to detect the presence of *Perkinsus* spp. in mollusks. Specific LAMP primers were designed targeting the conserved internal transcribed spacer 2 (ITS-2) region of the rRNA gene of *Perkinsus* spp. Using ITS-2 recombinant plasmid as a template, we optimized the LAMP reaction system and conditions and then evaluated the analytical sensitivity and specificity of the assay. The LAMP assay was validated using clam samples collected from coastal areas in eastern China and oysters imported to China and compared with the traditional Ray's fluid thioglycollate culture method (RFTM). Our results showed that the LAMP detection method for *Perkinsus* was successful. The detection limit was 10 copies of plasmid DNA. Compared to the RFTM assay, the LAMP detection method was more sensitive (56 versus 52 positive out of 60 samples). *P. olseni* and *P. marinus* from infected hosts were successfully detected by this method. The LAMP method is rapid, sensitive, and specific for *Perkinsus* spp. detection, and could be used to screen for perkinsosis both on farms and at ports.

KEY WORDS: *Perkinsus olseni* · *Perkinsus marinus* · Mollusk · LAMP assay · Ray's fluid thioglycollate medium culture assay · Detection method

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INTRODUCTION

Perkinsosis is a worldwide parasitic disease of mollusks caused by *Perkinsus* species (Villalba et al. 2004). Among the 6 recognized *Perkinsus* species, *P. olseni* (also named *P. atlanticus*) and *P. marinus* are the primary causes of severe economic losses in a number of shellfisheries (Villalba et al. 2004). Conventional methods for the detection of *Perkinsus* spp. in oysters and clams include histological observation and pathogenic examination after culture of host tissues by Ray's fluid thioglycollate culture method (RFTM; Ray 1952). Although recommended by the World Organization of Animal Health (OIE) as the 'gold standard' for *Perkinsus* spp. diagnosis, RFTM is known to lack specificity. Some *Perkinsus*-like parasites, particularly dinoflagellates, can yield false positive results in the RFTM assay. RFTM is time consuming, taking 4 to 7 d (Robledo et al. 1998), and therefore a rapid field diagnostic test is needed.

Specificity and sensitivity are the main principles for the establishment of new diagnostic methods. The application of nucleic acid amplification methods combined with sequencing tools has largely overcome the specificity and sensitivity limitations associated with the RFTM assay (Robledo et al. 1998, Yarnall et al. 2000, Park et al. 2005). Having multiple occurrences in the genome of *Perkinsus* sp., the

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internal transcribed spacer (ITS) regions (including ITS1 and ITS2) have become one of the most frequently adopted gene markers in the determination of genus and species of *Perkinsus* (Kotob et al. 1999). Audemard et al. (2004) reported that a single cell of *P. marinus* could be detected using a conventional PCR assay targeting the ITS region. Other protocols, such as detection of *P. marinus* in host tissues and environmental samples by polyclonal antibodies, have also been attempted. Results of these methods showed that cross-reaction with several free-living, phototrophic, and parasitic dinoflagellate species was unavoidable (Dungan & Roberson 1993, Bushek et al. 2002).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies a DNA template under isothermal conditions. This method employs a *Bacillus stearothermophilus* (*Bst*) DNA polymerase and a set of 4 specially designed primers that recognize a total of 6 distinct regions on the target DNA sequence. It can amplify the target sequence (either DNA or RNA template) in a very short time with high sensitivity, specificity, and efficiency (Notomi et al. 2000). For these reasons, the LAMP assay has been widely applied in the detection of pathogenic microorganisms (Tatibana et al. 2009), genetically modified ingredients (Fukuta et al. 2004), and tumors (Horibe et al. 2007), as well as in embryo sex identification (Hirayama et al. 2004). In this study, we developed a LAMP assay for the detection of *Perkinsus* spp.

MATERIALS AND METHODS

Sample collection

Live clams *Ruditapes philippinarum* were collected from farms in the Yellow Sea and East China Sea during the summers of 2010 and 2011. The samples were labeled and shipped to our lab on ice. Suspected *Perkinsus marinus*-infected oysters were imported from Australia in May 2011 and August 2012; these samples were first inspected by PCR and sent to our lab for confirmation by the Guangdong Entry-Exit Inspection and Quarantine Bureau, PR China.

Parasite-infected tissue controls

The status of *Bonamia ostreae*-infected oysters was confirmed by PCR and subsequent endonuclease digestion assay (OIE 2012a). The DNA of *Ichthyophthirius multifiliis* from infected carp was kindly provided by Dr. Xinzhong Chen from the Xiamen Entry-Exit Inspection and Quarantine Bureau, PR China. *Haplosporidium nelsoni*-positive oyster *Crassostrea gigas* samples were collected from coastal areas of the Yellow Sea in northeast China during the routine aquatic parasite surveillance program performed by our lab (unpubl. data).

RFTM performance

In total, 60 clams were selected using the diagonal method from the collected samples. After shucking, part of the gill was excised and stored at −20°C for later PCR examination. The remaining parts of each clam tissue were cultured separately with RFTM following the methods of Wu et al. (2011). In brief, the remaining clam tissue was homogenized and then placed in fluid thioglycollate medium (Fluka) supplemented with 2.5% chloromycetin (Amresco) and 1% nystatin (Amresco). After incubation at 22 to 25°C in the dark for 7 d, the pellets were harvested by centrifugation at 1500 × *g* (5 min) and further digested in 1 ml of 2 M sodium hydroxide for 2 h. The digested cellular materials were then centrifuged and the pellets were rinsed 3 times with 0.1 M phosphate-buffered saline containing 0.1% bovine serum albumin. The pellets were suspended in 2 ml of deionized water, and the suspension was mounted onto a glass slide, stained with Lugol's iodine solution according to Choi et al. (1989), and then examined under a light microscope. The presence of hypnospores in each clam was recorded.

DNA extraction

The DNA of the above excised part of the gill and of each control parasite-infected tissue was isolated using a DNeasy Blood & Tissue Kit, following the manufacturer's instructions (Qiagen). The total DNA of oysters was isolated in the same manner with excised gill tissue. All DNA isolates were eluted into 50 µl elution buffer and stored at −20°C before use.

Construction of recombinant plasmid for positive control

The 673 bp ITS-2 fragments were amplified from RFTM-positive samples by PCR using the primers PerkITS85 (5′-CCG CTT TGT TTG GAT CCC-3′)

and PerkITS750 (5′-ACA TCA GGC CTT CTA ATG ATG-3′; Casas et al. 2002). The amplified products were purified and cloned into pGEM-T vector (Promega). After sequencing, the sequence information of these amplicons was subjected to BLAST analysis online (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

LAMP performance

A set of 6 primers (2 outer primers, 2 inner primers, and 2 loop primers) was designed to target the conserved ITS-2 region of *Perkinsus* spp., using the online software Primer Explorer V4 (http://primerexplorer.jp). The ITS-2 sequence of *Perkinsus* isolates from China (GenBank accession number FJ841985) was chosen as the reference sequence for primer design. Considering lower dimer formation, less cross-reaction, and higher amplification efficiency, the ultimately selected primer sets were as follows: forward outer primer (F3), TGG ATT TTG GTA TTT CAA AAC GA; backward outer primer (B3), ATG TTT GTA TCG GGA AGA AGA G; forward inner primer (FIP), AGT GCT TAT CGC ACT TCG CTC AAA CTC TCA ACG ATG GAT GC; backward inner primer (BIP), GAT TTG CAG AAT TCC GTG AAC CAG CAC TGA TAT GTA TGT ACA AAG AGG; loop forward primer (LF), TCC TTC ATC GAT TCT CGA GC; loop backward primer (LB), TCA ACG CAT ACT GCA CAA AG.

To acquire an ideal amplification system, we tried different dosages of each ingredient. Betaine (range from 0 to 0.896 M), $MgSO_4$ (4 to 32 mM), dNTP (0.4 to 1.6 mM), *Bst* DNA polymerase (1.6 to 20.8 U), outer primers (0.1 to 0.6 µM), inner primers (0.8 to 2.8 µM), and loop primers (with or without) were optimized. Turbidity was used as the index for judging quality, and was measured by a Loopamp® real-time turbidimeter (LA-320C, Eiken). Double-distilled water (dd H_2O) was used as template in blank controls. The detection limit of the assay was determined by using 10-fold serial dilutions of plasmid (ranging from 10^7 to 10^0 copies) as template. The results were judged using a parabola chart, an amplification efficiency chart, or a histogram.

To avoid cross reaction with other protozoan parasites and host tissues, DNA from *Bonamia ostreae*, *Haplosporidium nelsoni*, *Ichthyophthirius multifiliis*, and healthy clams was used as templates in the specificity verification of the *Perkinsus* spp. LAMP assay. To ensure that the developed *Perkinsus* LAMP assay could work on other *Perkinsus* species besides *P. olseni*, suspected *P. marinus*-infected oysters were chosen for LAMP detection. To determine the actual infection status of these samples, PCR amplification of a *P. marinus* sequence combined with histological observation as further performed according to Audemard et al. (2004).

Evaluation of the LAMP assay

As recommended by OIE, RFTM has been considered the 'gold standard' in the diagnosis of *Perkinsus* infection. The above 60 clams examined by RFTM were further evaluated by the LAMP assay. Positive plasmid (1.0×10^6 copies) was used as the positive control and healthy clam DNA as the negative control in the LAMP assay. The result of LAMP detection was compared with that of the RFTM assay.

Samples with different results from RFTM and LAMP detection were further examined. As one *Eco*RI digestion site was found within the targeted DNA sequence of *Perkinsus* spp., restriction endonuclease *Eco*RI digestion was carried out. The digestion solution (20 µl) consisted of 2 µl 10×H Buffer, 15 U *Eco*RI, 2 µl LAMP products, and 15 µl ddH_2O. The solution was incubated at 37°C for 3 h. The digested solution was visualized under UV light after 2% agarose gel electrophoresis. The digested LAMP fragments were also sequenced and then analyzed by BLAST online after separation and purification from the gel.

Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of the LAMP assay were calculated as: DSe = TP/(TP + FN) and DSp = TN/(TN + FP) (OIE 2012b), where TP is true positive, FN is false negative, TN is true negative, and FP is false positive.

RESULTS

RFTM culture assay

The pellets of each sample cultured in the RFTM assay were observed under a light microscope. The hypnospores of *Perkinsus* were about 10 to 50 µm in diameter, bluish-black, and spherical, and were easily observed (Fig. 1). Of 60 samples, 52 were determined to be *Perkinsus* positive, and the 8 remaining samples were deemed *Perkinsus* negative (Table 1).

PCR and recombinant plasmid construction

Electrophoresis of the PCR products revealed an approximate 673 bp amplicon, shown in Fig. 2.

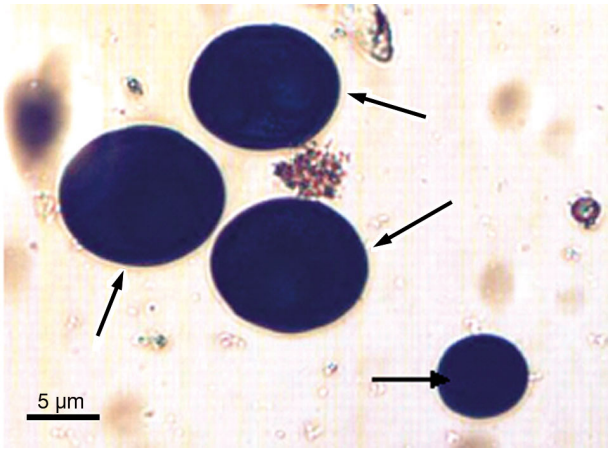

Fig. 1. Results of the RFTM assay. Arrows indicate the Lugol's iodine-dyed hypnospores of *Perkinsus* (100×)

Table 1. Diagnostic sensitivity and diagnostic specificity of the *Perkinsus* LAMP assay. Totals are given in parentheses. TP: true positive, FP: false positive, FN: false negative, TN: true negative

Method	RFTM positive (52)	RFTM negative (8)
LAMP positive (56)	52 (TP)	4 (FP)
LAMP negative (4)	0 (FN)	4 (TN)

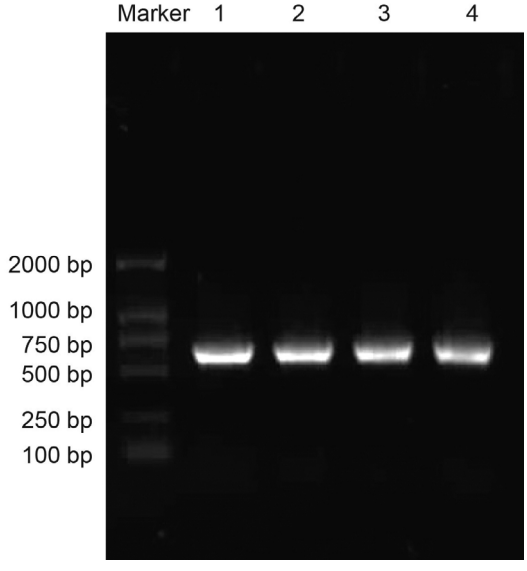

Fig. 2. Results of 4 representative *Perkinsus olseni*-infected clams *Ruditapes philippinarum* amplified by PCR assay. All 4 samples showed 673 bp fragments as indicated. Lane 1, marker; lanes 2 to 5, PCR products from 4 clams

Sequencing results revealed that all PCR products were consistent with the targeted ITS2 sequence of *Perkinsus olseni* (GenBank accession number FJ841985).

Optimization of *Perkinsus* LAMP assay

Our experiment revealed that the concentration of some reagents can influence the efficiency of amplification. The optimal concentration of betaine and Mg^{2+} was 0.128 M and 8 mM, respectively (Fig. 3A,B), whereas the concentration of dNTP, *Bst* DNA polymerase, and outer primers had little effect on the LAMP amplification efficiency. An increased concentration of the inner primer could improve amplification efficiency. Supplementation with loop primers could considerably speed up the reaction, as shown in Fig. 3C,D. The optimal reaction system (25 µl volume) was thus 1× Thermo buffer, 0.128 M betaine, 8 mM $MgSO_4$, 1 mM dNTP mix, 8 U *Bst* DNA polymerase, 0.2 µM primer F3 and B3, 2 µM primer FIP and BIP, 0.8 µM primer LF and LB.

Sensitivity and specificity of *Perkinsus* LAMP assay

Our results indicated that the detection limit of the developed *Perkinsus* LAMP assay was 10 copies of plasmid DNA with loop primers. The detection time was 49.8 min (Fig. 3D). There was no amplification with one copy of plasmid template and the negative control within 90 min. In the specificity test for the *Perkinsus* LAMP assay, DNA template from *Bonamia ostreae*, *Ichthyophthirius multifiliis*, *Haplosporidium nelsoni* and healthy clam tissue did not yield any positive result (Fig. 4). With the developed LAMP assay, 18 of 40 oyster samples were shown to be *Perkinsus* sp. positive, which was consistent with the results of PCR and histological identification for *P. marinus*.

Evaluation of the *Perkinsus* LAMP assay

Of 60 samples, 56 yielded a positive result in the LAMP assay (Table 1). These 56 samples included 52 positive and 4 negative samples as determined by RFTM. To confirm the infection status of the 4 samples which were negative by RFTM but positive by LAMP, amplified DNA of the 4 LAMP products was digested with the endonuclease *Eco*RI. All showed 87 bp and 101 bp fragments in electrophoresis, which was in agreement with the predicted fragment size

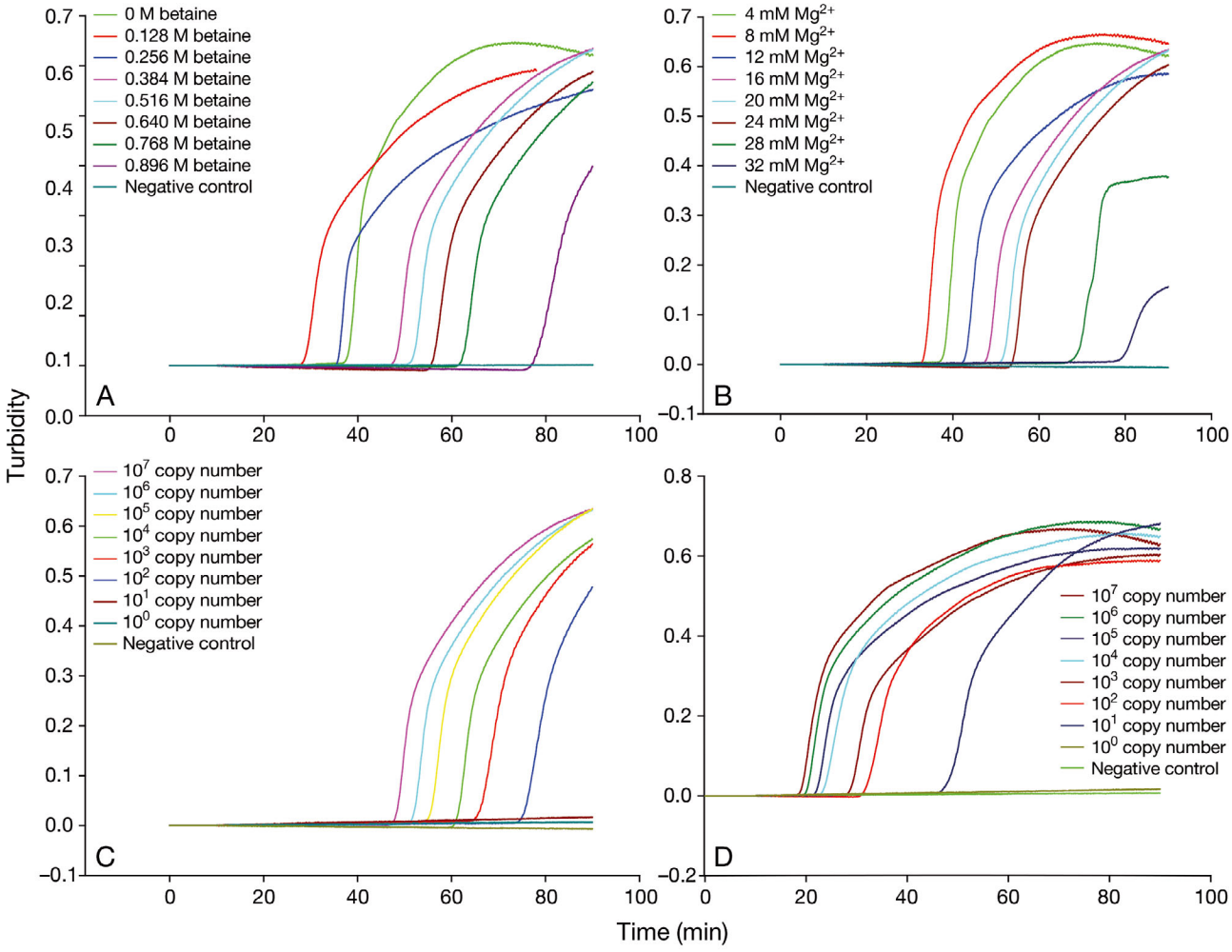

Fig. 3. Optimization of the LAMP reaction system: (A) betaine concentration; (B) Mg^{2+} concentration. (C) LAMP reaction without loop primers, and using 10-fold serial dilutions of plasmids from 10^7 to 10^0 copies as templates; (D) LAMP reaction with loop primers, using the same templates as in panel C

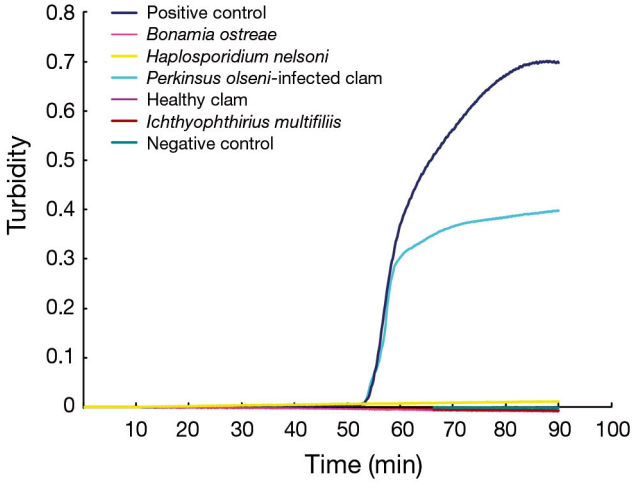

Fig. 4. Determination of the specificity of the *Perkinsus* LAMP assay

(Fig. 5). Further sequencing and BLAST of the digested products showed that all belonged to *Perkinsus* spp. Thus, the 4 RFTM-negative samples were in fact infected with *Perkinsus*. The DSe and DSp for the *Perkinsus* LAMP method were 100 and 50 %, respectively (Table 1).

DISCUSSION

Being responsible for mass mortality of some commercially valuable shellfish, *Perkinsus* has received much attention in recent years. The association of *Perkinsus* spp. with large mortalities has required detection methods for surveillance and quarantine in the field and at ports. The LAMP assay is known for its simplicity. The LAMP reaction can be carried out by mixing the template and reaction reagents in a single

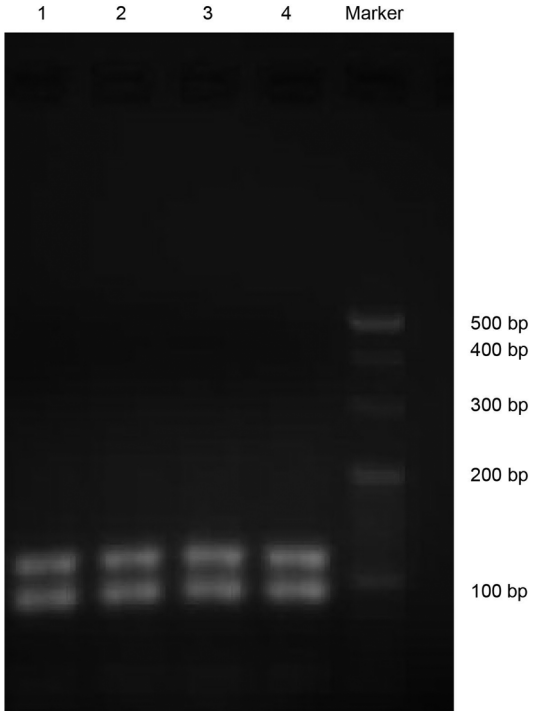

Fig. 5. Electrophoresis of endonuclease digestions of 4 LAMP products of LAMP-positive but RFTM-negative samples

tube and then incubating at stable 60–65°C for a short time. The only laboratory equipment needed for the LAMP reaction is a regular water bath or a heating block that can provide a constant temperature. With these considerations, a LAMP assay for the detection of *Perkinsus* spp. was developed in this study.

The primers for the LAMP assay were designed based on the ITS2 region of *Perkinsus* spp. ITS regions in the rRNA gene of the protozoan represent a domains that accumulate a high degree of sequence variability between genera but a relatively conserved region between species of *Perkinsus*, and are commonly used as gene markers (Kotob et al. 1999). The consistency and specificity of the primers were analyzed using BLAST online. The region amplified by our LAMP assay was 100% conserved between *P. olseni, P. marinus*, *P. mediterraneus*, and *P. honshuensis*, which indicated theoretically that this assay could be applied to detect several *Perkinsus* spp. In this study, we detected 2 OIE notifiable species of *Perkinsus*, viz. *P. olseni*, and *P. marinus*, by the LAMP assay. Due to the limited sample materials in our lab, more *Perkinsus* species should be tested in the future.

As indicated in our data, Mg^{2+} and betaine could affect the reaction efficiency (Fig. 3A,B). We used 8 mM Mg^{2+} in our LAMP, which showed little difference with other reports, while 0.128 M betaine was substantially lower compared to other reports (Gunimaladevi et al. 2005, Zhao et al. 2010). These results offer a new range of betaine concentration for future studies. The loop primers could also accelerate the amplification efficiency of the LAMP. The lowest detection limit of the LAMP assay was 100 copies without loop primers but improved to 10 copies with loop primers (Fig. 3C,D). The loop primers could hybridize with and help the formation of stem-loops, which greatly enhance the velocity of the LAMP reaction (Nagamine et al. 2002). Mori et al. (2001) showed that the DNA template could be increased up to 10^9 times within 10 to 15 min when adding loop primers to the reaction solution. To enhance the reliability of the LAMP examination in the field, the gills of infected clams were selected as the material to extract DNA. Gills of infected clams have the highest concentration of *Perkinsus,* as reported by Alderman & Gras (1969).

Given the short time for temperature changes during the reaction (Nagamine et al. 2001), the LAMP assay could be finished within 49.8 min in our study (Fig. 3D), which is faster than conventional PCR methods. This is very important for the quarantine at ports, where shorter times for import and export admission are necessary to ensure high quality of the goods.

No cross-reactivity to clam tissue itself and other aquatic parasites was observed for the LAMP assay (Fig. 4). This result is consistent with previous studies (Liu et al. 2008, Chen et al. 2010), and might be due to the use of a set of 6 primers targeting 8 distinct regions on template DNA and the selection of the properly targeted DNA region. Up until now, the LAMP assay has been extensively used in pathogen detection (Liu et al. 2008, Yamazaki et al. 2008, Chen et al. 2010).

The RFTM assay has been recommended as the 'gold standard,' but it only detects *Perkinsus* spp. from fresh host tissue. Combined with the DNA extaction method, the LAMP assay in this study could detect *Perkinsus* spp. from fresh or frozen tissues and environments like sea water and dirt, which the RFTM assay cannot (Audemard et al. 2004). The RFTM assay was reported to be only able to detect >1000 *P. marinus* cells per gram of wet oyster tissue (Bushek et al. 1994). As shown by our research data, the lowest detection limit of the LAMP assay was 10 copies of plasmid without considering that there are multiple regions of ITS2 in the genome of one *Perkinsus* cell, which was similar to real-time PCR (10 versus 26 plasmid copies; Wu et al. 2009). LAMP was also reported to be more sensitive than conventional

PCR (Z. Liu et al. 2008, A. Liu et al. 2012). Other reports showed that the LAMP assay could detect a limit of 10 copies of the apical membrane antigen-1 (AMA-1) gene of parasitemia (Lau et al. 2011). The detection limit of the LAMP assay developed for the detection of *Theileria luwenshuni* and *T. uilenbergi*, parasites of small ruminants, was down to 0.1 pg of parasite DNA (Liu et al. 2008).

Reclassification of 4 samples from RFTM by the LAMP assay showed that the LAMP assay was more sensitive than the RFTM assay in perkinsosis diagnosis, even though they had the same value of Dse when RFTM was selected as the 'gold standard.' For this reason, the Dsp of the LAMP assay was only 50% of that of the RFTM assay.

The LAMP also showed great potential for application in the field with or without special equipment. The white turbidity can be observed by the naked eye, and observations could be simplified by pulse-spinning the precipitate into the bottom of the tube. Other methods for the convenient observation of LAMP products, such as dyeing with calcein (Tomita et al. 2008), SYBR Green, or HNB (Venkatesan et al. 2012) have also been developed as alternatives. Being a one-tube reaction, the LAMP assay minimizes potential environmental contamination, is convenient for detection on-scene and can potentially be used in quarantine/surveillance programs.

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