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Full Length Research Paper

Sequential method for rapid early diagnosis of white spot syndrome virus in crayfish

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We developed a practical method to rapidly detect and diagnose latent white spot syndrome virus (WSSV) in infected crayfish that were non-symptomatic for WSSV. This method included a simplified extraction of DNA template, optimized loop-mediated isothermal amplification (LAMP), and final visualization of the product by means of staining with SYBR green I. Using this method, WSSV was detected in crayfish that had been artificially infected in two ways: at 5 h after injection, and 24 h after feeding with tissue from WSSV-infected crayfish (at a stage when such infected crayfish were non-symptomatic), and a thousand times or more dilution can omit fluorescent background when SYBR green I was used. Results indicate that this was a rapid, convenient, and highly sensitive method for the early diagnosis and detection of WSSV. The whole detection procedure took less than one hour to complete.

Key words: White spot syndrome virus, loop-mediated isothermal amplification, SYBR green I, *Procambarus clarkii*, early diagnosis.

INTRODUCTION

White spot syndrome virus (WSSV), an envelope dsDNA virus in the genus *Whispovirus* (Nimaviridae*)* (Leu et al., 2009), can infect many crustacean species, including penaeids and other shrimp and crabs (Lo et al., 1996a; Supamattaya et al., 1998; Peng et al., 1998; Chen et al., 2000), often causing extensive economic losses to the shrimp aquaculture industry in most major shrimp-producing countries. Certain clinical symptoms caused by bacterial infection may hinder diagnosis of WSSV infections and lead to misdiagnosis, and the WSSV genome may reside within hosts in a quiescent state or remain as a persistent infection. The persistence of such infections is the main reason for the high prevalence of WSSV in the ecosystem, which explains why WSSV is regarded as one of the most hazardous viruses in shrimp aquaculture (Khadijah et al., 2003). Detection of WSSV has been primarily based on molecular methods such as single-step polymerase chain reaction (PCR) (Lo et al., 1996b; Nunan et al., 1997) and nested PCR (Thakur et al., 2002), but assay detection using these techniques may require four to six hours to complete the procedures of DNA extraction and PCR amplification, as well as electrophoresis that would be required for the confirmation of results. In contrast, the loop-mediated isothermal amplification (LAMP) test for nucleic acids under isothermal conditions is well known for its specificity and sensitivity, higher than that of PCR. It can amplify a target nucleic acid to 10^9 copies at 60 to 65°C within one hour. LAMP and modified-LAMP, such as reverse transcription-loop-mediated isothermal amplification (RT-LAMP) have been developed for the detection of viral and bacterial pathogens such as (hepatitis B virus) HBV DNA (Li and Liu, 2005), SARS RNA (Hong et al., 2004) and Campylobacter fetus (Wataru and Masumi, 2009). Certain pre-clinical detection of pathogens, such as classical swine fever virus (CSFV) has also employed

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Abbreviations: WSSV, White spot syndrome virus; **LAMP,** loop-mediated isothermal amplification.

a LAMP protocol, combined with agarose gel electrophoresis (Chen and Zhang, 2009). This LAMP combined with electrophoresis method has been developed for detection of shrimp pathogens such as yellow head virus (YHV) (Mekata et al., 2009), Taura syndrome virus (TSV) (Kiatpathomchai et al., 2007), the *Macrobrachium rosenbergii* nodavirus (MrNV) (Pillai et al., 2006), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun et al., 2006) and WSSV (Kono et al., 2004). At least 30 min is, however, required to detect the LAMP product by means of gel electrophoresis staining with carcinogenic ethidium bromide (EtBr). It has also been shown that LAMP, combined with lateral flow dipstick, can be used for diagnosis of WSSV (Jaroenram et al., 2009). This approach is complicated however, since it requires extra loop LAMP primers and DNA probe design, and lateral flow dipstick has to be prepared in advance for its application.

It is possible to improve DNA extraction methods, since the procedure involving DNA purification from samples can be omitted by making use of rapid boiling methods (Kaneko et al., 2007). However, WSSV virions had to be isolated before the DNA of WSSV was extracted (Kaneko et al., 2007; Jaroenram et al., 2009) to insure the purity of WSSV-DNA, but this procedure costs time and money. SYBR green I has strong DNA binding affinity. The exceptional sensitivity of this dye makes it useful in many applications that involve a limited amount of DNA, and the fluorescence yielded by DNA and SYBR green I complex can be detected (Invitrogen, USA). LAMP products can be stained with SYBR green I, enabling visual identification of LAMP amplicons simply by observing fluorescence emitted under ultra violet (UV) irradiation. SYBR green I will also combine double strand DNA, except the target of LAMP amplicons, if WSSV-DNA template.

Shrimp and crayfish are members of *Decapoda*, some of them have been found the host of WSSV. *Procambarus clarkii* has a high economic value in China, and it was used as an experimental animal model for virus proliferation and immune responses (Zhang and Shu, 2009; Mo et al., 2002). Recently, recombinant oral vaccine has been used for protection of *P. clarkii* from WSSV in *Pichia pastoris* (Jha et al., 2007). Therefore, it was selected as the experimental model. A sequential method for the detection of WSSV that includes simplified DNA template preparation, high efficient LAMP and visualization under UV irradiation, is sensitive and efficient, simple and convenient, as well as safe. This rapid method can be used to detect WSSV before the infected shrimp becomes symptomatic. Therefore, it has the capacity to diagnose WSSV at early stage of infection, and we can use it to predict whether cross-infection exists among neighboring shrimp-cultivating pools. This new WSSV-detection method also has the advantage of being rapid, having high specificity and efficiency, and limits the detection within one hour. This makes it suitable for use in both the laboratory and the field.

MATERIALS AND METHODS

Samples and viral stock preparation

Healthy *P. clarkii* crayfish, weighing approximately 15 g, used for WSSV experimental challenge, were obtained from a local market in Suzhou, China, and were acclimatized in the laboratory for seven days in continuously-aerated aquaria containing fresh water, kept at about 25°C. A WSSV viral stock solution prepared from naturally-infected *P. clarkii* was obtained from a farm in Nanjing in which an outbreak of WSSV had occurred. A tissue sample weighing 1.0 g was mixed with 1000 μl of Tris EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 25°C). After centrifugation at 5000 rpm for 5 min, the supernatant was used as the experimental viral stock solution.

DNA template preparation

The total DNA was extracted directly from tissue of diseased crayfish by means of the rapid boiling method (Kiatpathomchai et al., 2001), but with the specially developed buffer. To select the best solvent associated with rapid boiling method, five solvent were prepared: buffer I (20 mM Tris-HCl, 10 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100, pH 7.5, 25°C), buffer II (20 mM Tris-HCl, 10 mM KCl, 0.1%Triton X-100, PH 8.8, 25°C), buffer III (20 mM Tris-HCl, 10 mM KCl, 0.1% Triton X-100, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, pH 8.8, 25°C), TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 25°C), and double-distilled water. An equivalent amount of WSSV-infected crayfish tissue was mixed with each of these solvent to make five different mixtures. The mixture was then heated at 95°C for 5 min, and then centrifuged at 5000 rpm, at a temperature of 4°C for 5 min. The supernatant was used as the DNA template for LAMP, and the rapid boiling method associated with the best solvent was selected for DNA template preparation for subsequent procedures.

LAMP primer design and LAMP conditions

Four LAMP primers were designed according to the published sequence of open reading frame (ORF) 234 of the WSSV genome (GeneBank accession no. AF332093) (Yang et al., 2001). Forward inner primer (FIP) of WSSV ORF 234, named WSSV-FIP-234, included F1C-234, a linker of TTTT, and F2-234 (5′-CGC ATA TTT CCC TCT ATC GCT ATT ATT TTTT AGC ACA GAT TTT TTG AT-3′). Backward inner primer (BIP), named WSSV-BIP-234, contained B1C-234, a linker of TTTT, and B2-234 (5′-GGT CTG AAA TAT ACA TGG GTG CCT TTTT GAA AAT GGG GTT TAC GAC AA-3′). Primer WSSV-F3-234 and WSSV-B3-234 of WSSV ORF 234 were 5′-GGA AAA GGA ACG TTT TGT TG-3′ and 5′-GCA ATG GGA ATG ATA ACT CTT-3′, respectively (Figure 1). All of the primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Company Limited. LAMP was carried out in a total volume of 25 μl containing 2.0 μl (20 pmol) of each WSSV-FIP-234 and WSSV-BIP-234, 1.0 μl (5 pmol) of WSSV-F3-234 and WSSV -B3-234, 2.5 μl of 10 × reaction buffer, 1.0 μl of 10 mM dNTPs, 1.0 μl of DNA template and 14.5 μl of double-distilled water. The mixture was kept at 95°C for 5 min, then chilled on ice, after which 1.0 μl (8 U) of *Bst* DNA polymerase large fragment (New England Biolabs Limited) was added. Tests were carried out at three different temperatures (60, 63 and 65°C) to determine the optimum reaction temperature, and the LAMP products were

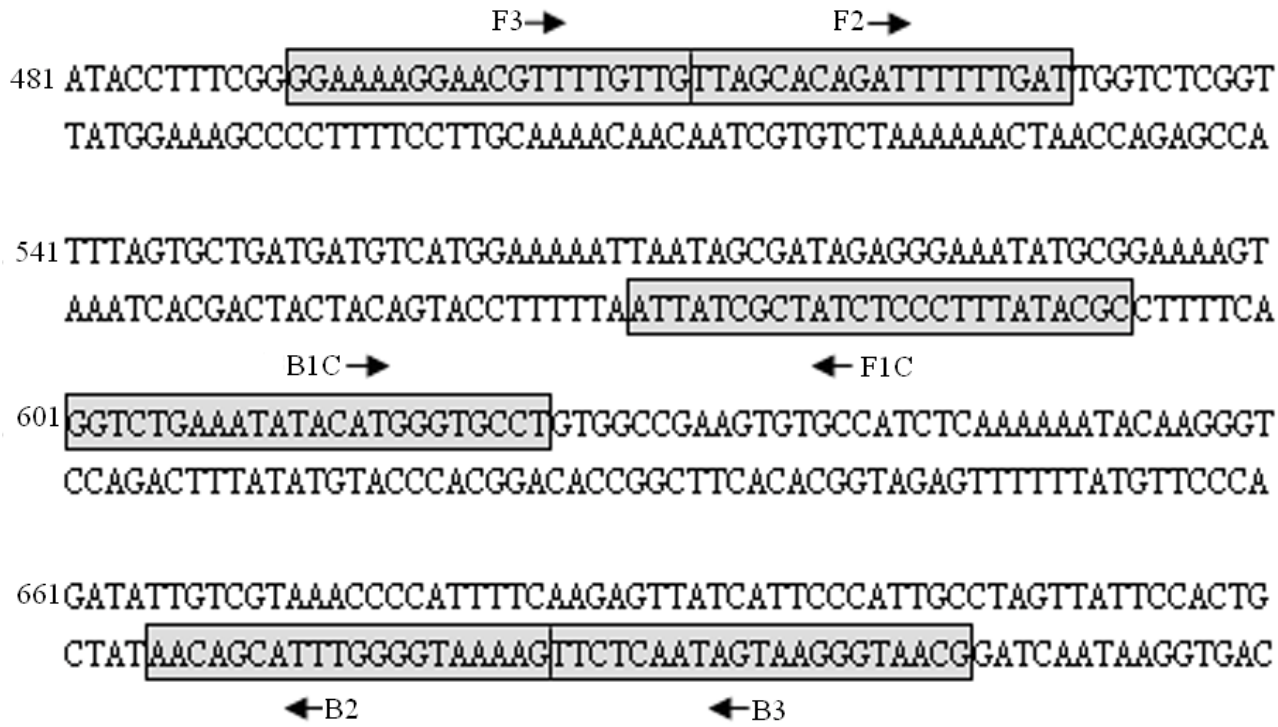

Figure 1. Sequences of LAMP primers. Boxes and arrows show LAMP primers of ORF 234 of the WSSV genome (GenBank accession no. AF332093) (Yang et al., 2001).

electrophoresed on 2% agarose gel to determine optimal conditions.

Single-step PCR for diagnosis of WSSV

Single-step PCR was performed in a total volume of 25 μl containing 2.5 μl of 10× *Taq* polymerase buffer, 0.5 μl of DNA template, 0.25 μl of each primer: WSSV-P1 (5′- CCG AAT TCA CCA TGG AGT ATA TAG GGG-3′) and primer WSSV-P2 (5′- CGA AGC TTG ATA CAG TGA CCG TCC CTG-3′), 1.0 μl of 0.25 mM of dNTPs, 1 U of *Taq* DNA polymerase and double-distilled water. DNA template for single-step PCR was optimized using the rapid boiling extraction method with buffer III. The reaction mixture was heated and kept at 94°C for 4 min (for initial denaturation), followed by 35 cycles comprising 50 s of denaturation at 94°C, 50 s of annealing at 51°C, and 1 min of elongation at 72°C, followed by a final extension of 10 min at 72°C.

Comparative analysis of detection limits of single-step PCR and LAMP and sensitivity of LAMP combined with SYBR green I dyeing

To determine the detection limit of single-step PCR and LAMP for WSSV, a 10-fold serial dilution of total DNA extracted from WSSV-infected crayfish by rapid boiling method with buffer III was used as a template. Single-step PCR and LAMP reaction, and analysis on agarose gel electrophoresis were then undertaken. To evaluate effectiveness and sensitivity of LAMP combined with SYBR green I dyeing, LAMP product stained with SYBR green I inner tubes were observed directly under UV irradiation, and analysis of LAMP product on 2% agarose gel electrophoresis was undertaken as well.

Artificial infection and early diagnosis of WSSV in challenged *Procambarus clarkii*

15 healthy crayfish obtained from a natural environment, were randomly divided into three groups of five: in the first group, the crayfish were each injected with 2.5 μl of viral stock solution; in the second group, the crayfish were given 0.5 g of tissue from WSSV-infected crayfish and in the third group, the crayfish were each injected with 2.5 μl of viral stock solution, which was devitalized at 100°C for 5 min. The water in which all the challenged crayfish were kept was fully-refreshed on a daily basis. Pleopods (about 0.1 g) of the challenged crayfish were cut, and at 4 h post-injection, to obtain a sample for viral checking. Starting at a time of 24 h after feeding with WSSV-infected tissue, the challenged crayfish were checked. The last five injected crayfish were sampled as a negative control. LAMP results were demonstrated by 2% agarose gel electrophoresis and by directly observing the products stained with SYBR green I inner tubes under UV irritation, respectively.

RESULTS

Determination of LAMP conditions for WSSV detection

To determine LAMP reaction temperature, the LAMP reactions were carried out at 60, 63 and 65°C, using total DNA extracted from WSSV-infected crayfish by means of the rapid boiling method, for use as a template.

Electrophoresis results indicated the following performance results of LAMP at various temperatures: at 63°C,

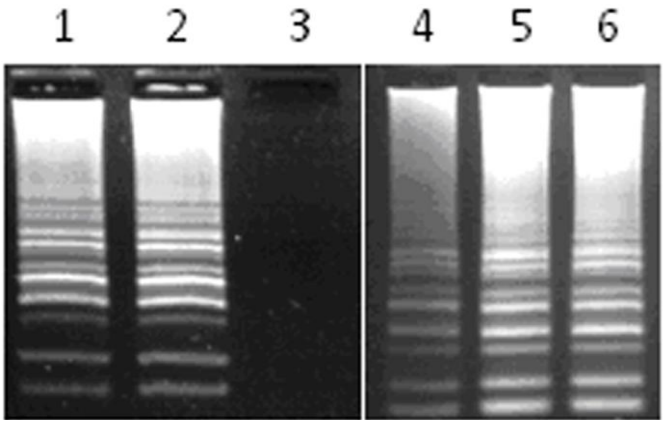

Figure 2. Determination of LAMP conditions. Lanes 1 to 3, LAMP reaction at 60, 63 and 65°C for 75 min, respectively. Lanes 4 to 6, LAMP reaction at 63°C for 45, 60 and 75 min, respectively.

performance was highly successful; performance at 60°C was satisfactory, but no strap was produced at 65°C, and the ladder-like straps of amplification at 63°C for 45 min were clearly visible (Figure 2). Therefore, LAMP at 63°C for 45 min was determined as the LAMP conditions for detecting ORF 234 of the WSSV genome.

Comparison of five DNA extraction methods

LAMP was performed using DNA template extracted from an equivalent amount of tissue of WSSV-infected crayfish, and the template was extracted using the rapid boiling method with five different solvents, respectively. Results indicate that LAMP, using DNA templates extracted through the rapid boiling method with buffer III, performed the best on gel electrophoresis, which indicated that rapid boiling method with buffer III was superior to that of the four other methods, and that the rapid boiling method was improved by using buffer III when used to detect ORF 234 of the WSSV genome (Figure 3).

Detection limit of single-step PCR and LAMP for WSSV

10-fold serial dilutions of total DNA extracted from WSSV-infected shrimp using the rapid boiling method were used as the respective template for comparison between LAMP and single-step PCR (Figures 4A and B). Analysis on agarose gel electrophoresis showed that LAMP products performed well-formed straps at 1 to 10^9 dilutions, while the detection limit of single-step PCR was at 10^3 dilutions. Results indicate that the LAMP method for detecting WSSV was 10^6 more sensitive than that of single-step PCR.

Sensitivity of LAMP combined with SYBR green I dyeing

The remarkable sensitivity of SYBR green I binding to DNA suggest that it may also bind to LAMP primers and DNA templates, resulting in non-ignorable background fluorescence in tubes. It was, however found that serial diluting could greatly reduce background fluorescence, but did not obviously limit the recognition of LAMP amplicons. Diluting of LAMP product to 10^{-3} or even lower, could also result in the emission of strong visible green fluorescence under UV excitation after the addition of SYBR green I. A thousand-fold dilution of product without the production of amplicons would lower the concentration of previously-added DNA (primers and DNA template) to such an extent as to result in negligible emissions under UV, after the addition of SYBR green I, as seen in the left of Figure 5. Using 10^6 to 10^9 ten-fold serial dilutions of total DNA extracted from WSSV-infected crayfish, by means of the rapid boiling method as respective template for LAMP, 10^3 dilution of LAMP product stained with SYBR green I could emit green fluorescence inner tubes under UV irradiation, upper right of Figure 5. The result was then confirmed by the satisfactory performance of LAMP product performed on 2% agarose gel electrophoresis stained with EtBr, downright of Figure 5. It indicated that LAMP combined with SYBR green I dyeing was simple, sensitive and with high effectiveness.

Early diagnosis of WSSV in challenged crayfish

Crayfish were sampled at 4, 5, 6, 12, 18, and 24 h post-injection to detect WSSV in the first injection group. Using the improved rapid boiling method, the supernatant solution after centrifugation was taken for use as a DNA template for LAMP. Result of LAMP product by gel electrophoresis indicated that the crayfish was infected with WSSV at 5 h post-injection, and at 24 h after feeding with tissue from WSSV-infected crayfish. It indicated that through injection, the proliferation of WSSV was easier than that through *per os*. Single-step PCR was able to detect WSSV after injecting for 24 and 42 h post-feeding at the same time (Figures 6A and B). Meanwhile, dilutions of 10^3 of LAMP product stained with SYBR green I were noted to emit green fluorescence under UV irradiation when the crayfish were injected with 2.5 μl of viral stock solution from 5 h, and from 24 h after feeding with tissue from WSSV-infected crayfish. Results obtained from LAMP combined with SYBR green I dyeing analysis was in accordance with the above results (Figures 6A and B). The result from a DNA template extracted from a deactivated viral injection sample was negative. Results indicate that LAMP was more sensitive than single-step PCR when used for early diagnosis of WSSV, and LAMP combined with SYBR green I dyeing

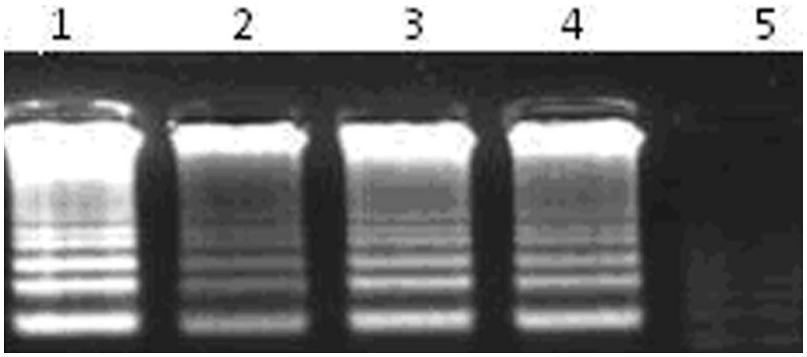

Figure 3. Determination of DNA template preparation by LAMP. Lanes 1 to 5, Respective electrophoresis results of DNA prepared using the rapid boiling method with buffer III, buffer I, buffer II, TE buffer and double-distilled water, respectively.

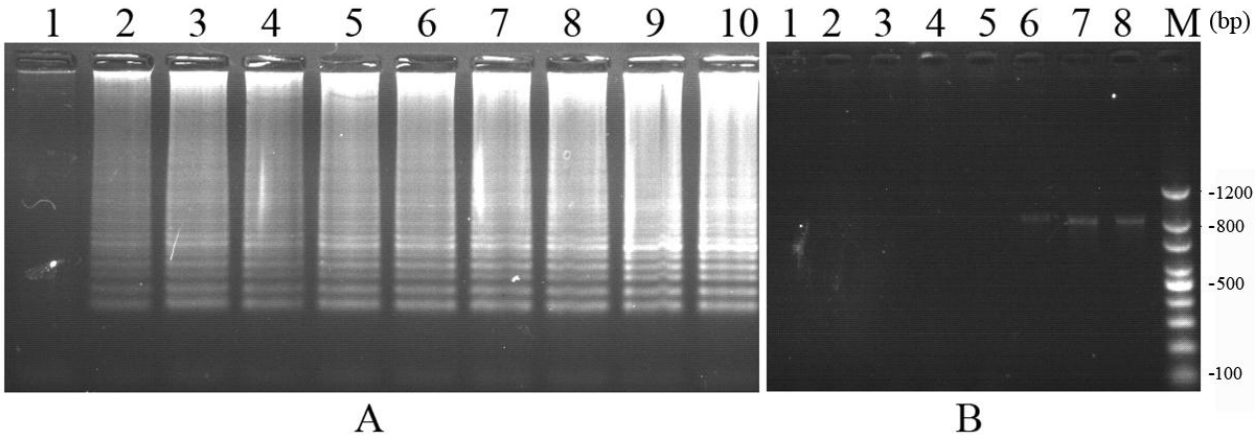

Figure 4. Comparison of sensitivity of LAMP and single-step PCR for WSSV detection. Lanes 1 to 10 (A), LAMP amplification with template of 10^{10} to 10^{1} ten-fold serial dilutions; lanes 1 to 8 (B), single-step PCR amplification with template of 10^{8} to 10^{1} ten-fold serial dilutions; lane M, molecular marker.

analysis was sensitive and effective in early diagnosis of WSSV.

DISCUSSION

Kono et al. (2004) previously reported that heat-extracted DNA could be used in LAMP to speed up DNA extraction. Since the LAMP is less sensitive to inhibition by sample components than is the case for PCR, the DNA purification step can be omitted (Kaneko et al., 2007; Teng et al., 2007). In the experiment, we have compared five different DNA extraction methods for DNA preparation and the LAMP results indicated that the quality of DNA extracted by the rapid boiling method with buffer III was higher than those extracted using the other four methods. Under optimal conditions for LAMP (ORF 234 of WSSV), we found that pre-heating and terminating steps at a high temperature (Nagamine et al., 2001) were not essential because when these two steps were omitted, LAMP performed well on gel electrophoresis, compared with similar LAMP procedures in which these two steps were included. Efforts were also made to concentrations optimization of WSSV-FIP-234, WSSV-BIP-234, WSSV-F3, WSSV-B3, and the DNA template amount was optimized to 1.0 μl. LAMP was therefore standardized in this experiment as described earlier.

The presence of betaine can improve DNA amplification through the induction of DNA conformational changes (Rees et al., 1993) and it was also reported that betaine provided catalytic proficiency to restriction enzymes (Sugimoto et al., 2005). Taken together, supplementation with betaine improved functioning in an efficient enzymatic reaction. In our experiment, however, adding betaine did not improve LAMP amplification. In some cases, no strap was shown on gel electrophoresis when betaine was added, whereas performance of LAMP, when betaine was omitted, was satisfactory under

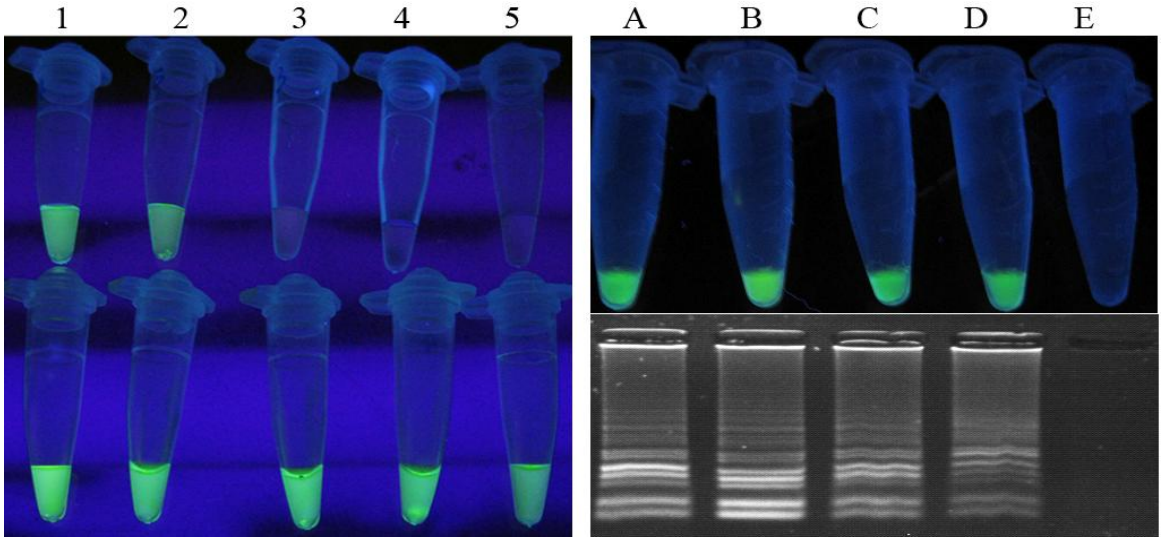

Figure 5. LAMP product stained with SYBR green I under UV irradiation and compared with gel electrophoresis. Lanes 1 to 5 (upper left), 10^1 to 10^5 ten-fold serial dilutions of LAMP product with DNA template prepared from healthy shrimp; lanes 1 to 5 (down left), 10^1 to 10^5 ten-fold serial dilutions of LAMP product with DNA template prepared from WSSV-infected crayfish; right lines (upper), 10^3 dilutions of LAMP product stained with SYBR green I under UV irradiation; right lines (lower), LAMP product performed on gel electrophoresis staining with EtBr; lanes A to D, LAMP using 10^6, 10^7, 10^8, 10^9 serial dilutions of template extracted from WSSV-infected crayfish; lane E, negative result using template extracted from healthy crayfish.

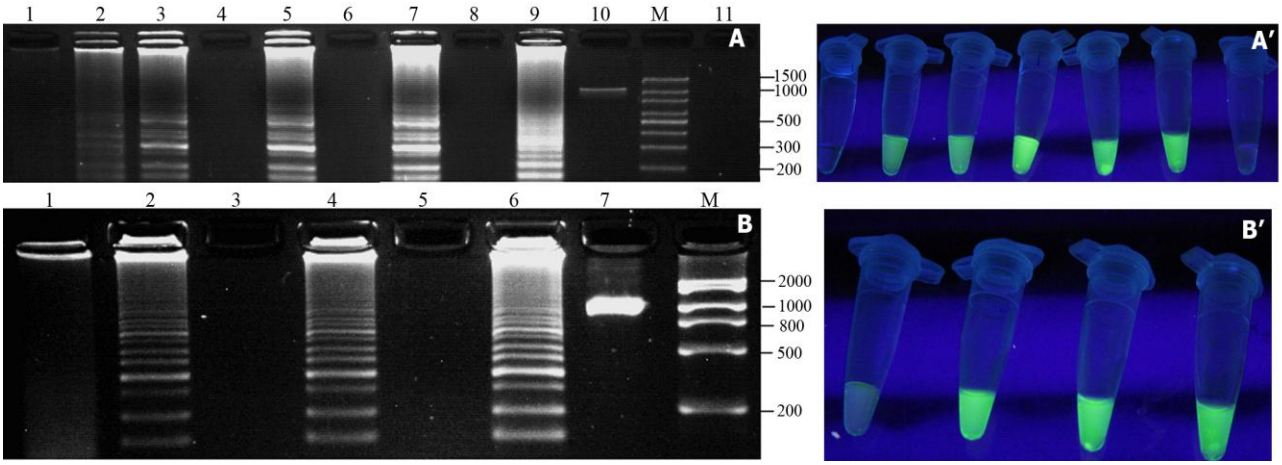

Figure 6. Result of early diagnosis of WSSV in injected and in tissue-feeding crayfish. A. Lanes 1, 2, 3, 5, 7, 9, LAMP result of sample from crayfish at 4, 5, 6, 12, 18, 24 h post-injection, respectively; lanes 4, 6, 8, 10, single-step PCR result of sample from shrimp at 6, 12, 18, 24 h post-injection, respectively; lane 11, LAMP result of sample from devitalized viral injected crayfish. A'. Samples corresponding with A visualized in tubes with SYBR green I dyeing. B. Lanes 1, 2, 4, 6, Detection of WSSV by LAMP from the challenged crayfish after feeding for 24, 30, 36 and 42 h, respectively; lanes 3, 5, 7, detection of WSSV by single-step PCR from the challenged crayfish after feeding for 30, 36 and, 42 h, respectively. B'. Samples corresponding with B visualized in tubes with SYBR green I dyeing. Lane M, molecular marker.

the same conditions. This might be attributed to the low GC-content in the detected DNA fragment (Henke et al., 1997), since the GC-content of ORF 234 of WSSV is less than 40%. The true reason is however still unclear. Further investigations into the omission of betaine therefore require further examination of LAMP reactions under various conditions.

WSSV is a systemic virus. When it infects crayfish through *per os*, it has to cross the epithelial cells and basal membrane of the digestive tract to reach the coelomic cavity where it would be able to infect permissive cells. Contrarily, when it infected crayfish through injection, virions were dispersed in the haemocoel by haemolymph circulation, and were distributed quickly in

different tissues, constituting the first foci of infection. Therefore, under the same condition, the injection might cause the WSSV disease occuring earlier than that caused by *per os* infection. In the present study, in the challenged crayfish, the LAMP was able to detect WSSV at 5 h post-injection or at 24 h after feeding with tissue from WSSV-infected crayfish; it should be due to the different infection routes. Whereas, no WSSV was detected from the crayfish injected with deactivated virions even at 24 h post-injection, suggesting that by haemolymph circulation, it was also quickly distributed in different tissues after injection, but were unable to truly multiplicate, and therefore resulted in a negative result. Single-step PCR could detect WSSV after injecting for 24 and 42 h post-feeding, which were of 19 and 18 h later, respectively, compared to LAMP combined with SYBR green I dyeing analysis. It indicated that LAMP was more sensitive than single-step PCR when used for early diagnosis of WSSV. LAMP was often combined with gel electrophoresis staining with carcinogenic EtBr. Certain disadvantages are however associated with such method; they are potentially harmful to laboratory researchers and also require long assay times. We employed LAMP, combined with SYBR green I dyeing. SYBR green I is a highly sensitive, fluorescent nucleic acid stain. Originally designed as a DNA gel stain (Haugland, 2002), it has been used successfully in many other applications, for example in the determination of DNA in solution (Vitzthum et al., 1999), capillary electrophoresis (Zabzdyr and Lillard, 2001), real time PCR (Ricketts, 2004) and staining virus-like particles (Rinta-Kanto et al., 2004). SYBR green I stain is a weak mutagen and appears to be less mutagenic than EtBr (Singer et al., 1999). In addition, sensitivity of DNA stained with SYBR green I is higher than that of DNA stained with EtBr for visualizing nucleic acids (Zipper et al., 2003).

One of the most practical characteristics of LAMP is that it allows visual detection of amplification through the addition of fluorescent dyes such as SYBR green I (Poon et al., 2006). The LAMP amplicons can be visually inspected by adding fluorescent dye SYBR green I under UV irradiation, since fluorescent complexes are formed by SYBR green I intercalation and emit green fluorescence (Theilmann et al., 2005). The production of non-ignorable background fluorescence has not however yet been reported in the absence of DNA of LAMP amplicons in tubes. Such background fluorescence can be attributed to the exceptional affinity of SYBR green I for DNA, and a large fluorescence enhancement upon DNA binding. It was reported that visual inspection can be used to distinguish positive or negative result in the detection of *Karenia mikimotoi* (Dinophyceae), by intercalating SYBR green I with LAMP product (Zhang et al., 2009). LAMP combined with SYBR green I dyeing has also been applied for the detection of *Schistosoma japonicum* DNA (Xu et al., 2010), but neither the production of background fluorescence nor detection sensitivity were recorded in the descriptions of these combined methods.

In the present study, we succeeded in eliminating background fluorescence. Since SYBR green I is one of the most sensitive stains available for detecting low quantities of dsDNA, (for example, in one thousand-fold dilutions of LAMP amplicons), and diluting will eliminate the background fluorescence produced by SYBR green I binding to LAMP primers and template, this may lead to a misinterpretation of the result. Therefore, LAMP combined with SYBR green I dyeing is a more reliable method for detection of WSSV; simply by a thousand-fold diluting of LAMP product. This simplifies the whole procedure by making the WSSV-specific amplicons easily and rapidly inspected by visualization under UV irradiation, or using the naked eye. In practice, even a simple mini UV irradiator is suitable when necessary. This LAMP-based sequential method described here has many practical advantages such as enabling the easy and efficient extraction of DNA, it does not require expensive equipment, analysis time is very short, and amplicons confirmation is achieved by simple visualization. These factors represent key advantages of this LAMP-based sequential method for WSSV detection. Compared with single-step PCR, LAMP is 10^6 times more sensitive for detecting WSSV. Using this sequential method, WSSV was detected at 5 h after artificial injection, and WSSV was detected at 24 h after feeding with tissue from WSSV-infected crayfish. In the early diagnosis of WSSV, the same result was given by single-step PCR almost 18 h later. WSSV was noted to proliferate rapidly in challenged crayfish after infection, which proved that the virus that had been detected was not the same as that which we injected, but had been proliferated in the challenged individual. Such a simple, sensitive and rapid detection method will be especially beneficial for confirmation of a WSSV-infected state prior to crayfish becoming symptomatic, and would therefore be an important factor when preparing for an outbreak of viral disease. This test platform can also be adapted for the rapid detection of other shrimp infectious agents by designing appropriate sets of LAMP primers. This sequential method also assists in exploring the multiplication cycle of WSSV, and would therefore benefit research on the diagnosis of spreading and cross-infection of WSSV. Applying this method during early diagnosis of WSSV under natural conditions will be beneficial and will help to significantly reduce the economic losses associated with this disease.

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