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Loop-mediated isothermal amplification for rapid detection of Acute viral necrobiotic virus in scallop *Chlamys farreri*

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Summary. – Loop-mediated isothermal amplification (LAMP) assay for rapid and sensitive detection of Acute viral necrobiotic virus (AVNV) in scallop *Chlamys farreri* was developed and evaluated. Four primers recognizing six targets on distinct AVNV DNA sequences were designed and the LAMP reaction was carried out in a water bath. Reaction temperature and time were optimized at 64°C for 60 mins and LAMP products were detected using agarose gel electrophoresis and visual assessment. Confirmation of the expected LAMP products was performed with *Mbo*I restriction enzyme analysis. The detection limit of LAMP assay was as low as 1 fg AVNV DNA and accordingly, this assay was 100 times more sensitive than conventional PCR technique. A comparative evaluation of 20 samples using the LAMP and PCR assays revealed a complete accord in positivity or negativity for AVNV. These results indicate that the LAMP assay is simple, sensitive, specific, and has a great potential for detection of AVNV in the laboratory and field.

Keywords: Acute viral necrobiotic virus; scallop Chlamys farreri; loop-mediated isothermal amplification

Introduction

AVNV is an enveloped, spherical DNA virus with spike-like surface protrusions (Wang *et al.,* 2002a, 2004). The species name Acute viral necrobiotic virus is only preliminary and the virus has not been approved by the International Committee on Taxonomy of Viruses (ICTV) yet. AVNV has caused an acute viral necrobiotic disease in scallop *C. farreri* in China (Song *et al.,* 2001; Wang *et al.,* 2002b; Ai *et al.,* 2003) since 1997. The mortality of scallops infected with AVNV can be as high as 90% within 5 to 8 days after the infection. It was estimated that the outbreak of the disease resulted in over 60% mortality of cultured *C. farreri.* This disease has been presenting the most severe threat to scallop industry in China. To the best of our knowledge, a successful cure against AVNV infection has not available been so far. Therefore, a development of preventive strategies, especially simple, rapid, and inexpensive screening technology for AVNV infection is of special urgency in the scallop farming.

At present, several methods including ultrastructural examination by electron microscopy (Wang *et al.,* 2002a, 2004), ELISA (Wang *et al.,* 2003; Fu *et al.,* 2005a), and immunofluorescence (Fu *et al.,* 2005b; Wang *et al.,* 2005) have been developed for the detection of AVNV. Usually, these methods are time-consuming, laborious, and insensitive. PCR assay is a powerful and sensitive tool for the detection of various pathogens, but it requires a thermal cycler for amplification and appropriate equipment for electrophoresis, what limits its use in hatcheries and farms. Real-time PCR assay has been recently developed for the rapid identification and quantification of AVNV with high precision and sensitivity (Ren *et al.,* 2009). However, it can not be routinely used in regular laboratories and scallop aquaculture facilities,

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Abbreviations: AVNV = Acute viral necrobiotic virus; BIP = backward inner primer; CyHV-3 = Cyprinid herpesvirus 3; FIP = forward inner primer; LAMP = loop-mediated isothermal amplification; OsHV-1 = Ostreid herpesvirus 1; WSSV = White spot syndrome virus

because of the expense of thermal cycler and fluorescence detector required for this assay. Therefore, a simple, rapid, and sensitive assay that could be used as a routine diagnostic tool in the regular laboratories and farms would be very critical for monitoring of AVNV infection.

LAMP is a new technique developed recently for amplification of nucleic acid under isothermal conditions (Notomi *et al.,* 2000). The method employs a set of four specific primers that recognize a total of six distinct sequences on the target gene and uses an auto-cycling strand displacement DNA synthesis performed by *Bst* DNA polymerase. In the auto-cycling strand displacement method**,** the outer forward primer hybridizes and displaces the synthesized first strand with the forward inner primer (FIP) linked complementary strand resulting in a stem-loop DNA structure at one end. This strand initiates a process in which a backward inner primer (BIP) hybridizes to the other end of the target DNA sequence and results in strand displacement by the backward outer primer to form a dumbbell shape. This process continues in subsequent LAMP resulting in the final products of multiple stem-loop DNAs. The whole procedure of LAMP reaction can be completed in less than 1 hr under isothermal conditions ranging from 60**–**65°C. Gene amplification products can be detected by visual observation of turbidity (Mori *et al.,* 2001) or by a color change when fluorescent dsDNA intercalating dye is added (Iwamoto *et al.,* 2003). Thus, LAMP assay is faster and easier to perform (Nagamine *et al.,* 2002) as well as being more sensitive and specific (Hara-Kudo *et al.,* 2005; Goto *et al.,* 2007). LAMP assay was developed and used for detection of various pathogens (Caipang *et al.,* 2004; Thai *et al.,* 2004; Saito *et al.,* 2005; Yeh *et al.,* 2005; Soliman and El-Matbouli, 2006; Blomström *et al.,* 2008; Saleh *et al.,* 2008). To date this method has not been applied to the specific detection of AVNV. In this paper, a highly sensitive, specific, and rapid diagnostic protocol for the detection of AVNV in scallop *C. farreri* is described.

Materials and Methods

Samples. Samples of scallop *C. farreri* infected and non-infected with AVNV were collected from scallop culture farms in Shazikou of Qingdao, Shandong Province, P.R. China in 2007. All samples were stored at -80°C.

Preparation of DNA template. AVNV virions were purified from tissues of scallop *C. farreri* infected with AVNV following the sucrose continuous density gradient method (Le Deuff and Renault, 1999). DNA from AVNV virions and kidney tissues of scallops were extracted with TaKaRa Universal Genomic DNA Extraction Kit Ver.3.0 (Takara Bio Dalian) following the manufacturer's protocol. The concentration of AVNV DNA was determined by A_{260} measurement. DNA sample was adjusted to 10 ng/μl in Tris-EDTA buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0, TE). Ten-fold serial dilutions from 1 ng/μl to 0.1 fg/μl were prepared in TE buffer.

DNA from White spot syndrome virus (WSSV) (the genus *Whispovirus*, the family *Nimaviridae*), Infectious hypodermal and hematopoietic necrosis virus (unassigned virus), Turbot reddish body iridovirus (unassigned virus; Shi *et al.,* 2004), Cyprinid herpesvirus 3 (CyHV-3) (unassigned virus), and Ostreid herpesvirus 1 (OsHV-1) (unassigned virus in the family *Herpesviridae*) were also included in the analysis to test for the specificity of LAMP assay.

Primers design for LAMP. The primers were designed from AVNV genomic DNA sequence (GQ153938) following the method of Notomi *et al*. (2000) and using the LAMP primer designing software PrimerExplorer V4 (http://primerexplorer.jp/e/). A set of four primers (two inner primers and two outer primers) recognizing six distinct regions on the target sequence (F1c, F2, F3, B1c, B2, and B3) was designed. Forward inner primer (AVNV-FIP) consisted of F1c, a TTTT linker and F2, and backward inner primer (AVNV-BIP) consisted of B1c, a TTTT linker and B2. The two outer primers AVNV-F3 and AVNV-B3 were located outside the F2-B2 region, respectively. The location and sequence of each primer are shown in Fig. 1 and Table 1.

Determination of LAMP conditions. LAMP was carried out with AVNV DNA as a template and the reaction was carried out in a 25 μl reaction mixture containing 1.6 μmol/l AVNV-FIP and AVNV-BIP primers, 0.2 μmol/l AVNV-F3 and AVNV-B3 primers, 1.6 mmol/l dNTPs, 1 mol/l betaine (Sigma-Aldrich), 4 mmol/l $MgSO_4$, 20 mmol/l Tris-HCl (pH 8.8), 10 mmol/l KCl, 10 mmol/l $(NH_4)_2SO_4$, 0.1% Triton X-100, 8 U of *Bst* DNA polymerase (New England Biolabs), and 1 μl AVNV DNA template (10 ng/μl) in 0.2 ml tube. The reaction mixture was incubated at 62, 63, 64, and 65°C in a water bath for 60 mins, respectively. Then, the reaction was terminated by heating at 80°C for 2 mins. Reaction time of LAMP assay (15, 30, 45, and 60 mins) was also tested. The LAMP products were analyzed by 2% agarose gel electrophoresis to determine the optimal reaction conditions.

Table 1. Primers used for LAMP

Primer	Length of oligonucleotide (bp)	Sequence (5'-3‘)
AVNV-F3	18	GTGCT GAGAC GGAAT GTG
AVNV-B3	18	TGGAT GACAC CCTTA TGC
AVNV-FIP (F1c+TTTT+F2)	49	GACAT CTGGC GGTAT TTTCA ATATG +TTTT+TGCAG TTTAA ATCTC CCAAC
AVNV-BIP (B1c+TTTT+B2)	46	GGTTA GATCT ACAAT TGCGC CA +TTTT+CACCA TTCTT TTGAC ACAGG

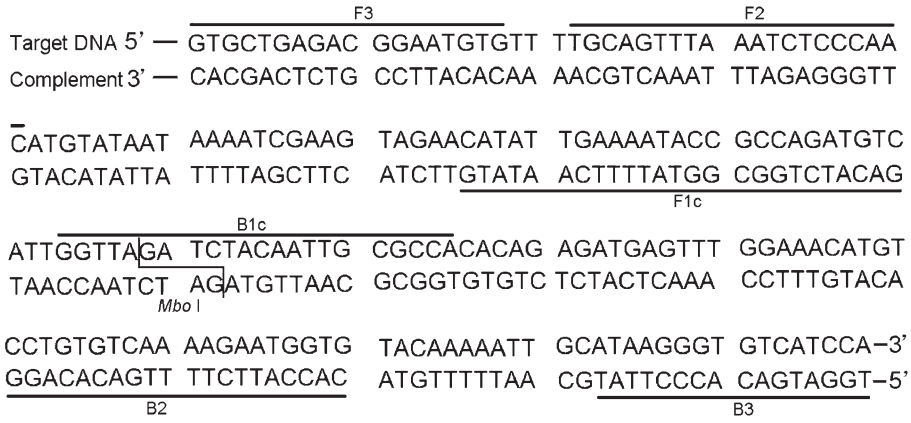

Fig. 1

Nucleic acid sequence of target DNA used to design inner and outer primers for LAMP assay

The nucleic acid sequences used for the primers design and their positions are marked by solid lines.

Detection of LAMP products. The products were detected by visual observation of color change of mixture after addition of 1 µl of 1:100 diluted GeneFinder™ (Biov Bio Xiamen). The mixture turned green in the presence of LAMP amplicons, while remained orange in their absence. Alternatively, the LAMP products could be observed visually through the white precipitate of magnesium pyrophosphate that was produced as a byproduct of DNA amplification. LAMP amplicons were digested with the *Mbo*I restriction enzyme (Takara Bio Dalian) and analyzed by 2% agarose gel electrophoresis for confirmation. In addition, LAMP amplicons after digestion and cloning were sequenced in order to confirm the amplification of a correct target sequence according to the method previously described (Notomi *et al.*, 2000).

PCR assay for detection of AVNV. To assess the validity of LAMP assay for detection of AVNV, PCR was performed with the same DNA template using one primer set. The sequences of the primers were 5'-TACCGCCAGATGTCCTCCTA-3' and 5'-TAC CCAATTCCAACCCTGTT-3', respectively. A product of 314 bp was expected to be amplified by the conventional PCR assay. PCR reaction was carried out using PCR Amplification Kit (Takara Bio Dalian) in a 25 µl reaction mixture containing 10 ng AVNV DNA and 25 pmol of each primer. Initial denaturation was done at 94°C for 4 mins followed by 35 cycles of denaturation (30 secs at 94°C), annealing (30 secs at 50°C), and extension (60 secs at 72°C), then final extension for 5 mins at 72°C. The PCR products were then analyzed by 2% agarose gel electrophoresis and stained with GeneFinder™.

Sensitivity and specificity of the AVNV LAMP assay. The sensitivity of the assay was determined by testing of ten-fold serial dilutions (1 ng to 0.1 fg) of AVNV DNA template prepared from purified virus particles. The products from each reaction were analyzed by 2% agarose gel electrophoresis and the sensitivity was compared with the conventional PCR assay. To assess the specificity of the LAMP, potential cross-reactions with DNA of WSSV, Infectious hypodermal and hematopoietic necrosis virus, Turbot reddish body iridovirus, CyHV-3, and OsHV-1 were examined. AVNV DNA was used as a positive control and the reaction mixture without DNA template was included as a negative control. After LAMP, the products were analyzed by 2% agarose gel electrophoresis.

Applicability of the AVNV LAMP assay. The applicability of the LAMP assay for the detection of AVNV was validated by testing 10 AVNV-infected and 10 non-infected samples of scallop *C. farreri*. The LAMP and PCR assay were carried out simultaneously for the same samples using the primers described above. The reaction mixture containing AVNV DNA was used as a positive control, while the reaction mixture without AVNV DNA was used as a negative control.

Results and Discussion

Optimization of reaction conditions for AVNV LAMP assay

LAMP was carried out with AVNV DNA as the template to optimize reaction temperature and time. LAMP ladder-like bands could be detected at 63, 64, and 65°C. However, the strongest and clearest bands were recorded at 64°C, and therefore this temperature was considered as the optimal reaction temperature (Fig. 2a). No ladder-like bands were found when the reaction was carried out at 64°C for 15 mins. For the reaction time of 30, 45, and 60 mins at 64°C, the LAMP ladder-like bands were detected (Fig. 2b). However, for the complete amplification, the reaction time of 60 mins was selected as the optimal reaction time. It was found that the AVNV LAMP reaction could be terminated within 30–45 mins. These results showed that the LAMP detection of AVNV was possible also in a shorter time (<1hr) under isothermal conditions at 64°C. However, the conventional PCR assay takes 2–3 hrs.

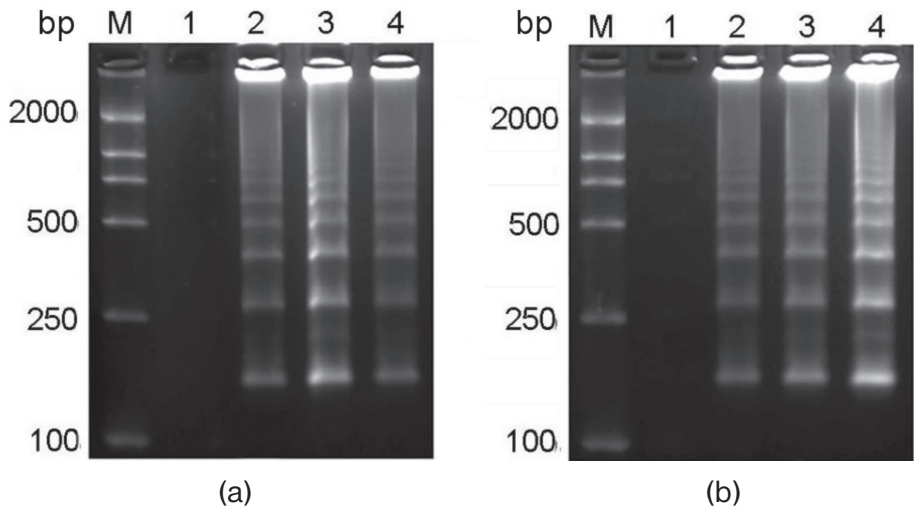

Fig. 2

Evaluation of LAMP reaction conditions by agarose gel electrophoresis

(a) Assay was carried out at 62, 63, 64, and 65°C (lanes 1–4, respectively), (b) Assay was carried out for 15, 30, 45, and 60 mins (lanes 1–4, respectively). DNA size marker (lane M).

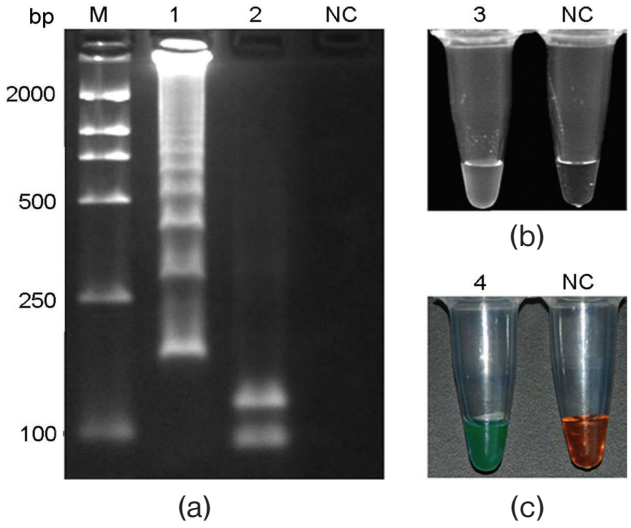

Fig. 3

LAMP assay products detected by electrophoretic analysis (a) and by visual assessment (b and c)

LAMP products of AVNV (lane 1), LAMP products digested with *Mbo*I restriction enzyme (lane 2), positive LAMP reaction visualized by turbidity (3), and positive LAMP reaction visualized using GeneFinder™ (4), DNA size marker (lane M), no template control (NC).

Detection of LAMP products by alternative methods

LAMP products were subjected to the agarose gel electrophoresis showing characteristic ladder-like multiple bands (Fig. 3a). The ladder pattern is characteristic of the LAMP (Notomi *et al.,* 2000). It was also observed that large amounts of pyrophosphate ions were produced in the reaction mixture yielding a white precipitate of magnesium pyrophosphate that could be visible by naked eye (Mori *et al.,* 2001). The production of magnesium pyrophosphate was seen as distinct white turbidity in the positive sample (Fig. 3b). Alternatively, a presence of LAMP amplicons in the reaction mixture was confirmed by using fluorescent dsDNA intercalating dye (Iwamoto *et al.,* 2003; Dukes *et al.,* 2006). Reaction mixture containing AVNV-specific amplicons turned green in the presence of GeneFinder™, whereas the original orange color did not change in the negative control tube (Fig. 3c).

The two visual inspections of LAMP products (Fig. 3b and c) turned to be a better detection method when compared with the gel electrophoresis running and staining with carcinogenic ethidium bromide. Moreover, it was found that the samples tested as positive/negative using fluorescent dsDNA intercalating dye were also positive/negative in the electrophoretic analysis (En *et al.,* 2008). Moreover, the visual observation of the orange-to-green color change with aid of GeneFinder™ was easily accomplished and gave a clear-cut result in comparison with the observation of white precipitate. These findings could facilitate the application of LAMP especially as a field test in the regular laboratories or scallop aquaculture facilities.

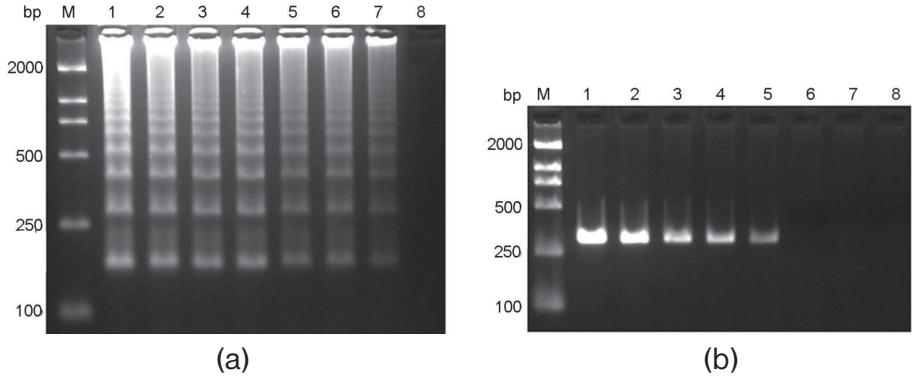

Fig. 4

Evaluation of LAMP (a) and PCR assay (b) sensitivity for detection of AVNV by agarose gel electrophoresis

Assays were carried out with AVNV DNA in concentrations ranging from 1 ng to 0.1 fg (lanes 1–8, respectively), DNA size marker (lane M).

Sensitivity of the AVNV LAMP assay

Sensitivity of the assay is essential for the development of an optimal protocol for detection of the virus. LAMP is a highly sensitive assay that amplifies DNA with high efficiency under isothermal conditions and its cycling reaction continues for 1 hr with the accumulation of 10^9 of the target nucleic acid sequences (Notomi *et al.,* 2000). In this study, LAMP assay was tested using ten-fold serial dilutions of AVNV DNA and compared with the conventional PCR test. The lower detection limit of AVNV DNA by LAMP assay was about 1 fg, whereas conventional PCR assay was only able to amplify 100 fg AVNV DNA (Fig. 4a and b). Thus, the sensitivity of LAMP assay was 100 times higher than that of the conventional PCR assay. This increased sensitivity makes the LAMP assay a better choice than the conventional PCR for detection of AVNV in asymptomatic samples where lower levels of virus are expected. Thus, scallops infected with AVNV can be identified in early stage of the infection and consequently, the spreading of the disease can be effectively controlled.

Specificity of the AVNV LAMP assay

After electrophoresis**,** the LAMP products showed ladder-like bands that represented mixtures of stem-loop DNAs with various sizes of stem and head between alternately inverted repeats of the target in the same strand (Notomi *et al.,* 2000). To confirm the specificity of LAMP reaction, the AVNV-specific amplicons were digested with the restriction enzyme *Mbo*I, which cut the sequence in B1c (Fig. 1). DNA fragments of approx. 75 bp and 134 bp length were observed (Fig. 3a), which were in accord with their predicted size. Further confirmation of the correct target sequence of LAMP products was also carried out by sequencing, wherein the sequence obtained perfectly matched with the expected nucleic acid sequence (data not shown).

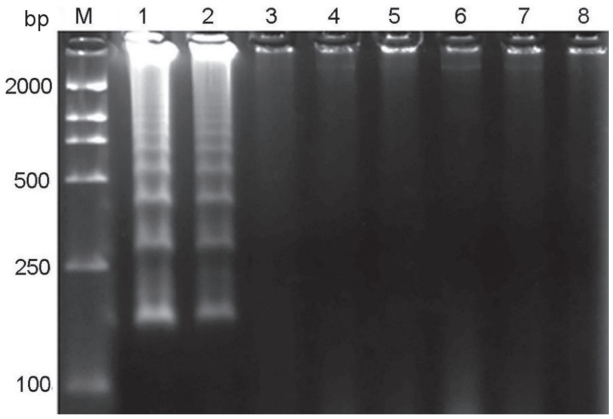

Fig. 5

Specificity of LAMP assay examined by electrophoretic analysis

AVNV DNA (lane 1), DNA from AVNV-infected scallop (lane 2), DNA from WSSV, Infectious hypodermal and hematopoietic necrosis virus, CyHV-3, Turbot reddish body iridovirus, and OsHV-1 (lanes 3–7, respectively), no template control (lane 8), DNA size marker (lane M).

Using LAMP assay followed by electrophoretic analysis of products, no cross-reactions were found with other DNA viruses such as WSSV, Infectious hypodermal and hematopoietic necrosis virus, CyHV-3, Turbot reddish body iridovirus, and OsHV-1 found commonly in China. In addition, no amplification products were detected in the negative control (Fig. 5). These results indicate that the LAMP assay was highly specific and selective for the target gene (Notomi *et al.,* 2000).

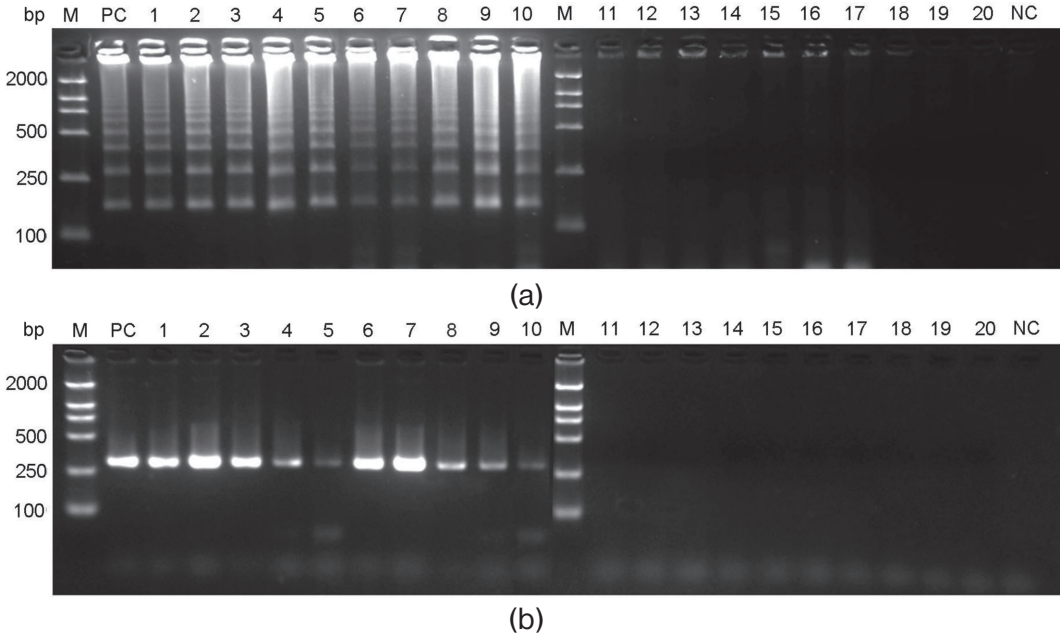

Fig. 6

Comparison of LAMP (a) and PCR assay (b) for detection of AVNV in scallop

DNA from AVNV-infected scallop (lanes 1–10), DNA from non-infected scallop (lanes 11–20), AVNV DNA (lane PC), no template control (lane NC), DNA size marker (lane M).

Use of AVNV LAMP assay as a diagnostic technique

With the optimized reaction conditions, AVNV LAMP assay was used for the detection of AVNV in scallop. A total of 10 AVNV-infected and 10 non-infected scallops were analyzed. All AVNV-infected samples tested by LAMP assay were positive for presence of AVNV, whereas none of the non-infected samples showed any LAMP ladder-like bands (Fig. 6a). The same positivity/negativity of the samples was obtained with PCR assay (Fig. 6b). These results showed that these two assays are equivalent to their specificity and reliability. Hence, the incidence of a false positivity or negativity in the LAMP was excluded and the assay could be applied with confidence for detecting of AVNV in scallop.

Taken together, a simple, sensitive, and specific LAMP protocol for the detection of AVNV was developed and evaluated. In this simple diagnostic assay, the reaction was carried out in a single tube containing buffer, primers and *Bst* DNA polymerase and by incubation of the mixture for 30–60 mins in a water bath or heating block to provide a constant temperature of 64°C. In addition to the high specificity for AVNV, the sensitivity of the LAMP assay was 100 times higher than that of PCR assay with the detection limit of about 1 fg AVNV DNA. LAMP products could be detected by agarose gel electrophoresis or alternatively by visual observation of the turbid magnesium pyrophosphate suspension or color reaction with Genefinder™. The visual inspections of LAMP products required very simple reagent or equipment and could facilitate the broad application of the LAMP assay. Thus, LAMP assay is advantageous for the detection of AVNV due to its simplicity, sensitivity, specificity, and easy detection. It can be extensively used for the rapid diagnosis of AVNV infection in scallop in the laboratories and farms that lack the resources needed for molecular diagnostic techniques.

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