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# Optimisation of reverse transcriptase loop-mediated isothermal amplification assay for rapid detection of *Macrobrachium rosenbergii* noda virus and extra small virus in *Macrobrachium rosenbergii*

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A B S T R A C T

The standardisation and optimisation of a one step single tube reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) procedure is described for rapid diagnosis of white tail disease, a viral disease caused by *Macrobrachium rosenbergii* noda virus (*Mr*NV) and extra small virus (XSV), in giant fresh water prawn, *M. rosenbergii*. Time, temperature and quantity of each reagent were optimised for the detection of the two viruses. This method was more sensitive than the conventional reverse transcriptase polymerase chain reaction (RT-PCR) for detecting the two viruses. The RT-LAMP reaction is highly suited for disease diagnosis in developing countries. Amplification of DNA can be detected without the use of agarose gel electrophoresis, by the production of a whitish precipitate of magnesium pyrophosphate as a by-product. The cost of RT-LAMP for one reaction is nearly 4 times less than that of RT-PCR.



## 1. Introduction

The giant fresh water prawn *Macrobrachium rosenbergii* is a farmed crustacean which is important economically. A virus-borne disease affecting this crustacean called white tail disease (WTD) was first observed and reported in a hatchery in Guadeloupe, then in Martinique, French West Indies (Arcier et al., 1999) and in Taiwan (Tung et al., 1999). Later, WTD was reported in China (Qian et al., 2003; Sri Widada et al., 2003), India (Sahul Hameed et al., 2004) and most recently in Bangkok, Thailand (Yoganandhan et al., 2006). Clinical signs in infected postlarvae include lethargy, opaqueness of abdominal muscles, degeneration of telson and uropods. The mortality may reach 100% within two or three days after the appearance of the clinical sign of opaqueness (Arcier et al., 1999; Sri Widada et al., 2003; Sahul Hameed et al., 2004).

Two viruses, the *M. rosenbergii* nodavirus (*Mr*NV) and the extra small virus (XSV), were identified as causative pathogenic agents of WTD (Arcier et al., 1999; Qian et al., 2003). *Mr*NV is a small icosahedral non-enveloped particle, 26–27 nm in diameter, identified in the cytoplasm of connective tissue cells. It contains a genome consisting of two linear single stranded positive sense RNA segments, RNA1 of 2.9 kb, which encodes the viral part of the RNA-dependent RNA polymerase (RdRp) and RNA2 of 1.3 kb that encodes the capsid protein. The capsid contains a single polypeptide of 43 kDa (cp-43) (Bonami et al., 2005). XSV is also an icosahedral virus with a diameter of 14–16 nm. Its genome consists of a linear single stranded positive sense RNA about 0.9 kb coding for a capsid protein, cp-17 (Qian et al., 2003; Sri Widada et al., 2004). Because of its extremely small size and the absence of a gene which codes for enzymes required for viral RNA replication, it has been suggested that XSV may be a satellite virus, while *Mr*NV plays the role of a helper virus (Sri Widada et al., 2004). The linear correlation between *Mr*NV and XSV genome copies in infected prawns confirmed that XSV is a satellite virus, dependent on *Mr*NV, but its role in the pathogenicity of WTD remains unclear (Zhang et al., 2006).

As there are no effective treatments for these viral pathogens, the spread and impact of white tail disease can be minimized only by adoption of better management practices in hatcheries and farms. Early diagnosis of pathogens in hatcheries is the only way of preventing the development of disease. It is essential, therefore, that sensitive, specific and rapid diagnostic methods be developed for early detection of both pathogenic agents of this disease (Pillai et al., 2006). Various diagnostic methods have been developed for detection of these viruses including histopathology, serological methods, and genome based detection methods. Sandwich ELISA (Romestand and Bonami, 2003) and three genome based methods, i.e., dot blot hybridization, in situ hybridization and RT-PCR (Sri Widada et al., 2003, 2004), are available for detection of WTD.

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Of these, RT-PCR was found to be the most sensitive and highly specific method for detection of *Mr*NV and XSV (Sri Widada et al., 2003). Although very sensitive and specific, RT-PCR requires the use of a thermal cycler and hence needs a well-equipped laboratory for performance. It is a time consuming process, which takes at least 3–5 h to complete. Apart from the sensitivity and rapidity, any detection method developed for *M. rosenbergii* needs to be economical as unlike the tiger shrimp *Penaeus monodon*, which has a high fecundity of 0.5–1.0 million eggs, *M. rosenbergii* has a much lower fecundity of 20,000–30,000 (Vadher, 2003).

Loop-mediated isothermal amplification (LAMP), described originally by Notomi et al. (2000), was developed for rapid diagnosis of white tail disease in *M. rosenbergii* (Pillai et al., 2006). There are very few reports of the application of LAMP method for detection of RNA viruses. It is a specific nucleic acid amplification technique that can amplify target nucleic acid to $10^9$ copies at 60–65 °C in 1 h (Notomi et al., 2000; Parida et al., 2004; Savan et al., 2005). The method relies on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment, a DNA polymerase with high strand displacement activity. Using this technique, the presence of the virus can be detected successfully in 1 h by observing the formation of a white precipitate of magnesium pyrophosphate. As the reaction is carried out under isothermal conditions, it can be conducted with a simple and inexpensive water bath (Notomi et al., 2000; Parida et al., 2004; Savan et al., 2005). There is no time loss of thermal change due to the isothermal reaction and the inhibition reaction at the later stage of amplification is less likely to occur, compared with the PCR.

In the present study, extensive standardisation of RT-LAMP method was carried out for rapid detection of both *Mr*NV and XSV in infected white tail prawns. The purpose of this study is to optimise the RT-LAMP reaction in terms of time and quantity of ingredients used and establishes the advantages of this cost effective farmer friendly technique.

## 2. Materials and methods

### 2.1. Extraction of total RNA

Samples showing clinical signs of white tail disease (WTD)/unhealthy, moribund postlarvae and juveniles were collected from different parts of India and the presence of the two viruses was confirmed by RT-PCR. Total RNA was extracted from infected and healthy postlarvae using trizol reagent (Invitrogen Life Technologies, Carlsbad, USA) as described by the manufacturer and stored at −80 °C until use. 30–50 mg of postlarvae was homogenized in 1 mL of trizol reagent. Following 5 min incubation at room temperature, 200 μl of chloroform (Sigma–Aldrich, Hyderabad, India) was added to the sample, mixed well, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube and the RNA precipitated by mixing with 500 μl of isopropyl alcohol (Sigma–Aldrich, Hyderabad, India). The samples were incubated for 10 min at room temperature and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The RNA pellet was dissolved in RNase free water, after a wash with 75% ethanol followed by centrifugation at 4 °C.

### 2.2. Design of primers for RT-LAMP

Based on the sequence of *Mr*NV (GenBank accession nos AY222839, AY222840) and XSV (accession no. AY247793), seven sets of LAMP primers were designed for *Mr*NV and two sets for XSV using Primer Explorer V software. Each set consisted of four primers, two outer primers (F3 and B3) and two inner primers (FIP and BIP). 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, the BIP consisted of the complementary sequence of B1 (B1c), a TTTT spacer and the sense B2 sequence. The FIP and BIP initiate LAMP cycling by structuring the loop while the outer primers initiate the strand displacement of DNA (Notomi et al., 2000). The sequence of primers for *Mr*NV specific to RNA2 and XSV is shown in Tables 1 and 2.

### 2.3. Standardisation of RT-LAMP reaction

#### 2.3.1. Optimum temperature and time

The RT-LAMP reaction was standardised initially for the detection of *Mr*NV, testing with all the seven sets of primers to determine the set of LAMP primers that would give the maximum precipitate and the best result in terms of specificity in minimum time. Each reaction was carried out at two temperatures, viz., 63 °C and 65 °C for 1 h, followed by heat inactivation at 80 °C for 2 min to terminate the reaction. The reaction mixture contained 2 μM each of inner primers FIP and BIP, 0.2 μM each of outer primers F3 and B3 (Bioserve, Bangalore, India), 1400 μM of dNTP mix (Bangalore Genei, Bangalore, India), 0.6 M betaine (Sigma–Aldrich, Hyderabad, India), 6 mM MgSO4 (Merck, Mumbai, India), 8 U of *Bst* DNA polymerase large fragment (New England Biolabs, Ipswich, USA) together with 1× of the buffer supplied, 0.125 U of AMV RTase (Bangalore Genei, Bangalore, India) and the specified amount of template RNA in a final volume of 25 μl (Pillai et al., 2006). Uninfected samples and the reaction mix without the template served as the negative controls. To determine the optimum time for amplification, the LAMP reactions were carried out at different time periods (15 min, 30 min, 45 min and 60 min) with all primers. Turbidity measurement of RT-LAMP products at all conditions was carried out by recording optical density at 600 nm in a spectrophotometer (Eppendorf, Hamburg, Germany).

#### 2.3.2. Optimum quantity of reagents

Optimum quantity of each ingredient to be added was also standardised. The following changes were attempted in the reaction to optimise the quantity of whitish precipitate obtained. Optimisation of (i) amount of template: the volume (concentration) of the template RNA was increased progressively from 1 μl to 5 μl i.e., 0.3 μg, 0.6 μg, 0.9 μg 1.2 μg and 1.5 μg; (ii) inner–outer primer ratio—different ratios of inner and outer primers viz., 1:2, 1:4, 1:6, 1:8, 1:10 were tried; (iii) betaine concentration—two different concentrations viz., 0.6 M and 0.8 M were tried; (iv) $MgSO_4$ concentration—different concentrations of $MgSO_4$, viz., 8, 10, 12 and 14 mM (including the 2 mM $MgSO_4$ in the buffer) were tried; (v) concentration of the reverse transcriptase enzyme—T-LAMP was carried out at different concentrations of RTase (1.25 U, 0.125 U, 0.05 U, 0.04 U, 0.03 U, 0.02 U, 0.01 U); (vi) concentration of the *Bst* DNA polymerase—two concentrations, viz., 6 U and 8 U were attempted.

### 2.4. Detection of RT-LAMP product

The presence/absence of a whitish precipitate was analysed visually and also by recording the optical density at 600 nm to determine the turbidity using a spectrophotometer. To confirm the amplification of DNA, 1.0 μl of $10^{-1}$ diluted SYBR GreenI (Invitrogen Life Technologies, Carlsbad, USA) was added to the reaction mixture and the colour change was observed. Aliquots of 5 μl of LAMP products were analysed by electrophoresis on a 2% ethidium bro-

**Table 1**
Primers for *Macrobrachium rosenbergii* noda virus (LAMP1–LAMP7) used for RT-LAMP reaction.

| Primer name | Type | Sequence (5–3′) |
|---|---|---|
| LAMP1 B3 | Backward outer | CAGGCTACGTCACAAGTG |
| LAMP1 F3 | Forward outer | CCAATCTAAATATAGTGAACCTG |
| LAMP1 BIP | Backward inner | GTACAATTGATCATCACGCCTGACATTTTGTGCGCGGCAGTGGAATTTC |
| LAMP1FIP | Forward inner | CGTTCGTTTCTCTCAACAGGGTGTTTTATGGCTTCACCACTAATATTAAG |
| LAMP2 B3 | Backward outer | GTACTGCAGAAGTGCACAATC |
| LAMP2 F3 | Forward outer | TTATTGCCGACGATAGCTCTG |
| LAMP2 BIP | Backward inner | ACCCATCACATTTGATAGCGCTTTTCTTGTAATGAGGTTGACGCAG |
| LAMP2 FIP | Forward inner | GAAGCAACCTTCACCTTAGTGTTTTCCTATACTGATAGGGTTTAGGG |
| LAMP3 B3 | Backward outer | ACTGATAGTTGACTCTACTCAA |
| LAMP3 F3 | Forward outer | ACATCCTAGGTATTCTTAGCATTA |
| LAMP3 BIP | Backward inner | CGGGCCTTAGTTTGGGGATTTTTTCACGTGCGTTCATCTCAA |
| LAMP3 FIP | Forward inner | GTTCGGACAATCCACCCAGGTTTTGTGGCTAAGTTTCTTTGG |
| LAMP4 B3 | Backward outer | GCTCCAGGAGTTTTGTGTC |
| LAMP4 F3 | Forward outer | CTTCCTATGTGGTAATCGGA |
| LAMP4 BIP | Backward inner | TGCGTACTGACTGGTACCCATTTTCATTTTCAGATAAGCGGTGAG |
| LAMP4 FIP | Forward inner | CAAATCAACCATGGCCCTTTTGTATTTTCCTTATGAAGCTATTGCTAAAAGC |
| LAMP5 B3 | Backward outer | CAATTTCAGACGCCAATGG |
| LAMP5 F3 | Forward outer | CTTAGATGTTGTTCTAGGCTT |
| LAMP5 BIP | Backward inner | ACGGTTTGTCAGTTCTACAACAGTTTTTGGACAGGTGGTTGTGAA |
| LAMP5 FIP | Forward inner | GCCAAAGTTTGTGGTTGCTTGTTTTCCATGCAGTCAGTTACCT |
| LAMP6 B3 | Backward outer | ACGAAAAGAAGTTGTAGGCT |
| LAMP6 F3 | Forward outer | GCTTAAGTACCATGCAGTCA |
| LAMP6 BIP | Backward inner | GTGCCGCTTATGATATACTAAGCTGTTTTTCAATTTCAGACGC |
| LAMP6 FIP | Forward inner | GAACTGACAAACCGTAGCCAAATTTTCTTCAAGACTTTTACAAATGGGTT |
| LAMP7 B3 | Backward outer | CCATGGTAACACGATGTTCT |
| LAMP7 F3 | Forward outer | CTACAACAGTGCCGCTTA |
| LAMP7 BIP | Backward inner | TACTCCAACAATATTGTGACCTGTGTTTTACTTAAAACCTCCCA |
| LAMP7 FIP | Forward inner | CAATTTCAGACGCCAATGGCCTTTTTGTAGCATAGTTGCTAGA |

mide stained agarose gel and visualised on a UV-transilluminator (Biorad, Hercules, USA) at 302 nm.

### 2.5. Specificity of the RT-LAMP reaction

To determine the specificity of RT-LAMP detection, reactions were carried out with uninfected samples from various sources including *M. rosenbergii*, *P. monodon* and *Fenneropenaeus indicus* and shrimp samples infected with WSSV. Samples without a template were also tested for all the seven sets of primers.

### 2.6. Sensitivity of RT-LAMP reaction

To compare the sensitivity of RT-LAMP with RT-PCR, 10-fold serial dilutions of the total RNA containing *Mr*NV/XSV were used as the template. Dilutions ranged from 0.3 μg of total RNA (RNA content of 1.5 mg of tissue) to 3 fg. RT-PCR was carried out with 25 μM of the outer primers F3 and B3 used for the LAMP reaction, in a total reaction volume of 25 μl, with reaction conditions as follows: reverse transcription at 52 °C for 30 min followed by an initial denaturation at 94 °C for 2 min and then 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 68 °C for 30 s and a final extension at 68 °C for 10 min. 10 μl of the reaction products were analysed by electrophoresis on a 2% agarose gel and visualised on a UV-transilluminator.

### 2.7. Cost comparison of RT-LAMP with RT-PCR

The cost of various ingredients used in RT-LAMP was compared with those used in RT-PCR for a single reaction.

## 3. Results

### 3.1. Determination of optimum conditions of RT-LAMP reaction

*Mr*NV and XSV could be detected successfully by RT-LAMP with the different primer sets. RT-LAMP assay was carried out for *Mr*NV detection with the seven sets of four primers at different temperatures as described in Section 2. Among the different primer sets, primer set VII gave the best result in terms of maximum precipitate in the minimum time. The specificity of amplification of the template was optimum at 63 °C and 65 °C with set VII primer as observed by agarose gel electrophoresis and visually by the formation of a whitish precipitate (Fig. 1). Uninfected *Macrobrachium* sample collected from the wild showed no visible precipitate at these temperatures. However, a slight absorbance was observed in the uninfected sample in the spectrophotometer reading. The results indicated that set I and set II primers were very specific for XSV detection at 63 °C as well as at 65 °C. The optimum time required for amplification by RT-LAMP was studied for both *Mr*NV with set VII primers and XSV with set II primers. The results

**Table 2**
Primers for extra small virus (XSV1 and XSV2) used for RT-LAMP reaction.

| Primer name | Type | Sequence (5–3′) |
|---|---|---|
| XSV1 FIP | Forward inner | GTCATTAGTAATCCTCGGAAAACTTTTGTGT AAATCCCGGACTCTTCAC |
| XSV1 BIP | Backward inner | TATGGTATAGGGACTGGGTGAATTTTTTGGGA CGCGGTAGGACAATG |
| XSV1 F3 | Forward outer | ACGGTATGACCCAACCGCC |
| XSV1 B3 | Backward outer | ACGGTATGACCCAACCGCC |
| XSV 2 FIP | Forward inner | GAGCCGCAGTAGGTATTCTTTATTTTGTGTGC CTGTTGCTGAAATAC |
| XSV2 BIP | Backward inner | CATCAACGACTTGTATGCCAGTTTTGCATACG CTGCACCAGGAG |
| XSV2 F3 | Forward outer | GGATCTTTAGCAGTGGTAGTG |
| XSV2 B3 | Backward outer | TAACTTTATTATTGAGAAGGTTGC |

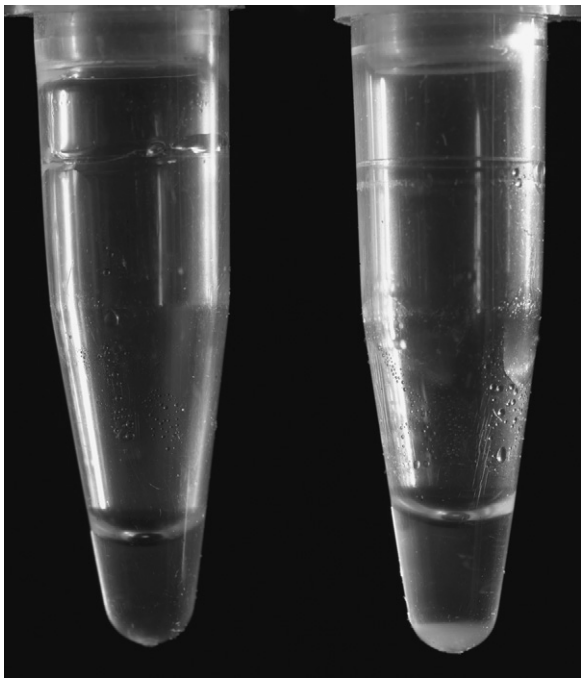


**Fig. 1.** Detection of whitish precipitate in WTD infected sample—positive and negative reactions of the RT-LAMP were distinguished by simple eye inspection of white precipitate caused by magnesium pyrophosphate.

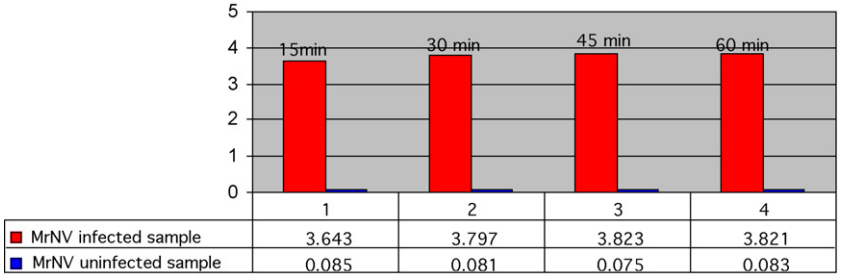
suggested that efficient amplification of template RNA could be obtained within 15–45 min (Fig. 2). A white precipitate of magnesium pyrophosphate started to form within 15 min with lamp primer set VII at 63 °C (Fig. 2). The time required for initiation of amplification in the case of XSV was also the same as for *Mr*NV (figure not shown).

### 3.2. Optimum quantity of reagents

The quantity of precipitate increased visibly when the volume of the template added was increased from 1 μl to 4 μl (0.3–1.2 μg), which was confirmed by turbidity measurement of RT-LAMP products using a spectrophotometer (Fig. 3). There was no apparent difference in the quantity of precipitate produced when the template was increased to 5 μl (1.5 μg) [not shown in the graph]. No visible precipitate was formed in the uninfected sample even when 4 μl or 5 μl of template were added. But, a slight, almost static amplification was observed in the uninfected sample in spectrophotometer reading (Fig. 3). RT-LAMP worked in all inner–outer primer ratios tried, i.e., 1:2 to 1:10 (figure not shown). But the maximum precipitate and minimum non-specificity was observed with 1:6 and 1.8 ratios. The maximum precipitate was obtained with 12 mM $MgSO_4$ (10 mM $MgSO_4$ + 2 mM $MgSO_4$ present in the buffer) and 14 mM $MgSO_4$. Hence, the addition of 10 mM $MgSO_4$ to the reaction mix to obtain a total concentration of 12 mM was taken as the optimum concentration. RT-LAMP worked well both with 0.6 M and 0.8 M betaine. But the latter concentration, i.e., 0.8 M, appeared to be slightly better in terms of a visual precipitate (data not shown). RT @ 0.125 U was found to be sufficient for amplification of DNA by RT-LAMP. When higher RT concentration (1.25 U) was used, non-specific amplification was observed in negative samples (figure not shown) and even in reagent controls. There was no significant difference in the visible perception of whitish precipitate with 6 U and 8 U of *Bst* polymerase. Hence, 6 U was selected for the reaction mix.

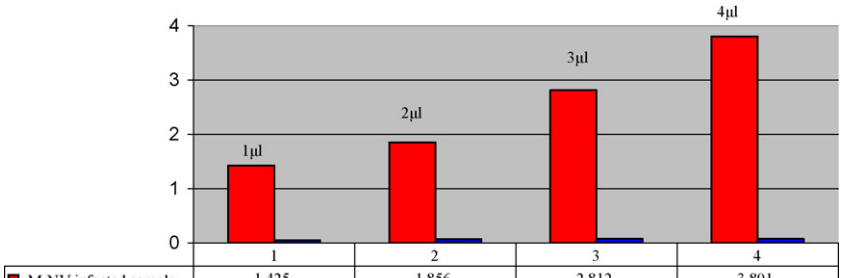
### 3.3. Detection of RT-LAMP product

Positive and negative reactions of the RT-LAMP were clearly distinguishable by the naked eye. The whitish precipitate of magnesium pyrophosphate, a by-product of LAMP reaction was observed in all infected samples (Fig. 1). The positive reaction was also detected by recording the optical density of LAMP products at 600 nm. DNA amplification in the positive sample was confirmed further by agarose gel electrophoresis and also by the addition of




| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| MrNV infected sample | 3.643 | 3.797 | 3.823 | 3.821 |
| MrNV uninfected sample | 0.085 | 0.081 | 0.075 | 0.083 |

**Fig. 2.** Turbidity measurement of RT-LAMP product of *Mr*NV at different periods of time (at 600 nm)—the time required for initiation of amplification of template RNA was 15 min. Primer VII was used (OD values are in Angstrom unit).




| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| MrNV infected sample | 1.425 | 1.856 | 2.812 | 3.801 |
| MrNV uninfected | 0.054 | 0.074 | 0.078 | 0.081 |

**Fig. 3.** Turbidity measurement of RT-LAMP product of *Mr*NV at different volume of template (at 600 nm)—the turbidity increased when the volume of template added was increased from 1 μl to 4 μl (0.3–1.2 μg). A slight, almost static amplification was observed in the uninfected sample (OD values are in Angstrom unit).

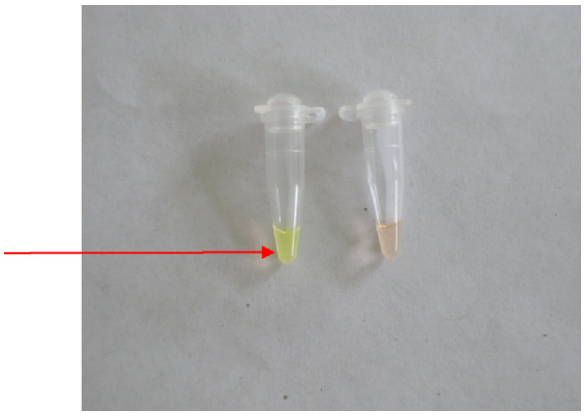


**Fig. 4.** Visual detection using SYBR green 1—the original orange colour of SYBR green turned to green in the infected sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

SYBR Green I, which changed to green from the original orange colour (Fig. 4).

### 3.4. Specificity of RT-LAMP

The specificity of the RT-LAMP reaction was determined using suitable controls such as uninfected samples, samples without a template, and DNA viruses such as WSSV. *Mr*NV and XSV were amplified very specifically by the RT-LAMP reaction. RT-LAMP reaction without a template did not provide any precipitate in either visible or spectrophotometric analysis. Very slight amount of absorbance was detected in uninfected and the sample infected with WSSV in spectrophotometer reading. However, it was negligible compared to the amount of visible precipitate formed with the infected sample (Fig. 5).

### 3.5. RT-LAMP vs. RT-PCR

The detection limit of RT-LAMP was established by using successive 10-fold dilutions of total RNA extracted from an infected sample. Comparison of the sensitivity of RT-LAMP with that of RT-PCR showed that LAMP reaction was 10 times more sensitive than RT-PCR for *Mr*NV detection. Amplified DNA was observed at dilution 6 in RT-LAMP (Fig. 6A), which corresponded to 0.3 pg of total RNA, while amplification was observed only upto dilution 5 (Fig. 6B) corresponding to 3 pg of total RNA. These studies have shown clearly that RT-LAMP is as sensitive as nested/2nd step RT-PCR.

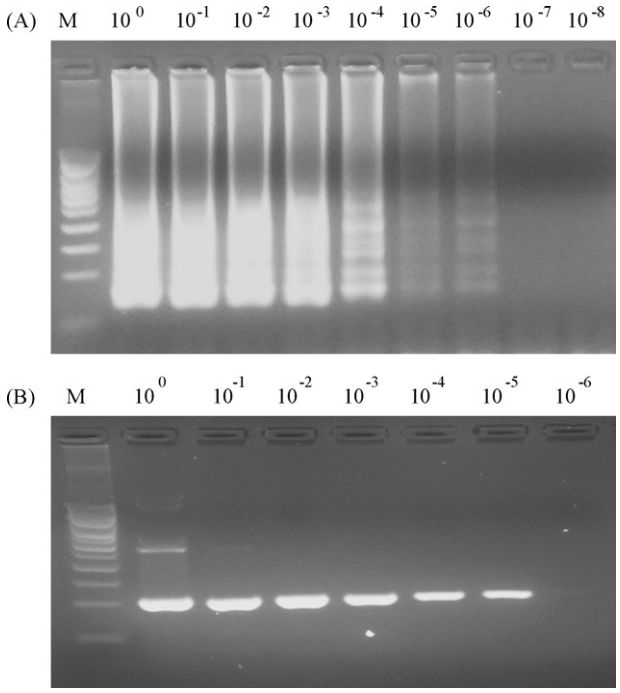


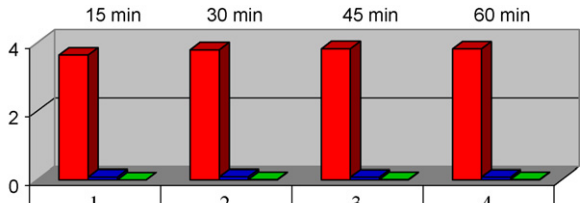

**Fig. 6.** (A and B) Comparative sensitivities of RT-LAMP and RT-PCR—amplifications were performed with 10-fold dilutions of the virus-infected sample RNA, ranging from 0.3 μg to 3 fg. Amplification products of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) by RT-LAMP (A) and RT-PCR (B). Molecular weight marker (M). Amplified DNA was observed in dilution 6 ($10^{-6}$) in RT-LAMP (A). Amplification was observed only upto dilution 5 ($10^{-5}$) in RT-PCR (B).

### 3.6. Cost comparison of RT-LAMP with RT-PCR

The cost of various ingredients used in RT-LAMP was compared with those used in RT-PCR for a single reaction. Taking into account the cost of ingredients alone, RT-PCR by conventional method costs USD 13.8 and the same using RT-PCR kit costs USD 21, while RT-LAMP for one reaction costs only USD 3.8. In addition, RT-PCR requires the use of expensive equipment such as a thermal cycler, transilluminator and electrophoresis unit, and their operation requires trained technical personnel. RT-LAMP, on the other hand, requires only a simple water bath.

## 4. Discussion

There are relatively few published reports on the use of LAMP for the detection of RNA viruses. The first report on the use of RT-LAMP was for the detection of the Japanese Yam mosaic potyvirus from infected leaves, propagules and roots of Japanese Yam (Fukuta et al., 2003). Subsequently, protocols for the detection of tomato




| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| WTD infected sample | 3.643 | 3.797 | 3.823 | 3.821 |
| WTD uninfected sample | 0.081 | 0.084 | 0.076 | 0.078 |
| WSSV infected sample | 0.002 | 0.01 | 0.012 | 0.012 |

**Fig. 5.** Turbidity measurement of RT-LAMP products of DNA virus (WSSV) infected, WTD infected and uninfected samples (at 600 nm)—*Mr*NV was amplified very specifically in RT-LAMP reaction (OD values are in Angstrom unit).

spotted wilt virus (Fukuta et al., 2004), severe acute respiratory syndrome (SARS) coronavirus (Hong et al., 2004; Poon et al., 2004) and West Nile virus (Parida et al., 2004) have been developed. In fish, RT-LAMP has been reported for infectious haematopoietic necrosis virus (IHNV) (Gunimaladevi et al., 2005). It has been shown that this technique can also be applied for the rapid detection of *M. rosenbergii* noda virus and the extra small virus in giant fresh water prawn, *M. rosenbergii* (Pillai et al., 2006).

For field diagnosis, the detection system should not only be economical, rapid and easy to operate, but must also meet the requirements of specificity and sensitivity. In this study, extensive standardisation of the RT-LAMP method has been carried out to increase its efficiency and rapidity for the detection of *Mr*NV and XSV in *M. rosenbergii*. In an earlier study, it was established that the RT-LAMP reaction for the detection of *Mr*NV and XSV works well at 63 °C and 65 °C (Pillai et al., 2006). Further studies with different primer set combinations have again reaffirmed that the optimum temperature for RT-LAMP assay is 63 °C or 65 °C. However, since reverse transcriptase is used in the RT-LAMP assay, and considering the convenience of use of the LAMP method for detection of the two viruses simultaneously, all further reactions were carried out at 63 °C for both *Mr*NV (with set VII primers) and XSV (with set II primers). In their study on detection of West Nile virus by RT-LAMP, Parida et al. (2004) observed the efficiency of amplification to be optimal at 63 °C.

Earlier studies had demonstrated the time for detection of visible precipitate as 60 min (Parida et al., 2004; Pillai et al., 2006). The present studies with primer set VII for *Mr*NV showed that amplification begins to occur in 15 min. In the present study, the time to obtain a white precipitate has been reduced to 15 min by the choice of appropriate primers. The time required for initiation of amplification in the case of XSV was also the same as *Mr*NV. The earlier studies by us showed that the accelerated LAMP reaction by the use of loop primers could reduce the time for appearance of the white precipitate to 20 min (Pillai et al., 2006). However, non-specific amplification appeared to be a problem with the use of additional primers (Pillai et al., 2006). Since highly visible whitish precipitate could be obtained with LAMP primer VII by the use of 4 primers in 15 min, loop primers were not used in the present study. The RT-LAMP assay (without loop primers) for *Mr*NV and XSV reported here showed exceptionally high rapidity in comparison with LAMP assays of other RNA and DNA viruses.

Following the standardisation of temperature and time, optimisation of the RT-LAMP assay was also carried out with regard to the effect of the primer ratio, template and other reagent concentrations on the reaction kinetics and formation of white magnesium pyrophosphate in the reaction mixture. The quantity of white precipitate produced was found to increase with the amount of template used as observed by Caipang et al. (2004). Even though a slight amplification was observed in uninfected sample in spectrophotometer reading, it remained more like background amplification and was not found to increase with the increasing amounts of template. With the LAMP reaction, there was no inhibition of amplification with increasing amounts of template used. This is an advantage of RT-LAMP over RT-PCR since increase in template beyond the optimal level could actually inhibit detection by RT-PCR (Sri Widada et al., 2003; Sahul Hameed et al., 2004).

Although RT-LAMP worked in all inner–outer primer ratios tried, maximum precipitate was observed in 1:6 and 1:8. This observation suggests that primer ratio influences the formation of a white precipitate. As suggested in a previous study (Parida et al., 2004), no significant improvements could be observed with regard to reaction kinetics when the primer or template concentration was increased. Experiments proved that the concentration of $MgSO_4$, betaine, reverse transcriptase enzyme and *Bst* DNA polymerase play a role in the formation of a white precipitate of magnesium pyrophosphate.

The data presented in this study suggest that the RT-LAMP assay is 10 times more sensitive than RT-PCR for *Mr*NV detection. Most studies using the LAMP reaction for detection have found sensitivity to be equal to (Gunimaladevi et al., 2004) or 10 times more than the conventional PCR/RT-PCR (Caipang et al., 2004; Kono et al., 2004; Pillai et al., 2006; Saleh et al., 2008).

Although the RT-LAMP reaction showed high degree of specificity in the formation of a white precipitate, insignificant amount of non-specific DNA amplification was observed in spectrophotometer reading of uninfected sample. However, increasing the template volume (1–4 μl) did not have any effect in increasing non-specific amplification of DNA in uninfected sample. Spectrophotometer reading of turbidity in uninfected sample was always constant and irrelevant. Further studies are required to determine the mechanisms underlying the non-specific or background amplification of DNA.

RT-LAMP was found to be very economical compared to RT-PCR. Cost comparison of RT-LAMP with RT-PCR suggests that the cost of RT-LAMP is nearly 4 times less than RT-PCR. The ability of LAMP method to amplify DNA from fewer copies of initial target DNA compared to PCR has been demonstrated conclusively (Gunimaladevi et al., 2004, 2005; Kono et al., 2004; Savan et al., 2004; Pillai et al., 2006). Though RT-LAMP has many advantages over RT-PCR, our experience has shown that RT-LAMP needs highly purified template RNA for assay. In an earlier trial, non-specific DNA amplification, including formation of white precipitate, was observed in uninfected samples using RNA without prior DNAse and proteinase treatment. This problem was solved by the use of ultra pure RNA. In their study on detection of food borne salmonella in raw milk by LAMP, Wang et al. (2008) demonstrated very clearly the importance of nucleic acid purity in the LAMP reaction. Another disadvantage of the LAMP assay is that the high sensitivity and very large amount of amplified DNA produced makes it susceptible to false positive reactions because of cross-contamination. Hence, the necessary precautions should be taken during extraction of RNA and preparation of the reaction mix.

RT-LAMP is a cost effective, rapid and sensitive diagnostic tool for the detection of white tail disease of *M. rosenbergii*. This technique is "farmer friendly" as the detection can be carried out in any simple field laboratory. Visualisation of the result is convincing to the farmer. It is suitable as a routine diagnostic tool in private clinics and field laboratories where equipment such as a thermal cycler and electrophoresis apparatus are not available.

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## References

Arcier, J.M., Herman, F., Lightner, D.V., Redman, R., Mari, J., Bonami, J.R., 1999. A viral disease associated with mortalities in hatchery-reared postlarvae or the giant fresh water prawn *Macrobrachium rosenbergii*. Dis. Aquat. Org. 38, 177–181.

Bonami, J.R., Shi, Z., Qian, D., Widada, J.S., 2005. White tail disease of the giant fresh water prawn *M. rosenbergii*, separation of the associated virions and characterization of *Mr*NV as a new typed noda virus. J. Fish Dis. 28, 23–31.

Caipang, C.M.A., Haraguchi, I., Ohira, T., Hirono, I., Aoki, T., 2004. Rapid detection of a fish iridovirus using loop-mediated isothermal amplification (LAMP). J. Virol. Methods 121, 155–161.

Fukuta, S., Iida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., Ishimoto, Y., 2003. Detection of Japanese yam mosaic virus by RT-LAMP. Arch. Virol. 148, 1713–1720.

Fukuta, S., Ohishi, K., Yoshida, K., Mizukami, Y., Ishida, A., Kanbe, M., 2004. Development of immunocapture reverses transcription loop-mediated isothermal

amplification for the detection of tomato spotted wilt virus from chrysanthemum. J. Virol. Methods 121, 49–55.
Gunimaladevi, T., Kono, T., Venugopal, M.N., Sakai, M., 2004. Detection of koi herpesvirus in common carp, *Cyprinus carpio* L., by loop-mediated isothermal amplification. J. Fish Dis. 27, 583–589.
Gunimaladevi, I., Kono, T., Lapatra, S.E., Sakai, M., 2005. A loop mediated isothermal amplification (LAMP) method for detection of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*). Arch. Virol. 150, 899–909.
Hong, T.C., Mai, Q.L., Cuong, D.V., Parida, M., Minekawa, H., Notomi, T., Hasebe, F., Morita, K., 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. 42, 1956–1961.
Kono, T., Savan, R., Sakai, M., Itami, T., 2004. Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. J. Virol. Methods 115, 59–65.
Notomi, T., Okayama, H., Masubuchi, H., Yonakawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop mediated isothermal amplification of DNA. Nucleic Acids Res. 28, 63.
Parida, M., Hong, T.C., Mai, Q.L., Notomi, T., Hasebe, F., Morita, K., 2004. Development and evolution of a novel loop mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome corona virus. J. Clin. Microbiol. 42, 1956–1961.
Pillai, D., Bonami, J.R., Widada, J.S., 2006. Rapid detection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV), the pathogenic agent of white tail disease of *Macrobrachium rosenbergii* (*De Man*), by loop mediated isothermal amplification. J. Fish Dis. 29, 275–283.
Poon, L.L., Leung, C.S., Tashiro, M., Chan, K.H., Wong, B.W., Yuen, K.Y., Guan, Y., Peiris, J.S., 2004. Rapid detection of the severe acute respiratory syndrome (SARS) coronavirus by a loop-mediated isothermal amplification assay. Clin. Chem. 50, 1050–1052.
Qian, D., Shi, Z., Zhang, S., Cao, Z., Liu, W., Li, L., Xie, Y., Cambournac, I., Bonami, J.R., 2003. Extra small virus like particle (XSV) and nodavirus associated with whitish muscle disease in the giant fresh water prawn *Macrobrachium rosenbergii*. J. Fish Dis. 26, 521–527.
Romestand, B., Bonami, J.R., 2003. A sandwich enzyme linked immunosorbent assay (S-ELISA) for detection of *Mr*NV in the giant fresh water prawn *Macrobrachium rosenbergii*. J. Fish Dis. 26, 71–75.
Sahul Hameed, A.S., Yoganandhan, K., Widada, J.S., Bonami, J.R., 2004. Studies on the occurrence and RT-PCR detection of *Macrobrachium rosenbergii* noda virus & extra small virus-like particles associated with white tail disease of giant fresh water prawn *Macrobrachium rosenbergii* in India. Aquaculture 238, 127–133.
Saleh, M., Hatem, S., Mansour, E.I.M., 2008. Loopmediated isothermal amplification as an emerging technology for detection of *Yersinia ruckeri* the causative agent of enteric red mouth disease in fish. BMC Vet. Res. 4 (31), 1–10.
Savan, R., Igrashi, A., Matsuoka, S., Sakai, M., 2004. Sensitive and rapid detection of edwardsiellosisin fish by a loop mediated isothermal amplification method. Appl. Environ. Microbiol. 70, 621–624.
Savan, R., Kono, T., Itami, T., Sakai, M., 2005. A review on loop mediated isothermal amplification: an emerging technology for detection of fish and shellfish pathogens. J. Fish Dis. 28, 573–581.
Sri Widada, J., Durand, S., Cambournac, I., Qian, D., Shi, Z., Dejonghe, E., Richard, V., Bonami, J.R., 2003. Genome based detection methods of *Macrobrachium rosenbergii* nodavirus, a pathogen of the giant fresh water prawn, *Macrobrachium rosenbergii*: dot blot, in situ hybridization and RT-PCR. J. Fish Dis. 26, 583–590.
Sri Widada, J., Richard, V., Shi, Z., Bonami, J.R., 2004. Dot blot hybridization and RT-PCR detection of extra small virus (XSV) associated with white tail disease of prawn, *Macrobrachium rosenbergii*. Dis. Aquat. Org. 58, 83–87.
Tung, C.W., Wang, C.S., Chen, S.N., 1999. Histological & electron microscopic study on *Macrobrachium* muscle virus (*M*MV) infection in the giant fresh water prawn. *Macrobrachium rosenbergii* (de Man), cultured in Taiwan. J. Fish Dis. 22, 1–5.
Vadher, K.H., 2003. Effect of eyestalk ablation on the rematuration of female *Macrobrachium rosenbergii* (De Man) in captivity. Master of Fisheries Science (MFSc) Thesis. Faculty of Fisheries, Kerala Agricultural University, Kerala, India, 124 pp.
Wang, D., Huo, G., Wang, F., Li, Y., Ren, D., 2008. Drawback of loop mediated isothermal amplification. Afr. J. Food Sci. 2, 083–086.
Yoganandhan, K., Learivibhas, M., Sriwongpuk, S., Limsuwan, C., 2006. White tail disease of the giant fresh water prawn *Macrobrachium rosenbergii* in Thailand. Dis. Aquat. Org. 69, 255–258.
Zhang, H., Wang, J., Yuan, J., Li, L., Zhang, J., Bonami, J.R., Shi, Z., 2006. Quantitative relationship of two viruses (*Mr*NV and XSV) in white tail disease of *Macrobrachium rosenbergii*. Dis. Aquat. Org. 71, 11–17.