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# Application of high resolution melt (HRM) analysis for duplex detection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in shrimp

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ABSTRACT

In this work, a probe-free, multiplex RT-PCR was combined with high resolution melt (HRM) analysis for the simultaneous detection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) infection in the freshwater prawn *Macrobrachium rosenbergii*. This first application of HRM multiplex RT-PCR in shrimp reveals a new potential for rapid and sensitive detection of multiple pathogens. In addition, sequence variation in XSV could be observed from the high resolution melt peaks, as confirmed by sequence analysis. In 19 field samples of the freshwater prawn *M. rosenbergii* the technique revealed samples negative for both viruses, positive for both viruses or positive for *Mr*NV alone. No sample was found positive for XSV alone. Comparison of these results to those obtained using the same samples in analysis by traditional nested RT-PCR combined with gel electrophoresis revealed that HRM multiplex RT-PCR was more sensitive. Thus, the latter technique allows for rapid and sensitive, simultaneous detection of *Mr*NV and XSV and also has the potential to be adapted for simultaneous detection of other mixed viral infections in shrimp.



## 1. Introduction

White tail disease (WTD) can be lethal for the freshwater prawn *Macrobrachium rosenbergii* and can cause serious losses in the prawn culture industry. It was first reported from the French West Indies [2], and it was later found to be associated with a mixed infection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) [3, 14]. Although, the exact role of each of these viruses in causing WTD is still unclear [14, 31], recent work indicates that *Mr*NV may be the major cause of mortality. XSV is probably a satellite virus, dependent on *Mr*NV for its replication [28, 29] and that it may be less significant in causing disease [31]. Juveniles of the marine shrimp *Penaeus* (*Penaeus*) *monodon*, *Penaeus* (*Marsupenaeus*) *japonicus* and *Penaeus* (*Fenneropenaeus*) *indicus* were shown to be susceptible to non-lethal, laboratory infections of *Mr*NV/XSV [25], while natural, larval infections of *Penaeus monodon* and *Penaeus indicus* were lethal [15].

The initial methods used to determine infection of *Mr*NV and XSV employed conventional RT-PCR [17, 19, 21, 22]. Later, multiplex RT-PCR methods were introduced for simultaneous detection of *Mr*NV and XSV [27, 30]. More sensitive was a probe-based, real-time RT-PCR analysis, but the assay was performed in a two-tube format that comprised an initial cDNA synthesis step and a subsequent amplification step [31]. Apart from PCR based detection methods, other techniques for *Mr*NV and XSV detection include loop-mediated isothermal amplification, *in situ* hybridization, dot blot hybridization, and a double-antibody, sandwich enzyme-linked immunosorbent assay [12, 16, 21].

High resolution melt (HRM) analysis is based on the determination of changes in fluorescence as a result of melting double-stranded PCR products in response to 0.05 °C steps of increasing

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temperature (i.e., high resolution melting pattern). The melting temperature ($T_m$) and the characteristic shape in the melting curve profile of amplified products are highly dependent on nucleotide sequence. A single base substitution can change the $T_m$ of an amplified product and this change can be detected using instrumentation capable of real-time fluorescence monitoring combined with high resolution temperature change.

HRM has been applied widely in clinical diagnostics and medical research especially for human genotyping and mutation analysis [1, 7–9, 18]. In addition, HRM has been applied for research on detection and typing of human pathogens [10, 13, 20, 24, 26]. In aquaculture, common carp (*Cyprinus carpio* L.) was the first species subjected to HRM analysis for polymorphisms in mitochondrial DNA sequences to assess evolutionary and population relationships [5]. The purpose of this study was to develop a rapid and sensitive, duplex method for detection of *Mr*NV and XSV as an example of the potential for HRM application to detect pathogens in shrimp.

## 2. Materials and methods

### 2.1. Sample collection and total RNA isolation

Several stages of *Macrobrachium rosenbergii* were obtained from commercial shrimp farms in Thailand and Myanmar and stored in 70% ethanol until processed (Table 1). Pools of 4–5 whole post larvae (PL) from each source or tail muscle tissue from later life stages were subjected to RNA extraction. Total RNA isolation was carried out using TriZol (Invitrogen) according to the manufacturer's protocol. RNA concentration in the extracts was measured by spectrophotometry and adjusted to a concentration of 100 ng/μl before use in assays.

### 2.2. Primer design for nested RT-PCR and HRM multiplex RT-PCR

Primers used in multiplex RT-PCR and HRM analysis (HRM multiplex RT-PCR) were selected based on criteria recommended by Corbett's protocol, i.e. primers with nearly identical melting temperatures ($T_m$), with lengths of 18–30 nucleotides, with target sequences of 50–350 bp, etc. The targets for the selected *Mr*NV-specific and XSV-specific primers (Table 2) were located at 482–597 and 257–372 in the respective viral genome sequences (GenBank accession numbers NC_005095 and AY247793). The expected amplicons were of equal size (both 116 bp).

**Table 1**
Source of *M. rosenbergii* samples used in this study.

| Sample no. | Life Stage | Source | Year of collection |
|---|---|---|---|
| 1 | Post larvae (PL) | Kanchanaburi, Thailand | 2006 |
| 2 | PL | Kanchanaburi, Thailand | |
| 3 | PL | Kanchanaburi, Thailand | |
| 4 | PL | Kanchanaburi, Thailand | |
| 5 | PL | Kanchanaburi, Thailand | |
| 6 | PL | Kanchanaburi, Thailand | |
| 7 | PL | Bago, Myanmar | |
| 8 | Broodstock | Phetchaburi, Thailand | |
| 9 | Juvenile | Chachoengsao, Thailand | |
| 10 | Broodstock | Ratchaburi, Thailand | |
| 11 | Broodstock | Ratchaburi, Thailand | |
| 12 | Broodstock | Ratchaburi, Thailand | |
| 13 | Broodstock | Ratchaburi, Thailand | |
| 14 | Broodstock | Chanthaburi, Thailand | |
| 15 | PL | Chanthaburi, Thailand | |
| 16 | PL | Chanthaburi, Thailand | |
| 17 | PL | Chachoengsao, Thailand | |
| 18 | PL | Chachoengsao, Thailand | |
| 19 | Adult | Ratchaburi, Thailand | 2007 |

**Table 2**
Primers used in this study.

| Virus | Primer name/Sequence | Expected product size (bp) | Reference |
|---|---|---|---|
| *Mr*NV | MrNV-F/GAT ACA GAT CCA CTA GAT GAC C | 681 | [30] |
| | MrNV-R/GAC GAT AGC TCT GAT AAT CC | | |
| | MrNV-HRM-116F/CCC AAC GTT ATC CTC GAT GT | 116 | This study |
| | MrNV-HRM-116R/TCA TCA CGC CTG ACA ATC A | | |
| XSV | XSV-F/GGA GAA CCA TGA GAT CAC G | 507 | [30] |
| | XSV-R/CTG CTC ATT ACT GTT CGG AGT C | | |
| | XSV-HRM-116F/GTC CGG GAT TTA CAC ATT GG | 116 | This study |
| | XSV-HRM-116R/AAT CTC CTG GTG CAG CGT AT | | |

### 2.3. Preparation of in vitro transcribed RNA

Amplicons generated from the first round PCR (681 bp for *Mr*NV and 507 bp for XSV, see Table 2 for primer sequences) were cloned downstream of T7 promoter of pDrive cloning vector (QIAGEN) to prepare reference plasmids. The plasmid containing an *Mr*NV insert was linearlized with *Xho*I restriction enzyme while that containing an XSV insert was linearlized with *Hin*dIII. An *in vitro* transcription reaction of 200 μl contained 10 μg of respective digested plasmid DNA, 50 units of T7 RNA polymerase (Promega), 10 mM DTT and 0.75 mM each ribonucleoside triphosphates in 1X reaction buffer. After incubation at 37 °C for 4 h, the DNA templates were removed by DNase I treatment. The synthesized RNAs were subsequently quantified by spectrophotometry and stored at −80 °C to serve as positive control templates in both nested RT-PCR and HRM RT-PCR experiments.

### 2.4. Nested RT-PCR detection of MrNV and XSV

The first step RT-PCR was carried out as described in a previous report [30]. However, detection was performed separately for each virus. Subsequent nested RT-PCR assays were performed using primers described above (Table 2). A reaction mixture of 20 μl contained 2 μl of first step PCR product template, 0.25 U of Taq polymerase (Invitrogen), 200 μM of dNTP mix, 250 nM of each primer, and 1X buffer. The nested PCR protocol for both viruses comprised heating at 94 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 30 s, followed by a final elongation step at 72 °C for 5 min. PCR amplicons were analyzed by 1.2% agarose gel electrophoresis followed by ethidium bromide staining and visualization using a gel documentation system (SYNGENE). Reactions without a template served as negative controls while reactions containing either one of the respective *in vitro* transcribed RNAs or a mixture of them served as positive controls.

### 2.5. Probe-free multiplex RT-PCR with high resolution melt (HRM multiplex RT-PCR)

Multiplex RT-PCR and HRM analysis (HRM multiplex RT-PCR) were performed using Rotor Gene-6000™ (kindly provided by Cybeles Thailand Co., Ltd). The reaction mixture contained two pairs of primers that were used in nested PCR reactions as described above. Reaction components consisted of 100 ng RNA template, 200 nM of each primer, 0.4 μl of SuperScript One-Step RT/Platinum Taq mix (Invitrogen), 1 μl of 1:200 diluted SYTO9 Green (Invitrogen), and 1X reaction buffer in a total volume of 20 μl. The thermocycling protocol comprised one cycle at 50 °C for 20 min and 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 30 s. Control reactions were included as in the assays