A new nodavirus is associated with covert mortality disease of shrimp

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INTRODUCTION

A major disease that occurred in the shrimp farming industries in China before 2009 was generally named ‘covert mortality disease’ due to the most moribund shrimp hiding on the bottom in deep water rather than swimming to the surface or in shallow water, as did shrimp suffering from white spot disease (Zhang, 2004; Xing, 2004; Song & Zhuang, 2006; Xu & Ji, 2009; Gu, 2012; Huang, 2012). The shrimp suffering the disease exhibited clinical signs including hepatopancreatic atrophy and necrosis, empty stomach and guts, soft shell, slow growth, and in many cases abdominal muscle whitening and necrosis (Zhang, 2004; Huang, 2012). The farmers noted that dead shrimp could be found in the diseased population every day and mortality increased during 60–80 days post-stocking with a cumulative mortality up to 80%. Early mortality syndrome (EMS)/acute hepatopancreas necrosis disease (AHPND) that emerged in the south of China in 2010 (Lightner et al., 2012) was once thought to represent severe cases of covert mortality disease as its clinical signs were similar to that of covert mortality disease to some degree.

Infection with several RNA viruses has been found to cause typical muscle necrosis in cultured shrimp. Infectious myonecrosis virus (IMNV) and Penaeus vannamei nodavirus (PvNV) infect Litopenaeus vannamei and result in muscle necrosis (Poulos et al., 2006; Tang et al., 2007; Flegel, 2012). Macrobrachium rosenbergii nodavirus (MrNV) can infect freshwater prawns and L. vannamei (Qian et al., 2003; Bonami et al., 2005; Senapin et al., 2011, 2013; NaveenKumar et al., 2013), causing muscle necrosis in tail muscles. However, IMNV, MrNV and PvNV were not detectable in shrimp suffering from covert mortality disease using relevant reverse transcription (RT)-PCR methods. As has been observed for IMNV, MrNV and PvNV, histological examination of shrimp revealed coagulative necrosis of...
the skeletal muscle and pyknosis was found in the muscle. In addition, eosinophilic inclusions were identified in the hepatopancreas and lymphoid organ. The histological characteristic suggested the disease might be caused by a virus.

In the present study, a new nodavirus, tentatively named covert mortality nodavirus (CMNV), was identified as the aetiological agent of covert mortality disease through construction of a cDNA library and sequencing, virus extraction, fluorescence in situ hybridization (FISH), histopathology, transmission electron microscopy (TEM), and challenge testing.

RESULTS

Observation of clinical signs of farmed shrimp

The farmed shrimp of *L. vannamei* suffering from covert mortality disease exhibited obvious clinical signs, including hepatopancreatic atrophy with colour fading, empty stomach and guts, soft shell, and slow growth, and in many cases were accompanied by uneven slightly whitish muscle lesion areas in the abdominal segments or slightly pale body (Fig. 1a, b). The moribund shrimp sank to the bottom of deep water and were rarely found in shallow water or swimming under the surface. Farmers monitor the bottom of ponds for dead shrimp by throwing out and drawing back a convenient iron-made tetrahedroid with a net at the bottom triangle and a drawstring at the top. Dead shrimp were also often observed on the bottom downstream of aerators. Moribund and dead shrimp could be found every day in diseased ponds. The mortality began 1 month post-stocking and increased after 60–80 days post-stocking, accompanied by an increase of nitrite nitrogen (NO₂⁻-N) with a cumulative mortality up to 80 %.

Histopathology of CMNV infection

Histological examination showed that muscle fibres comprising the whitish muscle lesions had muscle fragmentation tending towards coagulative, muscular lysis, and myonecrosis (Fig. 2a). Multifocal myonecrosis in the striated muscle was accompanied by haemocytic infiltration and karyopyknosis of haemocytes (Fig. 2b). Vacuolation in the cytoplasm of hepatopancreocytes and eosinophilic inclusions were observed within the tubular epithelium of the hepatopancreas (Fig. 2c, d). In some cases, swollen nucleoli could be found in hepatopancreocytes. Inclusions and nuclear pyknosis were observed in the lymphoid spheroids (Fig. 2e, f). Additionally, specimens of *Fenneropenaeus chinensis* and *Marsupenaeus japonicus* with whitish abdominal muscle collected from diseased ponds exhibited the same gross histopathological characteristics.

TEM of virus particles

TEM of ultrathin sections of the hepatopancreas of CMNV-infected shrimp revealed the presence of spherical unenveloped virus-like particles with a diameter of ~24.9 ± 1.8 nm (n=21) (Fig. 3a, b). Unenveloped virus-like particles were also observed with TEM of negatively stained grids coated with the inoculation extracted from diseased shrimp for challenge tests and purified virions obtained by sucrose-gradient centrifugation (Fig. 3c, d). Small spherical particles were also observed by TEM. The larger virus-like particles were 32.1 ± 5.5 nm (n=37) in diameter and the smaller spherical particles were 19.0 ± 1.9 nm (n=15) in diameter (Fig. 3c, d).

Sequencing and phylogenetic analyses

In total, 326 clones and 221 sequences were obtained through the cDNA library constructed with the anchor random primers from the total RNA of the hepatopancreas of diseased shrimp. A BLAST search showed the amino acid sequence of a 1185 bp fragment clone insert from the cDNA library (designated CMNV-7; GenBank accession number KM112247) shared 54, 53 and 39 % identity with the amino acid sequences of RNA-dependent RNA polymerase from Flock House virus (FHV), black beetle virus (BBV) and MrNV, respectively (Table 1). Nucleotide alignment by BLAST showed no significant similarity in GenBank when the nucleotide fragment of CMNV-7 was submitted. The resulting phylogenetic tree inference revealed that the deduced amino acid sequence of the
RNA-dependent RNA polymerase gene of CMNV (CMNV-7) clustered in the genus Alphanodavirus, which includes MrNV, PeNV, Nodamura virus, Boolarra virus, FHV, BBV, Drosophila melanogaster American nodavirus and Pariacoto virus (Fig. 4). Bootstrap values indicated that the node support of the CMNV-7 isolate was 98 %, strongly suggesting that the isolate was a novel virus within the genus Alphanodavirus. The isolate was therefore tentatively named CMNV.

**Confirmation of the causative pathogen by experimental challenge**

To further identify whether CMNV was the aetiological agent, experimental infections were conducted. The results of the challenge test showed that cumulative mortality of shrimp in the 0.22 µm filtered injection, unfiltered injection and per os infection groups was 100, 100 and 84.85 ± 2.14 %, respectively, by day 10 post-injection. In contrast, there was no mortality of shrimp in the control group injected with PBS (Fig. 5). Further, histopathological examination showed striated muscle necrosis and the presence of karyopyknosis, and inclusions in the hepatopancreas and lymphoid organ. All of the observations were almost identical to those in clinical specimens. In addition, RT-PCR analysis indicated that all shrimp from the infected group were strongly positive for CMNV, whereas shrimp from the control group were negative.

**FISH of CMNV**

A 165 bp PCR-amplified product from CMNV-7 was labelled with fluorescein and used as a probe to detect CMNV by *in situ* hybridization in sections of infected *L. vannamei* from the experimental infection. The results showed that fluorescent signals were evident in the hepatopancreas, lymphoid organ and striated muscle of shrimp of the filtered injection group (Fig. 6). No reaction was observed in tissues prepared from uninfected shrimp (data not shown). The probe reacted most intensely with the inclusions in the tubular epithelium and myoepithelial cells surrounding the hepatopancreatic tubules (Fig. 6a–c). For striated muscle, a few inclusion-like bodies showed a very strong fluorescent signal. Diffusely distributed fluorescence was also observed within the muscle (Fig. 6d–f). The probe also reacted intensely with the inclusions of the lymphoid organ cells (Fig. 6g–i).

In addition, when the probe was used to detect CMNV in shrimp that were collected from diseased ponds characterized with obvious whitish abdominal muscles, similar fluorescent signals could also be found in the lymphoid organ, striated muscle and hepatopancreas of those shrimp.

**CMNV RT-PCR assay**

A nested RT-PCR assay was developed for highly sensitive detection of CMNV. The amplification result showed that
Fig. 3. Transmission electron micrographs of ultrathin section of the hepatopancreas and negative-stained CMNV virions. (a) Viral inclusion (big black arrow) and CMNV particles (white arrows) in the inclusion can be observed in the ultrathin section of the hepatopancreas. (b) Magnified micrograph of the virus particles in the ultrathin section of the hepatopancreas. The white arrows show the virus particles assembled in the inclusion. (c) Virus-like particles collected from sucrose-gradient fractionation. The size of the virus-like particles is not homogeneous. The larger virus-like particles (black arrows) are ~32 nm in diameter and the smaller virus-like particles (white arrows) are ~19 nm in diameter. (d) Magnified micrograph of the virus-like particles. The black arrows indicate the larger virus-like particles and the white arrows indicate the smaller virus-like particles. Bar, 100 (a) and 50 (c, d) nm.

Table 1. Percentage similarity of the amino acid sequence of RNA-dependent RNA polymerase of CMNV compared with other nodaviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Abbreviation</th>
<th>GenBank accession number</th>
<th>Sequence similarity to CMNV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphanodaviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Covert mortality nodavirus</td>
<td>CMNV</td>
<td>AIL48199</td>
<td>100</td>
</tr>
<tr>
<td>Flock House virus</td>
<td>FHV</td>
<td>NP_689444</td>
<td>54</td>
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<tr>
<td>Black beetle virus</td>
<td>BBV</td>
<td>YP_053043</td>
<td>53</td>
</tr>
<tr>
<td><em>Macrobrachium rosenbergii</em> nodavirus</td>
<td>MrNV-CN</td>
<td>AAQ54758</td>
<td>39</td>
</tr>
<tr>
<td>(China strain)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Macrobrachium rosenbergii</em> nodavirus</td>
<td>MrNV-AU</td>
<td>AEY63648</td>
<td>39</td>
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<tr>
<td>(Australia strain)</td>
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<tr>
<td><em>Macrobrachium rosenbergii</em> nodavirus</td>
<td>MrNV-MY</td>
<td>AEQ39078</td>
<td>39</td>
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<tr>
<td>(Malaysia strain)</td>
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<tr>
<td><em>Peneaus vannamei</em> nodavirus</td>
<td>PvNV</td>
<td>YP_004207810</td>
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<tr>
<td><em>Drosophila melanogaster</em> American nodavirus</td>
<td>DmANV</td>
<td>ACU32794</td>
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<tr>
<td>Nodamura virus</td>
<td>NoV</td>
<td>NP_077730</td>
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<td>Boolarra virus</td>
<td>BoV</td>
<td>NP_689439</td>
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<tr>
<td>Pariacoto virus</td>
<td>PaV</td>
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<td>35</td>
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<tr>
<td>Betanodaviruses</td>
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<tr>
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<td>SJNNV</td>
<td>NP_599247</td>
<td>33</td>
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<tr>
<td>Tiger puffer nervous necrosis virus</td>
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<td>YP_003288759</td>
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</tr>
<tr>
<td>Atlantic halibut nodavirus</td>
<td>AHNV</td>
<td>AAY34458</td>
<td>32</td>
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<tr>
<td>Golden pompano nervous necrosis virus</td>
<td>GPNNV</td>
<td>ACX54065</td>
<td>32</td>
</tr>
<tr>
<td>Atlantic cod nodavirus</td>
<td>ACNV</td>
<td>ABR23192</td>
<td>32</td>
</tr>
<tr>
<td>Japanese flounder nervous necrosis virus</td>
<td>JFNNV</td>
<td>ACN58225</td>
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<td>Dragon grouper nervous necrosis virus</td>
<td>DGNVV</td>
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<tr>
<td>Barfin flounder nervous necrosis virus</td>
<td>BFNNV</td>
<td>YP_003288756</td>
<td>31</td>
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<tr>
<td>Redspotted grouper nervous necrosis virus</td>
<td>RGNVV</td>
<td>ACX69744</td>
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the first step of the PCR produced a 618 bp amplicon and the second step of the RT-PCR produced a 165 bp amplicon. The RNA samples extracted from purified virus, and shrimp in the filtered group and unfiltered group were all strongly CMNV-positive after the first step of the RT-PCR (Fig. 7, lanes 1–3). The RNA samples extracted from the healthy shrimp were CMNV-negative after the first and second step of the RT-PCR. Specificity analysis against the extracted nucleic acid samples from shrimp infected with yellow head virus (YHV), MrNV, white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) indicated that the nested RT-PCR was specific for CMNV.

When the newly developed nest RT-PCR was used to amplify RNA from the hepatopancreas and muscle of different shrimp for epidemiological investigation, some specimens of *L. vannamei*, *F. chinensis* and *M. japonicus* with gross signs of atrophied hepatopancreas, soft shell, slow growth or whitish abdominal muscle were CMNV-positive.

**DISCUSSION**

The name ‘covert mortality disease’ (or ‘bottom death’) was broadly used in China before 2009 to describe the disease in which mortality was hidden under deep water in shrimp farms (Xing, 2004; Zhang, 2004). It was suggested that environmental factors, such as NO$_2^-$-N, high temperature, etc., caused the disease (Xu & Ji, 2009). AHPND,
for which the causative agent was demonstrated to be virulent strains of *Vibrio parahaemolyticus* (FAO, 2013; Tran *et al.*, 2013), hit the shrimp farming industries in 2010 in China (NACA, 2012). Many cases of EMS/AHPND were still called covert mortality disease, as the new syndrome looks like a severe case of covert mortality disease. However, the present study reveals that the covert mortality disease was caused by a suspected viral infection, with typical histopathology of inclusions and pyknosis in the tubular epithelium of the hepatopancreas, muscle and lymphoid spheroids, which does not match the histopathology of infection with any known viruses. Phylogenetic analysis of the sequenced clone insert in the cDNA libraries from diseased shrimp revealed a new nodavirus belonging to the genus *Alphanodavirus*. FISH with the probe amplified from the fragment showed consistent signals with the inclusions observed in the haematoxylin and eosin (HE)-stained histopathology in the hepatopancreas, lymphoid organ and muscle. The challenge test, which proved that both intramuscular injection with bacterium-free filtered inoculum of tissue homogenates prepared from the shrimp suffering from covert mortality disease and per os infection could successfully pass on the disease to healthy *L. vannamei*, indicated a virus-like infectious agent as the most probable cause of the disease. The challenged shrimp *L. vannamei* became infected and exhibited the signs and lesions associated with the disease. TEM examination of ultrathin sections of the lesions of diseased shrimp and negative staining of virus suspensions indicated particles with a mean diameter of 32 nm (measured from the negative staining of purified virions; a mean diameter of 25 nm was measured with ultrathin sections). These results fulfill Rivers’ postulates for the demonstration of a viral aetiology (Rivers, 1937). Based on the findings of the current study, the aetiological agent of the disease has been designated CMNV.

In this study, TEM showed that the size of CMNV virus particles is larger than those of MrNV and PvNV; however,
Fig. 7. RT-PCR detection of the presence of CMNV in the experimentally infected *L. vannamei*, and the spontaneously infected *F. chinensis* and *M. japonicas* collected from diseased ponds. M, DL2000 molecular mass marker. Lanes 1–6: result of the first step of RT-PCR; lanes 7–12: result of the second step of RT-PCR. Lanes 1 and 7, 2 and 8, and 3 and 9: amplification results of cDNAs from purified virus, and shrimp of the filtered group and unfiltered group, respectively. Lanes 4 and 10 and 5 and 11: amplification results of cDNAs from *F. chinensis* and *M. japonicas*, respectively. Lanes 6 and 12: negative controls.

It is in the diameter range of 32–33 nm, which is the typical size of alphanodaviruses (Thiéry et al., 2012). In addition to the major virus-like particles with a mean diameter of 32 nm, smaller particles were found in the virus preparations with a mean diameter of 19 nm. Given the size of the smaller particles (i.e. 19 nm) and the tissue of viral isolation (i.e. the hepatopancreas), it is highly likely that a densovirus-like hepatopancreatic parovirus (HPV) or perhaps even infectious hypodermal and haematopoietic necrosis virus (IHHNV) has been co-isolated with CMNV. Nevertheless, the smaller particles were negative for HPV and IHHNV when they were analysed by PCR methods recommended by the World Organisation for Animal Health (OIE, 2012) (data not shown). Extra small virus particles of 14–16 nm diameter were also found in preparations of MrNV, which causes whitish muscle disease in *M. rosenbergii* (Qian et al., 2003). The present study may provide the second instance for the nodavirus-associated satellite virus recognized by the International Committee on Taxonomy of Viruses (Briddon et al., 2012).

The bootstrap values of cluster nodes of CMNV compared with insect and crustacean nodaviruses were 98 and 94 %, respectively. The fact that CMNV-7 shared the higher bootstrap value with some of the insect nodaviruses rather than the crustacean nodaviruses might be attributed to the phylogenetic analysis, which was based on the partial sequence of the RNA-dependent RNA polymerase gene of CMNV (CMNV-7).

The clinical signs of CMNV infection have some similarities with the other two crustacean nodaviruses (MrNV and PvNV) and IMNV (abdominal muscle whitening) in terms of muscle lesions; however, these four viruses differ in virulence. MrNV can cause 100 % mortality in the infected post-larval and juvenile *M. rosenbergii* (Arcier et al., 1999; Sahul Hameed et al., 2004; NaveenKumar et al., 2013). PvNV does not cause mortality in *L. vannamei* in laboratory infections and the survival was reduced from a mean of 74 % in 2003 (before PvNV was detected) to 50 % after the appearance of PvNV in 2004 (Tang et al., 2007). IMNV causes 20 % mortality in laboratory-infected *L. vannamei* (Tang et al., 2005). Typically, the disease caused by IMNV progresses slowly, with low mortality rates that persist throughout the growing season, and the infection can cause 40–60 % mortality in affected ponds (Nunes et al., 2004). In contrast, our result showed that CMNV cumulative mortality of *L. vannamei* in the *per os* infection group is 84.85 ± 2.14 % by day 10 post-injection and the median lethal time (LT_{50}) is 107 h. In the CMNV-infected shrimp ponds, a slowly developing mortality was found during daily management and cumulative mortality of *L. vannamei* may be variable up to 80–90 %. High CMNV-related mortality indicates that CMNV possesses higher virulence than PvNV and IMNV.

In addition, the host range of MrNV, PvNV, IMNV and CMNV appears to be different. MrNV was isolated originally from *M. rosenbergii* (Arcier et al., 1999) and several species of marine penaeid shrimp (*Fenneropenaeus indicus*, *M. japonicus* and *P. monodon*) have been identified as reservoir hosts (Sudhakaran et al., 2006; Mello et al., 2011). PvNV appears to have a limited host range; it was isolated originally from *L. vannamei* (Tang et al., 2007) and did not infect *M. rosenbergii* in a 4 week injection bioassay as determined by RT-PCR analysis (Tang et al., 2011). IMNV can infect *L. vannamei*, *Litopenaeus stylirostris* and *P. monodon*, and it caused moderate mortality (20 %) in infected *L. vannamei* and 0 % mortality in *L. stylirostris* or *P. monodon* over a period of 4 weeks (Tang et al., 2005). Our results from histopathology and nested RT-PCR showed that CMNV can infect *L. vannamei*, *F. chinensis* and *M. japonicas*, which indicated that the host range of CMNV is wide in cultured shrimp species.

The target tissues of CMNV are different from those of MrNV, PvNV, IMNV. Until now, there was no evidence or report that demonstrated that MrNV, PvNV and IMNV can cause hepatopancreatic atrophy and necrosis. In our study, the shrimp with clinical signs collected from both CMNV-infected ponds and from the experimental infection showed that CMNV caused hepatopancreatic atrophy and necrosis. FISH of the hepatopancreas of CMNV-infected shrimp revealed that the CMNV infected the tubular epithelium of the hepatopancreas. Early studies on tissue tropism of fish nodavirus indicated that nodavirus was detected primarily in nervous tissues (Nguyen et al., 1997; Skliris & Richards, 1999; Grove et al., 2003; Nopadon et al., 2009), whereas recent studies have shown that RNA and viral proteins of nodavirus are present in the liver and non-nervous organs, with the highest persistence of viral antigens in the liver (Lopez-Jimena et al., 2011, 2012). Virus detection in the liver may support epitheliotrophism of nodaviruses (Lopez-Jimena et al., 2012). In addition, the obvious fluorescent signal in myoepithelial cells surrounding the hepatopancreatic tubules indicated that myoepithelial cells were also targeted cells and the
destruction of myoepithelial cells might result in the atrophy of hepatopancreatic tubules.

Additionally, similar to PnNV to some degree, CMNV-related mortality may be sporadic in ponds, but the mortality will be aggravated by environmental stressors, such as NO$_3^-$–N and high temperature. When the air temperature is $>$30 °C and water temperature is $>$28 °C, survival in CMNV-infected ponds decreases sharply. Higher mortality can be found after 60–80 days post-stocking during the summer crop (Zhang, 2004; Xing, 2004; Song & Zhuang, 2006; Xu & Ji, 2009; Gu, 2012).

In conclusion, through isolation, reinfection and histopathology, we revealed that CMNV, a new nodavirus, is the causative agent of shrimp covert mortality disease. Additionally, we developed a FISH and nested RT-PCR method for the detection of CMNV. The results of the current study indicate that CMNV is a new and highly virulent nodavirus. These findings underscore the need for professionals versed in aquatic animal health and farmers in the shrimp aquaculture industries to pay close attention and take measures to prevent disease outbreaks and economic losses caused by CMNV.

**METHODS**

**Shrimp.** Penaeid shrimp exhibiting signs of whitish abdominal muscle and hepatopancreatic atrophy were collected for virus isolation from farms in Fujian, Shandong and Hebei Provinces in China during 2010–2013. All farms were suffering from an outbreak of covert mortality disease. For RT-PCR tests, *L. vannamei*, *F. chinensis* and *M. japonicus* were sampled from shrimp farms from the coastal provinces of China during 2012 and 2013. Apparently healthy subadult *L. vannamei* shrimp (10 g mean weight) for challenge testing were purchased from a shrimp farm in Changyi, Shandong Province. The shrimp were tested and demonstrated to be free of WSSV, YHV, TSV, IHHNV, *Enterocytococcus hepatopenaei* and AHPNS by PCR or RT-PCR methods recommended by the World Organisation for Animal Health (OIE, 2012; Helig & Lo, 2013; Suesbsng et al., 2013).

**Virus isolation.** Virus isolation was conducted according to the method reported by Bonami et al. (2005) with a minor revision. Sampled cephalothoraxes and whitish abdominal muscle were homogenized in TN buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7.4) and clarified at 1400 g for 15 min. The supernatant was clarified for a second time at 10 000 g for 25 min. The supernatant of the second centrifugation was then centrifuged at 130 000 g for 4 h. The final supernatant was centrifuged for a second time at 10 000 g for 25 min and divided into two parts in two tubes. The supernatant in the first tube was injected directly into healthy shrimp in the lateral area of the fourth abdominal segment. The supernatant in the second tube was filtered through a 0.22 µm membrane and then centrifuged at 130 000 g for 4 h. The precipitation in the second tube was resuspended with 1 ml PBS (pH 7.4) at 10% of total body weight after 24 h starvation. Thereafter, the shrimp were maintained with pellet feeds for 10 days. At the end of the bioassay, shrimp were sampled for nested RT-PCR analysis. The healthy shrimp were raised similar to the control group.

**Construction of a cDNA library and sequencing.** Total RNA was extracted from the cephalothoraxes of challenged shrimp by using a QIAamp viral RNA kit (Qiagen) following the manufacturer’s protocol. The cDNA was synthesized using an anchor random primer (P17N8: 5'-GTTTCCAGTTGCTCTCNNNNNNNNNNN-3') and dsRNA was synthesized by PCR amplification according to the method of Hang et al. (2012). The amplification products were examined by agarose gel electrophoresis and then purified with a PureElute GX DNA Gel Extraction and Cleanup kit (EdgeBio). A rapid amplification pyrosequencing library for the purified, anchored, random RT-PCR products was prepared by using a GS FLX Titanium Rapid Library Preparation kit (Roche 454 Life Sciences) (Hang et al., 2012). Clones containing the insert were sequenced and the amino acid sequenced was deduced.

**Phylogenetic analysis.** The sequence of CMNV (CMNV-7) was submitted for a BLAST (National Center for Biotechnology Information) search and highly similar matches were included in the dataset for phylogenetic analysis. A total of 20 RNA-dependent RNA polymerase sequences (Table 1) were aligned with CLUSTAL W as implemented in MEGA5.0 (Tamura et al., 2011) using the default settings. The alignment file was checked visually for alignment gaps and missing data. A phylogenetic tree was then reconstructed by the neighbour-joining method with bootstrap analysis (1000 replicates) using MEGA5.0.

**Probe labelling and FISH.** A pair of primers designed from the cDNA clone was used to label the fluorosecent probe. PCR reaction mixture (25 ml) contained 10 ng CMNV-7 plasmid, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 4 mM MgCl$_2$, 2 mM dATP/dCTP/dGTP, 1.5 mM dTTP, 0.5 mM Fluorescein-12-DUTP, 0.4 µM primers (CMNV-7Probe-F: 5’-GCCGATGACGGCTTGA-3’ and CMNV-7Probe-R: 5’-GGCGATGACGGCTTGA-3’) and 2.5 U Taq DNA polymerase (PCR Fluorescein Labelling Mix; Roche Applied Science). The PCR was carried out according to the protocol supported by the
PCR Fluorescein Labelling Mix. Following PCR, the fluorescent-labelled DNA probe was precipitated with ethanol, resuspended in water and stored at −20 °C.

Tissues originating from CMNV-infected ponds or experimental challenge tests were fixed with RNA-friendly fixative (Hasson et al., 1997) for 24 h and then transferred into 70% ethanol for storage. The fixed tissues were embedded in paraffin blocks and sectioned (6 μm thick) in accordance with standard methods (Lightner, 1996). The sections were then subjected to in situ hybridization assays according to the protocol supported by the PCR Fluorescein Labelling Mix with minor modifications adopted by Alcivar-Warren et al. (2006) and Panphut et al. (2011). The PCR amplification product with Fluorescein-12-dUTP and nucleic acid from healthy shrimp were used in negative control hybridization. After incubation, the specimens were washed and viewed under a confocal microscope. The cytoplasm was stained by Evans blue.

**Nestted RT-PCR**. Total RNA was extracted from haemolymph and cephalothorax of shrimp using RNAiso Plus (TaKaRa). The extracted RNA was denatured initially at 65 °C for 5 min and then cooled on ice for 2 min. The denatured RNA was then reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) at 50 °C for 60 min and then denatured at 70 °C for 15 min. The reaction mixture contained 2 μl cDNA, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 1.5 mM dNTP, 0.4 μM primers (CMNV-7F1: 5'-AAATAGCGCGATGACG-3' and CMNV-7R1: 5'-ACGAAGTGCACAGAC-GC-3'), and 2.5 U TaKaRa Ex Taq DNA polymerase (TaKaRa). The PCR was performed at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 40 s, ending with 72 °C for 7 min. In the first step, the RT-PCR amplified a 619 bp amplicon from the viral genome. For the second step of the PCR, the PCR mixtures were the same as that described above, except different template and primers (CMNV-7F2: 5'-CACACGGCTTAAACC-3' and CMNV-7R2: 5'-GGTAAAACGGGAAGG-3') and were included in the reactions. The amplification was performed with the following cycling parameters: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 20 s, and a final extension at 72 °C for 7 min. A 165 bp amplicon was amplified by the second step of the PCR.

To test the specificity of the RT-PCR, nucleic acids from shrimp infected with YHV, MrNV, WSSV and TSV were used as template in the RT-PCR. PCR products (5 μl) were analysed in a 1.5% agarose gel containing GeneFinder (Bio-V).

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CMNV causes covert mortality disease of shrimp


