Advances in the study of nodavirus

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Nodaviruses are small bipartite RNA viruses which belong to the family of *Nodaviridae*. They are categorized into alpha-nodavirus which infects insects and beta-nodavirus which infects fishes. Another distinct group of nodavirus infects shrimps and prawns, which has been proposed to be categorized as gamma-nodavirus. Our current review focuses mainly on recent studies performed on nodaviruses. Nodavirus can be transmitted vertically and horizontally. Recent outbreaks have been reported in China, Indonesia, Singapore and India, affecting the aquaculture industry. It also decreased mullet stock in the Caspian Sea. Histopathology and transmission electron microscopy (TEM) are used to examine the presence of nodaviruses in infected fishes and prawns. For classification, virus isolation followed by nucleotide sequencing are required. In contrast to partial sequence identification, profiling the whole transcriptome using next generation sequencing (NGS) offers a more comprehensive comparison and characterization of the virus. For rapid diagnosis of nodavirus, assays targeting the viral RNA based on reverse-transcription PCR (RT-PCR) such as microfluidic chips, reverse-transcription loop-mediated isothermal amplification (RT-LAMP) and RT-LAMP coupled with lateral flow dipstick (RT-LAMP-LFD) have been developed. Besides viral RNA detections, diagnosis based on immunological assays such as enzyme-linked immunosorbent assay (ELISA), immunodot and western blotting have also been reported. In addition, immune responses of fish and prawn are also discussed. Overall, in fish, innate immunity, cellular type I interferon immunity and humoral immunity cooperatively prevent nodavirus infections. Whereas prawns and shrimps adopt different immune mechanisms against nodavirus infections, through upregulation of superoxide anion, prophenoloxidase, superoxide dismutase (SOD), crustin, peroxinectin, anti-lipopolysaccharides and heat shock proteins (HSP). Potential vaccines for fishes and prawns based on inactivated viruses, recombinant proteins or DNA, either delivered through injection, oral feeding or immersion, are also discussed in detail. Lastly, a comprehensive review on nodavirus virus-like particles (VLPs) is presented. In recent

years, studies on prawn nodavirus are mainly focused on *Macrobrachium rosenbergii* nodavirus (*Mr*NV). Recombinant *Mr*NV VLPs have been produced in prokaryotic and eukaryotic expression systems. Their roles as a nucleic acid delivery vehicle, a platform for vaccine development, a molecular tool for mechanism study and in solving the structures of *Mr*NV are discussed intensively.

1 **Advances in the study of nodavirus**

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19 **ABSTRACT**

21 Nodaviruses are small bipartite RNA viruses which belong to the family of *Nodaviridae*. They

22 are categorized into alpha-nodavirus which infects insects and beta-nodavirus which infects

23 fishes. Another distinct group of nodavirus infects shrimps and prawns, which has been proposed

24 to be categorized as gamma-nodavirus. Our current review focuses mainly on recent studies

25 performed on nodaviruses. Nodavirus can be transmitted vertically and horizontally. Recent

26 outbreaks have been reported in China, Indonesia, Singapore and India, affecting the aquaculture

27 industry. It also decreased mullet stock in the Caspian Sea. Histopathology and transmission

28 electron microscopy (TEM) are used to examine the presence of nodaviruses in infected fishes

29 and prawns. For classification, virus isolation followed by nucleotide sequencing are required. In

30 contrast to partial sequence identification, profiling the whole transcriptome using next

31 generation sequencing (NGS) offers a more comprehensive comparison and characterization of

32 the virus. For rapid diagnosis of nodavirus, assays targeting the viral RNA based on reverse-

33 transcription PCR (RT-PCR) such as microfluidic chips, reverse-transcription loop-mediated

34 isothermal amplification (RT-LAMP) and RT-LAMP coupled with lateral flow dipstick (RT-

35 LAMP-LFD) have been developed. Besides viral RNA detections, diagnosis based on

36 immunological assays such as enzyme-linked immunosorbent assay (ELISA), immunodot and

37 western blotting have also been reported. In addition, immune responses of fish and prawn are

38 also discussed. Overall, in fish, innate immunity, cellular type I interferon immunity and humoral

39 immunity cooperatively prevent nodavirus infections. Whereas prawns and shrimps adopt

40 different immune mechanisms against nodavirus infections, through upregulation of superoxide

41 anion, prophenoloxidase, superoxide dismutase (SOD), crustin, peroxinectin, anti-

42 lipopolysaccharides and heat shock proteins (HSP). Potential vaccines for fishes and prawns

43 based on inactivated viruses, recombinant proteins or DNA, either delivered through injection,

44 oral feeding or immersion, are also discussed in detail. Lastly, a comprehensive review on

45 nodavirus virus-like particles (VLPs) is presented. In recent years, studies on prawn nodavirus

46 are mainly focused on *Macrobrachium rosenbergii* nodavirus (*Mr*NV). Recombinant *Mr*NV

47   VLPs have been produced in prokaryotic and eukaryotic expression systems. Their roles as a

48   nucleic acid delivery vehicle, a platform for vaccine development, a molecular tool for

49   mechanism study and in solving the structures of *Mr*NV are discussed intensively.

51   **INTRODUCTION**

52   The current review discusses recent studies related to nodaviruses. Recent reported outbreaks of

53   nodaviruses, diagnostic assays, host immunological responses, vaccines, and virus-like particles

54   (VLPs) are emphasized. To the best of our knowledge, there are only 8 review articles related to

55   nodavirus which had been published within the past 5 years: immunological-based detection of

56   shrimp viruses (Chaivisuthangkura, Longyant & Sithigorngul, 2014); recombinant nodavirus-like

57   particles as delivery system (Jariyapong, 2015); the life cycle of beta-nodaviruses (Low et al.,

58   2017); viral encephalopathy and retinopathy in aquaculture (Doan et al., 2017); interaction

59   between beta-nodavirus and its host for development of prophylactic measures for viral

60   encephalopathy and retinopathy (Costa & Thompson, 2016); reactive oxygen species-mediated

61   cell death (Reshi, Su & Hong, 2014); mitochondrial disruption and necrotic cell death (Hong,

62   2013); and immunity to beta-nodavirus infections of marine fish (Chen, Wang and Chen, 2014).

63   Another two review articles published within the past 10 years are about the biology and

64   biomedical applications of Flock House virus (Venter and Schneemann, 2008), and white-tail-

65   disease (WTD) in *Macrobrachium rosenbergii* (Bonami & Sri Widada, 2011). However, none of

66   these articles review the recent advances in the study of nodaviruses as presented in the current

67   review.

68

SURVEY METHODOLOGY

"PubMed" and "Scopus" were used to search for journal articles published within the recent 5 years using the keyword "nodavirus". These articles were screened and used as references for the current review. Additional information was obtained through the "Google" search engine with more specific keywords for older publications.

Nodavirus

Nodavirus belongs to the family of *Nodaviridae*. Generally, nodaviruses are classified into alpha-nodavirus and beta-nodavirus based on their hosts. Alpha-nodaviruses such as Nodamura virus (NoV), Flock House virus (FHV), black bettle virus (BBV), Pariacoto virus (PaV), and a recently discovered mosinovirus (MoNV) (Schuster et al., 2014) infect insects, whereas beta-nodaviruses such as striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), redspotted grouper nervous necrosis virus (RGNNV), and tiger puffer nervous necrosis virus (TPNNV) infect fishes. Another type of nodavirus infects prawn, it is distinctive from alpha- and beta-nodaviruses (NaveenKumar et al., 2013). This prawn nodavirus includes *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and *Penaeus vannamei* nodavirus (*Pv*NV). NaveenKumar et al. (2013) proposed that the *Mr*NV and *Pv*NV should be categorized into gamma-nodaviruses, based on their distinct genomic sequences compared with that of both the alpha- and beta-nodaviruses. More recent studies have identified another two prawn nodaviruses, namely the covert mortality nodavirus (CMNV) (Zhang et al., 2014; Zhang et al., 2015) and *Farfantepenaeus duorarum* nodavirus (*Fd*NV) (Ng et al., 2013), infecting *Litopenaeus vannamei* and *F. duorarum*, respectively. Although nodaviruses are usually named

91   after their native hosts, nodaviruses are often capable of infecting multiple species. RGNNV has

92   been reported to infect Asian seabass, *Lates calcarifer* (Banerjee et al., 2014); Nile tilapia,

93   *Oreochromis niloticus* (Keawcharoen et al., 2015); and *Amphiprion sebae* Bleeker, a marine

94   clownfish (Binesh et al., 2013), whereas *Mr*NV has also been reported to infect *Penaeus indicus*,

95   *Penaeus monodon*, and *P. vannamei* (Ravi et al., 2009; Senapin et al., 2012).

96

97   **Fish nodavirus**

98   The fish nodavirus, also known as Nervous Necrosis Virus (NNV), infects fishes and

99   causes viral encephalopathy and retinopathy (VER). The first outbreak occurred in 1985 (Costa

100   & Thompson, 2016). The infection was first described by Yoshikoshi & Inoue (1990) in the

101   Japanese parrot fish *Oplegathus fasciatus*. Fishes infected by nodavirus suffer neurological

102   disorders, which are characterized by intensive vacuolization of retina and central nervous

103   systems, culminating in abnormal swimming pattern and darkening of fish color (Munday &

104   Nakai, 1997). In fish, Nodavirus can be detected in many organs but central nervous system

105   including the brain, spinal cord and retina are the main targets (Ghiasi et al., 2016). The fish

106   nodavirus seriously affects aquaculture industry worldwide, resulting in great economic losses.

107   Infection by this virus is often associated with high mortality rate, up to   100% in fish larvae

108   and juveniles (Skliris et al., 2001). To date, nodavirus is known to affect over 120 fish species,

109   particularly groupers and seabass such as the Asian seabass *Lates calcarifer* and European

110   seabass *Dicentrarchus labrax* (Breuil et al., 1991; Costa & Thompson, 2016; Frerichs et al.,

111   1996; Munday et al., 2002; Parameswaran et al., 2008). Although nodavirus mostly affects

112   marine fishes, nodavirus infections in freshwater fishes such as European eels (*Anguilla anguilla*

113 L.), yellow-wax pompano (*Trachinotus falcatus*), firespot snapper (*Lutaanus erythropterus* B.),

114 cobia (*Rachycentron canadum*) and Chinese catfish (*Parasilurus asotus*) have been reported in

115 Taiwan (Chi, Shieh & Lin, 2003). In addition, outbreaks in hybrid striped bass x white bass

116 (*Morone saxalitis* x *Morone chrysops*) and largemouth bass (*Micropterus salmoides*) have also

117 been reported in Italy (Bovo et al., 2011). Apart from horizontal transmission, fish nodavirus can

118 be transmitted vertically through infections at the gonads, passing the virus to their progenies

119 (Breuil et al., 2002; Kocan et al., 2001; Valero et al., 2015a).

120

121 **Prawn nodavirus**

122 Like fish nodavirus, prawn nodavirus has significant economic impact on the prawn

123 aquaculture industry. Prawn nodavirus can be isolated from cephalothoraxes and whitish

124 abdominal muscle (Zhang et al., 2014) of infected prawns. The most studied prawn nodavirus is

125 the *Mr*NV. It is a non-zoonotic nodavirus which infects *M. rosenbergii*, commonly known as the

126 giant river prawn. *Mr*NV was first isolated and reported in 1999 (Arcier et al., 1999) from *M.*

127 *rosenbergii*. Infection by *Mr*NV causes white tail disease (WTD) or white muscledisease

128 (WMD), where infected cells undergo necrosis and turn whitish. The rate of mortality is

129 extremely high (up to 100%) in larvae and post-larvae of *M. rosenbergii* (Qian et al., 2003; Ravi

130 et al., 2009), causing great economic losses to *M. rosenbergii* hatchery and nursery farm

131 industries. Despite the high mortality rate in larvae and post-larvae prawns, *Mr*NV does not

132 cause death in adult prawns. However, the adult prawns still serve as the virus carriers,

133 transmitting the virus vertically to their offsprings (Sudhakaran et al., 2007), and horizontally to

134 other prawns during cannibalization (Sahul Hameed et al., 2004). Another prawn virus, *Pv*NV

135 was first isolated in 2005 from a *P. vannamei* farm in Belize (Tang et al., 2007; Tang et al.,

136 2011). Being a prawn nodavirus, *Pv*NV shares 83% similarities with *Mr*NV in its viral genome

137 (Tang et al., 2011). It causes muscle necrosis, resulting in white, opaque lesions in the tail,

138 similar to the symptoms of *Mr*NV infection. However, the virulence of *Pv*NV is not as high as

139 *Mr*NV, in which the former normally resulted in approximately 50% production loss in an

140 infected farm (Tang et al., 2007). Apart from its native host, *Pv*NV has also been demonstrated

141 to be able to infect *Penaeus monodon* in an experimental infection (Tang et al., 2007).

142

143 **Insect nodavirus**

144   Unlike fish and prawn nodaviruses, insect nodavirus does not have a direct impact on

145 global economy. Despite that, insect nodavirus, especially the FHV and BBV have served as

146 excellent models to study the mechanisms of other positive-strand RNA viruses, such as those of

147 *Caliciviridae, Flaviviridae, Picornaviridae,* and *Togaviridae,* due to their small genome size and

148 high level of replication in compatible hosts (Ball & Johnson, 1998). FHV was originated from

149 grass grub, *Costelytra zealandica* (Dearing et al., 1980). FHV has been demonstrated to be able

150 to infect a wide variety of hosts, including insects, yeasts, plants, and mammalian cells. Apart

151 from its original host *C. zealandica*, FHV also infects the common fruit fly, *Drosophila*

152 *melanogaster*. Therefore, cell-lines derived from *D. melanogaster* such as Schneider Line 1

153 (DL1) have been established for the propagation of insect nodaviruses (Dearing et al., 1980;

154 Miller, Schwartz & Ahlquist, 2001). When the yeast *Saccharomyces cerevisiae* was transfected

155 with FHV, the viral genomic RNA induced the production of infectious virion capable of

156 infecting *Drosophila* cells (Price, Rueckert & Ahlquist, 1996). In addition, FHV was also

157 reported to infect the whole plants of barley, cowpea, chenopodium and tobacco (Selling, Allison

158 & Kaesberg, 1990), as well as mammalian cells such as the baby hamster kidney cell (BHK21)

159 (Ball, Amann & Garrett, 1992). Due to its wide host range, FHV has been an excellent model to

160 study the mechanisms of other economically important RNA viruses. Another well-studied insect

161 virus, BBV, was isolated from *Heteronychus arator*. BBV propagates well in *Drosophila* line 1

162 cells, but not in BHK21, mouse L-cell, mosquito cells (*Aedes albopictus* and *A. aegypti*),

163 cabbage looper (*Trichoplasia ni*), fall armyworm (*Spodoptera frugiperda*) and line GM1 of *D.*

164 *melanogaster* (Friesen et al., 1980). Selling & Rueckert (1984) established a plaque assay for

165 nodaviruses using *Drosophila* cell-adapted BBV, which greatly facilitates the isolation and

166 reassortant of nodaviruses (Kopek et al., 2010; Settles and Friesen, 2008). BBV's structure has

167 been studied intensively using electron microscopy and crystallization followed by small-angle

168 x-ray scattering (Hosur et al., 1984). As in other nodaviruses, BBV appeared to form icosahedral

169 structure with a triangulation number of *T*=3. Furthermore, the RNA3 of nodavirus was

170 identified to be a subgenomic mRNA of the viral RNA1 by studying BBV, and it can be isolated

171 from cells infected by BBV (Friesen and Rueckert, 1982; Guarino et al., 1984). Another insect

172 nodavirus, Boolarra virus was isolated from ghost moth *Oncopera intricoides* (Reinganum,

173 Bashiruddin & Cross, 1985). The viral morphogenesis was shown to be restricted to the

174 cytoplasm of cultured *Drosophila* cell lines (Bashiruddin & Cross, 1987). A more recent Wuhan

175 nodavirus was isolated from *Pieris rapae* larvae (Liu et al., 2006a). A study of its subgenomic

176 RNA3 has provided an insight into the RNAi inhibitory property of the nodavirus B2 protein

177 (Cai et al., 2010).

178

179 **General features of nodavirus**

180   In general, nodaviruses are non-enveloped zoonotic viruses with icosahedral structures.

181   Their genomes comprise of two linear, positive-sense, single-stranded RNA. RNA 1 is

182   approximately 3.1-3.2 kilobases (kb) in length, whereas RNA2 is approximately 1.2 − 1.4 kb.

183   Both of which lack a poly-A tail at their 3' ends (Comps, Pepin & Bonami, 1994; Mori et al.,

184   1992). RNA 1 encodes for the RNA-dependent RNA polymerase (RdRP), which functions in

185   replicating the viral RNA genome without involving an intermediate DNA. RNA 3, a

186   subgenomic transcript of RNA 1, it encodes for a non-structural B2-like protein (Cai et al., 2010;

187   Hayakijikosol & Owens, 2012; Lingel et al., 2005). B2 functions as a suppressor for the post-

188   transcriptional gene silencing of host defense mechanisms through non-specific binding to

189   double-stranded RNA generated during the virus replication (Fenner et al., 2006). RNA 2

190   encodes for the viral capsid protein, which forms the core of nodavirus. The nodavirus capsid

191   protein assembles into virus particles with icosahedral structures, approximately 30 nm in

192   diameter, with a triangulation number of 3 (T=3) containing 180 capsid subunits. The virus

193   particles package only the RNA 1 and RNA 2, forming simple but infectious virions.

194

195   **Transmission of nodavirus**

196   It has been confirmed that vertical transmission is the main mechanism of nodavirus

197   spreading (Murwantoko et al., 2016; Zhang et al., 2017). This vertical transmission in the

198   aquaculture industry can be overcome by good biosecurity practices in hatchery-reared larvae

199   and juveniles of some fish species. Besides vertical transmission, nodavirus may also infect the

200   cultured fish even at the grow-out stages through horizontal transmission. Although nodaviruses

201   detected in aquaculture farms are often with relatively low sequence variations, PCR based

202   molecular analyses (including RT-PCR and nested PCR) have revealed different betanodaviruses

203   with high numbers of sequence variations in wild fishes and even seawater samples. This implies

204   that, nodavirus with different virulence may be shed by the less susceptible wild fish in water

205   and consequently virulent forms of nodavirus in the seawater would infect the susceptible

206   cultured fish (Nishi et al., 2016).

207

208   **Recent incidence of nodavirus**

209   Nodavirus infection has a great negative impact on the aquaculture industry. To date,

210   more than 40 marine and freshwater fish species have been identified susceptible to nodavirus

211   (particularly betanodavirus) infection (Nishi et al., 2016). It has been detected in freshwater

212   prawn hatcheries in Indonesia (Murwantoko et al., 2016) and marine shrimp farms located in

213   Fujian, Shandong and Hebei Provinces in China (Zhang et al., 2014). In addition, nodavirus

214   caused mass mortality in cage-reared freshwater guppy *Poicelia reticulate* in Singapore (Hegde

215   et al., 2003), larval rearing facility of marine clownfish, *Amphiprion sebae* in India (Binesh et al.,

216   2013) and Asian seabass in India (Banerjee et al., 2014). Apart from affecting aquaculture

217   industry, nodaviruses detected in wild golden grey mullet *Liza aurata* and sharpnose mullets *Liza*

218   *saliens* were correlated to the dramatically decrease of mullets stock in Caspian Sea

219   (Zorriehzahra et al., 2014; Ghiasi et al., 2016).

220

221   **Detection of nodavirus**

222   **General identification**

223   Histopathology and Transmission Electron Microscopy (TEM) examinations were used

224   to observe the presence of nodaviruses in fishes (Ghiasi et al., 2016) and shrimps (Zhang et al.,

225   2014). In terms of histopathological analysis of nodavirus infected shrimps, necrotic epithelium

226   and inclusions in the hepatopancreatic tubular epithelium are commonly observed in a nodavirus

227   infected shrimp. In addition, viral inclusion and viral particles are commonly observed in the

228   hepatopancreas using TEM (Zhang et al., 2014). Moreover, severe anemia associated with

229   increase of neutrophil populations, decrease of lymphocyte populations, raise of liver enzyme

230   profile and decline of total protein, albumin and total immunoglobulin levels were also observed

231   in fishes infected with nodaviruses (Ghiasi et al., 2016).

232

233   **Molecular identification**

234   For the phylogenetic analysis, conventional and real-time reverse transcription PCR (RT-

235   PCR) that amplify the RNA-dependent RNA polymerase (RdRp) of RNA 1 (Murwantoko et al.,

236   2016) or the T4 region of RNA2 of nodavirus (Nishizawa et al., 1994; Hegde et al., 2003;

237   Banerjee et al., 2014; Overgård et al., 2012), random shotgun metagenomic sequencing (Ng et al.,

238   2013) and Illumina whole transcriptome metagenomic sequencing were able to detect the

239   presence of nodavirus in infected organisms (Greninger & DeRisi, 2015) or even from seawater

240   (Nishi et al., 2016). For example, tombunodavirus that shares nucleotide sequence similarity with

241   that of nodavirus and tombuvirus family members was identified in the weekly metagenomic

242   sequencing of organisms in San Francisco wastewater (Greninger and DeRisi, 2015).

243   Nevertheless, whether this phenomenon was due to co-infection of nodavirus and tombuvirus or

244   the real existence of tombunodavirus needs further validation. In another study by Conceição-

245   Neto et al. (2015), putative novel member of nodavirus was detected in the fecal samples of otter

246   (*Lutra lutra*) in Portugal based on the identification of RdRp in the metagenomic analysis.

247   However, this nodavirus identified in the gut of the otter maybe originated from a fish diet,

248   which doubted the report of a new host for nodavirus (Conceição-Neto et al., 2015).

249   To improve molecular identification, virus isolation coupled with either the Sanger

250   sequencing or next generation sequencing (NGS) allow specific characterization of a particular

251   strain of nodavirus (Zhang et al., 2014). Based on the International Committee on Taxonomy of

252   Viruses (ICTV), isolated nodaviruses can be classified according to the genetic diversity of the

253   RNA2 segment by the simple and cost-effective Sanger sequencing method (Conceição-Neto et

254   al., 2015). Pairwise identity of the RNA2 with less than 80% at the nucleotide level and less than

255   87% at the amino acid level is classified as a novel species (Schuster et al., 2014). Comparing to

256   the partial sequence identify determined by the Sanger sequencing method, profiling the whole

257   transcriptome of a nodavirus offers a more comprehensive comparison and characterization of

258   the virus classification. For example, CMNV, an alphanodavirus that shares only 31-54%

259   nucleotide sequence similarity with other nodaviruses in the GenBank, was successfully

260   characterized by sequencing the cDNA library using the Roche 454 sequencer (Zhang et al.,

261   2014). In addition, fluorescence *in situ* hybridization (FISH) and nested RT-PCR assays that

262   detect a specific nodavirus can be designed (Zhang et al., 2014). Another study by Schuster et al.

263   (2014) reported the identification of Mosinovirus (MoNV), a novel member of the family

264   *Nodaviridae*, neither belongs to alpha- nor beta-nodaviruses. Without the isolation of the virus,

265   recombination that was detected by the whole transcriptome 454 pyrosequencing in MoNV

266   would not be accepted (Schuster et al., 2014). Although nodavirus can be detected in water

267   samples, quantitative isolation of the nodavirus remains challenging with the current available

268   protocols (Nishi et al., 2016).

269

270   **Diagnosis of nodaviruses**

271   Most of the diagnostic assays for nodaviruses are based on detection of the viral RNA

272 through RT-PCR. In recent years, efforts have been focused to establish diagnostic assays which

273 require minimal laboratory setup. A rapid and sensitive automated microfluidic chip system for

274 the detection of piscine nodavirus in groupers has been developed (Kuo et al., 2012). The

275 microfluidic chip contains an RT-PCR module capable of processing extracted RNA samples,

276 and a capillary electrophoresis module. This microchip has been field-tested in an

277 epidemiological investigation of NNV in Taiwan (Kuo et al., 2012).

278   Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) is another

279 potential point-of-care diagnostic assay, as a laboratory setup such as thermocycler and

280 electrophoresis equipment can be omitted. Suebsing, Prombun & Kiatpathomchai (2013)

281 developed an RT-LAMP with colorimetric gold nanoparticle probe assay for the detection of

282 PvNV in *P. vannamei* and *P. monodon*. This assay is 10x more sensitive than the nested RT-PCR

283 established by Tang et al. (2007). On the other hand, Zhang et al. (2017) used the RT-LAMP as a

284 rapid and quantitative diagnostic assay for the detection of CMNV in *P. vannamei*. This assay is

285 capable of detecting as little as 6.3 pg of total RNA from infected shrimps.

286   Assays based on lateral flow strips have also been deployed for diagnosis of nodavirus.

287 Lin et al. (2014) combined RT-LAMP with a lateral flow dipstick (RT-LAMP-LFD) for the

288 detection of *Mr*NV, targeting six distinct regions of *Mr*NV RNA2. The sensitivity of this RT-

289 LAMP-LFD is 10x higher than that of the RT-LAMP. Toubanaki, Margaroni & Karagouni

290 (2015b) also developed a lateral flow paper biosensor for the detection of NNV in European

291 seabass. Instead of RT-LAMP, this lateral flow biosensor detects the viral RNA through RT-

292 PCR using a 5'-biotin-tagged primer, a probe containing a poly-A tail, and gold nanoparticles

293 conjugated to a poly-T oligonucleotide, with streptavidin forming the test line. This assay was

294 reported to detect 270 pg of initial total RNA, which is less sensitive than the RT-LAMP based

295 method (6.3 pg).

296   Apart from simply detecting the presence of nodavirus infection, it is also important to

297 identify the genotype of the infecting virus, either for epidemiological study, or for specific

298 strategy design to eliminate virus infection in an aquaculture farm. Toubanaki, Margaroni &

299 Karagouni (2015a) developed a tetra-primer PCR which can amplify specifically RGNNV or

300 SJNNV cDNA, thereby generating short PCR products of different sizes which can distinguish

301 between RGNNV and SJNNV infections in European seabass.

302   Apart from detecting the virus at RNA level, the presence of nodavirus can also be

303 evaluated by immunological methods, such as western blotting, indirect florescent antibody,

304 enzyme-linked immunosorbent assay (ELISA) and immunodot blot tests (Ghiasi et al., 2016;

305 Hegde et al., 2003; Sri Widada et al., 2003). *Mr*NV infection has been diagnosed with western

306 blotting, dot blot and ELISA using polyclonal antibodies against the recombinant *Mr*NV capsid

307 protein raised in rabbit (Farook et al., 2014a). Wang, Chang & Wen (2016) used an immunodot

308 blot assay to detect *Mr*NV with a polyclonal antibody raised against the recombinant viral capsid

309 in a Wistar rat. In addition, Wangman et al. (2012) successfully produced monoclonal antibodies

310 that bind specifically to *Mr*NV capsid protein. These antibodies can be used to detect *Mr*NV

311 without cross-reaction with other common shrimp viruses. Although the immunological methods

312 are less sensitive compared with the viral RNA-based detection methods, the former remains a

313 viable alternative for many laboratories.

314

315 ***In vitro* model for nodavirus studies**

316   Cell lines are important models in virology, toxicology and gene expression studies. In

317   virology, cell lines have been widely used to determine the infectivity, pathogenicity and

318   infectious mechanisms of nodavirus (Abdul Majeed et al., 2013; Nishi et al., 2016). Currently,

319   *Channa striatus* kidney (CSK) (kidney of *Channa striatus*), GB (brain of *Epinephelus coioides*),

320   GF-1 (fin of *Epinephelus coioides*), SSN-1 (fry of *Ophicephalus striatus*), E-11 (clone of SSN-1),

321   SISK (kidney of *Lates calcarifer*), SISS (spleen of *Lates calcarifer*), SIGE (eye of *Epinephelus*

322   *coioides*), ICF (fin of *Clarias batrachus*), IEE (eye of *Etroplus suratensis*), IEG (gill of *Etroplus*

323   *suratensis*), IEK (kidney of *Etroplus suratensis*), and IGK (kidney of *Epinephelus coioides*) fish

324   cell lines were proven susceptible to nodavirus infections and thus suitable for *in vitro*

325   propagation and studies of the viral infectious mechanisms (Abdul Majeed et al., 2013; Chi, Hu

326   & Lo, 1999; Sarath Babu et al., 2013; Kai, Wu & Chi, 2014; Nishi et al., 2016). Among these

327   cell lines, SISK, SISS and SIGE were found more susceptible to nodavirus infections and thus

328   they are suitable models for nodavirus propagation, diagnostic reagent and vaccine productions

329   (Sarath Babu et al., 2013).

330

331   **Immune response against nodavirus infection**

332   Immunity plays an important role in the prevention and recovery of nodavirus infection in

333   aquatic animals. Overall, activation of innate immunity (such as NK cells and antimicrobial

334   peptides), cellular T cell type I interferon immunity, and humoral immunity (immunoglobulins

335   antibodies) cooperatively prevent nodavirus infection (Chen, Wang & Chen, 2014; Costa &

336   Thompson, 2016). Nodavirus mainly affects fishes at larval stage, which may be due to the lack

337   of well-developed adaptive immune cells as present in adult fishes that restrict the viral

338   replication, thus minimize the development of pathological and clinical signs (Overgård et al.,

339   2012).

340   Pathogen-associated molecular patterns (PAMPs) on RNA viruses are first recognized by

341   pattern-recognition receptors (PRRs). This process subsequently induces intracellular signals to

342   activate defensive mechanisms (Chen et al., 2014). Toll-like receptors (TLRs) and retinoic acid-

343   inducible gene I (RIG-I)-like receptors (RLRs) are the two important classes of PRRs that sense

344   the PAMPs of RNA viruses such as fish nodaviruses (Chen et al., 2014; Costa and Thompson,

345   2016). The RLR family consists of RIG-1, melanoma differentiation-associated gene 5 (MDA-5)

346   and Laboratory of Genetics and Physiology 2 (LGP2). Activation of RLRs and TLRs

347   subsequently promotes interferon type I antiviral immune response (Costa and Thompson, 2016).

348   RLRs, MyD88-dependent TLRs (Chen et al., 2015) and TLR7 (Takano et al., 2011) were found

349   upregulated and correlated with the production of type I interferon and pro-inflammation

350   response. MDA5 is a member of RLRs that promotes transcription of interferon related immune

351   factors, which include interferon regulatory factor 3 (IRF3), IRF7 (Yu et al., 2017), and

352   interferon-stimulated response element (ISRE) such as interferon-stimulated gene 15 (ISG15)

353   (Huang et al., 2013) and proinflammatory cytokines (Huang et al., 2016b). Unlike RIG-1 and

354   MDA-5, the exact functions of LGP2 in different virus infections are controversial. LGP2 is

355   generally reported as a positive regulator of RIG-1 and MDA-5 (Chen et al., 2014). However, a

356   recent study showed a contrary function of LGP2, in which the overexpression of LGP2

357   suppressed the expression of MDA5, Mx promoter, ISRE, pro-inflammatory cytokines and type I

358   interferon genes, thus resulting in a high viral load (Yu et al., 2016).

359   Tripartite motif-containing (TRIM) proteins are multi-domain proteins that exert

360   important immune regulatory roles on TLR and RLRs mediated antiviral innate immunity

361   (Huang et al., 2016a). The innate antiviral immunities of fish against nodavirus infection are

362   summarized in Fig. 1. In fish, TRIM proteins play an important role in recognition and initiation

363   of protection against nodavirus infection. To date, fish TRIM25, TRIM32 and TRIM39 exerted

364   positive antiviral responses via activation of MDA5 expression (Wang et al., 2016b; Yang et al.,

365   2016; Yu et al., 2017). On the other hand, TRIM13 exerted negative regulation on antiviral

366   immunity against nodavirus infection through downregulation of MDA5 and the downstream

367   IFN signaling pathway (Huang et al., 2016a).

368   Type I (α/β) and type II (γ) interferons play important roles in the innate immune

369   responses against nodavirus infection in fish. Post detection by PRRs, IFN type I will be secreted

370   and picked up by IFN receptor of neighboring cells, activating its Janus kinases 1 (JAK1)/signal

371   transducer and activators of transcription (STAT) pathway which leads to the transcription of

372   IFN-stimulated genes (ISGs) (Chen et al., 2014) and pro-inflammatory cytokines, such as IL-1β,

373   IL-6 and TNF-α (Costa and Thompson, 2016). Activation of ISGs via type I interferon

374   subsequently promotes Mx promoter activity, which increases host resistance to nodavirus

375   infection (Chen et al., 2014). Mx is one of the downstream antiviral effectors under type I

376   interferon immunity (Sadler and Williams, 2008). The researchers demonstrated that the

377   sevenband grouper *Epinephelus septemfasciatusu* pre-treated with non-lethal aquabirnavirus

378   (ABV) developed a protection against the RGNNV nodavirus infection prior to the activation of

379   type I interferon. This protection was attributed to the overexpression of the type I interferon

380   downstream effector, the Mx gene in head kidney and brain of the fish (Pakingking Jr et al.,

381   2005). Furthermore, inoculation of gilthead seabream *Sparus aurata* with lipopolysaccharides

382   from *Vibrio alginolyticus* also stimulated the Mx gene expression in liver, which effectively

383   reduced the load of nodavirus in the brain (Bravo et al., 2013).

384   Halibuts and groupers are known reservoirs of nodavirus but resistant to the virus and vertically

385   transmit the disease (Chaves-Pozo et al., 2012; Overgård et al., 2012). Although the early

386   proinflammatory type 1 interferon response helps to control the nodavirus infection in the

387   juvenile Atlantic halibut *Hippoglossus hippoglossus*, the viral RNA was still detected in the brain,

388   eye and head kidney of the fish even after 14 weeks post infection by the virus. This was

389   accompanied by more drastic T cell mediated responses including upregulation of the T cell

390   markers (CD4, CD8α and CD8β), ISG15, Mx and IFNγ genes expression (Overgård et al., 2012).

391   However, elevation of proinflammatory cytokines without significant changes in CD4, CD8α

392   and CD8β T cell markers was observed in the brain, eye and head kidney of Atlantic halibut at

393   the early stage of nodavirus infection (Overgård et al., 2012). On other hand, susceptible species

394   such as European seabass was detected with delayed but stronger proinflammatory response,

395   resulting in an irreparable brain damage (Poisa-Beiro et al., 2008; Valero et al. 2015b). In

396   addition, a challenge study on sensitive seabass species with low titer of nodavirus induced early

397   but short-term type I interferon response (Scapigliati et al., 2010). Moreover, Valero et al. (2015a)

398   showed that nodavirus replicated more in the reservoir seabream's testis than the susceptible

399   seabass, through modulation of reproductive system that favor the transmission and shedding of

400   the virus in the reservoir species. This result indicates that proinflammatory type I interferon did

401   not involve in stimulating T cell proliferation at the early stage of the viral infection, and thus T

402   cell type I interferon response is not sufficient to clear the nodavirus, resulting in vertical virus

403   transmission in the reservoir species. The researchers proposed that antimicrobial peptides

404   (AMPs) play an important role in vertical transmission of nodavirus in resistant fish species

405   (Valero et al., 2015b). AMPs are a major component of the innate immune system in fish that

406   activate antiviral effects upon nodavirus infection (Xie, Wei & Qin, 2016; Valero et al., 2015b).

407   Grouper epinecidin-1 (CP643-1), complement factor 3 (c3), lysozyme (lyz), hepcidin (hamp),

408   dicentracin (dic), piscidin (pis) or b-defensin (bdef) are AMPs found to be activated during

409   nodavirus infection in both susceptible and resistant fish species (Valero et al., 2015b). CP643-1

410   was also reported to induce the Mx gene expression during nodavirus infection in fish (Chia et al.

411   2010). In addition, Tachyplesin I has been reported as an AMP found in resistant grouper strains

412   which activates the antivirus activity through promotion of ISRE and IFN-β expression (Xie,

413   Wei & Qin, 2016). Histones (H1 to H4) are another type of potential AMPs that may play some

414   roles in the antiviral effects in fish. However, more studies are needed to investigate the function

415   of histones in protecting fish against nodavirus infection (Valero et al., 2016a). Production of

416   AMPs in resistant gilthead seabream and susceptible European seabass differs significantly, in

417   which the AMPs were highly expressed in the brain but low in the gonad of gilthead seabream,

418   whereas in European seabass it was highly expressed in the gonad but low in brain. These results

419   indicate that vertical transmission of nodavirus by the resistant gilthead seabream could be

420   attributed to the poor AMP response in the gonad. The European seabass containing a high level

421   of AMPs in the gonad did not survive nodavirus infection as the AMP expression level in the

422   brain was low (Valero et al., 2015b).

423   There are other immune factors contribute to the protection of fish against nodavirus

424   infection. Esteban et al. (2013) reported that nodavirus strain 411/96 (RGNNV) induced early

425   (day 1 post-challenged) expression of the peroxiredoxin natural killer enhancing factor A

426   (NKEF-A), which involved in inflammation and innate immunity in both the brain and head

427   kidney of gilthead seabream, but not in European seabass. This result shows that an early

428   expression of NKEF-A which activates immune cells including the NK cells and macrophages is

429   an important anti-nodavirus mechanism in resistant species. On the other hand, the involvement

430   of CD83 gene in the immune response of fish during nodavirus infection was also evaluated.

431   Downregulation of CD83-like molecule expression was observed in the head kidney of European

432   seabass post-infected with nodavirus. Although CD83 was known as a marker for matured

433   human dendritic cells, active thymic T cells and even B cells, the exact function of CD83 in fish

434   lymphocytes is still unknown (Buonocore et al., 2012). Thus, more studies have to be performed

435   to investigate the involvement of CD83 expression in immunity against nodavirus infection.

436   Nodavirus is a simple RNA virus with only 3 genes. However, it has developed some

437   virus-host interaction properties, which include hijacking the host system and escaping host

438   defense mechanism (Chen et al., 2014). Overexpression of heat shock transcription factor 1

439   (HSF1) promoted the replication of nodavirus at the initial stage of the viral infection, which

440   could be due to an increase of fish body temperature as the expression of Mx protein was

441   suppressed at high temperature condition (Wang, Chen & Chen, 2016). Suppression of HSF1 by

442   the heat shock protein 90 (HSP90) thereby reduced the replication of nodavirus during the initial

443   stage of the viral infection (Chen et al., 2010; Wang, Chen & Chen, 2016). Moreover, fish is

444   more susceptible to nodavirus infection in the present of immunosuppressive agents. Lawrence,

445   Reid & Whalen (2015) reported that an organotin compound, tributyltin, which is commonly

446   used as antifouling paints for ships and fishing nets caused immune suppression in fishes.

447   Exposure of Japanese medaka *Oryzias latipes* larvae to tributyltin increased their susceptibility

448   towards SGWak97 nodavirus (RGNNV) infection, which resulted in a higher mortality in a

449   dosage dependent manner. This phenomenon could be attributed to the immunosuppression

450   caused by tributyltin on fish NK cell activity (Kitamura et al., 2017).

451   Besides innate and cell mediated immunity, humoral immunity also plays an important

452   role in protecting fish against nodavirus infection (Chen et al., 2014), especially the two

453 immunoglobulins, IgM and IgT. Infection of susceptible seabass with low titer of nodavirus did

454 not significantly alter the expression of IgM and IgT in the gills and spleen (Buonocore et al.,

455 2017) but only induced a marginal increase of serum IgM (Scapigliati et al., 2010). On the other

456 hand, activation of IgM$^+$ and IgT$^+$ B cells in the brain and overexpression of soluble IgT$^+$ by B

457 cells in the head kidney by early inflammatory response in the central nervous system reduced

458 nodaviral replication in the resistant aquaculture-relevant fish species (Lopez-Munoz, 2012;

459 Piazzon et al., 2016). Thus, activation of IgM and IgT expression by vaccination can protect the

460 fish from nodavirus infection (Costa and Thompson, 2016).

461   Unlike vertebrates including fish, the understanding of prawn immunity against nodavirus

462 infection is even limited. Based on current findings, prawns generally fight infections through

463 non-specific innate immune responses including prophenoloxidase-activating system (Ourth &

464 Renis, 1993; Popham et al., 2004) and over accumulation of superoxide anion (Ravi et al., 2010),

465 which were known to inactivate DNA and RNA viruses. However, the basic understanding on

466 the prawn immunity against nodavirus infection has ignited a spark of interest among researchers

467 to produce vaccines against the prawn nodavirus. Instead of the whole virus, Farook et al. (2014b)

468 introduced a recombinant *Mr*NV capsid protein (r-MCP) produced in *E. coli* into *M. rosenbergii*

469 as a potential vaccine against the WTD. This r-MCP increased the level of prophenoloxidase,

470 superoxide anion, and other anti-viral compounds such as crustin, peroxinectin, anti-

471 lipopolysaccharides and heat shock proteins (HSP21, HSP70, HSP90), which protected the *M.*

472 *rosenbergii* post-larvae from *Mr*NV challenge up to 76%.

473

474 **Advances in nodavirus vaccine development**

475   Vaccination has been proposed as a solution to control and prevent nodavirus outbreaks

476   in aquaculture industry (Pakingking Jr et al., 2010). Table 1 summarizes the studies on nodavirus

477   vaccines.   Among different types of vaccines, virus-like particles (VLPs) show the highest

478   potential to induce a long lasting and protective humoral immunity (Liu et al., 2006b).

479   Intramuscular administration of recombinant DGNNV VLPs produced in *E. coli* induced a high

480   antibody titer which is capable to neutralize the virus *in vitro*. Even without an adjuvant,

481   neutralizing antibodies induced by the DGNNV VLPs lasted over 5 months, further justifying the

482   potential application of nodavirus VLPs as a vaccine. A recombinant betanodavirus capsid

483   protein r-FNCP42 was generated by Vimal et al. (2014) based on the gene sequence of a fish

484   nodavirus isolated from Asian seabass (*L. calcarifer*) larvae. Intramuscular injection of 50 µg r-

485   FNCP42/fish resulted in 75% survival of juveniles of Asian seabass challenged with $1x10^{6.5}$

486   TCID$_{50}$ of nodavirus. As the genome sequence of r-FNCP42 shares more than 98-99% similarity

487   with other fish nodaviruses including red spotted grouper nervous necrosis virus, *Dicentrarchus*

488   *labrax* encephalitis virus, Asian seabass nervous necrosis virus, and *Epinephelus tauvina* nervous

489   necrosis virus (ETNV), thus cross protectivity of r-FNCP42 against these nodaviruses should be

490   tested. Naveen Kumar, Karunasagar & Karunasagar (2013) immunized *M. rosenbergii* through

491   oral administration of inactivated bacteria encapsulated dsRNA of *Mr*NV and XSV, where a

492   post-feeding virus challenge showed promising results. The *Mr*NV challenge at 24 h and 72 h

493   post-feeding showed relative high percentage of survival at 80% and 75%, respectively,

494   indicating a regulation via RNA interference. Ramya et al. (2014) used chitosan conjugated

495   DNA vaccine, where XSV antisense (XSVAS) nucleotide sequence was cloned into the pcDNA

496   plasmid vector. The presence of plasmid pcDNA-XSVAS was confirmed after 30 days of

497   administration through oral feeding, where it provided approximately 50% protection to prawns

498   challenged with crude extract of WTD-prawns. In addition, introduction of recombinant *Mr*NV

499   capsid protein through 24 h immersion followed by *Mr*NV challenge boosted the relative percent

500   survival of prawns by 76.03% (Farook et al., 2014b).

501

502   **Virus-like particles**

503   After decades since nodaviruses were first discovered, studies on their VLPs continue.

504   DGNNV VLPs produced in *E. coli* were crystallized and studied with x-ray diffraction, revealing

505   a T=3 icosahedral structure approximately 38 nm in diameter, closely resembling the native

506   virion (Luo et al., 2014). Recombinant FHV capsid protein produced in *E. coli* was also used in

507   an *in vitro* assembly study (Bajaj & Banerjee, 2016). The capsid protein possesses additional N-

508   terminal tag which hinders the assembly of the capsid protein into VLPs. Cleavage of this N-

509   terminal region *in vitro* in the presence of Ca^{2+} ion allows the capsid protein to assemble into

510   VLPs of different sizes. Despite the heterogenicity, these VLPs were capable of membrane

511   disruption, a property required by the nodavirus to penetrate its host cells (Bajaj & Banerjee,

512   2016).

513   In addition, FHV VLPs have also been used to display foreign epitopes, such as that of

514   hepatitis C virus (HCV) (Peng, Dai & Chen, 2005). Chen et al. (2006) used FHV VLPs to

515   display the epitopes of HCV core protein and hepatitis B virus (HBV) surface antigen, where the

516   displayed epitopes were shown to be immunogenic in guinea pigs. In another study, Manayami

517   et al. (2007) fused the protective antigen-binding von Willebrand A domain of ANTXR2 cellular

518   receptor to FHV VLPs. The researchers demonstrated that the fusion protein inhibited lethal

519   anthrax toxin, and at the same time induced toxin-neutralizing antibody which protected rats

520   from anthrax lethal toxin challenge. All of these studies demonstrated the potential of insect

521 nodavirus VLPs as a foreign epitope presenting agent. In recent years, however, studies on

522 nodavirus VLPs focused more on the prawn nodavirus, particularly *Mr*NV.

523   The first prawn nodavirus VLPs were produced by Goh et al. (2011) via recombinant

524 DNA technology. The recombinant *Mr*NV capsid protein expressed in *E. coli* self assembles into

525 VLPs of approximately 30 nm in diameter (Goh et al., 2011). Recently, they reported that 20-29

526 amino acids (a.a.) at the N-terminal region of *Mr*NV capsid protein are responsible for RNA

527 binding during the VLPs assembly through ionic interaction, where mutation of positively

528 charged a.a. at this region to alanine abolished the RNA binding of the *Mr*NV capsid protein

529 (Goh et al., 2014). Despite the role of RNA binding, the N-terminal region (1-29 a.a.) is not

530 required for the assembly of the VLPs, as demonstrated by Goh et al. (2014). Jariyapong et al.

531 (2014) demonstrated the ability of the *Mr*NV VLPs to encapsidate plasmid DNA in 0.035 −

532 0.042 mol ratio (DNA/ protein) through particle disassembly and reassembly, with the use of

533 EGTA [ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid] and Ca^{2+} ion, thereby

534 opening a path for the *Mr*NV VLPs to be used for the delivery of nucleic acid based therapeutic

535 agents, such as DNA vaccine or siRNA.

536   VLPs have been widely used for displaying foreign epitopes, for instance the VLPs of

537 human papilloma virus (Matic et al., 2011), HBV (Ibañez et al., 2013; Murray and Shiau, 1999;

538 Yap et al., 2012), as well as bacteriophages (Hashemi et al., 2012; Kok et al., 2002; Tan et al.,

539 2005; Wan et al., 2001). VLPs are known to enhance the immunogenicity of small epitopes

540 displayed on the particles (Murata et al., 2003; Quan et al., 2008). We have displayed the

541 immunodominant region of HBV on the surface of the *Mr*NV VLPs through fusion at the C-

542 terminal end of *Mr*NV capsid protein and confirmed the fusion protein with immunogold TEM

543 (Yong et al., 2015a). When introduced into BALB/c mice, this recombinant VLPs induced the

544 production of anti-HBV antibodies, as well as the cellular immune responses including natural

545 killer cells, cytotoxic T lymphocytes (CTL) and IFNγ. In addition, we have fused and displayed

546 multiple copies of influenza A virus matrix 2 ectodomain (M2e) on the surface of the *Mr*NV

547 VLPs (Yong et al., 2015b). The displayed M2e epitopes were highly antigenic and

548 immunogenic, where they correlated well with the copy number of M2e displayed on the surface

549 of the VLPs. Most recently, Somrit et al. (2017) showed that the C-terminal region of *Mr*NV

550 capsid protein is exposed on the surface of the VLP, constituting the core of the viral capsid

551 protrusion, through a homology-based modeling based on cucumber necrosis virus. When the C-

552 terminal region was removed with chymotrypsin digestion, the internalization capability of the

553 truncated VLPs into Sf9 cells reduced significantly, suggesting the importance of the C-terminal

554 region in the viral infection.

555 In an attempt to discover the possible mechanism of *Mr*NV infection pathway, we used

556 the *Mr*NV VLPs labeled with fluorescein as a model to study the *Mr*NV entry and localization in

557 Sf9 cells (Hanapi et al., 2017). Through the use of endosomal inhibitors coupled with laser

558 confocal microscopy and live cell imaging, we demonstrated that the internalization of *Mr*NV

559 VLPs was facilitated by clathrin- and caveolae-mediated endocytosis. We have also identified a

560 potential nuclear localization signal (NLS), which could aid in the localization of *Mr*NV capsid

561 protein to the nucleus based on the importin-α pathway (Hanapi et al., 2017).

562 Apart from the *Mr*NV VLPs produced in *E. coli*, we have also produced the *Mr*NV

563 capsid protein in Sf9 cells through baculovirus expression system (Kueh et al., 2017). This

564 eukaryotic produced *Mr*NV capsid protein self-assembles into VLPs significantly larger than

565 their prokaryotic counterparts. The Sf9 produced *Mr*NV VLPs are structurally more homogenous

566 as observed by TEM, representing a better candidate to be used in structural study. Subsequently,

567 we used this *Mr*NV VLPs produced in Sf9 for 3D structure reconstruction using the images

568 obtained from cryogenic electron microscopy (Ho et al., 2017). The 3D structure of *Mr*NV

569 capsid at 7 Angstroms resolution reveals a *T*=3 icosahedral structure distinctive to other insect

570 and fish nodavirus capsids, characterized by large dimeric blade-like spikes exposed on the

571 surface of the VLPs. This finding supports the assertion that prawn nodavirus should be

572 classified into a new genus.

573

574

575 **CONCLUSIONS**

576 Prawn nodaviruses are relatively new compared to the typical alpha- and beta-nodaviruses.

577 Despite their genomic and structural differences with the two established genera, prawn

578 nodaviruses have yet been classified into a new genus. There are likely more prawn nodaviruses

579 unknown to men, such as the recently discovered CMNV and FdNV. Isolation and

580 characterization of these new prawn nodaviruses could contribute in creating a new genus of

581 *Nodaviridae*, which is the gamma-nodavirus. In addition, there is yet an effective mid- or long-

582 term vaccine for shrimps and prawns against nodavirus infections. Due to the lack of adaptive

583 immune response in crustacean, antigens that can induce protection against the infections have to

584 be administrated from time to time, especially during the larval stage. Therefore, nodavirus

585 vaccines based on recombinant proteins incorporated into feeds would be more relevant. Better

586 yet, self-replicating DNA expression vector-based vaccines would be more cost-effective to be

587 utilized in shrimp and prawn aquaculture industries.

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1116   **Figure Legends**

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1118   **Figure 1:**

1119   Innate antiviral immunity of fish against nodavirus infection. **(A)** Positive regulation which

1120   inhibits viral replication. TRIM25, TRIM32 and TRIM39 upregulate the expression of MDA5,

1121   which in turn induces ISRE, IRF3 and IRF7. The upregulation of ISRE also induces the

1122   expression of ISG15 and proinflammatory cytokines, cooperatively reducing the viral load.

1123   Other elements known to inhibit virus replication include HSP90, 2-C Type I IFN and

1124   Tachyplesin I, which downregulates HSF1, upregulates Mx promoter and IFN- β, respectively.

1125   **(B)** Negative regulation which promotes viral replication. TRIM13 and LGP2 downregulate

1126   MDA5, thereby reduce the ISRE. LGP2 also downregulates Mx promoter, proinflammatory

1127   cytokines and Type I IFN. TRIM: Tripartite motif-containing protein; MDA5: Melanoma

1128   differentiation-associated gene 5; IRF: Interferon regulatory factor; ISRE: Interferon-stimulated

1129   response element; HSF1: Heat shock transcription factor 1; HSP90: Heat shock protein 90; ISG:

1130   Interferon-stimulated gene.

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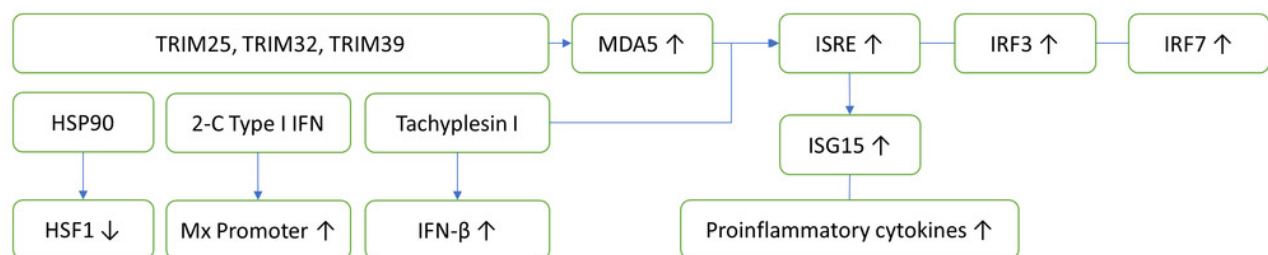
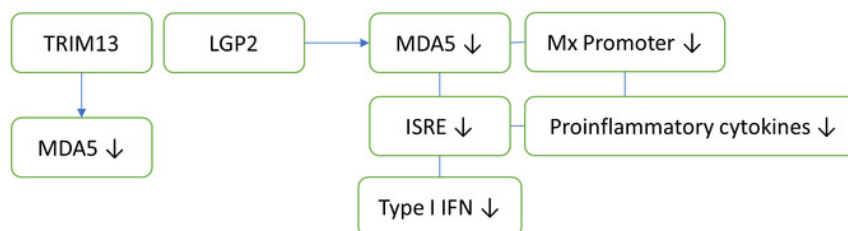
Figure 1

Innate antiviral immunity of fish against nodavirus infection.

(A) Positive regulation which inhibits viral replication. TRIM25, TRIM32 and TRIM39 upregulate the expression of MDA5, which in turn induces ISRE, IRF3 and IRF7. The upregulation of ISRE also induces the expression of ISG15 and proinflammatory cytokines, cooperatively reducing the viral load. Other elements known to inhibit virus replication include HSP90, 2-C Type I IFN and Tachyplesin I, which downregulates HSF1, upregulates Mx promoter and IFN- β, respectively. **(B)** Negative regulation which promotes viral replication. TRIM13 and LGP2 downregulates MDA5, thereby reduce the ISRE. LGP2 also downregulates Mx promoter, proinflammatory cytokines and Type I IFN. TRIM: Tripartite motif-containing protein; MDA5: Melanoma differentiation-associated gene 5; IRF: Interferon regulatory factor; ISRE: Interferon-stimulated response element; HSF1: Heat shock transcription factor 1; HSP90: Heat shock protein 90; ISG: Interferon-stimulated gene.

Vaccines, route of administration and their protectivity.

Type of vaccine	Route of vaccination	Protectivity	Remarks	References
Recombinant betanodavirus of RNA2 capsid protein r-FNCP42	IM	75% higher survival rate of juveniles of Asian seabass challenged with $1\times10^{6.5}$ $TCID_{50}$ of nodavirus/fish	As the genome sequence analysis of r-FNCP42 has more than 98-99% of similarity with other fish nodavirus including red spotted grouper nervous necrosis virus, Dicentrarchus labrax encephalitis virus, Asian seabass nervous necrosis virus, and Epinephelus tauvina nervous necrosis virus (ETNV), thus cross protectivity of r-FNCP42 against other strains of nodavirus shall be tested.	Vimal et al. (2014; 2016)
Recombinant r-FNCP42-DNA	IM	77% higher survival rate of juveniles of Asian seabass challenged with $1\times10^{6.5}$ $TCID_{50}$ of nodavirus/fish	Capsid protein was highly expressed in the heart, muscle and liver of the vaccinated fish.	Vimal et al. (2016)
Recombinant capsid protein MGNNV virus like particles (VLPs)	IM	~70% higher survival rate of juvenile European seabass (Dicentrarchus labrax) challenged with 10^{5} $TCID_{50}$/fish	MGNNV induced humoral immunity against nodavirus.	Thiery et al. (2006)
DNA vaccine pVHSV-G encoding glycoprotein of Viral hemorrhagic septicaemia virus	IM	~54% higher survival rate of juvenile turbot (Scophthalmus maximus) challenged with $10^{6.3}$ $TCID_{50}$	DNA vaccine induced inflammatory response that cross protect nodavirus infection.	Sommerset et al. (2003)
Synthetic peptides (N-terminal regions) of	IM	~ 27% higher survival rate of seabass challenged with 10^{9}	Peptides induced humoral immunity	Coeurdacier, Laporte &

nodavirus DIEV RNA2 protein		FCU/fish		P´epin (2003)
Heat inactivated S1 and Sb2 nodavirus	IM	~33% and 26% higher survival rate of seabass challenged with 9×10^9 FCU/fish, respectively	Induced humoral immunity	Coeurdacier, Laporte & P´epin (2003)
Virus-like particles (VLPs) of grouper nervous necrosis virus	IM	-	Induced humoral immunity. No challenge test was performed.	Liu et al. (2006b)
Recombinant RGNNV-CP	IM	~60% higher survival rate of humpback grouper challenged with $10^{5.5}$ $TCID_{50}$/fish, respectively	Induced humoral immunity.	Yuasa et al. (2002)
Recombinant ETNNV-CP (Epinephelus tauvina nervous necrosis virus-capsid protein)	IM	-	Induced stronger humoral immunity than formalin inactivated nodavirus. No challenge test was performed.	Hegde, Lam & Sin (2005)
Formalin inactivated nodavirus	IP	60% higher survival rate of brown-marbled grouper challenged with $10^{6.5}$ $TCID_{50}$/fish of OSGBF1E	Induction of humoral immunity.	Pakingking Jr et al. (2010)
Recombinant capsid protein, recAHNV-C	IP	29% higher survival rate of juvenile turbot (Scophthalmus maximus) challenged with	Fishes vaccinated with plasmid DNA expressing the recombinant capsid protein were not protected as the plasmid DNA only induced cellular but not humoral	Sommerset et al. (2005)

		10^6 TCID$_{50}$/ml AHNV	immunity.	
Formalin inactivated SGWak97	IP	Not reported	Inactivated SGWak97 induced humoral immunity.	Pakingking Jr et al. (2009)
Recombinant rT2 SJNNV-CP (Scophthalmus maximus nervous necrosis virus-capsid protein)	IP	~36% higher survival rate of humpback grouper challenged with 6.3x10^7 TCID$_{50}$/fish, respectively	Induced humoral immunity.	Húsgağ et al. (2001)
Chitosan-encapsulated DNA vaccine (CP-pNNV)	Oral	55% higher survival rate of juvenile European seabass (Dicentrarchus labrax) challenged with 10^6 TCID$_{50}$/fish	CP-pNNV failed to induce humoral immunity but activated interferon pathway and cell-medicated cytotoxicity.	Valero et al. (2016b)
Chitosan conjugated DNA vaccine pcDNA-XSVAS	Oral	Approximately 50% higher survival rate of prawn challenged with crude extract of prawn with WTD.	XSV with nodavirus caused white tail disease (WTD) in prawn. The challenge experiment shall consider using isolated virus instead of crude one.	Ramya et al. (2014)
Recombinant yeast expressing RGNNV-CP (red-spotted grouper necrosis virus capsid protein)	Oral	-	Induced humoral immunity in mice. No challenge test was performed.	Kim et al. (2013)
Artemia-encapsulated recombinant pET24a-	Oral	~34% higher survival rate of grouper larvae challenged	Induced humoral immunity	Lin et al. (2007)

NNV VP E. coli expressing nodavirus capsid protein		with 10^5 TCID$_{50}$/fish, respectively		
Inactivated bacteria encapsulated dsRNA of *Mr*NV and XSV	Oral	*Mr*NV challenge 24 h and 72 h post-feeding showed relative percent survival of 80% and 75% respectively	Protection through RNA interference with capsid and B2 genes of *Mr*NV, and capsid gene of XSV.	Naveen Kumar, Karunasagar & Karunasagar (2013)
Solid lipid nanoparticles encapsulated binary ethylenimine inactivated nodavirus	Bath and Oral	45% higher survival rate of grouper larvae challenged with 1×10^6 TCID$_{50}$/ml HGNNV	Simple vaccination procedure that fit for larvae. Both routes of vaccinations induced pro-inflammatory cytokines expression, type I IFN response, humoral immunity and cellular immunity.	Kai & Chi (2008); Kai, Wu & Chi (2014)
Recombinant *Mr*NV capsid protein	Bath	Immersion for 24 h followed by *Mr*NV challenge showed 76.03% survival in 15 days post-challenge	Protection is believed to be through upregulation of prophenoloxidase, superoxide anion and SOD activity.	Farook et al. (2014b)

1   **Notes:**

2   IM – intramuscular injection; IP – intraperitoneal injection; Oral – oral feeding; Bath – immersion