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Successful yellow head virus infection of *Penaeus monodon* requires clathrin heavy chain

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**A B S T R A C T**

Viral disease caused by the Yellow head virus (YHV) had great impact on economic loss in the aquaculture industry. Prevention or curing YHV disease is still not possible due to the lack of understanding of the basic mechanisms of YHV infection. In this report, the endocytosis inhibitors (chlorpromazine (CPZ), amiloride and methyl-β-cyclodextrin (MβCD)) were used to identify the cellular entry pathway of YHV. Pretreating shrimp with CPZ but not amiloride or MβCD followed by YHV challenge resulted in a significant reduction of YHV levels, suggesting that YHV entered the shrimp cells via clathrin-mediated endocytosis. Next, the major component of the clathrin-coated vesicle, *Penaeus monodon* clathrin heavy chain (*PmCHC*) was cloned and characterized. The complete coding sequence of *PmCHC* is 5055 bp encoding a putative protein of 1684 amino acids. Specific silencing of *PmCHC* mRNA by dsRNA-PmCHC showed an inhibition of YHV replication for 48 h post YHV injection as well as exhibiting a delay in shrimp mortality. These results indicated that *PmCHC* was an essential component for YHV infection of shrimp cells.

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1. Introduction

Yellow head virus (YHV) is one of the most devastating shrimp pathogens as it causes mortality within 3 days. This virus was first identified in 1992 from moribund shrimp from southern Thailand (Boonyaratpalin et al., 1993). The virus was named from symptoms of the disease in which the moribund shrimp would present a yellowish cephalothorax and very sallow overall coloration. YHV can infect various penaeid shrimp such as *Penaeus aztecus*, *Penaeus duorarum*, *Penaeus merguiensis*, *Penaeus monodon*, *Penaeus setiferus*, *Penaeus stylirostris* and *Litopenaeus vannamei* (Flegel, 1997). The infected moribund shrimp shows nuclear condensation as well as pyknosis and karyorrhexis which are signs of cell apoptosis (Khanobdee et al., 2002). YHV has a positive-sense single-stranded RNA genome of approximately 27 kb with a poly (A) tail. It belongs to the genus Okavirus, family Roniviridae in the order Nidovirales. The morphology of YHV reveals an enveloped bacilliform which has a particle size about 50–60 × 190–200 nm, containing the internal helical nucleocapsid which is closely surrounded by an envelope studded with prominent peplomers or spikes (Nadala et al., 1997; Sitthidikratna et al., 2008). YHV contains two major structural transmembrane glycoproteins (gp116 and gp64) and a nucleoprotein (p20) (Jitrapakdee et al., 2003). Antiserum against the gp116 but not gp64 can neutralize YHV infectivity in the primary lymphoid cells of *Penaeus monodon* (Assavalapsakul et al., 2005). In addition, injection of double-stranded RNA (dsRNA) targeting to the YHV-binding protein, YRP65, inhibited YHV replication (Assavalapsakul et al., 2006). Also, suppression of PmRab7 (a late endosomal marker involved in trafficking) resulted in inhibition of YHV replication (Ongvarrasopone et al., 2008). These results imply that for successful infection, YHV requires receptor mediated endocytosis as a route of entry and takes advantage of the host cell machineries for endosomal trafficking and replication. Therefore, better understanding of the endocytosis and trafficking pathway of YHV will shed light on the mechanism of YHV infection and replication and thus will lead to the development of an antiviral agent or strategy to combat YHV infection.

Many viruses utilize well-characterized cellular endocytic mechanisms including clathrin-mediated endocytosis, lipid raft caveolae-dependent endocytosis and macropinocytosis to internalize into host cells. For instance, several positive-sense ssRNA viruses such as severe acute respiratory syndrome virus (Inoue et al., 2007), hepatitis C, dengue and semliki forest virus use clathrin-mediated endocytosis (Marsh and Helenius, 2006; Meertens et al., 2006; Mercer et al., 2010) as a route of entry. In clathrin-mediated endocytosis, the cargo is trafficked through the cell via the coated vesicle which is surrounded by the polymerized clathrin in a basket-like structure. The formation of the endocytic clathrin-coated vesicles occurs through the interaction of clathrin, heterotetrameric adaptor protein-2 (AP-2), and several...
accessory proteins such as Epsin, Eps15, AP180/CALM and dynamin. Clathrin, in which the light chain and heavy chain form a unique structure called the clathrin triskelion, as well as AP-2 are recruited by epsin to the plasma membrane in response to receptor-mediated internalization signals. Epsin then mediates the assembly of a clathrin cage, which results in membrane curvature induced by the coated-pit formation. Once assembled, the clathrin-coated pits are pinched off from the plasma membrane by dynamin to form clathrin-coated vesicles which are then trafficked to endosomes (Doherty and McMahon, 2009; Mousavi et al., 2004). Whether YHV utilizes this pathway as a route of entry into shrimp cells remained to be investigated. Therefore, one purpose of this study was to characterize the YHV entry pathway by using several trafficking inhibitors. *P. monodon* clathrin heavy chain (*PmCHC*), a major protein component in the clathrin-dependent pathway was cloned and characterized. The suppression effect of *PmCHC* in YHV-challenged shrimp also was investigated.

2. Materials and methods

2.1. *P. monodon* (black tiger shrimp) culture

Juvenile shrimp were obtained from Manoach's farm in Nakhon Pathom province, Thailand. Shrimp were sampled to determine that they were free from YHV and white spot syndrome virus (WSSV) infection using Diagnosis Strip Test YHV + WSSV (Pacific Biotech Co., Ltd., Thailand). In addition, shrimp were acclimatized for at least 5 days before use in experiments. They were maintained in large containers with oxygenated sea water at 5 ppt salinity before the experiment and fed with commercial feed every day. Half of the water was changed every 2 days.

2.2. Yellow head virus preparation

Virus stock was prepared from hemolymph of YHV infected moribund shrimp. The moribund shrimp showed signs of YHV infection for example, a yellowish cephalothorax. To confirm that the hemolymph was collected from the moribund shrimp infected with YHV not other viruses, total RNA was extracted and reverse transcription-PCR analysis was performed to detect the helicase gene of YHV and other viruses such as VP28 gene of WSSV. To prepare the viral stock, hemolymph was collected with AC-1 solution (27 mM Sodium citrate, 34.33 mM NaCl, 104.5 mM Glucose, 198.17 mM EDTA, pH 7.0), ratio 1:1, and the virus was obtained by ultracentrifugation (100,000 × g) for 1 h. Viral pellet was dissolved in 150 mM NaCl and stored at −80 °C until use. The virus titer that causes 100% mortality within 3 days 0.25 mM g−1 shrimp (9 shrimp per group). The inhibitors were subjected to restriction enzyme digestion. The purified fragment of the sense-loop was cloned into the linearized fragment of pGEM-T easy vector (Promega). The recombinant plasmid containing *PmCHC* was sequenced by using *PmCHC* cDNA and were designed to amplify PmCHC from hemocytes of *P. monodon* and were designed based on the nucleotide sequences obtained from *Marsupenaeus japonicus* clathrin heavy chain. The cDNA of *PmCHC* coding sequences was amplified by using Taq DNA polymerase (New England Biolabs). The PCR was performed by hot-start at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 68 °C for 6 min; followed by 68 °C for 7 min. The cDNA was cloned into pGEM-T easy vector (Promega). The recombinant plasmid containing *PmCHC* was sequenced using T7, T3 primers, cdfullCHC-F and cdfullCHC-R (Table 1), were used to amplify PmCHC from hemocytes of *P. monodon* and were designed based on the nucleotide sequences obtained from *Marsupenaeus japonicus* clathrin heavy chain. The cDNA of *PmCHC* coding sequences was amplified by using Taq DNA polymerase (New England Biolabs). The PCR was performed by hot-start at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 68 °C for 6 min; followed by 68 °C for 7 min. The cDNA was cloned into pGEM-T easy vector (Promega). The recombinant plasmid containing *PmCHC* was sequenced using T7, SP6, walkCHC-F1, walkCHC-F2, walkCHC-R1 as primers (Table 1) by First Base Co., Ltd. (Malaysia).

2.3. Screening of YHV entry pathways by using drug inhibitors

The entry pathways of YHV were screened by using drug inhibitors. Various inhibitors of clathrin-dependent endocytosis, macropinocytosis and caveolar endocytosis such as chlorpromazine (CPZ), amiloride and various inhibitors of clathrin-dependent endocytosis, macropinocytosis were performed by VectorNTI program (Invitrogen). Phylogenetic analysis was performed with the neighbor-joining method (Dereeper et al., 2008, 2010).

2.4. Construction of the recombinant plasmid expressing dsRNA-PmCHC

Recombinant plasmid containing stem-loop of dsRNA was constructed in pGEM-3Zf+ (Promega) and pET-17b (Novagen) vectors. Sense-loop region of the dsRNA was amplified from the first-strand cDNA by specific primers, sCHC-F1 and lpCHC-R1 (Table 1). The anti-sense region was amplified by asCHC-F2 and asCHC-R2 (Table 1). Both PCR fragments were gel-purified and subjected to restriction enzyme digestion. The purified fragment of the sense-loop was cloned into the linearized fragment of pGEM-3Zf+ (digested by Xhol and KpnI). Then, this recombinant plasmid containing the sense-loop region of *PmCHC*...
was digested by KpnI and EcoRI and ligated with KpnI and EcoRI digested antisense fragment. The recombinant plasmid containing sense-loop-antisense fragments of PmCHC (pGEM-3Zf-+PmCHC) was obtained. Then, the sense-loop-antisense fragment of PmCHC was subcloned into pET17b vector at XbaI and EcoRI sites to construct recombinant plasmid pET17b-PmCHC which was used for dsRNA production by in vivo bacterial expression.

2.7. Production of dsRNA by in vivo bacterial expression

Recombinant plasmid pET17b-PmCHC was transformed into a RNase III mutant HT115 Escherichia coli strain. The strain is modified to express T7 RNA polymerase from an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter. Therefore, dsRNAs can be produced in the HT115 bacterial host after induction with IPTG. Double-stranded RNA was extracted and purified as previously described (Ongvarrasopone et al., 2007; Posiri et al., 2013). The quality of dsRNA was characterized by ribonuclease digestion assay using RNase A and RNase III. dsRNA concentration was estimated by agarose gel electrophoresis by comparing to the intensity of 100 bp DNA marker.

2.8. Suppression of PmCHC by dsRNA-PmCHC

The knockdown effect of dsRNA-PmCHC was tested by injection of dsRNA into hemolymph. Shrimp were injected with 2.5 μg g⁻¹ shrimp of dsRNA-PmCHC or an unrelated gene of dsRNA-GFP dissolved in 150 mM NaCl. Injection of 150 mM NaCl was used as control. After 24 h post dsRNA injection, gills of individual shrimp were collected to extract total RNA. Suppression effect of dsRNA was analyzed by reverse-transcription PCR (RT-PCR) to determine PmCHC mRNA level. To study the knockdown effect of PmCHC in YHV-challenged shrimp, shrimp were injected with dsRNA-PmCHC for 24 h before YHV challenge. Forty-eight hours later, gills of individual shrimp were collected to extract total RNA to detect YHV (helicase gene) and PmCHC mRNA expression levels. PmActin mRNA was used as an internal control.

2.9. Shrimp mortality assay

The mortality of shrimp injected with dsRNA followed by YHV challenge were observed every 6 h. Shrimp were about 1 g with 15 shrimp per group. Three independent experiments were performed. Shrimp were injected with 150 mM NaCl, 2.5 μg g⁻¹ shrimp of dsRNA-PmCHC or dsRNA-GFP. After 24 h post injection, shrimp were challenged with YHV. Mortality was plotted every 12 h.

2.10. RNA isolation and RT-PCR analysis

Total RNA from gill tissues was isolated by Trizol® reagent (Molecular Research Center) following the manufacturer’s procedure. The RNA concentration was measured by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Total RNA (2 μg) was used to generate first-strand cDNA by Improm-II™ reverse transcriptase (Promega) and PRT primer (Table 1). PmCHC mRNA level was amplified by primers; PmCHC-F1 and PmCHC-R1 (Table 1). PmActin mRNA expression, used as internal control, was amplified by specific primers, PmActin-F and PmActin-R1 (Table 1). Multiplex PCR for PmCHC and PmActin was performed according to this condition: 95 °C for 5 min; 25 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s; followed by 72 °C for 7 min. YHV mRNA level was amplified using primers, YHV[hel]-F and YHV[hel]-R (Table 1). The multiplex PCR condition for YHV and PmActin was as above except that the annealing temperature was changed to 55 °C for 30 s. The PCR products were analyzed on 1.5% agarose gel. The intensity of each band after subtracting the background was quantified by using ImageJ analysis program (version 1.46r). The relative expression level of PmCHC and YHV was normalized with PmActin levels and expressed as an arbitrary unit.

2.11. Quantitative real time PCR (qPCR)

Total RNA 2 μg was used for the first-strand cDNA synthesis by Improm-II™ reverse transcriptase (Promega) using PRT primer. Dilution of cDNA at 1:32 was mixed with qPCR reaction using KAPA™ SYBR® Fast master mix (2×) ABI Prism™ (KAPA Biosystems) following the manufacturer’s protocol. qYHV-F and qYHV-R specific primers (Table 1) were used to amplify YHV mRNA; EF1-α (EF1α-F and EF1α-R) is used as internal control: 95 °C for 3 min; 40 cycles of 95 °C for 5 s, 60 °C for 30 s. The qPCR was analyzed in an ABI 7500 real-time detection system (Applied Biosystems). The cycle threshold (Ct) value of YHV and EF1-α was compared and calculated by 2⁻ΔΔCt method (Livak and Schmittgen, 2001).

2.12. Statistical analysis

The relative mRNA levels of PmCHC or YHV normalized with PmActin and fold change in PmCHC expression were presented as mean ± SEM. Cumulative percent mortality was plotted as mean ± SEM. In addition, significant differences of each experimental group were tested by using analysis of variance (ANOVA). A probability (P) value of 0.05 was used to define significant difference.

3. Results

3.1. Analysis of YHV entry pathway

To identify the pathway for YHV internalization into shrimp cell, shrimp of about 2 g were pretreated with inhibitors of clathrin-mediated endocytosis (chlorpromazine, CPZ), macropinocytosis (amiloride) or the caveolar dependent pathway (methyl-β-cyclodextrin, MβCD), and this was followed by YHV challenge. All shrimp receiving inhibitors survived throughout the experiment. Quantitative RT-PCR of the shrimp pretreated with chlorpromazine showed a significant reduction of YHV mRNA levels, approximately 77%, when compared to the PBS-YHV injected group, whereas shrimp pretreated with amiloride and MβCD demonstrated no significant difference to the YHV control group (Fig. 1).

3.2. Cloning of PmCHC coding region and sequence analysis

The sequence of M. japonicus clathrin heavy chain was used to design specific-primers to amplify the full-length coding region of P. monodon.
clathrin heavy chain from hemocytes. The open reading frame of PmCHC is 5055 bp, encoding a protein of 1684 amino acids, with an estimated molecular weight of 192.5 kDa and a pI of 5.53. The nucleotide sequence is deposited in the GenBank database under the accession number KJ700941. The protein contains several conserved domains: a seven CHC repeat homology, one CHC H-linker and one CHC linker. One clathrin propeller repeat is observed in plants including Zea mays, Theobroma cacao, P. monodon, Aedes aegypti, Drosophila melanogaster, Homo sapiens and Danio rerio (Table 2). Phylogenetic tree analysis clustered organisms in three groups which are plants, invertebrates and vertebrates. PmCHC was clustered into an invertebrate group (Fig. 3).

### Table 2

| Organisms                      | Accession number |
|-------------------------------|------------------|
| Acronyrmex echinuator         | EGI06013         |
| Aedes aegypti                 | XP_001656878     |
| Bombyx mori                   | NP_001136443     |
| Bos taurus                    | AAC45824         |
| Ciona intestinalis            | XP_002130279     |
| Culex quinquefasciatus        | ED540945         |
| Dananus plexippus             | EHJ79063         |
| Danio rerio                   | NP_001005391     |
| Drosophila melanogaster       | NP_001096993     |
| Gallus gallus                 | NP_001073586     |
| Glycine max                   | AAC90294         |
| Homo sapiens                  | NP_004850        |
| Medicago truncatula           | AE571175         |
| Paeus monodon                 | KJ700941         |
| Populus trichocarpa           | EFO2372          |
| Rattus norvegicus             | AAA40874         |
| Riportus pedestris            | BAN20627         |
| Sus scrofa                    | NP_001139599     |
| Theobroma cacao               | EYO34523         |
| Tribolium castaneum           | XP_967829        |
| Zea mays                      | AGC62051         |

#### 3.3. Production of dsRNA-PmCHC and its suppression effect

Recombinant plasmid pET17b containing a stem loop of PmCHC was constructed and transformed into HT115 E. coli strain to be used as template for PmCHC dsRNA production (dsRNA-PmCHC) by in vivo bacterial expression. Then, hairpin dsRNA-PmCHC was extracted using an ethanol method (Posiri et al., 2013). The quality of dsRNA-PmCHC was characterized by ribonuclease III (RNase III) and by ribonuclease A (RNase A) digestion assay. dsRNA-GFP was also produced for nonspecific dsRNA injection. The hairpin dsRNA-PmCHC and dsRNA-GFP could be cleaved by RNase III but not by RNase A, suggesting that good quality dsRNA were obtained (Fig. 4A). Injection of dsRNA-PmCHC resulted in a significant reduction of PmCHC mRNA levels, approximately 90% when compared to the NaCl injected group (P < 0.01; n = 5). In contrast, no significant difference in PmCHC expression levels was observed between dsRNA-GFP- and NaCl-injected groups (Fig. 4B and 4C).

#### 3.4. Suppression effect of PmCHC in YHV-challenged shrimp inhibited YHV mRNA levels

To determine whether PmCHC is required for YHV internalization into shrimp cells, the PmCHC-knockdown shrimp were challenged with YHV. After 4 h, gills were collected for total RNA extraction and RT-PCR analysis to determine mRNA expression of YHV and PmCHC. The results demonstrated the suppression effect of PmCHC, by dsRNA-PmCHC, and resulted in almost complete inhibition of YHV mRNA expression. However, high levels of YHV mRNA were still observed in both NaCl-injected and dsRNA-GFP injected groups (Fig. 5A and B).

#### 3.5. Suppression of PmCHC in YHV-challenged shrimp delayed mortality

Shrimp receiving dsRNA-PmCHC, followed by YHV challenge, reached 100% cumulative mortality at 120 h post YHV injection (hpi) whereas NaCl- and dsRNA-GFP-injected groups gave 100% mortality at 108 hpi. Furthermore, shrimp injected with dsRNA-PmCHC followed by YHV challenge showed significant reduction in cumulative percent mortality at 84–108 hpi when compared with the NaCl injection group (Fig. 6A). This result suggested that clathrin heavy chain is required for YHV infection. However, shrimp injected with dsRNA-PmCHC at 2.5 μg g⁻¹ shrimp without YHV challenge reached 100% mortality at 156 h post dsRNA injection whereas shrimp injected with NaCl or dsRNA-GFP showed no mortality (Fig. 6B). In addition, shrimp injected with dsRNA-PmCHC at lower dosage, 1.25 μg g⁻¹ shrimp, 

![Fig. 2](https://example.com/image2.png)

Comparison of the clathrin heavy chain protein domains among several organisms. The protein domains comprising of clathrin propeller repeat (⩾), clathrin heavy chain linker (●), clathrin-H-link (†), clathrin heavy chain repeat homology (◇). Aedes aegypti, Drosophila melanogaster, Homo sapiens, Danio rerio, Zea mays and Theobroma cacao were compared to Penaeus monodon.
without YHV challenge reached 100% mortality at a later time, 180 h post dsRNA injection. A 75% mortality was observed in shrimp injected with dsRNA-PmCHC at 0.63 μg g⁻¹ without YHV challenge during 144–240 h post dsRNA injection (Supplementary Fig. 2). Dead shrimp were sampled for viral load detection. The results showed that dsRNA-PmCHC → YHV group (Fig. 6C (b)) demonstrated lower YHV mRNA levels when compared to NaCl → YHV group (Fig. 6C (a)) and dsRNA-GFP → YHV group (Fig. 6C (c)). All dead shrimp except samples from lanes 2, 3, 8, 11, and 12 in the dsRNA-PmCHC → YHV group showed the presence of YHV. This suggested that the shrimps in lanes 2, 3, 8,
11, and 12 were protected from YHV infection but were dead from the PmCHC knockdown. Taken together, the results strongly suggested that clathrin heavy chain is essential for YHV infection.

4. Discussion

Clathrin heavy chain (CHC) is a major protein in clathrin-coated pit and clathrin-coated vesicle. Clathrin heavy and light chains are assembled to form a three-legged structure, called a triskelion. Clathrin heavy chain together with adaptor and accessory proteins, including AP2, EPS15, AP-180, epsin or dynamin (McMahon and Boucrot, 2011; Mousavi et al., 2004; Young, 2007) are involved in endocytosis. The functions of clathrin heavy chain are believed to be involved in sorting cargo protein in the membrane and membrane curvature.

In black tiger shrimp, the role of clathrin heavy chain in virus infection has not been previously investigated. In this study, we have cloned \( P. \) monodon clathrin heavy chain (\( \text{PmCHC} \)). The function of PmCHC especially for yellow head virus (YHV) infection was tested to study the major route for YHV internalization. RNA interference (RNAi) was used as a tool to study the function of PmCHC. Shrimp injected with dsRNA-PmCHC showed reduction of PmCHC mRNA levels up to 90% when compared to the NaCl-injected control group. In addition, knockdown of PmCHC mRNA in YHV-challenged shrimp exhibited low levels of YHV expression (Figs. 5 and 6C) and a delay in shrimp mortality up to 24 h post YHV injection (84–108 hpi) (Fig. 6A). These effects may be due to suppression of PmCHC gene (Fig. 6B and Supplementary Fig. 2). After knocking down of PmCHC mRNA, low levels of PmCHC present may slow down the entry of YHV into the cell, thus resulting in a delay of shrimp mortality. Moreover, the shrimp death may also be due to the depletion of clathrin heavy chain (Fig. 6B and Supplementary Fig. 2). This is because clathrin heavy chain has diverse cellular functions such as transportation of cargo inside the cell, cytokinesis and glucose metabolism (Brodsky, 2012). Depletion of PmCHC may inhibit some cellular functions which are essential for cell survival. Several studies have shown that some viruses require clathrin heavy chain for infection (Bhattacharyya et al., 2010; Hongliang and Chengyu, 2009; Hussain et al., 2011). For instance, confocal microscopy demonstrated the colocalization between rhesus rhadinovirus and clathrin heavy chain (Zhang et al., 2010). Similar to this study, suppression of the clathrin heavy chain by small interfering RNA exhibited a reduction of viral load in the host cells, including \( \text{fl} \)uenza A virus, severe acute respiratory syndrome coronavirus or human enterovirus 71 (Hongliang and Chengyu, 2009; Hussain et al., 2011; Inoue et al., 2007). It has been shown that the cargo molecules that can be transported via clathrin-mediated endocytosis are size dependent, approximately 200 nm (Rejman et al., 2004) which nicely fit with the YHV virion, whose size is \( \sim 50 \times 60 \times 190\)–200 nm (Nadala et al., 1997). In addition, a recent report showed the reduction of YHV level in clathrin coat AP17 knockdown shrimp (Jatuyosporn et al., 2014). Moreover, by using various inhibitors (CPZ, M\( \beta \)CD and amiloride), only the YHV-challenged shrimp pretreated with CPZ showed a significant reduction of YHV levels. These results suggested that the major route of YHV into the shrimp cells was via clathrin-mediated endocytosis pathway.

In shrimp, a previous study has identified a protein in the endocytosis pathway, \( P. \) monodon Rab7 (PmRab7) (Sritunyauleksana et al, 2006). Rab7 is a small GTPase protein and plays a crucial role to regulate the
transportation from late endosome to lysosome during endosome maturation (Huotari and Helenius, 2011). In addition, PmRab7 is required for several shrimp viruses such as white spot syndrome virus (WSSV), taura syndrome virus (TSV) Laem-Singh virus (LSNV) or YHV for intracellular trafficking inside the cell (Ongvarrasopone et al., 2008, 2010, 2011). Therefore based on the previous evidence and this study, the

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**Fig. 6.** The cumulative percent mortality of shrimp injected with NaCl, dsRNA-PmCHC, or dsRNA-GFP followed by YHV challenge [A] or without YHV challenge [B]. (*) represents significant difference between dsRNA-PmCHC → YHV and NaCl → YHV group. (C) A representative gel of RT-PCR products of YHV and PmCHC mRNA levels of dead shrimp. a) NaCl → YHV group, b) dsRNA-PmCHC → YHV group and c) dsRNA-GFP → YHV group. PmActin was used as internal control. The number on the bottom of the each lane represents the time (hours of post YHV challenge, hpi) that the shrimp die. The expression of PmActin in dead shrimp samples that showed high expression levels of YHV were very faint. This result was also demonstrated in a previous study (Posiri et al., 2011).
mechanism of YHV entry and intracellular trafficking can be proposed. YHV enters the shrimp cells by using the envelop protein gp116 to bind to YHV binding protein, PmYRP65 (Assavalapsakul et al., 2006). Then, many adaptor proteins which are involved in clathrin-coated pit formation including AP2, AP17 and PmCHC are recruited to induce membrane invagination to form clathrin-mediated vesicles which then transport YHV to the early endosome which also may require Rab5 protein (Hutagalang and Novick, 2011; Steenmark, 2009). After that, YHV may be transported toward the late endosome and lysosome via the regulation by PmRab7 for viral uncoating (Hutagalang and Novick, 2011; Steenmark, 2009). Then, many adaptor proteins which are involved in clathrin-coated pit formation including AP2, AP17 and PmCHC are recruited to induce membrane invagination to form clathrin-mediated vesicles which then transport YHV to the early endosome which also may require Rab5 protein (Hutagalang and Novick, 2011; Steenmark, 2009). After that, YHV may be transported toward the late endosome and lysosome via the regulation by PmRab7 for viral uncoating (Hutagalang and Novick, 2011; Steenmark, 2009).

Taken together, this study demonstrated that PmCHC is an essential protein required for YHV internalization into the shrimp cell. Knockdown of PmCHC by dsRNA-PmCHC resulted in inhibition of YHV levels suggesting that clathrin-mediated endocytosis is a major route for YHV infection.

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