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# The *Penaeus stylirostris* densovirus capsid interacts with *Litopenaeus vannamei* troponin I

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ABSTRACT

The *Penaeus stylirostris* densovirus (PstDNV) (also known as infectious hypodermal and hematopoietic necrosis virus, IHHNV), a very small DNA virus, is a major shrimp pathogen. The PstDNV genome encodes only two nonstructural proteins and one capsid protein. This virus is thus an ideal, simple model for the investigation of virus-host interactions. To explore the role of the PstDNV capsid in viral infections, a yeast two-hybrid (Y2H) cDNA library was constructed based on Pacific white shrimp, *Litopenaeus vannamei* mRNA. The Y2H library was then screened, using the PstDNV capsid protein as bait. We identified a host protein that interacted strongly with the PstDNV capsid as *L. vannamei* troponin I (LvTnI). An *in vitro* co-immunoprecipitation experiment further supported this interaction. In addition, an *in vivo* neutralization experiment showed that the vaccination with anti-LvTnI significantly reduced PstDNV copies in PstDNV-challenged shrimp, indicating that the interaction between the PstDNV capsid and cellular LvTnI is essential for PstDNV infection. This result has important implications for our understanding of the mechanisms by which PstDNV infects shrimp.

## 1. Introduction

Viral capsids play various essential roles in viral infections [1]. In nonenveloped viruses, the capsid encases and protects the viral nucleic acid and provides the initial virus-host interaction [2]. After receptor engagement, viruses are internalized and the viral coat is disassembled to deliver the viral genome and accessory proteins into host cells [1]. Hence, to understand the mechanisms of viral infection, it is necessary to investigate the interaction between viral capsids and cellular proteins.

In recent years, viral diseases have seriously threatened the shrimp aquaculture industry [3,4]: epidemics of white spot syndrome virus (WSSV), taura syndrome virus (TSV), and *Penaeus stylirostris* densovirus (PstDNV) (also known as infectious hypodermal and hematopoietic necrosis virus, IHHNV) have resulted in huge economic losses for the global shrimp aquaculture industry [5–7]. Of these, PstDNV is one of the most important, commonly infecting both farmed and wild shrimp populations [8]. Although PstDNV causes mass shrimp mortalities, it more commonly causes a severe disease called 'runt deformity syndrome' (RDS) [9]. RDS is characterized by reduced irregular growth, as well as deformities of the cuticle and rostrum [8].

PstDNV is a very small, simple single-stranded DNA parvovirius, with nonenveloped, icosahedral virions averaging 22–23 nm in diameter [10,11]. The PstDNV genome includes only three open reading frames (ORFs), which encode two nonstructural proteins (NS1, NS2) and one capsid protein (CP) [12,13]. Thus, PstDNV is a simple model within which to investigate virus-host interactions. Recently, it was reported that the one of the PstDNV protein (NS2, encoded by ORF2) interacts with shrimp actin, facilitating viral infection of the host [14]. However, to our knowledge, no study has yet investigated the interaction between the PstDNV CP and the host cell proteins.

The Pacific white shrimp (*Litopenaeus vannamei*), also known as whiteleg shrimp or white shrimp, is one of the three most commonly cultivated shrimp species worldwide, and is the most commonly cultivated marine organism in China [15]. In L. *vannamei,* PstDNV infections may lead to RDS [16], resulting in severe economic losses [17,18]. After the virion invades the host, PstDNV interacts with the host protein to block normal physiological activities, thereby causing a series of disorders relating to gene and protein regulation [19]. Therefore, an understanding of the interaction between PstDNV-encoded proteins and

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shrimp host-encoded proteins is critical to an understanding of PstDNV pathogenesis in shrimp.

To further this understanding, we first aimed to identify the cellular protein that interacts with the PstDNV capsid by screening the *L. vannamei* cDNA library for previously identified *L. vannamei* proteins. We then aimed to confirm the interaction between the screened protein and the viral capsid via yeast two-hybrid (Y2H) and *in vitro* co-immunoprecipitation assays. Finally, we aimed to determine whether such interactions were essential for PstDNV infection using an *in vivo* neutralization experiment.

## 2. Materials and methods

### 2.1. Construction of the Y2H library

Total RNA was extracted from the muscles of specific pathogen-free (SPF) *L. vannamei* (obtained from the National and Guangxi Shrimp Genetic Breeding Center, Guangxi Province, China) using an RNAiso Plus kit (Takara, Japan). mRNA was isolated from total RNA using a PolyATtract mRNA isolation Kit (Promega, USA), and cDNA was synthesized using the SMARTer cDNA Synthesis Kit (Takara). The synthesized cDNA was used to construct a cDNA library using the cDNA Library Construction Kit (Takara). To create a Y2H library, the cDNA library plasmid was recombined with the yeast expression vector pGADT7 (Clontech, USA). The recombined pGADT7 plasmid was then transformed into yeast strain Y187 (Clontech) using the Yeastmaker Yeast Transformation System (Clontech).

### 2.2. Construction of the bait strain

The PstDNV strain was isolated from the *L. vannamei* shrimp collected in China, and was preserved in our laboratory. The partial PstDNV CP gene sequence (excluding transmembrane structure sequence) was amplified with polymerase chain reactions (PCRs) using the novel gene-specific primers Capsid-F and Capsid-R (Table 1), which were designed with Primer Premier (V 6.2) (http://www.premierbiosoft.com/) based on the genomic DNA sequence of PstDNV (GenBank accession EF633688.1) [20].

The capsid-F primer carried an EcoR-I site (GAATTC), and the capsid-R primer carried a BamH-I site (GGATCC). The amplicons were analyzed using agarose gel electrophoresis. Next, amplicons were digested with EcoR-I and BamH-I, and ligated into the EcoR-I and BamH-I sites of the pGBT9 plasmid (Clontech) to create a pGBT9-capsid (the bait plasmid). To create the bait strain, the pGBT9-capsid was then transformed into yeast strain Y2Hgold (Clontech) using the Yeastmaker Yeast Transformation System (Clontech). The toxicity and auto-activation of bait protein were measured with the Matchmaker Gold Yeast Two-Hybrid System (Clontech). In brief, Y2HGold yeasts that had been transformed with bait vectors were diluted at a 1/10 and 1/100 ratio and 100 μL was plated on SD/-Trp plates and cultured at 30 °C for 3–5 days until a single colony has grown. Then yeast colonies with a diameter > 2 mm and are growing well were inoculated into 3 mL SD/-Trp/Kan (20 μg/mL) liquid culture. After culturing at 200 r/min and 30 °C for 24 h, the OD600 was measured. Comparison of growth differences was used to determine whether the bait vector is toxic.

### 2.3. Y2H library screening

The Y2H library was screened using the Matchmaker Two-Hybrid System (Clontech), following the manufacturer's instructions. In brief, the capsid bait strain was cultured in 50 mL SD/-Trp liquid medium (Clontech) for 24 h in a shaker at 30 °C and 250 rpm. The library strain was cultured in 50 mL SD/-Leu liquid medium for 24 h in a shaker at 30 °C and 250 rpm. We then mixed 0.1 mL of the capsid bait strain with 1 mL of the library strain, and cultured this mixture in 100 mL of 2x YPDA liquid medium supplemented with 100 μg/mL kanamycin in a shaker for 24 h at 30 °C and 250 rpm. The mated culture was then spread on plates containing SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar. After 2–4 days, we sequenced the DNA of all blue clones growing on the SD/-Leu-Trp-His-Ade/X-α-Gal/Aba plates. The obtained sequences were searched against the NCBI GenBank database [21] using BlastX [22] to identify the positive clones.

### 2.4. Verification of the positive clones with Y2H

To verify the screening results, the prey plasmids (pGADT7-LvTnI) were co-transformed with the bait plasmids (pGBKT7-CP) into the yeast strain Y2HGold (Clontech). We considered blue colonies growing on SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar to indicate positive clones.

### 2.5. Verification of the positive clones by co-immunoprecipitation

The binding of the PstDNV capsid and the LvTnI protein was confirmed with a Pierce Co-immunoprecipitation kit (Thermo-Fisher Scientific, USA), following the manufacturer's instructions. In brief, the PstDNV CP gene sequence was amplified with PCR using the primers Capsid-V5-F and Capsid-V5-R (Table 1), which were designed with Primer Premier (V 6.2) based on the genomic DNA sequence of PstDNV (GenBank accession EF633688.1) [20]. The PCR product was verified using gel electrophoresis. The PCR amplicon was inserted in the pIZ/V5-His vector, using the appropriate restriction enzyme sites (*Bam*HI and *Xho*I), to generate plasmid Capsid-V5-His. The LvTnI gene was PCR amplified using the primers LvTnI-Flag-F and LvTnI-Flag-R (Table 1), which were designed with Primer Premier (V 6.2) based on the genomic DNA sequence of PstDNV (GenBank accession number JX683728.1) [23]. The PCR product was verified using gel electrophoresis. The PCR amplicon was inserted in the pIZ/Flag-His vector, using the appropriate restriction enzyme sites (*Kpn*I and *Eco*RI), to generate plasmid LvTnI-Flag-His. The plasmids were sequenced and the obtained sequences were searched against the NCBI GenBank database [21] using BlastX [22] to confirm the PCR product.

Using a Cellfectin II Reagent Kit (Invitrogen, USA), the plasmids of Capsid-V5-His and LvTnI-flag-His were co-transfected into High-Five cells. At 48 h post-transfection, cells were lysed in 0.1 M phosphate-buffered saline lysis buffer with 1% NP-40 (pH 6.0) at 4 °C for 15 min with occasional shaking. Cell lysates were centrifuged at 14,000 rpm for

**Table 1**
Primers used in this study.

| Primers | Sequence (5′-3′) |
|---|---|
| Capsid-F | CATGGAGGCCGAATTCATGTTATATCTCTATGGTCTAAAGAGC |
| Capsid-R | GCAGGTCGACGGATCCTTATTTAAGTTCGTCTCCATTTGGTCC |
| Capsid-V5-F | CGGGATCCATGGACATGTGCGCCGATTCAACAAGAGCA |
| Capsid-V5-R | CCGCTCGAGCGGGTATGCATAATATAACA |
| LvTnI-Flag-F | GGGGTACCATGGATTACAAGGATGACGACGATAAGATGGCGGACGAGAAAGCTAAG |
| LvTnI-Flag-R | CCGGAATTCCATTACGCAGCGGCTTCCTCT |
| 18S-F | GCCTGAGAAACGGCTACCACATC |
| 18S-R | GTAGTAGCGACGGGCGGTGTGT |

3 min. Next, each supernatant was mixed with anti-V5 epitope tag antibody (rabbit polyclonal) (sigma, USA) and rocked at 4 °C overnight. Immunoprecipitated proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF (Polyvinylidene Difluoride) membranes. We then performed additional western blot assays using antibodies against the His tag: V5-tagged PstDNV capsid fusion proteins were detected with anti-V5-HRP monoclonal antibodies (Sigma) and goat anti-rabbit IgGHRP conjugates (Sigma), while Flag-tagged LvTnI proteins were detected with mouse anti-Flag monoclonal antibodies (Sigma) and goat anti-mouse IgG-HRP conjugates (Sigma).

### 2.6. PstDNV neutralization assay

Recombinant LvTnI proteins were purified as described above (section 2.4). Polyclonal antibodies were generated by injecting the purified proteins into a rabbit (performed by Wuhan GeneCreate Biological Engineering Co., Ltd., China). At 60 days post-vaccination, rabbit-anti-LvTnI was successfully obtained. Enzyme linked immunosorbent assay (ELISA) showed that the antibody titer was greater than 1:10000.

For the neutralization assay, 240 three-month-old SPF *L. vannamei* (mass: 14–15 g) were obtained from the National and Guangxi Shrimp Genetic Breeding Center, and acclimated in salt-water tanks at 25 ± 1 °C for 7 days. After seven days, shrimp were divided into three groups, each with three biological replicates (20 shrimp per replicate). One group of shrimp (controls) were injected with 100 μL of viral suspension (1000 PstDNV copies/μL); shrimp in the second group were co-injected with 100 μL of viral suspension (1000 PstDNV copies/μL) and 10 μg rabbit-anti-LvTnI; and shrimp in the third group were co-injected with 100 μL of virus suspension (1000 PstDNV copies/μL) and 30 μg rabbit-anti-LvTnI. The experimental doses of viral suspension and rabbit-anti-LvTnI followed those used previously in our pre-experiments. At 72 h post treatment, we measured the PstDNV copies in the muscle of each shrimp using Real-Time PCR, which was carried out according to the Real-Time PCR Kit RR820A (TaKaRa). The internal reference was 18s RNA of *L. vannamei*, and the primers were 18s-F and 18s-R [24] (Table 1). The primers for measuring PstDNV were Capsid-F and Capsid-R (Table 1). The PCR was carried out at 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 30 s for 40 cycles. We tested the significance of differences between the experimental groups and the control group using *t*-test in SPSS 19.0 [25]. We considered $P < 0.05$ significant.

## 3. Results

### 3.1. Identification of PstDNV capsid-binding proteins

We constructed a *L. vannamei* cDNA library and Y2H library, and amplified a cDNA fragment putatively corresponding to the gene encoding the PstDNV CP. The size of the amplified fragment was about 600 bp (Fig. 1). We sequenced the amplicon and BlastX against GenBank, the result showed that the amplicon was 99.99% homologous to the previously published PstDNV capsid sequence [20].

To construct the bait strain, we ligated the amplicon into the pGBT9 plasmid to create a pGBT9-capsid, which was then successfully transformed into yeast strain Y2Hgold. The toxicity of the bait protein was measured, and an OD600 greater than 0.8 means that the bait vector is not toxic and can be used for Y2H experiments.

The mating of the bait strain (capsid) and the prey strain (Y2H library) produced 2 blue (positive) clones when cultured on SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar plates (Fig. 2). The positive clones were sequenced and sequence alignment by Blastx showed that the 2 positive clones encoded the full-length cDNA of a previously characterized protein *L. vannamei* troponin I (LvTnI; GenBank: JX683728.1) [23].

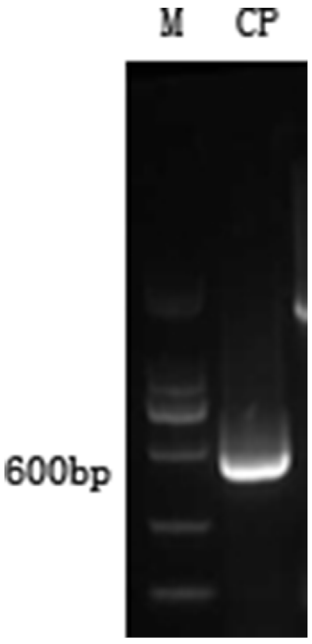


**Fig. 1. Agarose gel electrophoresis of the PstDNV capsid gene, as amplified by PCR.** Lane M: 1000-bp DNA ladder marker; lane CP: gene encoding the PstDNV capsid protein.

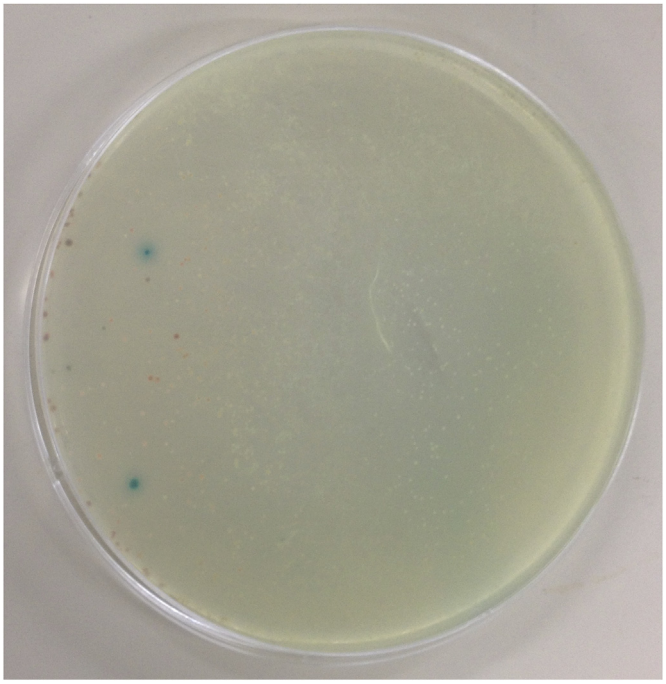

**Fig. 2. SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar plate, showing culture of yeast strain Y187, co-transformed with prey plasmids (carrying *Litopenaeus vannamei* cDNA library) and bait plasmids (carrying the PstDNV capsid protein).** Blue colonies are positive clones and colorless colonies are negative clones. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 3.2. Verification of the positive clones with Y2H

Our Y2H verification indicated that the yeasts carrying the pGBKT7-CP/pGADT7-LvTnI plasmid grew and presented as blue on SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar; representing a interaction between the CP and LvTnI. Although yeast carrying the pGBKT7-CP/pGADT7-LvTnI plasmid grew on SD/-Leu-Trp agar, they were colorless (Fig. 3).

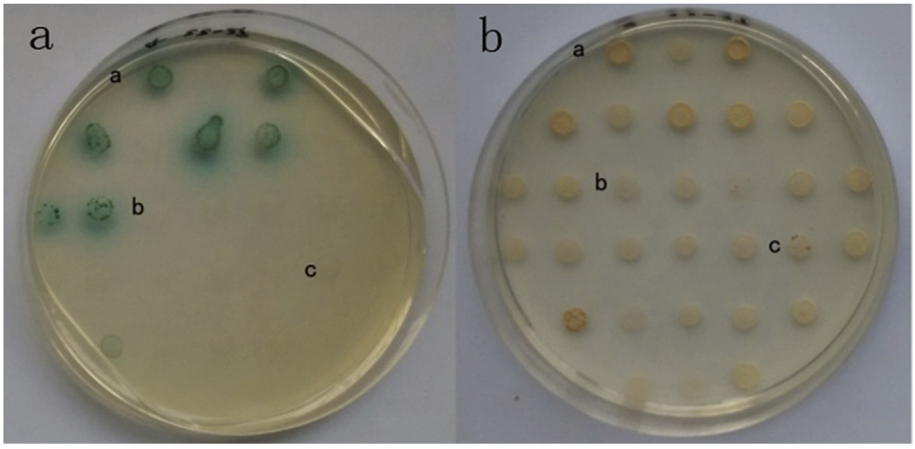


**Fig. 3. Culture of yeast strain Y2HGold co-transformed with prey plasmid pGADT7-LvTnI and bait plasmid pGBKT7-CP.** Yeast cells were spread on (a) a SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar plate, or (b) a SD/-Leu-Trp agar plate.

### 3.3. Verification of the positive clones by co-immunoprecipitation

The PCR products amplified by the Flag-F/Flag-R primer pair were about 990 bp and 650 bp, corresponding to Flag-CP and Flag-LvTnI, respectively (Fig. 4a). The PCR products amplified by the V5-F/V5-R primer pair were about 990 bp and 650 bp (Fig. 4b), corresponding to the recombinant vectors V5-CP and V5-LvTnI. In addition, our GenBank BlastX search indicated that Flag-CP and V5-CP were up to 99% homologous with previously published capsid protein sequences [20], and that Flag-LvTnI and V5-LvTnI were up to 99% homologous with previously published LvTnI sequences [23]. Therefore, we preliminarily assumed that these target genes had been correctly amplified. SDS-PAGE indicated that Flag-CP and V5-CP were about 37KD, while Flag-LvTnI and V5-LvTnI were about 25KD, consistent with sizes of the designed lengthes of target proteins. This indicated that the constructed recombinant vectors had been correctly expressed in the High Five cells.

After SDS-PAGE, we performed the CO-IP experiment. The plasmids of Capsid-V5-His and LvTnI-flag-His were co-transfected into High-Five cells. Then the cells were lysed and the lysate was mixed with anti-V5 antibody. Immunoprecipitated proteins were detected by western blots. The result (Fig. 5) showed that, when tested with anti-Flag antibody,

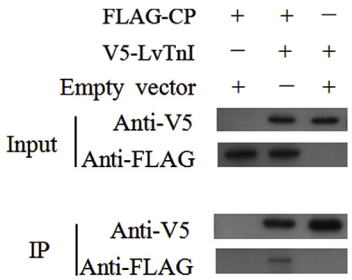


**Figure 5. Co-immunoprecipitation (CO-IP) of Flag-CP with V5-LvTnI.** High-Five cells were co-transfected with Flag-CP plasmids, V5-LvTnI plasmids, and empty plasmids (vector) as indicated. "Input" indicates the result of western blot analysis of cell lysates after 48 h of co-transfection, "IP″ indicates the result of western blot analysis of cell lysates that were mixed with anti-V5 antibodies. The immunoprecipitated complexes were analyzed by western blot with anti-FLAG antibody and anti-V5 as indicated. Black bands on the polyvinylidene difluoride membranes indicate western blot positive. "+" in the figure indicates that the vector was transfected, and "-" indicates that the vector was not transfected.

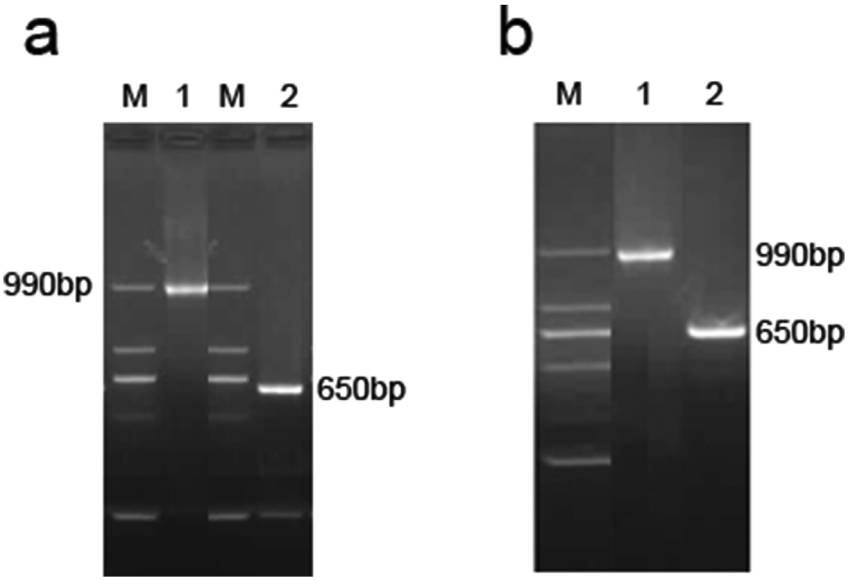


**Fig. 4. Agarose gel electrophoresis showing PCR amplicons.** (a) PCR amplicons corresponding to Flag-CP and Flag-LvTnI. Lane M: 1000-bp DNA ladder marker; lane 1: Flag-CP; lane 2: Flag-LvTnI; (b) PCR amplicons corresponding to V5-CP and V5-LvTnI. Lane M: 1000-bp DNA ladder marker; lane 1: V5-CP; lane 2: V5-LvTnI.

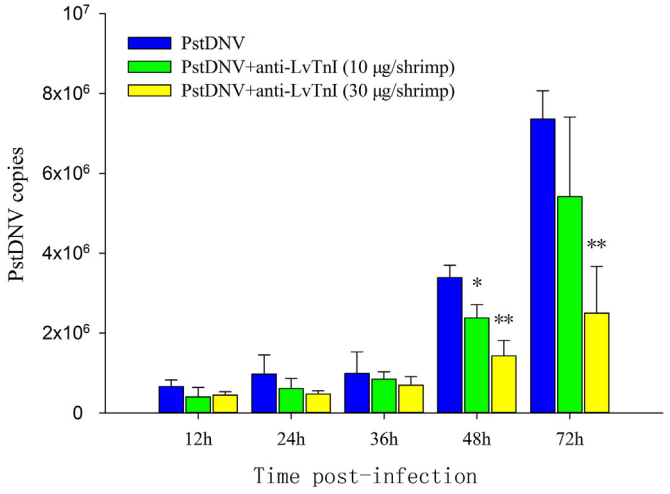


**Fig. 6. Viral titers in *Litopenaeus vannamei* after infection with 100 μL PstDNV suspension.** Blue bars: unvaccinated control; green bars: co-injection with 10 μg anti-LvTnI; yellow bars: co-injection with 30 μg anti-LvTnI. Bars represent means of 3 independent replicates; error bars represent standard deviations. Asterisks over the bars indicate significant differences, as compared to the unvaccinated control: *, $P < 0.05$; **, $P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

there is a specific band in the IP of the group Flag-CP + V5-LvTnI, which means that Flag-CP was immunoprecipitated with anti-V5 antibody via binding to V5-LvTnI and then was detected by anti-Flag antibody. The CO-IP analysis illustrated that Flag-CP could be immunoprecipitated with V5-LvTnI, confirming the interaction between the capsid protein and LvTnI.3.4 Neutralization assay *in vivo*.

At 24 h post infection with PstDNV, shrimp co-injected with 30 μg of the protein antibody had significantly lower viral titers than the unvaccinated control shrimp ($P < 0.05$; Fig. 6). At 48 h and 72 h post injection, the shrimp treated with either 10 μg or 30 μg of the protein antibody had significantly lower viral titers than the controls ($P < 0.05$). This indicated that LvTnI plays an essential role in PstDNV infection of shrimp.

## 4. Discussion

Viral capsid proteins play an important role in viral infection. It is critical to study the interactions between viral capsid proteins and host proteins in order to understand the mechanisms by which viruses invade the host. PstDNV is a good model for studying these interactions because it encodes only three proteins. However, little is known about the interaction between the PstDNV capsid and host proteins. In this study, we provide the first demonstration that the PstDNV capsid protein interacts with the shrimp protein LvTnI.

We successfully constructed a *L. vannamei* cDNA library and Y2H library, and amplified a cDNA fragment putatively corresponding to the gene encoding the PstDNV CP. The size of the amplified fragment was consistent with the designed product length. Our BlastX search found that the PCR product was 99.99% homologous to the previously published PstDNV capsid sequence [20], suggesting that the amplified sequence was correct. We then used the bait strain (capsid) to screen the Y2H library and found 2 blue (positive) clones grew on SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar plates. DNA sequencing and Blastx showed that the 2 positive clones encoded the full-length cDNA of a previously characterized protein *L. vannamei* troponin I [23]. Thus, we preliminarily identified a protein that interacts with CP.

The Y2H assay is a powerful technique that detects both transient and stable interactions between proteins [26]. This method has been used extensively for the high-throughput screening of protein-protein interactions, particularly between viral and host proteins [27]. However, the Y2H assay generates relatively large numbers of false positives [28]. Therefore, to confirm the interaction between the PstDNV capsid and LvTnI, we performed an additional prey/bait plasmid Y2H experiment. As expected, the clones of transformed yeast strain carrying the prey and bait plasmids appeared blue on SD/-Leu-Trp-His-Ade/X-α-Gal/Aba agar, representing a positive interaction between the PstDNV capsid protein and shrimp LvTnI. The yeasts also grew on SD/-Leu-Trp agar but they were colorless, which indicated that the pGBKT7-CP and pGADT7-LvTnI plasmid were successfully transformed into the yeasts.

Immunoprecipitation is an effective method that measures the physiological interaction between two proteins in intact eukaryotic cells [29], combining protein expression with the immune response. Immunoprecipitation represents an opportunity to analyze protein expression in a natural state, without the influence of human factors as in *in vitro* experiments [30]. Thus, to further test the interaction between the PstDNV CP and shrimp LvTnI, we performed an immunoprecipitation analysis. As no specific shrimp cell line is available, we used High-Five cells and the insect expression vector pIZ/V5-His for our co-immunoprecipitation experiment. Our western blots indicated that LvTnI and the PstDNV CP were correctly expressed in High Five cells. In addition, after the mixed recombinant vectors of LvTnI and the PstDNV CP were simultaneously transfected into High Five cells, the cell lysates were precipitated by the anti-Flag and anti-V5 antibodies, confirming the interaction between the PstDNV capsid protein and shrimp LvTnI.

Troponin is a complex of three regulatory proteins that is integral to muscle contraction in the cardiac and skeletal muscle, but not in the smooth muscle [31]. The troponin complex contains three a $Ca2^{+}$ binding subunit (troponin C, TnC), an inhibitory subunit (troponin I, TnI), and a tropomyosin binding subunit (troponin T, TnT) [32,33]. The binding of TnC and calcium ions produces a conformational change in TnI; TnT binds to tropomyosin and interlocks to form a troponin-tropomyosin complex; and TnI binds to actin to maintain the troponin-tropomyosin complex in place [34]. To date, shrimp TnI has been cloned only in the *L. vannamei* [23] and Chinese shrimp *Fenneropenaeus chinensis* [35], and it is unclear which cellular proteins interact with TnI.

To investigate whether LvTnI is involved in PstDNV infection, we performed an *in vivo* neutralization assay. We used neutralization methods rather than RNA interference because LvTnI is a structural protein instead of a secreted protein, and the neutralization method has previously been successfully applied to the interactions between viral and host proteins in shrimp [36–38]. Here, the anti-LvTnI antibody significantly inhibited PstDNV infection. Moreover, there were significant difference in PstDNV copies for different doses of anti-LvTnI. The PstDNV copies in the 30 μg anti-LvTnI treatment was significantly larger than those in the 10 μg anti-LvTnI treatment, showing that the anti-LvTnI antibody could effectively inhibit PstDNV infection. The result indicated that the interaction between the PstDNV capsid protein and host LvTnI is essential for PstDNV infection. However, the mechanism of this interaction during viral invasion remains unclear.

It is worth noting that the LvTnI/PstDNV interaction may have a physiological impact on the motor behavior of PstDNV-infected shrimp. That is, as troponin I plays a key role in muscle contraction [39,40], and as it has often been observed that PstDNV-infected shrimp swim slowly and irregularly [41], we hypothesize that the PstDNV capsid protein binds to host LvTnI, affecting shrimp motor function, but this possibility requires further investigation.

In conclusion, we demonstrated that the PstDNV capsid protein interacts with LvTnI, and this interaction is essential for PstDNV infection. The current study increases our understanding of the mechanisms by which PstDNV infects shrimp.

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