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PII: S1050-4648(19)30686-2

DOI: https://doi.org/10.1016/j.fsi.2019.06.039

Reference: YFSIM 6235

To appear in: *Fish and Shellfish Immunology*

Received Date: 22 February 2019

Revised Date: 12 June 2019

Accepted Date: 19 June 2019

Accepted refereed manuscript of: : Pang H, Wang G, Zhou S, Wang J, Zhao J, Hoare R, Monaghan SJ, Wang Z, Sun C (2019) Survival and immune response of white shrimp *Litopenaeus vannamei* following single and concurrent infections with WSSV and *Vibrio parahaemolyticus*, *Fish and Shellfish Immunology*, 92, pp. 712-718, doi: https://doi.org/10.1016/j.fsi.2019.06.039.

Survival and immune response of white shrimp *Litopenaeus vannamei* following single and concurrent infections with WSSV and *Vibrio parahaemolyticus*

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ABSTRACT

The survival and immune responses of *Litopenaeus vannamei* were evaluated during white spot syndrome virus (WSSV) or *Vibrio parahaemolyticus* single and concurrent infections. The mortality, WSSV load, activities of 4 immune enzymes: acid phosphatase (ACP), alkaline phosphatase (AKP), peroxidase (POD) and superoxide dismutase (SOD), and the transcription of Evolutionarily Conserved Signaling Intermediate in Toll pathways of *L.vannamei* (LvECSIT) were quantified at 0, 3, 6, 12, 24, 48, 72 and 96 h post-infection (pi). The results showed: (i) the cumulative mortality of the co-infection group (WSSV and *V. Parahaemolyticus* 83 %) was significantly lower than the WSSV infection group (97%) ($P < 0.05$) at 96 hpi; (ii) copies of WSSV in the co-infection group were significantly lower than that of the single infection group from 24 to 96 hpi ($P < 0.05$); (iii) ACP, AKP,POD and SOD activity in the gills of the co-infection group was higher than that of the WSSV group at12, 48 and 96 hpi ($P < 0.05$).The expression of LvECSIT mRNA in the co-infection group was significantly higher than in the WSSV infection group from 12 to72 hpi ($P < 0.05$).The results indicate that proliferation of WSSV is inhibited by *V.parahaemolyticus* infection. In addition, infection with WSSV alone causes a significant reduction in some immune responses of shrimp than co-infection with WSSV and *V.parahaemolyticus* occurs at 26 °C. Third, LvECSIT, an essential member of TLR signaling pathway might play a crucial role in shrimp defense against WSSV – *Vibrio* co- infection.

Keywords: *Litopenaeus vannamei*, Immune response, White spot syndrome virus (WSSV), *Vibrio parahaemolyticus*, Co-infection

1. Introduction

Shrimp aquaculture has developed very fast in China over the last two decades, but the production of shrimp has been seriously affected by white spot syndrome virus (WSSV) and *Vibrio* spp. [1]. WSSV - *Vibrio* co-infection is the normal manner of shrimp disease breakouts and shrimp infected with the virus are more susceptible to *Vibrio* spp.[2].It has been reported previously that *Vibrio alginolyticus* was isolated from shrimp during a breakout of white spot syndrome virus [3].Another study showed that during a WSSV and *Vibrio anguillarum* co-infection test in shrimp, WSSV increased more rapidly under co-infection conditions than in the single infection[4]. Similarly, the transcription of immune-related genes was suppressed in the co-infection groups, and the shrimp would suffer higher mortality in multiple infections [5]. Unlike the above observations, an outbreak of WSSV was postponed after co-infection with WSSV and *Vibrio harveyi* in *Penaeus vannamei* [6]. These studies about the WSSV - *Vibrio* co-infections in shrimp seem to be conflicting and the pathogenesis involved is unclear.

Although the defense mechanism of shrimp to WSSV - *Vibrio* co-infections remains unknown, it has been reported that bacterial infection could reduce the copies of virus in some arthropods [7-8].*Drosophila melanogaster* infected with *Wolbachia* appeared to inhibit the proliferation of Drosophila C virus[7].Furthermore, *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll/Toll-like receptor (TLR)-mediated signaling pathway to control dengue virus in the mosquito *Aedes aegypti*. Some Toll pathway-related genes (Spn27A, SPZ1, CECD, and DEFC)

were up-regulated in *Aedes aegypti* after co-infection with *Wolbachia* and dengue virus [8].Such virus suppression mechanisms may exist in shrimp, which warrants further exploration.

In shrimp, the innate immune system is the first line of defense against pathogenic infections [9]. When pathogens invade shrimp, they stimulate a series of immune responses including lymphatic hemocyte agglutination, melanisation, hemocyte phagocytosis, formation of cysts [10-12] and humoral immune factors (a variety of enzymes have been identified). It was reported that ACP, AKP, POD and SOD were susceptible to WSSV and *Vibrio* infections, and they could be used as indicators of immune response to these pathogens [13-15].

Under the stimulus of pathogens, various humoral and cellular immune responses of shrimp are activated through signaling pathways, among which Toll/Toll-like receptor (TLR)-mediated signaling pathway are the best known and can be activated by pathogenic related molecular patterns (PAMPs) [16]. After PAMP recognition, TLRs can either directly or indirectly trigger downstream signaling cascades, resulting in the regulation of cytokine gene expression [17].TRAF6 is an important downstream signal ligand of Toll-1 receptor protein and ECSIT is the first gene that has been approved to interact with TRAF 6 [18]. As an important adaptor protein of TLR, ECSIT have been demonstrated to be an immune-response gene since its transcript expression level is up-regulated after *Vibrio anguillarum* [19] or WSSV infection [20].

White spot syndrome virus (WSSV) is one of the most detrimental pathogens

affecting shrimp [21].It is a baculovirus with double stranded DNA [22], and the mortality rate of WSSV-infected shrimp can reach 100% in 7-10 days. Recently, researchers found another serious shrimp disease (acute hepatopancreatic necrosis disease AHPNS/early mortality syndrome EMS), which is characterised by empty stomach, severe atrophy of hepatopancreas and soft carapace. *Vibrio parahaemolyticus* is one of the causative agents of AHPNS / EMS, and it has caused big economic losses in the shrimp industry in China [23-25]. Nowadays, there is limited information available on molecular immune responses in shrimp under WSSV or *V. parahaemolyticus* single and concurrent infections.

In an attempt to provide a theoretical basis for the control of WSSV in *L. vannamei*, a number of parameters (mortality, WSSV load, the activities of the several immune enzymes, transcription of LvECSIT) were investigated following single infections and co-infection with WSSV and *V. parahaemolyticus*.

2. Materials and methods

2.1. Experimental animals and conditions

L. vannamei (size 7.66 ± 0.82 cm) were obtained from the East Sea Island Marine Biological Research Center in Guangdong Ocean University. Before the experiment, 20 shrimp were randomly selected to ensure that they were free of WSSV and *V. parahaemolyticus*, according to Sun *et al.*[14]. They were fed with artificial pellet diets twice a day and were kept at 26°C and salinity at 25 ‰. Filtered seawater was sterilized with 1.5 ppm trichloroisocyanuric acid and the residual chlorine was

112 detected to ensure that it was safe for shrimp. About 1/3 of the water was replaced and
113 un-eaten pellet diet was removed by siphoning daily.
114
115 2.2 Preparation of virus and *V. parahaemolyticus* suspension for injection
116 WSSV extracts were prepared from crude extracts of disease shrimp and stored
117 at - 80 °C. Healthy shrimp were injected intramuscularly with 3.3×10^{2} copies μL^{-1}
118 virus (in PBS) and mortalities occurred at 48 h post-injection (pi). Following removal
119 of the exoskeletons, WSSV infected shrimp were homogenized in cold PBS (KH_2PO_4
120 0.27g, Na_2HPO_4 0.01g, NaCl 8g, KCl 0.2g, diluted with water to 1 L and adjust pH to
121 7.4) (1 mL g^{-1}). After centrifugation at 12,000 g for 10 min, the crude viral
122 supernatant was filtered using a membrane filter (220 nm).
123 *V. parahaemolyticus* was obtained from the Economic Aquatic Animal Disease
124 Control Laboratory of the Guangdong Ocean University [26]. *V. parahaemolyticus*
125 was cultured in trypticase soy broth (TSB, Huankai Co Ltd., Guangzhou, China) at
126 28 °C for 18 h. The culture medium was centrifuged in an 8 mL tube at 4000 g for 15
127 min. The supernatant was removed and *V. parahaemolyticus* was re-suspended in
128 PBS to1.22×10^{6} CFU mL^{-1}.
129
130 2.3 Experimental design
131 The laboratory challenge test contained 4 treatments in triplicate (n=40 for each
132 sample group, n=10 for mortality group). For *V. prahaemolyticus* treatment, shrimp
133 were intramuscularly injected with 50 μL of *V. prahaemolyticus* (1.22×10^{6} CFU

134 mL^{-1}). For WSSV treatment, shrimp were intramuscularly injected with 50 μL of
135 WSSV viral suspension (3.3×10^2 copies μL^{-1}). For co-infection treatment, shrimp
136 were intramuscularly injected with 50 μL of cocktail suspensions containing *V.*
137 *prahaemolyticus* (1.22×10^6 CFU mL^{-1}) and WSSV (3.3×10^2 copies μL^{-1}). The PBS
138 treatment was injected with 50 μL of PBS. Tissues (muscle, gills) of one shrimp per
139 group were sampled individually at PBS 0 h post-infection (pi), and at each time point
140 (3, 6, 12, 24, 48, 72 and 96 hpi) from each group to measure virus load,
141 immune-related enzymes, and immune-related gene LvECSIT expression analysis
142 (Table 1-2). The experiments were repeated three times.
143
144 2.4Analysis of virus load
145 The muscle of the first abdominal segment (about 0.05 g) was dissected and
146 added to 45 μL 50 mM NaOH and homogenized on ice, mixed and then boiled in
147 water bath for 10 min. Then, 5 uL1M Tris solution was added, mixed and centrifuged
148 at 12,000 g for 10 min [14]. The supernatant was used as WSSV template for
149 quantitative PCR. The qPCR was carried out in 15 uL volume, and the primer
150 sequences are shown in Table 3. The standard curve was made according to the
151 method of Xin *et al*.[27].
152
153 2.5 Determination of activities of immune-related enzymes in the gills
154 The gills (0.2g) were cut off from the samples stored in liquid nitrogen and
155 homogenized on ice after adding 1.8 mL PBS. The samples were centrifuged at 3000g

for 10 min at 4 °C, the precipitate was removed and the supernatant was used for acid phosphatase (ACP), alkaline phosphatase (AKP), peroxidase (POD) and superoxide dismutase (SOD) immune enzyme analysis. Enzymatic activities for ACP, AKP, POD, SOD were determined using kits purchased from Jiancheng Bioengineering Institute (NJJCbio, Nanjing, China), according to the methods described by Sun *et al*. and Liu *et al*. [14,28].ACP and AKP activities are expressed in King unit (mg protein)$^{-1}$. POD and SOD activities are expressed in U (mg protein)$^{-1}$. Each enzymatic assay was performed in triplicate.

2.6 Immune-related gene LvECSIT expression analysis by real-time PCR

Gills from one shrimps were sampled [20] at PBS 0 h post-infection (pi) and at each time point (3, 6, 12, 24, 48, 72 and 96 hpi) from each group. The transcriptional level of LvECSIT was detected with real-time PCR. Primers for LvECSIT (Genbank accession No. is XM_027378031) were shown in Table 3. β-actin wasused as internal reference. RNA extraction, cDNA synthesis, real-time PCR for analysis of immune gene expression were as described by Li et al. [29].

2.7 Statistical analysis

Statistical analysis was carried out using the software SPSS 21. Results were analyzed using One-way ANOVA and Duncan's multiple comparisons of the means. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Effect of WSSV and *V. parahaemolyticus* infection on shrimp survival

Shrimp in each challenge group started to die at 12 hpi. The cumulative mortality reached peak at 96 hpi, and the mortality of WSSV group (97 %) was significantly higher than co-infection group (83 %) and *V. parahaemolyticus* group (34 %) ($P<0.05$) (Fig.1).

3.2 Effects of WSSV and *V. parahaemolyticus* infection on the proliferation of WSSV in *L. vannamei*

In the experiment, we collected the muscle of shrimp to detect the copies of WSSV by real time PCR. The results illustrated that WSSV could be detected in muscle within 3 h, and the maximum viral load in the WSSV infection group was 6.71×10^5 copies μL^{-1} at 72 hpi, significantly higher than that in co-infection group (1.80×10^4 copies μL^{-1}). The viral load in the WSSV infection group was approximately 10 times more than that in co-infection group at 24, 48, 72 and 96 hpi (Fig.2).

3.3 Effects of WSSV and *V. parahaemolyticus* infection on shrimp gill immune enzyme activity

The ACP activity in the gills of shrimp infected with *V. parahaemolyticus* alone and the co-infection groups showed an initial rise and subsequent fall, and reached maximum activity at 24 and 6 hpi respectively. In the *V. parahaemolyticus* group and

200 co-infection group, the maximum ACP activity was significantly higher than the PBS
201 group and WSSV group at 6, 12, 24, 48, 72 and 96 hpi ($P < 0.05$). By the end of the
202 experiment, the ACP activity of WSSV group remained at a low level, and was
203 consistently lower than both the *V. parahaemolyticus* and the co-infection groups.
204 Comparison of the degree of variation of each treatment group showed the following
205 trend: PBS group (0.14) < WSSV group (0.33) < *V. parahaemolyticus* group (0.45) <
206 co-infection group (0.58) (Fig.3A).

207 In the *V. parahaemolyticus* group and co-infection group, the AKP activity
208 decreased after the initial rise, and was higher than the WSSV group and PBS group
209 at all time points, and the maximum AKP activity was recorded at 6 h and 24 hpi
210 respectively. The AKP activity of WSSV group was significantly lower than the
211 co-infection group from 6-96 hpi. The AKP activity of *V. parahaemolyticus* group
212 varied over the course of the experiment whereas the AKP activity of the PBS group
213 was stable. Degree of variation: PBS group (0.18) < WSSV group (0.21) <
214 co-infection group (0.29) < *V. parahaemolyticus* group (0.45) (Fig.3B).

215 The POD activity of the PBS group remained higher than 3 challenge groups
216 until the end of experiment, and the difference was significant at 48 hpi ($P < 0.05$).
217 For the *V. parahaemolyticus* group, co-infection group and WSSV group, the
218 minimum POD activity occured at 3, 6 and 24 hpi respectively. The POD activity of
219 the co-infection group was higher than the WSSV group at 6, 12, 48 and 96 hpi, and
220 was significantly higher at 6 hpi. Degree of variation: PBS group (0.05) < *V.*
221 *parahaemolyticus* group (0.11) < co-infection group (0.14) < WSSV group (0.15)

(Fig.3C).

SOD activity of the WSSV and co-infection groups showed the lowest value at 96 h pi, which was significantly lower than PBS group ($P < 0.05$). The SOD activity of the co-infection group was significantly higher than the WSSV group at 48 hpi ($P < 0.05$). The SOD activity of *V. parahaemolyticus* group was significantly higher than WSSV group at 3, 6, 48and 96 hpi ($P < 0.05$). SOD activity in each group variation coefficient: PBS group (0.11) <*V. parahaemolyticus* group (0.18) < co-infection group (0.24) < WSSV group (0.32) (Fig.3D).

3.4 Effects of WSSV, *V. parahaemolyticus*, and WSSV and *V. parahaemolyticus* co-infection on LvECSIT expression in shrimp

In the challenge test, the expression of LvECSIT was detected in gill at 0, 3, 6, 12, 24, 48, 72 and 96 hpi. The transcription levels of LvECSIT in the PBS group up-regulated from 6 to 48 hpi. WSSV infection group showed a degree of fluctuation and reached maximum expression at 48h. Furthermore, LvECSIT expression up-regulated significantly in WSSV infection group more than co-infection group at 3hpi, and was significantly more up-regulated than *V. parahaemolyticus* group at 6 hpi. The LvECSIT expression was significantly up-regulated in *V. parahaemolyticus* group or co-infection group when compared with the WSSV infection group from12 to 72 hpi ($P < 0.01$). There was no significant difference between the *V. parahaemolyticus* group and co-infection group from 12 to 48 hpi. Each treatment group showed minimum LvECSIT expression at 96 hpi and was all significantly

lower than PBS group ($P < 0.05$) (Fig. 4).

4. Discussion

In complex aquaculture environments, the outbreak of shrimp disease is accompanied with sharply defined changes of physical factors or secondary infection and co-infection by pathogens [30-32]. Nonetheless, the conclusions about *Vibrio* spp. and WSSV co-infection in shrimp have been conflicting. Previous studies have shown that mortality in co-infections (39%) was significantly higher than in single WSSV infections (25%) and single infections with *Vibrio anguillarum* (25%) [5].However, other studies have revealed that the outbreak of WSSV was postponed after *P. vannamei* co-infection with WSSV and *V. harveyi* [6]. In this study, the mortality of WSSV group (97 %) was significantly higher than the co-infection group (83%) and *V. parahaemolyticus* group (34 %) ($P < 0.05$), which conflicted with the reported in *L. vannamei* after co-infection with WSSV and *V. anguillarum* [5], but was similar to previous findings in *P.vannamei* after co-infection with WSSV and *V. harveyi* [6]. The synergistic effect between WSSV and *Vibrio* may be influenced by the species of the *Vibrio* bacteria [6].

In this experiment, the WSSV copy number measured in the co-infection group was always lower than in the WSSV group. It might be the key factor of lower mortality in the co-infection group. The proliferation of WSSV result also demonstrated that the WSSV replication was controlled under co-infection conditions. It is possible that WSSV must make use of the metabolites in the host cell to assemble

nucleotides and proteins of the virus [33] after infection of the shrimp, but the metabolites were used by *V.parahaemolyticus* or the metabolism of shrimp was slowed down by *V.parahaemolyticus*. This suggests that virus couldn't replicate without the metabolites, hence the WSSV proliferation was inhibited.

ACP is a typical lysosomal enzyme and plays a key role in eliminating and hydrolyzing microbes [34]. In *Chlamys farreri* [35], the ACP activity was significantly increased at the early stage of *Vibrio anguillarum* challenge. In this experiment, the ACP activity is most sensitive to *V. parahaemolyticus* infection from 3 h after infection and reached the peak at 6 hpi. However, the ACP activity of the WSSV infected group declined at 3 hpi then increased and reached the peak at 12 hpi. The result was consistent with ACP activity in *Penaeus monodon* with WSSV in latent period on reinfection [36], but the time of appearance of the peak varied. The difference in the appearance of the peak might be associated with the dose of infection and environment. Furthermore, ACP activity in the virus infected group was always significantly lower than that of the co-injection group throughout the experimental period. In other words, the *V. parahaemolyticus* infection has, to some extent, affected ACP vitality of the shrimp. The ACP activity of the co-injection group from 3 to 96 h pi was always higher than the WSSV group. The ACP activity of the co-injection group from 6 to 24 hpi was significantly higher than that of the *V. parahaemolyticus* injected group which suggests that co-infection stimulates the immune response in *L. vannamei*. In the co-infection group, the ACP activity declined from 48 hpi, but remained significantly higher than the WSSV group. The co-infection may cause

disturbance of cell metabolism and immune function, which is consistent with the previous report in *Penaeus (Marsupenaeus) japonicus*[37].

AKP is a regulatory enzyme associated with the metabolism and can be seen as an important index in the assessment of the immune status of shrimp [38]. After an initial rise at 3 hpi, the AKP activity of WSSV-injected group decreased significantly at 6 hpi in this experiment which was similar to previous reports [39]. We observed that AKP activity in the gills of the shrimp is more sensitive to *V. parahaemolyticus* infection than WSSV infection; the AKP activity of the co-injection group varied in a similar manner.

Reactive oxygenspecies (ROS), including superoxide anion (O_2),hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) are an important part of the innate immune defense system that is produced to help eliminate invading microbes[40].Antioxidant enzymes such as peroxidases (POD) and superoxide dismutase (SOD) either convert $O_2$to H_2O_2(SOD), convert H_2O_2 to water and oxygen bycatalase (CAT), or use H_2O_2 to oxidize substrates by various peroxidases [41].POD activity can serve as an immune index to evaluate the immune status of crustacean[42].After infection with WSSV, the POD activity of *Cherax quadricarinatus* was shown to decrease significantly [43].In this study, the POD activity in gill decreased initially in all 3 challenge groups at 3 hpi. The minimum activity of the WSSV-injected groups was recorded at 6 hpi and was significantly lower than other groups. The POD activity in the co-infection group was significantly higher than WSSV group at 6 hpi, which may have contributed to enhancing the

ability of the co-infection group to resist the infection of WSSV at 6 hpi.

SOD is an enzyme that catalyses the rapid two-step dismutation of the toxic superoxide anion to molecular oxygen and hydrogen peroxide through the alternate reduction and oxidation of the active-site metal ion [44].A previous study indicated that a significant decrease in SOD activity occurred earlier at 3 hpi in white shrimp *L.vannamei* that received *V. alginolyticus* injection, followed by recovery after 96 hpi [45].In this study, the SOD activity of the *V. parahaemolyticus* -injected group significantly increased at 3 hpi which conflicted with the previous report[45]. A significant decrease in SOD activity occurred in WSSV –injected group at 6 hpi. It was consistent with reports in the shrimp *Penaeus monodon* [46] and *L. vannamei* [47], which showed a decrease of SOD activity after WSSV infection. According to the study in *Fenneropenaeus indicus* [48], the lower activities of SOD may have been due to inactivation of SOD by the oxidative stress generated singlet oxygen. In the present study, the SOD activity of co-infection group and *V. parahaemolyticus* group was significantly higher than that in WSSV group at 48 hpi, which suggests that the shrimp in the co-infection and *V. parahaemolyticus* group could clear the oxyradical more efficiently compared to WSSV group, and avoid the oxidative damage induced by pathogens. Previous studies have shown an increase in activity of antioxidant enzymes in shrimp during bacterial infections, with a decrease observed during viral infection with WSSV [41].

As far as we know, viral suppression mechanisms exist in arthropods [7].Studies had revealed that the proliferation of West Nile and chikungunya virus were

suppressed in individuals after infection with *Wolbachia* [50, 51]. In mosquito during co-infection with *Wolbachia* and dengue virus, the TLR signaling pathway was activated by ROS and expressed more immune factors than in the mosquito group infected with virus only [8]. ECSIT is a multifunctional adaptor protein of TLR signaling pathway, and represented a constitutive expression pattern in some tissues [51].In shrimp, MjECSIT was previously shown to be expressed in hemocyte, gill, hepatopancreatic, stomach, heart, intestinal, testicular, and ovarian tissues, and the expression level in gill was higher than in hemocyte [19]. The mRNA transcript of LvEcsit in gill was also higher than in hemocyte (Data will be showed in another paper), which are considered with the result in MjECSIT [19].So gill was chosen for the sample tissue in this study. TLR pathway is of major importance during innate immunity. Most genes in TLR pathway are reported to up-regulated in the stress of pathogen. ECSIT, an essential member of this pathway, was found to be significantly up-regulated after *Vibrio anguillarum* challenge in *Crassostrea gigas* [52], and by challenge with microorganisms (*Vibrio alginolyticus*, *Staphylococcus haemolyticus* and *Saccharomyces cerevisiae*) in the Hong Kong oyster *Crassostrea hongkongensis* (ChECSIT) [17]. In this study, the expression of LvECSIT was up-regulated by infection with *V. parahaemolyticus* (Fig.4). The transcription level of LvECSIT in the co-infection group was higher than WSSV group from 12h to 72hpi (Fig.4), which was consistent with the expression pattern of Toll pathway-related genes in *Aedes aegypti* [8]. Furthermore, the transcription levels of LvECSIT in the PBS group up-regulated from 6 to 48 hpi, was consistent with MjECSIT at 6 hpi [19], and

ChECSIT at 3 and 12 hpi [17].The difference in the kinetics of expression between these studies could be associated with the animal, dose of infection and environment. However, further study is required to elucidate the potential mechanism in shrimp.

In summary, this study demonstrated that 1) shrimp in co-infection groups suffered lower mortality than groups with single infection by WSSV only; 2) the amount of WSSV in co-infection group was always lower than that of WSSV single infection group over the course of the trial; 3) ACP and AKP activity in gills of shrimp co-infected with *V. parahaemolyticus* and WSSV was significantly higher than that of WSSV single infection group from 6 to72 hpi; ACP and AKP enzyme activity can be used as indicators of immune response to these pathogens; POD and SOD activity may not be the best indicators of immune response to WSSV - *Vibrio* infections.4) the transcription level of LvECSIT was up-regulated in *V. parahaemolyticus* infected and multiple infection groups. This study provided information for understanding the effect of WSSV - *Vibrio* infections on survival and immune responses in shrimp. Further study is needed to develop prevention and management strategies to reduce losses caused by multiple pathogens in aquaculture.

Acknowledgments

This work was funded by the National Key R & Development Program of China (2018YFD0900501)，National Natural Science Foundation of China (No. 31402344), Natural Science Foundation of Guangdong Province (No. 2017A030313174).

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553 Table1 Design of experiment for virus load, enzymes, and gene expression analysis.

Treatments	WSSV copies μL^{-1}	*V.pra* CFU mL^{-1}	No. of shrimp	Sampling Number of shrimp at hours post-injection (hpi)							
				0	3	6	12	24	48	72	96
1 PBS	-	-	40×3	1×3	1×3	1×3	1×3	1×3	1×3	1×3	1×3
2 *V. pra*	-	1.22×10^6	40×3	0	1×3	1×3	1×3	1×3	1×3	1×3	1×3
3 WSSV	3.3×10^2	-	40×3	0	1×3	1×3	1×3	1×3	1×3	1×3	1×3
4Co-infection	3.3×10^2	1.22×10^6	40×3	0	1×3	1×3	1×3	1×3	1×3	1×3	1×3

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Table2 Design of experiment for Lethality

Treatments	WSSV copies μL^{-1}	*V.pra* CFU mL^{-1}	No. of shrimp
1 PBS	-	-	10×3
2 *V. pra*	-	1.22×10^6	10×3
3 WSSV	3.3×10^2	-	10×3
4Co-infection	3.3×10^2	1.22×10^6	10×3

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609 Table 3 Sequences of primers used in this study.

Primer name	Primer sequence(5'-3')	references
WSSV-F	AAACCTCCGCATTCCTGTGA	[28]
WSSV-R	TCCGCATCTTCTTCCTTCAT	
LvECSIT-F	ATGATTCTTATGAACGCTT	This study
LvECSIT-R	AATTTGGGCATCCAGTAC	
β-actin-F	GAAGTAGCCGCCCTGGTTGT	This study
β-actin-R	GGATACCTCGCTTGCTCTGG	

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Figure 1.Cumulative mortality in shrimp. *L. vannamei* infected by intramuscular injection with *V. parahaemolyticus* only, by white spot syndrome virus (WSSV) only, or concurrently infected with *V. parahaemolyticus* and WSSV (Co-infection) at different time intervals pi (0, 3, 6, 12, 24, 48, 72, and 96 hours). Injection with PBS served as negative control. Groups that don't share a letter are significantly different ($P < 0.05$).

Figure 2. Effect of single injection (*V. parahaemolyticus* or WSSV) and co-infection injection (*V.parahaemolyticus* and WSSV) on the amount of WSSV (copies μL^{-1}) estimated in *L.vannamei* muscle at different time intervals pi (3, 6, 12, 24, 48, 72, and 96 hours). Values are expressed as mean ± SD. Groups that don't share a letter are significantly different ($P < 0.05$).

Figure 3. Effect of single injection(*V. parahaemolyticus*or WSSV) and co-infection injection (*V.parahaemolyticus* and WSSV) on the gill ACP(A), AKP(B), POD(C) and SOD(D) activity of *L.vannamei* at different time intervals pi (0, 3, 6, 12, 24, 48, 72, and 96 hours). Groups that don't share a letter are significantly different ($P < 0.05$).

Figure 4. Effect of single injection (*V. parahaemolyticus*or WSSV) and co-infection injection (WSSV and *V.parahaemolyticus*) on the mRNA expression of LvECSIT of *L.vannamei* at different time intervals pi (0, 3, 6, 12, 24, 48, 72, and 96 hours). Groups that don't share a letter are significantly different ($P < 0.05$).

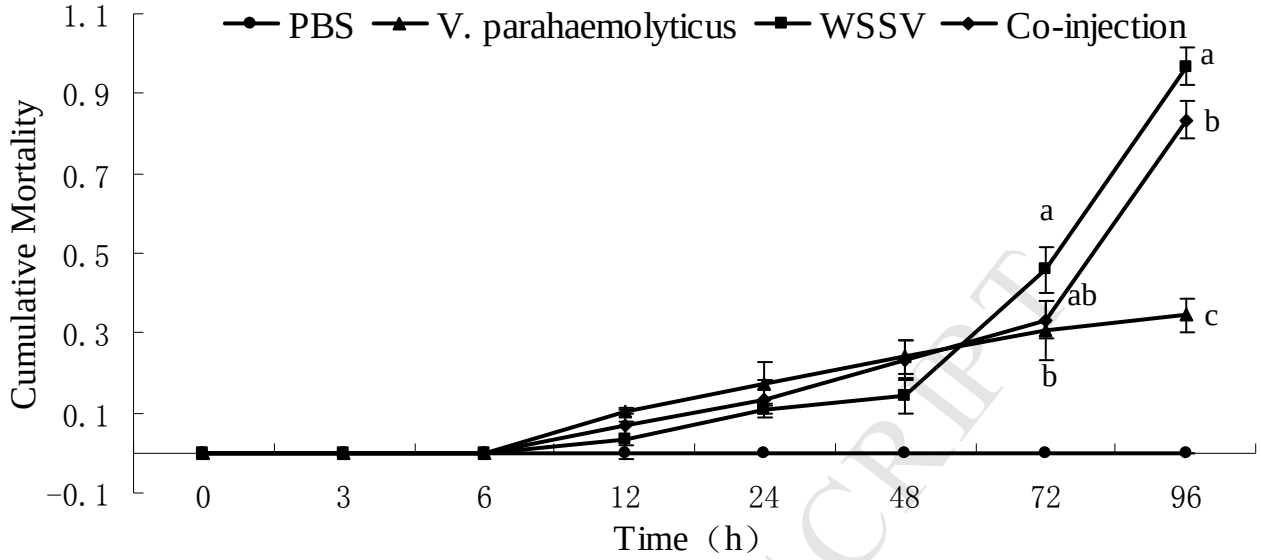
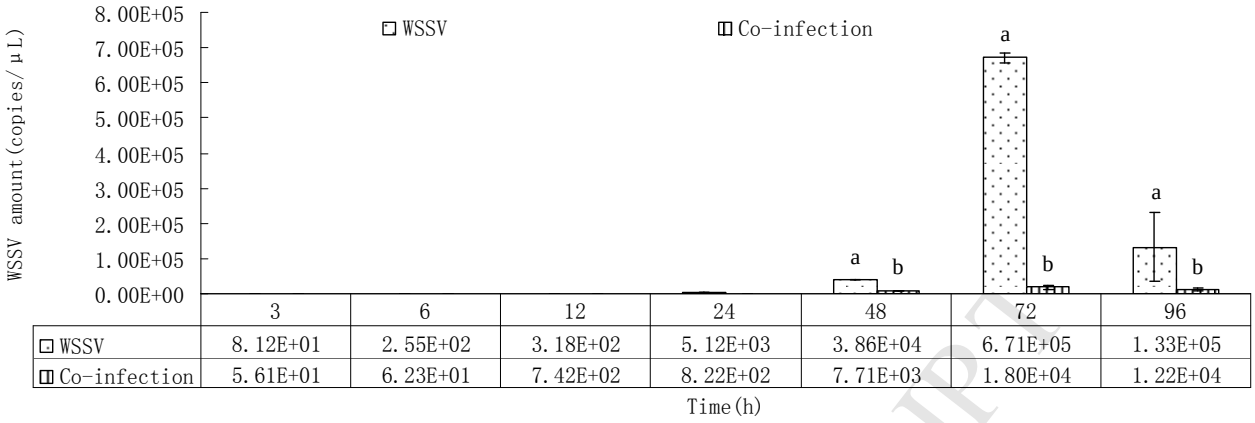
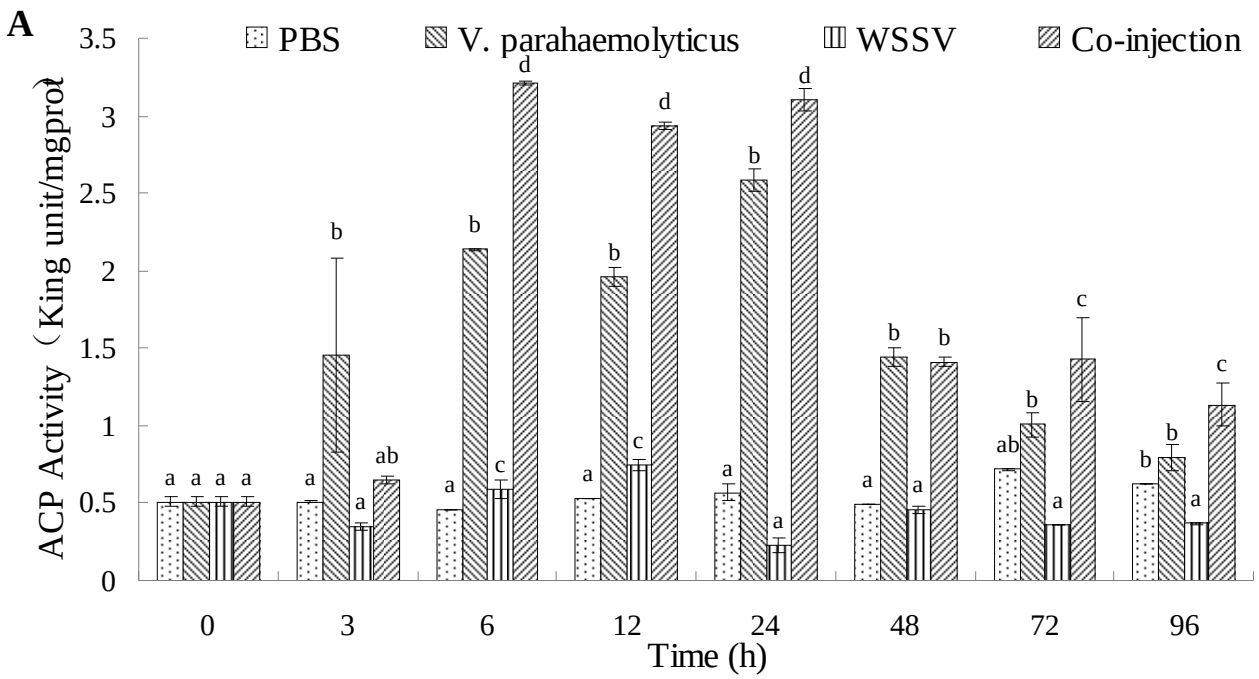


Fig.1



	3	6	12	24	48	72	96
WSSV	8.12E+01	2.55E+02	3.18E+02	5.12E+03	3.86E+04	6.71E+05	1.33E+05
Co-infection	5.61E+01	6.23E+01	7.42E+02	8.22E+02	7.71E+03	1.80E+04	1.22E+04

Fig.2



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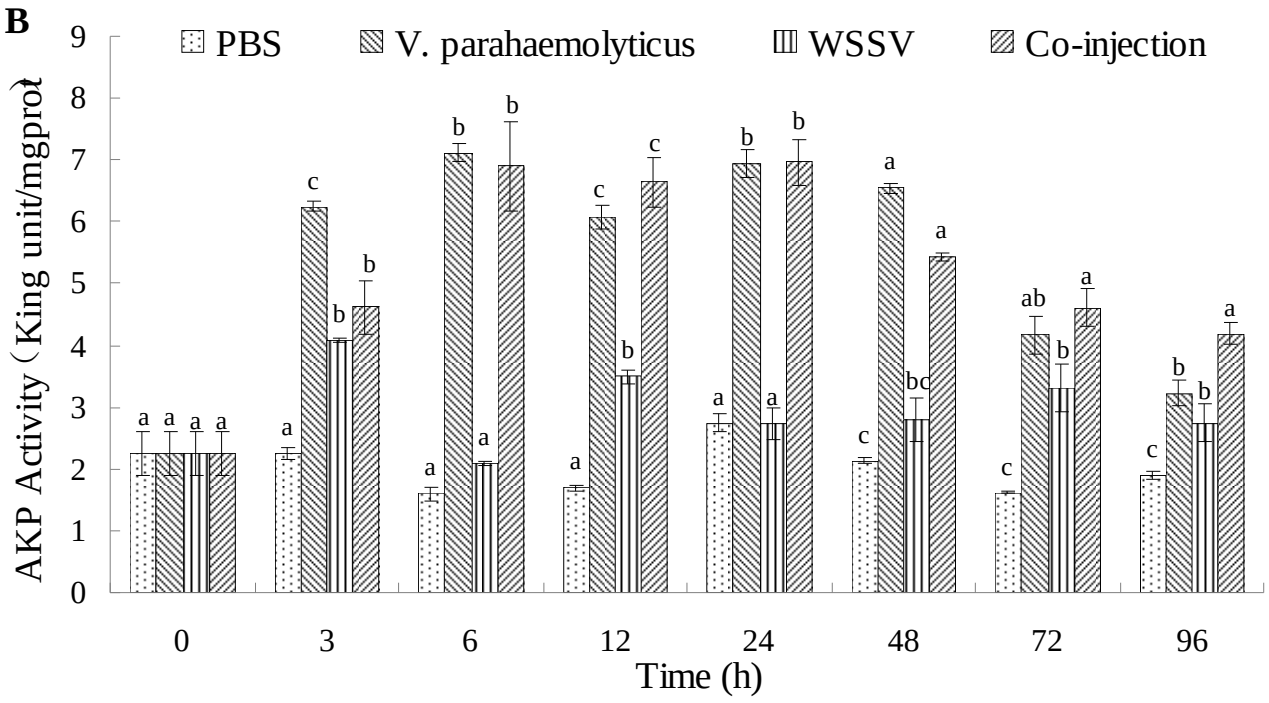

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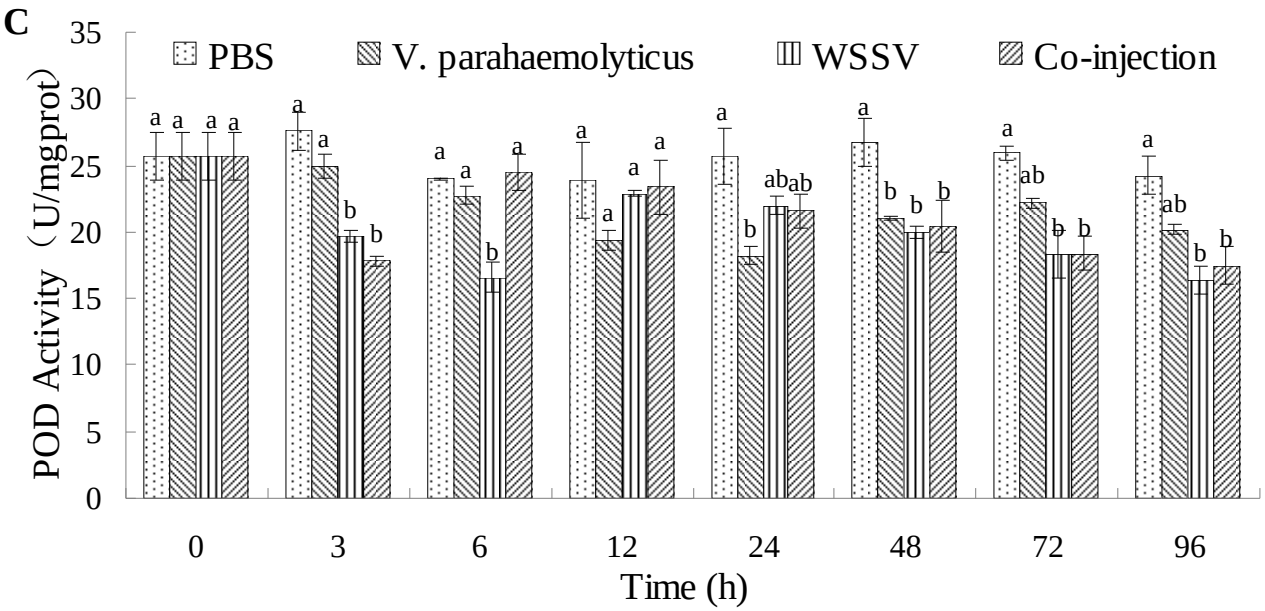

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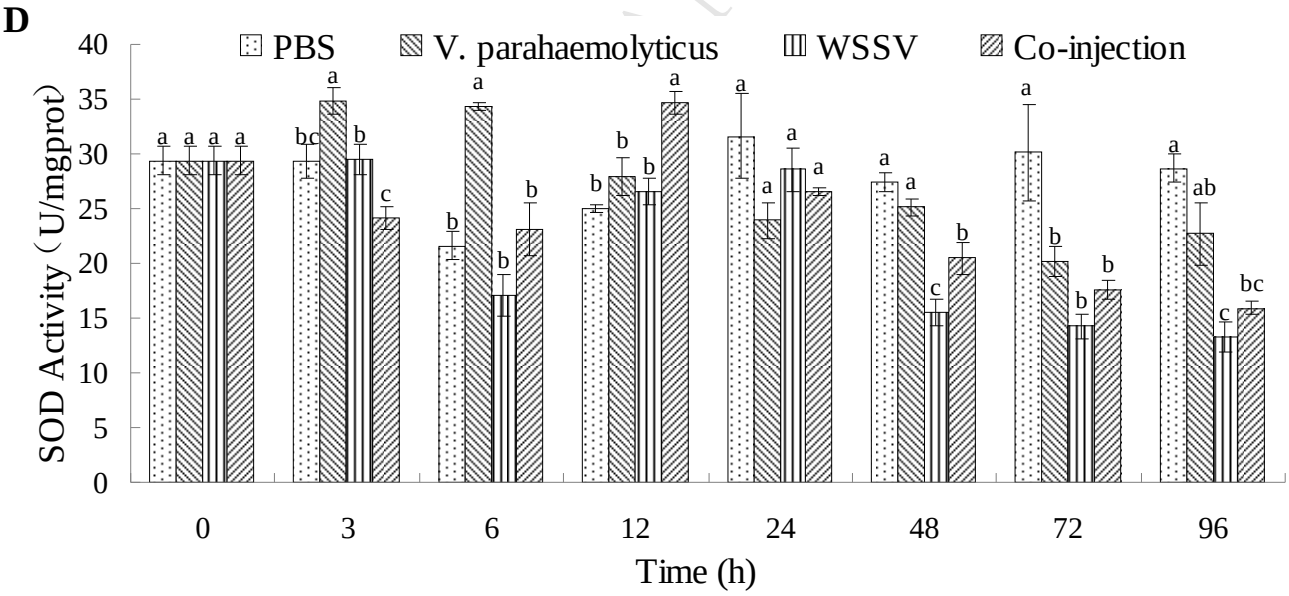

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727 **Fig.3**

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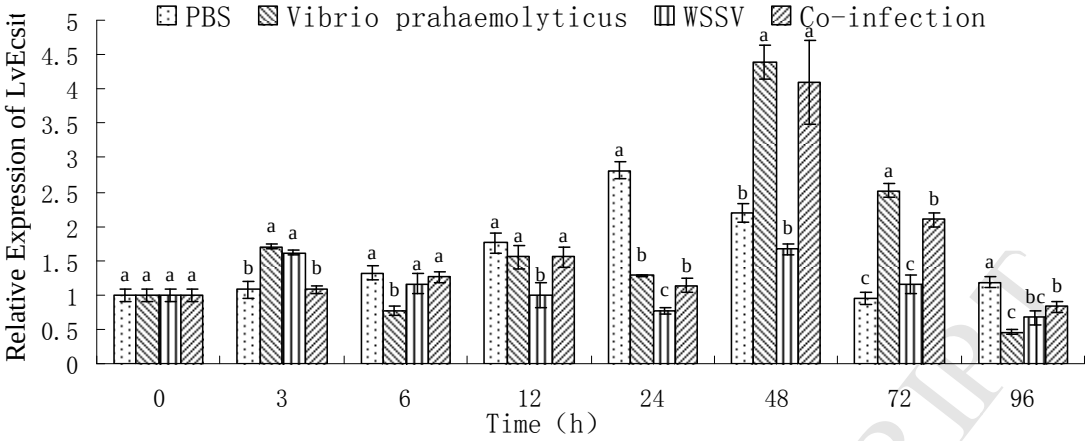

Fig.4

Shrimp in co-infection groups suffered lower mortality than WSSV group.

The amount of WSSV in co-infection group was lower than in WSSV group.

ACP and AKP enzyme activity can be used as indicators to co-infection.

The transcription level of LvECSIT was up-regulated in co-infection groups.