The utilization of VP28 gene to protect penaeid shrimps from white spot syndrome virus disease: a review

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ABSTRACT

White spot syndrome (WSS) is a viral disease which affects most of the commercially cultivated marine shrimp species all over the world causing significant losses. Deaths occur within days of symptoms and affect a diverse range of commercial crustacean species. A heavy mortality is usually observed in the entire infected population. Survival rates can be anywhere from 30% to zero. Consequently, tools with the capability to effectively and timely detect the diseases have been developed to prevent and contain these pathogens. White spot syndrome virus (WSSV) envelope protein VP28 gene is widely used because its ability to bind to the surface of shrimp epithelial cells and might promote innate immune recognition of WSSV. Its recombinant protein was expressed in various expression systems and used as recombinant vaccine or immunostimulant to increase shrimp survival against WSSV. An anti-body against VP28 was successfully used in virus neutralization assay. A DNA vaccine encoding VP28 gene also provided long-term and high levels of protection against WSSV. Moreover, RNA interference (RNAi) resulted in endogenous RNA sequence-specific degradation to silence VP28 gene expression as well as induced an immune response against virus using non-specific dsRNA in shrimp. However, in order to use VP28 on a commercial scale in the shrimp industry, there are several concerns that need to be addressed such as storage, delivery methodology, protection efficiency; protection period and ensuring that it can be produced in large scale. All these methods might lead to the new strategies to control WSSV using the VP28 gene in future.

Keywords: white spot syndrome virus (WSSV), VP28, penaeid shrimp

Mavichak, R., Kondo, H., Hirono, I. and Aoki, T. 2011. The utilization of VP28 gene to protect penaeid shrimps from white spot syndrome virus disease: a review, pp. 157-170. *In* Bondad-Reantaso, M.G., Jones, J.B., Corsin, F. and Aoki, T. (eds.). Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia. 385 pp.

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INTRODUCTION

Penaeid shrimp are regarded as the most economically important among the crustacean species. Penaeid shrimp are mainly farmed in Southeast Asia (Thailand, Indonesia, Philippines and Vietnam), Central America (Mexico and Panama) and South America (Ecuador, Peru and Venezuela). High demand for shrimp in the global market leads to the birth of shrimp farming in these developing countries. Shrimp aquaculture started in the 1970s as an industrial activity and developed rapidly with huge increase in number of hatcheries and farms. The demand for shrimps in global market is increasing every year. In 2006, the amount of total shrimp production was over 2.6 million tones and over US$10 billion (www.fao.org/fishery/statistics/global-production/en). However, with the rapid production and development, the shrimp aquaculture industry has encountered serious problems including non-infectious and infectious diseases in every major farming area throughout the world. Identified shrimp pathogens such as bacteria and virus are spreading and new diseases are continuously being identified.

White spot syndrome (WSS) is a viral disease that affects most of the commercially cultivated marine shrimp species all over the world (Flegel, 1997; Lotz, 1997). It was first observed in East Asia in 1992–1993 and has since spread to most shrimp culture countries (Yan *et al*., 2007). WSS manifests itself quickly and deaths occur with days of symptoms. It affects a diverse range of crustacean species (Lo *et al.*, 1996; Wang *et al.*, 1998) including *Fenneropenaeus chinensis*, *F. indicus*, *F. merguiensis*, *Penaeus monodon, Litopenaeus setiferus, L. stylirostris, and P. vannamei.* Within a week of the infection a heavy mortality begins. Survival rates can be anywhere from 30% to zero, but in many cases the entire pond is wiped out.

WSS is caused by an enveloped double-strand DNA virus called white spot syndrome virus (WSSV), which belong to the family of *Nimaviridae* and the monotypic genus *Whispovirus*. It has an ovoid or ellipsoid to bacilliform shape with a flagella-like appendage at one end of the virion (van Hultan *et al.*, 2001a). The genome of three WSSV isolates has been fully sequenced: Thailand 293 kbp (van Hulten *et al*., 2001b); China 305 kbp (Yang *et al*., 2001) and Taiwan 307 kbp (Chen *et al*., 2002). Its genome contain 181 non-overlapping open reading frames, consist of five major proteins named VP28, VP26, VP24, VP19 and VP15 (van Hultan *et al*., 2001b). Two of these major proteins (VP28 and VP19) were characterized as envelope protein (van Hultan *et al*., 2001b). VP28 appears to bind to the surface of shrimp epithelial cells (Yi *et al.,* 2004). Such binding is thought to be mediated by a shrimp VP28-binding protein, PmRab7, suggesting that shrimp possess innate immune recognition of WSSV (Sritunyalucksana *et al*., 2006; Tang *et al*., 2007). Therefore VP28 is wildly used for diagnosis and control WSS although its efficacy varies.

1. Recombinant protein VP28

VP28 recombinant protein (rVP28) has been expressed in various expression systems such as in Gram-negative bacteria, *Escherichia coli*, (Jha *et al*., 2006b; Namikoshi *et al.,* 2004; Witteveldt *et al*., 2004a; Witteveldt *et al*., 2004b; Witteveldt *et al*., 2006), Gram-positive

bacteria (Caipang *et al.*, 2008; Mavichak *et al.,* 2009) insect cells (Du *et al*., 2006), yeast (Jha, Xu and Pandey, 2006a; Jha *et al.,* 2007) and silkworms (Xu *et al*., 2006). In this first study, van Hulten *et al.* (2000) expressed the rVP28 in an insect cell. The difference between the actual weight (28 kDa) and the predicted weight (22 kDa) was attributed to the use of an insect expression system that may be the result of post-translational modifications (e.g. glycosylation, phosphorylation). However, using prokaryotic system to express and purify rVP28, it has been demonstrated to protect shrimp against WSSV. Namikoshi *et al.* (2004) demonstrated that *E. coli*-expressed rVP26, rVP28 and inactivated WSSV protected kuruma shrimp (*Marsupenaeus japonicus*). The result showed that injection with rVP28 twice (100µg/1g shrimp/ time) gave highest protection against WSSV with 95% relative percent survival (RPS) (Amend, 1981). Witteveldt *et al.* (2004b) evaluated the potential for vaccination using rVP28 N-terminally fused to maltose binding protein expressed in *E. coli*. The result showed that vaccination with rVP28 (4µg/1g shrimp) gave significantly better survival when challenged two days after vaccination with 33% (RPS), but not after 25 days.

Du *et al.* (2006) have compared the efficacy of rVP28 expressed in *E. coli* and insect cells in crayfish, *Procambarus clarkii*. They found that injecting shrimp with rVP28 expressed in insect cells (2.5µg/1g shrimp) provided greater protection than injection with rVP28 expressed in *E. coli* (2.5µg/1g shrimp). The rVP28 expressed in insect cells gave 95% RPS, while rVP28 expressed in *E. coli* gave 43.5% RPS. They suggested that posttranslational modifications of VP28 may have been correctly performed in insect cells. A similar experiment using prokaryote system expressed rVP28 was conducted by Jha *et al.* (2006a) who investigated the potential of injection vaccine using rVP28 expressed in yeast, *Pichia pastoris*, against WSSV in crayfish, *P. clarkii*. They found that injection of rVP28 2µg/ 1g of crayfish give the protection ability at 3 days post-vaccination at 91% RPS and at 21 days post-vaccination at 78% RPS. Also they investigated the effectiveness of oral, intramuscularly (IM) injection (2µg/ 1g of crayfish) and immersion vaccination against WSSV in crayfish, *P. clarkia* (Jha *et al*., 2006b). The rate of cumulative mortality was higher in case of immersion vaccination. The challenge test on 5^{th} and 21^{st} day post-immersion showed RPS value of 49% each followed by oral vaccination. The challenge test on 3^{rd} and 21^{st} day post-oral administration showed 52% and 49% RPS, respectively and injection vaccination. The challenge test on 3^{rd} and 21^{st} day post-immersion showed 50.5% and 43.1% RPS, respectively. However, there seem to be no significant difference based on the RPS value. Thus, oral administration of rVP28 might be a feasible method to administration because injecting individual shrimp is not practical when dealing with large number of shrimp.

Witteveldt and colleagues expressed VP28 in *E. coli* and demonstrated that, when mixed with commercial shrimp pellets, it significantly enhanced survival against WSS challenge in *P. monodon* (Witteveldt *et al*., 2004a) and *L. vannamei* (Witteveldt *et al.*, 2006). The level protection in *L. vannamei* was lower compared to *P. monodon* (RPS values of 50% in *L. vannamei* and 77% in *P. monodon*). Caipang *et al.* (2008) use Gram-positive bacterium, *Brevibacillus brevis*, to produce WSSV rVP28. This Gram-positive bacterium has three advantages: it secretes functional extracellular protein directly into the culture

medium, making it useful for large-scale commercial production and it doesn't produce lipopolysaccharide (LPS) which is normally present in Gram-negative bacteria and is endotoxic to many organisms. They found that feeding with rVP28 25µg/ 1g shrimp for 7 days before challenge with WSSV at 3 days post-feeding showed the highest RPS value at 92.8% RPS. The level of protection in shrimp against the disease significantly decreased at 2 weeks post-feeding and when the dose of feeding was decreased. They suggested that the protection ability of rVP28 might rely on the dose and duration of feeding. Also in our previous study (Mavichak *et al.,* 2009), we used Gram-positive bacteria; *B. choshinensis* expressed N- and C-terminus-lacking and N-terminus-lacking rVP28 to increase recombinant VP28 expression and compared the efficacy of oral administration and intramuscular (IM) injection of rN-VP28. Our finding showed that feeding with N-terminus-lacking recombinant VP28 (5µg/ 1g shrimp/day) for 7 days still protected shrimp against WSSV, while with the same feeding conditions of N-and C-terminus-lacking did not. Moreover, one time IM injection (5µg/ 1g shrimp) of VP28 provided greater protection than oral administration for 7 days (5µg/ 1g shrimp/day). Moreover, we coated rN-VP28 with liposomes to improve rN-VP28 uptake through oral administration. The determination of protection against WSSV showed that feeding with rN-VP28 coated with liposomes (rN-VP28-MLV) for 3 days gave greater protection than feeding with rN-VP28 treatment. However, feeding with rN-VP28-MLV for 7 days gave no significant difference to protection with rN-VP28 treatment (Mavichak *et al*., 2010). In addition, uptake of rN-VP28-MLV and rN-VP28 was compared by histology. In this experiment we coated rN-VP28-MLV and rN-VP28 with fluorescein isothiocyanate (FITC). At 1 h post-administration, a strong green-fluorescent signal was detected in the intestine of shrimp treated with FITC-rN-VP28-MLV but no signal was detected in shrimp treated with FITC-rN-VP28 and rN-VP28. Taken together, our results suggest that the use of liposomes can improve the efficiency of delivering rN-VP28 to shrimp.

Xu *et al.* (2006) determine the efficacy of rVP28 using silkworm expression system in crayfish, *P. clarkii*. The results showed that VP28 treated crayfish gave significantly better survival rate against WSSV than control treatment (94.7% RPS). Jha *et al.* (2007) also evaluated the efficacy of orally administration of rVP28 expressed in yeast, *P. pastoris* against WSSV in *P. clarkii*. They found that the highest RPS value was belonging to the sonicated yeast expressing rVP28 (86% RPS and 72% RPS when challenged with WSSV on 3rd and 21st day post-vaccination). These studies provided the information to protect shrimp using rVP28 against WSSV.

2. Antibody against VP28

Several groups have produced monoclonal and polyclonal antibodies against rVP28 as a tool for diagnosing WSSV (Anil, Shanker and Mohan*,* 2002; Chaivisuthangkura *et al.,* 2004; Liu *et al.,* 2002; Makesh *et al.,* 2006; Poulos *et al.,* 2001; Shih *et al.,* 2001; Zhan *et al.,* 1999). With the development of hybridoma technology, panels of monoclonal antibodies (MAbs) were produced and used in an indirect immunofluorescence assay (IFA) (Shih *et al.,* 2001; Zhan *et al.,* 1999), immunoperoxidase and whole-mount tissue assays (Poulos *et al.,* 2001) for rapid diagnosis of WSSV. However, tests are not suitable for use by farmers. Furthermore there is evidence that various WSSV geographic isolates differ at the molecular level

(Nadala and Lo, 1998; Lo *et al.,* 1999; Wang, Nunan and Lightner, 2000). Therefore, Anil, Shanker and Mohan. (2002) have produced MAbs to an Indian isolate of WSSV for antigen characterization and to develop a simple and sensitive immunodot test that can be used by farmers. Liu *et al.* (2002) produced MAbs specific to VP28 and developed a MAb-based antigen-capture enzyme-linked immunosorbent assay (Ac-ELISA) for detection of WSSV antigen from shrimp tissues and hemolymph. Makesh *et al.* (2006) developed MAbs that reacted with VP28, to diagnose WSSV infection in *P. monodon.* The test had an analytical sensitivity of 625pg of purified virus.

Moreover, antibodies raised against individual viral envelope proteins have been successfully used in neutralization assays to identify proteins involved in virus entry during infection (van Hulten *et al.,* 2001a; van Hulten *et al.,* 2001b: Wu, Wang and Zhang, 2005), as well as to prevent WSSV infection (Kim *et al.,* 2004; Musthaq *et al.,* 2006; Natividad *et al.,* 2007; Robalino *et al.,* 2006;). Kim *et al.* (2004) reported that egg yolk antibodies (IgY) against truncated VP28 fused with truncated VP19 WSSV enveloped proteins, TrVP28:19, were able to neutralize WSSV in challenge trials with *F. chinensis*. Robalino *et al.* (2006) used polyclonal and monoclonal antibodies against VP28 from rabbit serum to inactivate WSSV in *L. vannamei.* Mustaq *et al.* (2006) used various VP28 polyclonal antiserum concentrations to neutralize WSSV in *P. monodon.* The results indicate that WSSV can be neutralized by VP28 antiserum in a dose-dependent manner. Similarly, Natividad *et al.* (2007) demonstrated that a MAb against VP28 was effective in protection of *M. japonicus* from WSSV. These results indicate that antibody against VP28 will be useful for developing new strategies to control WSSV infection.

3. DNA vaccine

DNA-based vaccination constitutes one of the most recent approaches to vaccine development. Vaccination of plasmids carrying genes under the control of the CMV promoter was found to induce protective immunity to many pathogen diseases in farmed animals (Van Drunen *et al.,* 2000). Therefore, the potential of DNA vaccination strategy in shrimp using VP28 gene against WSSV was investigated. Rout *et al*. (2007) constructed DNA vaccines using vector pVAX1 (Invitrogen, Carlsbad, CA, USA), which containing CMV promoter. Each vector carried a WSSV gene (VP15, VP28, VP35 and VP281). The vaccines were injected IM to *P. monodon*. PCR was used to detect the plasmid carrying VP28 gene distribution in shrimp tissues (hemolymph, pleopods, telson, gills, hepatopancreas and gut). They found that on days 2-15 post vaccination, VP28 was detected in all tissues. However, on day 45 it was not detected in hemolymph and day 60 it was not detected in hemolymph and hepatopancreas. Rout *et al*. (2007) also compared the effects of rVP28 (2 IM doses of 1µg/ 1g of shrimp on days 1 and 5) and a DNA vaccine (single dose of 2µg/ 1g of shrimp). On day 14, RPS was better for the rVP28 group (65%) than for the DNA vaccine group (48%). However, the DNA vaccine provided longer protection (30% RPS on day 60 vs. -11.6% for the rVP28 group). These results suggested that a DNA vaccine could provide long-term antigen expression through a genetic immunization strategy. Therefore, it offered continuing protection ability against WSSV at least up to 60 days.

Similar results were obtained by Rajesh Kumar and co-workers (2008) using a DNA vaccine constructed from the pcDNA 3.1 expression vector, which also has the CMV promoter and the VP28 gene. The VP28 was detected by PCR in abdominal muscle, gill, hepatopancreas, pleopods and gut on days 3, 15 and 30 post-immunization. The DNA vaccine protected against WSSV up to 30 days post-immunization (RPS values were 90% on day 7 and 57% on day 30). Prophenoloxidase (proPO) and superoxide dismutase (SOD) activities were measured to assess the immunological response to the DNA vaccine encoding VP28. Both activities increased significantly on day 7 post-immunization and then gradually decreased. Both activities were higher than those in the control groups till day 30. However, the superoxide anion concentration did not change significantly during the experiment.

Attenuated *Salmonella typhimurium* have been used as a system for expressing foreign antigens under the control of a eukaryotic promoter for vertebrate host cells (Abudul Wahid and Faubert*,* 2007; Chen *et al*., 2006; Du and Wang, 2005; Li *et al.,* 2006). Ning *et al.* (2009) used attenuated *S. typhimurium* to express a DNA vaccine encoding VP28 and orally administered them to crayfish, *Procambarus clarkii*. The DNA vaccine was detected in hemolymph, ovary, male reproduction system, heart, hepatopancreas and abdominal muscle from day 4 to day 30 post-immunization. By the end of the experiment (day 30 post-immunization), the vaccine was detected by reverse transcription PCR only in the abdominal muscle. These results correlated with cumulative mortality data following WSSV challenge. The RPS values following WSSV challenge were 83% on day 7, 67% on day 15 and 57% on day 25 post-vaccination. Together, these findings suggest that VP28 DNA vaccine can be easily produced and can provide long-term protection against WSSV.

4. RNA interference

RNA interference (RNAi) is one of the new procedures to control WSS by induces sequence-specific antiviral silencing. Moreover, nonspecific double stranded RNA (dsRNA) was reported to induce shrimp innate immunity (Robalino *et al.,* 2004). Using VP28 dsRNA sequence-specific silencing was successfully demonstrated by Robalino *et al.* (2006). They found that injection with VP28 dsRNA (5µg/ 1g of *L. vannamei*) could increase shrimp survival rate against WSSV, while, sequence-nonspecific dsRNA caused only a delay in the onset of mortality. Westenberg *et al.* (2005) hypothesized that delivery of short interference RNA (siRNA), rather than large dsRNA provided a specific anti-viral response. Therefore, they injected siRNA against VP28 (1.6Mol/1g of *P. monodon*) and challenge with WSSV. The results showed that significantly lower mortality rate was observed in shrimp treated with VP28 siRNA but not significantly different from shrimp treated with sequence-nonspecific siRNA (GFP). Xu and co-workers (Xu, Han and Zhang, 2007) demonstrated the potential for the gene function research and therapeutic treatment of WSSV using four different VP28 siRNAs. However, only injected with VP28 siRNA (3.2g/ 1g of *M. japonicus)* consistent with the design rule for RNAi (Elbashir *et al.,* 2002) could increase the shrimp survival against WSSV. In order to produce high amount of dsRNA, *E. coli* HT115(DE3) the expression of VP28 dsRNA was demonstrated by Sarathi and colleagues (Sarathi *et al.,* 2008a; Sarathi *et al.,* 2008b). VP28 dsRNA was injected in to *P. monodon* (2.5g/ 1 g shrimp) (Sarathi *et al.,* 2008a). RT-PCR analysis was employed to confirm gene silencing effected

after challenge with WSSV. Shrimp treated with VP28 dsRNA did not present VP28 gene at the transcription level. VP28 dsRNA significantly increased shrimp survival rate against WSSV. In addition, they orally administed VP28 dsRNA using bacterially expressed VP28 dsRNA (Sarathi *et al.,* 2008b). The inactivated *E. coli* expressed VP28 dsRNA was mixed with the shrimp commercial pellets and fed to shrimp. Also the purified dsRNA from VP28 dsRNA expressed *E. coli* was coated with chitosan and mixed with shrimp commercial pellet before feeding to shrimp followed by challenge with WSSV. PCR and Western blot analysis were employed to confirm the gene silencing effected after challenged with WSSV. The RT-PCR and Western blot analysis result showed that inactivated *E. coli* expressed VP28 dsRNA and chitosan coated purified VP28 dsRNA treatments were not present VP28 after challenged with WSSV. However, inactivated *E. coli* expressed VP28 dsRNA and chitosan coated purified VP28 dsRNA treatments gave 32% and 63% mortality, respectively, while all control treatments gave 100% mortality after challenge with WSSV. All finding suggested that RNAi showed potential to increase shrimp survival rate against WSSV.

CONCLUSION AND DISCUSSION

Although PCR, which is used to screen post larvae for WSSV, is highly sensitive in WSSV detection, there are practical limitations to its widespread application, including the high cost involved in the setting up laboratory with special equipment and well-trained personnel (Sithigorngul *et al.,* 2000). Furthermore, Lo *et al.* (1998) have indicated that results positive by the highly sensitive 2-step PCR (nested PCR) are not always associated with pond outbreaks and may have limited value for field prognosis. However, 1-step PCR is strongly associated (Withyachumnarnkul, 1999). Some immunological protocols using polyclonal antibodies to whole virus antigens have also been reported (Nadala *et al.,* 1997) but their efficiency is affected by non-specific and false-positive reactions. Therefore, the use of rVP28 to produce monoclonal and polyclonal antibodies targeting rVP28 for diagnosis was suggested (Anil, Shanker and Mohan, 2002; Chaivisuthangkura *et al.,* 2004; Liu *et al.,* 2002; Makesh *et al.,* 2006; Poulos *et al.,* 2001; Shih *et al.,* 2001; Zhan *et al.,* 1999). *In vivo* neutralization experiments with neutralizing antibodies have been used for many viruses and have led to passive immunization strategies for WSSV (Kim *et al.,* 2004; Musthaq *et al.,* 2006; Natividad *et al.,* 2007; Robalino *et al.,* 2006; van Hulten *et al*., 2001b; Wu, Wang and Zhang, 2005).

Recombinant VP28 is wildly used to control WSS although the protection mechanism is unclear and its efficacy varies. One possible mechanism of rVP28 is that it binds to shrimp VP28 binding protein, preventing the binding of WSSV to cells (Xu *et al.,* 2006). Similarly, mixing anti-PmRab7 antibody with WSSV before it was inject to shrimp was found to increase survival rate against WSS (Srituyalucksana *et al.,* 2006) and by using dsRNA to suppress expression of PmRab7 (Ongvarrapone *et al.,* 2008). Another possibility suggested by many studies is that rVP28 stimulates shrimp immunity or acts as a vaccine (Bright Singh *et al.,* 2005; Caipang *et al.*, 2008; Chang *et al.,* 2003; Du *et al.,* 2006; Jha *et al.,* 2006a; Jha *et al.,* 2006b; Jha *et al*., 2007; Mavichak *et al*., 2009; Rout *et al.*, 2007; Song *et al.,* 1997; Takahashi *et al.,* 2000; Vaseeharan *et al.,* 2006; Witteveldt *et al.,* 2004a;

Witteveldt *et al.*, 2004b; Witteveldt *et al.,* 2006). Although, the evidence that rVP28 increases the shrimp survival rate against WSSV, the protection ability of rVP28 varies and seems to rely on the amount of proteins received (Caipang *et al.,* 2008; Jha *et al*., 2006b). However, most traditional rVP28 are not cost-effective because they cannot be stored at room temperature. Therefore, there is a need to improve the "storage" of protein-based vaccine to make it more convenient and effective. The alternative expression methods such as plant-based systems that would address "storage" problems should be pursued in future studies.

DNA vaccine is prepared using expression plasmid containing the protective gene for the antigen. It confers high and long-term protection against WSSV. Moreover, it can be stored at ambient temperature; it is easier than recombinant protein vaccine to be prepared at large scales and orally administrated which is practical for shrimp farming. For these reasons, DNA vaccine seems to be the best answer to control WSSV.

The sequence-specific effects of double-stranded RNA (dsRNA) that result in endogenous RNA degradation are widely conserved and probably present in most invertebrates. In contrast, inducing immune response against virus using non-specific dsRNA was demonstrated in shrimp (Robalino *et al.,* 2004). Therefore, dsRNA can engage both innate immune pathways and an RNAi-like mechanism to stimulate potent antiviral immunity in shrimp. From the previous reported, VP28 dsRNA showed the potential to prevent shrimp from WSSV and could be applied for commercial scale because it could produce in large scale using bacterially expressed dsRNA system (Sarathi *et al.,* 2008a; Sarathi *et al.,* 2008b). Moreover, the oral administration strategy is possible, although its efficacy is lower than IM injection method. Therefore, the delivery method needs to be studied using adjuvant or carrier to improve the efficacy of dsRNA oral administration.

In conclusion, the potential of using VP28 gene to diagnose and control WSSV has been studied recently. However, in order to use VP28 in commercial scale in the shrimp industry, there are several issues that need to be addressed including prolonged storage, improving delivery method, increasing its efficiency, the continues long-term protection and ensuring that it can be produced in large scale. Therefore, in the future studies, these issues and also new strategies to control WSSV should be studied to apply to shrimp commercial farming.

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