Application of Carriers and RNAi to Enhance the Antiviral Immune Response of Shrimp to WSSV

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Abstract

In aquaculture, vaccination is one of the approaches for disease prevention and control. The aim of the present study was to determine the efficacy of a VP28 double stranded RNA (VP28 dsRNA) and recombinant VP28 protein (rVP28) administered together as an antiviral treatment against WSSV. Double-stranded RNA was produced in RNAse-deficient *Escherichia coli* HT115 following published methods. To determine the appropriate dose, different concentrations of dsRNA ranging between 0.2 µg and 20 µg, were either injected intramuscularly or delivered orally to the shrimp via the feed ration. Thereafter, the shrimp were challenged with WSSV either by injection ($LD_{50}=10^{-7}$ dilution of the gill tissue filtrate) or bath immersion ($LD_{50}=10^{-4}$ dilution of the filtrate) in glass aquaria and transferred to fiberglass tanks for daily monitoring and recording of mortalities. Results showed significant differences in survival between PBS and the 0.2, 10, and 20 µg dsRNA/shrimp doses. Time to 100 % mortality significantly differed among the treatments with the control reaching mortality earlier (day 4) while shrimp injected with 0.2 and 10 µg dsRNA succumbed to WSSV much later on days 9–12. Different frequencies of dsRNA administration were also tested. The best result obtained was a dose of 20 µg/shrimp administered 4 times over 28 days (2 times before and 2 times during challenge for a total 80 µg/shrimp). Finally, VP28 dsRNA was combined with rVP28 at ratios of 1:1, 1:2, 1:3, 3:1, and 2:1, entrapped in chitosan microparticles and delivered per os via the feed according to the dose and frequency as previously determined. Following bath exposure challenge with WSSV, the best survival obtained in trials 1 and 2 was 40 % and 43 % at 1:3 VP28 dsRNA to rVP28 ratio.

Introduction

In shrimp aquaculture, a safe, effective, and inexpensive antiviral treatment is required to limit the impact of WSSV and other shrimp viruses. Successful vaccination in shrimp using whole formalin-killed virus or recombinant protein or plasmid DNA has been reported (Witteveldt et al., 2004; Rout et al., 2007). Vaccine vectors or carriers are examined based on their safety profile, economics, and practical utility. Vaccine carriers that protect antigens from degradation, ensure release of adequate antigen dose, contain immunomodulatory substances, and suitable for oral delivery in commercial grow-out systems such as ponds are deemed appropriate. Alginate and chitosan microparticles were previously tested as microparticle drug delivery systems in fish and freshwater prawn (Rodrigues

et al., 2006; Anas et al., 2008) and as recombinant vaccine carrier for giant tiger shrimp (*manuscript in preparation*). Owing to their properties, microparticles can deliver drugs or antigens comparable to or better than non-encapsulated inactivated whole virus when added to feed rations (Amar and Faisan, 2011; Amar *et al*., 2021). RNAi is an emerging technology that is based on gene silencing (Sagi *et al*., 2013). The silenced gene, by degrading its mRNA, is unable to produce the protein that performs an essential function. It has been applied in aquaculture in sex manipulation and control of reproduction (Ventura *et al*., 2009; Treerattrakool *et al*., 2011; 2013) and lately in disease control in crustaceans (Xu *et al*., 2007; Escobedo-Bonilla, 2011; Le Fauce and Owens, 2012). The antiviral effect of RNAi is based on silencing a viral or host gene that is primarily involved in viral pathogenesis. The main constraint of RNAi as an antiviral agent is production cost and a practical method of delivery. Thus, methods to reduce production cost of dsRNA as well as application of the microparticle method of delivery were examined. Moreover, a scheme where the two treatments are combined to enhance efficacy was explored. The main objective of the study was to apply emerging technologies in the management of WSSV infections in shrimp. Specifically, the study aimed to evaluate the efficacy of dsRNA treatment in protecting shrimp against WSSV, develop a prophylactic scheme combining the two treatments (rVP28+rVP28 dsRNA), and develop an inexpensive vaccine/drug delivery protocol for WSSV prevention in tanks and in ponds-based culture systems.

Materials and methods

Isolation of primary lymphoid organ (LO) cells and observation of CPE

Primary shrimp cells were isolated from lymphoid organ following the method of Assavalapsakul *et al*. (2003) with modifications. Briefly, the lymphoid organ was excised and pushed gently against a 100 µ mesh steel screen with a rubber-tipped syringe plunger to force the cells onto a sterile petri dish with Leibovit's (L15) medium. All materials used were sterilized by autoclaving. The isolated cells were washed several times in L15 by repeated pipetting and centrifugation, and the final cell pellet was suspended in L15 and counted under a microscope with a haematocytometer. Shrimp cells were plated in 96-well plate at an estimated density of 10^6 cells ml^{-1}. Then, 10^0-10^{-3} dilutions of the virus supernatant from homogenates of infected gill tissue were added and the cells were observed for the development of CPE for 7 days. The negative control wells contained cells without the virus. The number of cytopathic foci were assessed microscopically under 400x magnification.

Preparation of double-stranded RNA

Double-stranded RNA was prepared using a low-cost *in vivo* bacterially expressed dsRNA production method described by Ongvarrasopone et al. (2007). In this method, a strain of *E. coli* lacking RNAse III (HT115) was transformed with a plasmid containing the T7 RNA polymerase promoter and a DNA sequence (VP28

gene) homologous to a target viral protein (GenBank accession no. AF380842). The bacteria was then cultured and induced by IPTG to produce dsRNA that was then extracted from the bacterial cell by a combination of boiling in 0.1 % SDS, and protease and RNAse treatment to remove protein, single stranded RNA and total RNA of the host cell (**Figure 1**). The dsRNA was quantified using a nano spectrophotometer.

Evaluation of the efficacy of RNAi in protecting shrimp against WSSV infection

The efficacy of dsRNA treatments (both by intramuscular injection and by oral administration) was tested *in vivo* in tanks trials. dsRNA (100 µl) was first injected to 10 g shrimp at 0.2 and 10 µg/shrimp. Twenty-four hours after dsRNA injection, shrimp were injected with the virus (100 µl of 10^{-7} dilution of the infected gill tissue supernatant (LD_{50} as determined by the method of Reed and Muench (1938) from an earlier study) and returned to the tanks for observation and recording of mortality for 10 days. Semi-quantitative determination of the viral load by PCR in the hemolymph of the treated and control shrimp was also performed. The mean mortality values of dsRNA-treated and control shrimps were compared. Based on the results of the first trial, trial 2 was conducted using 0.2, 10, and 20 µg/shrimp (15 g) but instead of injection, dsRNA was administered orally through the feed for 14 days, and the shrimp challenged with WSSV by immersion at a dilution of 10^{-4} of the gill tissue supernatant (immersion LD_{50} as determined earlier). Finally, trial 3 was conducted using the best treatment obtained in trial 2 (20 µg/shrimp x 2 times before challenge and 2 times after challenge for a total of 80 µg/shrimp for 28 days). Different frequencies (8x, daily, 4x at 20 and 30 µg/shrimp over 28 days) were tested to determine whether a dose given once or divided into several smaller doses would result in better survival upon immersion challenge. A control group without dsRNA treatment and *E. coli* dsRNA were added to account for non-specific dsRNA treatment effects.

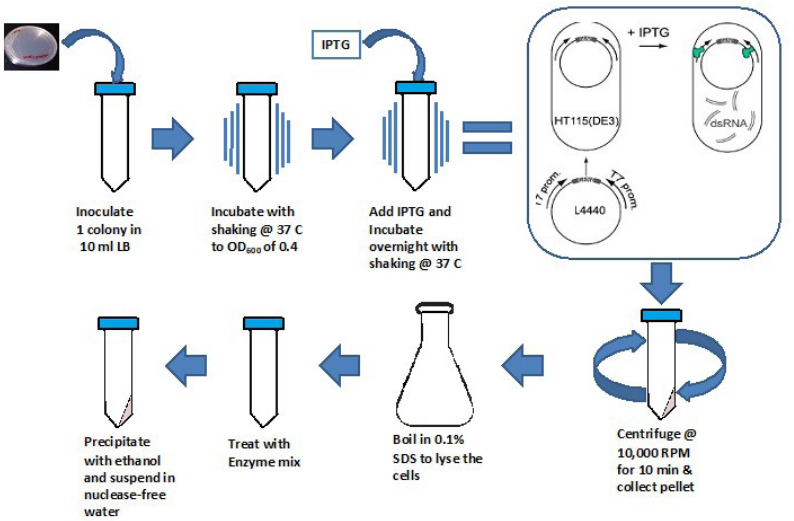

Figure 1. Preparation of VP28 double-stranded RNA. Cloning and transformation (a); culture of bacteria and extraction of bacterially-expressed dsRNA

Preparation of alginate and chitosan microparticles

Microparticles were prepared by ionotropic gelification following the method of Aral and Akbuga (2003) which was adapted and modified for the project. Briefly, chitosan solution was prepared by dissolving 0.35 g chitosan powder in 100 ml 1 % v/v Tween 80 and 2 % v/v Acetic acid, stirred for faster solubilization, and the dsRNA and/or solubilized inclusion bodies containing the VP28 protein were added. Ten milliliters 20 % sodium sulfate was added drop by drop and stirred continuously for 1h at the highest possible speed. The mixture was then transferred to a 50 ml blue cap tube and centrifuged at 1100 g for 30 min at room temperature (8,237 rpm in CR 21G centrifuge). The supernatant was decanted and the weight of the microparticles produced was determined. Alginate microparticles were prepared according to Rodriguez et al. (2006) and Tian et al. (2008). Alginate was dissolved in distilled water (3 % w/v) with dsRNA and/or solubilized rVP28 inclusion bodies. This is the aqueous phase. The oil phase was 2 % w/v Tween 80 in vegetable oil, whereas the gelification solution was 6.8 g CaCl2.2H2O+ 30 ml distilled water+ 30 ml ethanol+ 2 ml glacial acetic acid. The alginic suspension with dsRNA and protein was poured to 20 ml oil mixture to form the AS+P+O mix and stirred at maximum speed for 10 min in a magnetic stirrer. The AS+P+O mix was added to the gelification solution and stirred at maximum speed for 30 min. Layers were allowed to separate for at least 2 h. The top oil layer was removed by pipetting and the aqueous layer was centrifuged at 500 g for 25 min at 25°C to obtain the alginate microparticles. Microparticles were stored at 4°C until use. Encapsulation efficiency, loading percentage and yield of microsphere were evaluated both for alginate and chitosan microparticles using albumin as standard protein.

Tank evaluation of encapsulated dsRNA with rVP28 vaccine

A combined VP28 dsRNA and rVP28 protein vaccine encapsulated in alginate and chitosan microparticles and delivered orally through the diet was next tested to determine the proportion of rVP28 vaccine to VP28 dsRNA that could enhance overall efficacy of the treatment. This was done by incorporating the encapsulated dsRNA+ vaccine into the feeds, followed by feeding for 2 weeks at a dose determined earlier, tank challenge trial, and feeding for another 2 weeks until completion of the challenge test. Recombinant VP28 vaccine was prepared as described in previous reports. Encapsulation of VP28 dsRNA+rVP28 vaccine was done at different dsRNA to protein ratios using both alginate and chitosan microparticles. The WSSV challenge was conducted by bath-immersion in 1 LD_{50} or 10^{-4} dilution of the infected gill tissue supernatant.

Statistical analysis

Unless otherwise stated, data were expressed as means of 3 replicates ± standard error of the mean. The data were analyzed with Analysis of Variance (ANOVA) with post-hoc multiple comparison of means by Tukey's Highly Significant Difference Test (Tukey's-HSD). Percentage data were checked for normality and were arcsine-transformed before analysis. Differences were considered statistically significant when $p<0.05$.

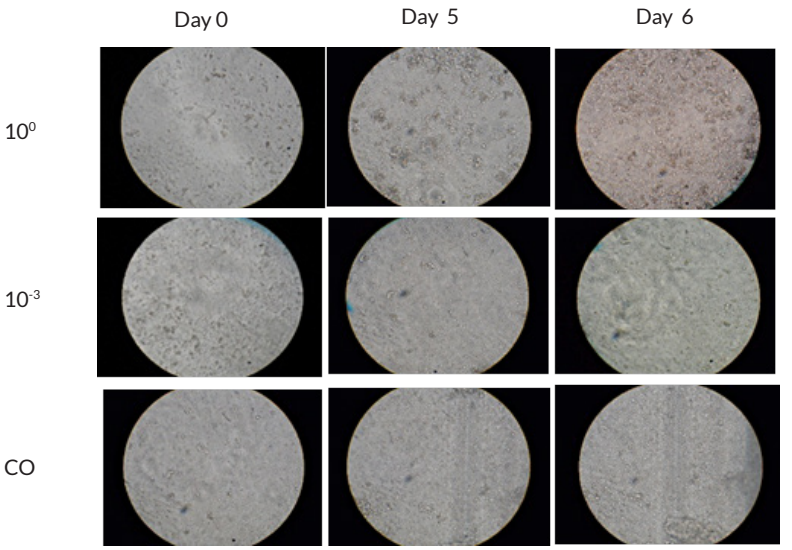

Figure 2. Primary cells of the *P. monodon* lymphoid organ of showing CPE upon in vitro infection with 10^0 and 10^{-3} dilution WSSV

Results

Isolation of primary lymphoid organ (LO) cells and observation of CPE

After 5 days of incubation, the cells showed signs of CPE (detachment from the well bottom and aggregation) due to WSSV infection. CPE was clearly found at 10^0 to 10^{-3} dilutions of the WSSV tissue filtrate whereas the higher dilutions had inconclusive results (**Figure 2**). The cell cultures could not be successfully observed for longer periods because of contamination. As the results indicated the need for further optimization of the primary cell culture, it was decided that *in vivo* evaluation would be employed from then on.

Preparation of double-stranded RNA

The agarose gel electrophoresis of the VP28 DNA that was PCR amplified from WSSV-infected shrimp gill tissues, the EcoRV-digested pL4440 plasmid, and the gel band of the colony PCR of the transformed bacteria containing recombinant plasmid (HT115/pL4440VP28) are shown in **Figure 2**. **Table 1** shows the quantity of dsRNA produced by representative colonies using the commercial kit and the SDS method.

Evaluation of the efficacy of RNAi in protecting shrimp against WSSV infection

Initial results showed significant differences in survival between PBS and 0.2 and 10 µg/shrimp dsRNA dose on day 3-7 post-challenge. Time to reach 100 % mortality also significantly differed among treatments with the control reaching 100 % mortality on day 4 while shrimp that received 0.2 and 10 µg/shrimp eventually died on days 9-12 (**Figure 3**). In trial 2, the best treatment was 20 µg/shrimp delivered 4x (2 x before challenge and 2x during challenge) which had 70 % cumulative mortality and significantly different from the control. Although not all shrimp died, those given 0.2 and 10 µg/shrimp delivered

Table 1. Quantity of dsRNA (µg/ml) produced by representative colonies C1-C7 using a commercial RNA extraction kit and the long method of SDS and enzyme treatment

Colony	SV RNA Kit	0.1% SDS and enzyme treatment for medium-scale prep
C1	0.772	164.5
C2	0.831	160.6
C3	0.506	158.8
C4	0.589	149.5
C5	0.341	131.0
C6	0.371	141.8
C7	0.498	148.0

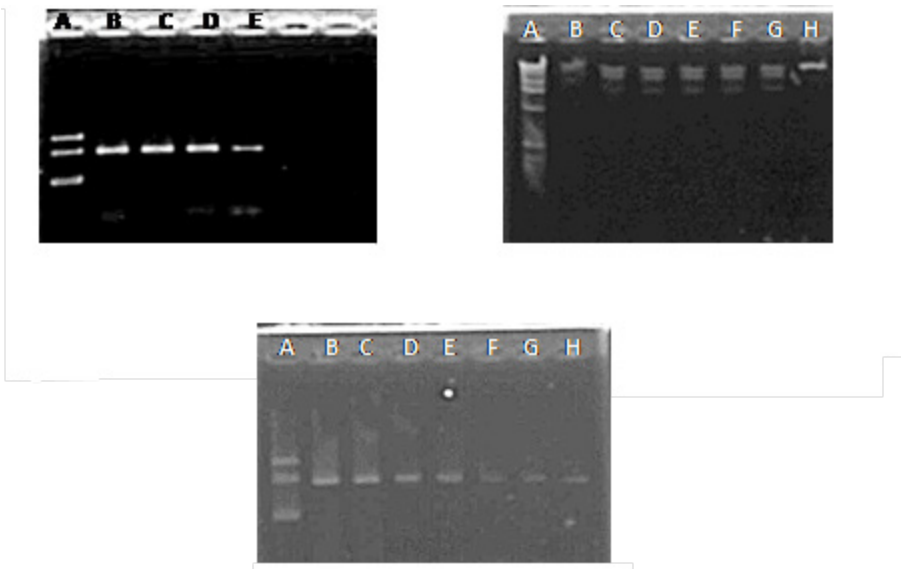

Figure 3. Agarose gel electrophoresis of VP28 DNA fragment after PCR of WSSV-infected gills of shrimp (A); EcoRV-digested pL4440 plasmid vector showing two bands (B-G) for the digested and one band only (H) for the undigested plasmid (B); and colony PCR of the transformed bacteria (HT115) harboring the recombinant plasmid pL4440VP28 (C)

4x (2 x before challenge and 2x during challenge) had survival that did not differ from the control (**Figure 4**). In trial 3, the frequencies from daily to 8 times over 28 days did not differ among treatments but these groups had cumulative mortalities ranging from 63-68% which were lower than the untreated control and unrelated dsRNA (100%) (**Figure 5**)

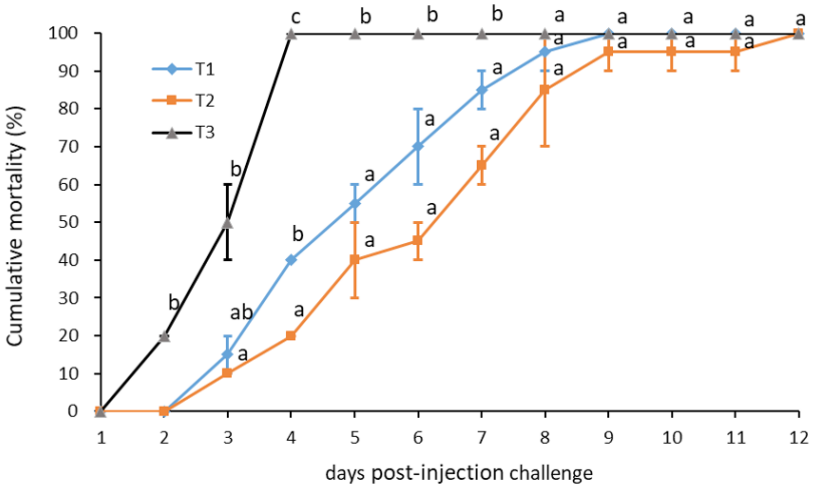

Figure 4. Cumulative mortality of the VP28 dsRNA-injected *Penaeus monodon* 12 days post-injection challenge with 1 LD_{50} of WSSV. T1, 0.2 µg/shrimp; T2, 10 µg/shrimp; T3, PBS. Line graphs represent means SEM (n=2 replicate tanks). Means at each time point with the same letter superscripts are not significantly different (p>0.05)

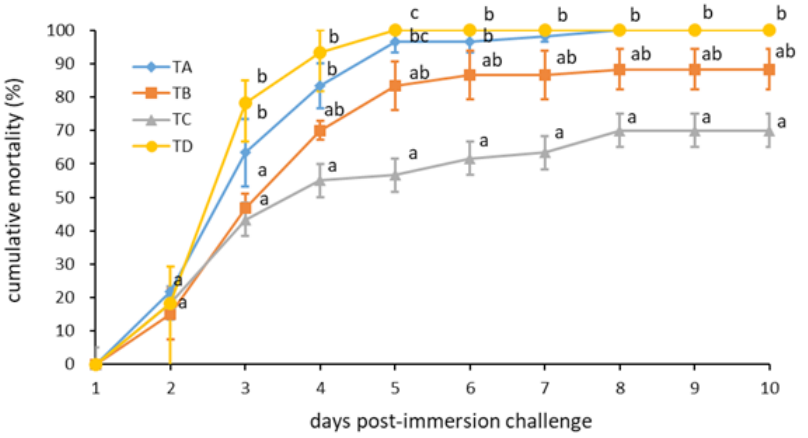

Figure 5. Cumulative mortality in *P. vannamei* fed with VP28 dsRNA and challenged with WSSV (TA, 0.2 µg/shrimp; TB, 10 µg/shrimp; TC, 20 µg/shrimp; TD, control). Line graphs represent means SEM (n=3 replicate tanks). Means with the same letter superscripts are not significantly different (p>0.05)

Preparation of microparticles and evaluation of their encapsulation efficiency, loading percentage, and yield

The chitosan and alginate microparticles as imaged by scanning electron microscope are shown in **Figure 6**. During the first 3 trials using albumin as the model protein, chitosan had 0.13 % loading percentage, 73.07 % encapsulation efficiency and 99.59 % yield as compared to alginate which had 0.21 %, 87.61 % and 98.72 %, respectively. Using both antivirals, alginate microparticles had higher encapsulation efficiency of 82.95 % as compared to 56.09 % for chitosan. Loading percentage for both antivirals was at 0.03 % and

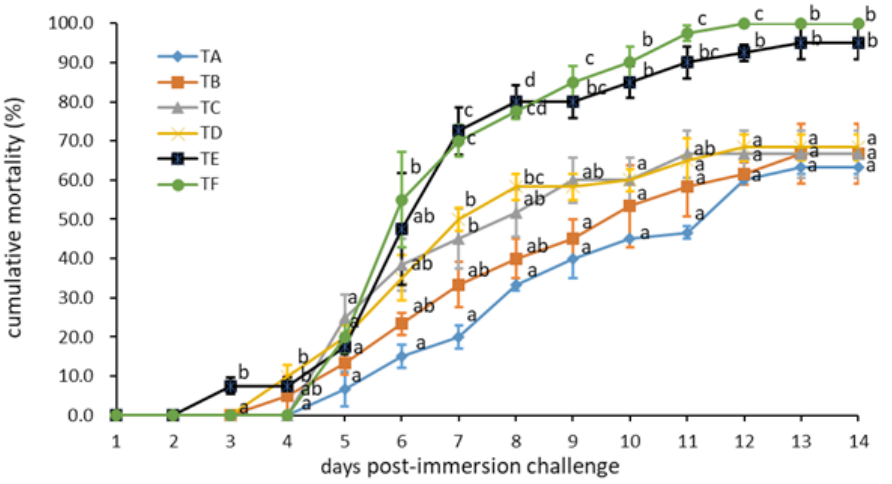

Figure 6. Cumulative mortality of *P. vannamei* fed dsRNA for 28 d at a single dose and different frequencies (TA, 2.86 µg/shrimp daily; TB, 10 µg/shrimp/ 8d; TC, 20 µg/shrimp/4d; TD, 30 µg/shrimp/4d; TE, E. coli non-specific dsRNA; TF, PBS. Line graphs represent means SEM (n=3 replicate tanks). Means at each time point with the same letter superscripts are not significantly different (p>0.05)

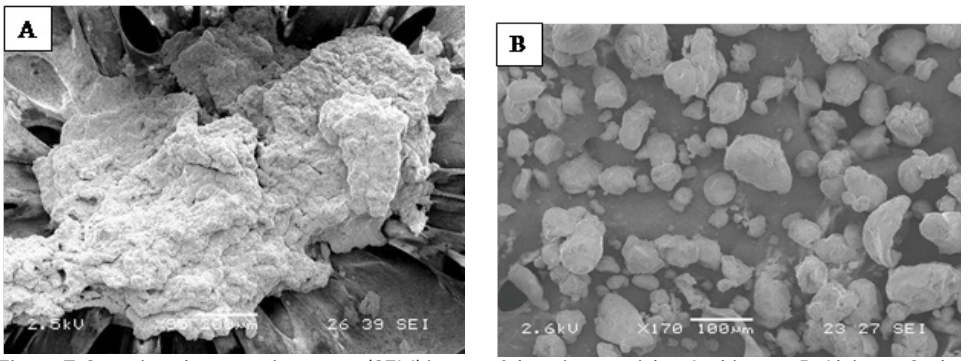

Figure 7. Scanning electron microscope (SEM) image of the microparticles. A, chitosan; B, Alginate. Scale bar: 100 µm

Table 2. Evaluation of the loading percentage, encapsulation efficiency, and yield of microspheres of dsRNA and rVP28 using chitosan and alginate. Albumin was used as the protein standard

	Chitosan	
Variables Calculated	**Albumin (%)**	**dsRNA and BL21 (%)**
Loading Percentage	0.13	0.03
Encapsulation Efficiency	73.07	56.09
Yield of Microspheres	99.59	99.97

	Alginate	
Variables Calculated	**Albumin (%)**	**dsRNA and BL21 (%)**
Loading Percentage	0.21	0.03
Encapsulation Effiency	87.61	82.95
Yield of Microspheres	98.72	99.98

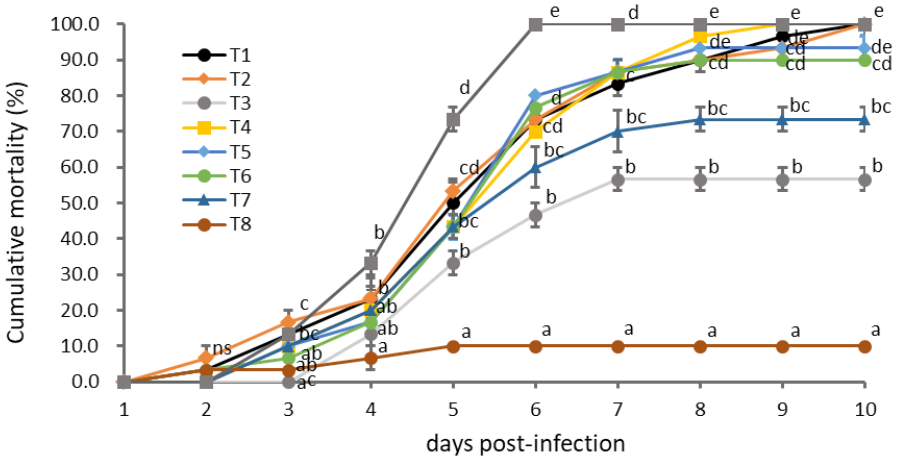

Figure 8. Cumulative mortality of *P. vannamei* fed for 28d at different VP28 dsRNA to rVP28 ratios: 1:1 (T1); 1:2 (T2); 1:3 (T3); 2:1 (T4); and 3:1 (T5); dsRNA only (T6); rVP28 only (T7); PBS Negative control (T8); virus positive control (T9). Trial 1. Line graphs represent means SEM (n=3 replicate tanks). Means at each time point with the same letter superscripts are not significantly different (p>0.05)

yield of microspheres was at 99.97 % for chitosan and 99.98 % for alginate (**Table 2**).

Survival of shrimp fed the combined VP28 dsRNA and rVP28 at different ratios

Survival of shrimp fed VP28 dsRNA plus rVP28 at different ratios ranging from 1:1 to 1:3 and 3:1 to 2:1 are shown in **Figure 7 (trial 1)** and **Figure 8** (**trial 2**). In both trials, shrimp fed dsRNA and protein at a ratio of 1:3 exhibited the highest survival after being challenged with WSSV at 1 immersion LD_{50} (10^{-4} dilution of the viral supernatant). However, in trial 1, this ratio had mortality that was significantly lower than dsRNA alone, the control, and the rest of the ratios but not lower than rVP28 alone (**Figure 7**). Similarly, in trial 2, the 1:3 ratio was significantly lower than the control but not lower than the rest of the treatments (**Figure 8**).

Discussion

In this study we found that dsRNA at a dose of 20 µg/shrimp administered 4x over 28 days gave the highest survival in shrimp challenged with 10^{-7} LD_{50} WSSV. For lower doses by injection of dsRNA, the results indicated that shrimp were protected from WSSV infection until day 7 post challenge. However, the shrimp eventually suffered 100 % mortality, although at different days post challenge. There are a few explanations for this result. The dsRNA dose might be too low

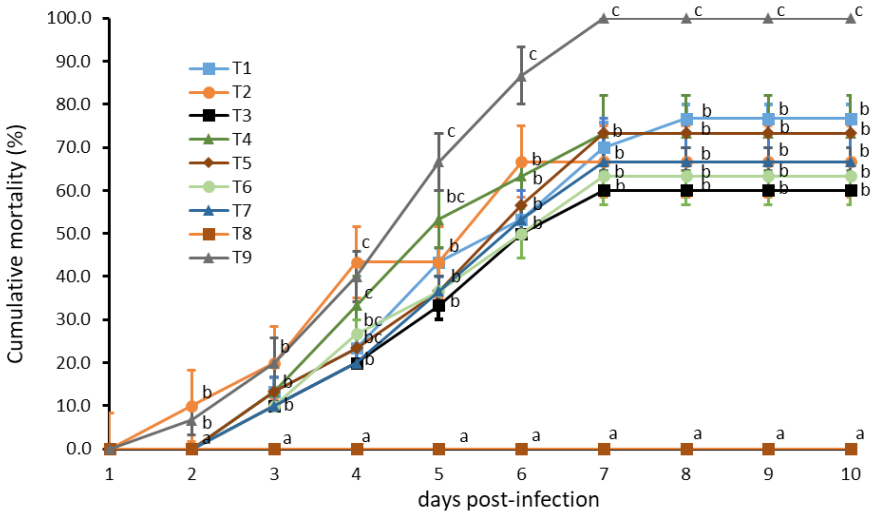

Figure 9. Cumulative mortality of *P. vannamei* fed for 28d at different VP28 dsRNA to rVP28 ratios: 1:1 (T1); 1:2 (T2); 1:3 (T3); 2:1 (T4); and 3:1 (T5); dsRNA only (T6); rVP28 only (T7); PBS Negative control (T8); virus positive control (T9). Trial 2. Line graphs represent means SEM (n=3 replicate tanks). Means at each time point with the same letter superscripts are not significantly different (p>0.05)

or that the injected dsRNA might not be able to persist in the tissue long enough to sustain its knockdown effect and exert protection. Against gill-associated virus, (GAV) injection but not oral administration protected shrimp from infection (Sellars et al., 2011), but oral administration was able to protect shrimp from WSSV (Sarathi et al., 2008). Subsequent experiments were then conducted with higher doses of dsRNA and frequency of administration. To examine if the persistence of dsRNA in the tissue might be affected by dose and frequency, dsRNA was administered orally by feeding it to the shrimp several times over the duration of the experiment. Also, the viral challenge method adopted was by bath-immersion as this method would likely give a more natural progression of mortality. Based on the results of the 3 trials, the best dose appeared to be 20 µg/shrimp administered 4x over 28 days. Increasing the frequency to 8 times or daily over 28 days did not further improve the results. The effect of dsRNA was specific to VP28 as non-specific *E. coli* dsRNA did not protect shrimp at all and had cumulative mortality similar to the untreated control. When VP28 dsRNA and rVP28 were combined, the best ratio obtained was 1part dsRNA to 3 parts rVP28 which exhibited the highest survival in the tank challenge trial.

The microparticle assay revealed that the alginate microparticles had a higher encapsulation efficiency (82.95 %) compared to chitosan microparticles (56.09 %) using both antivirals during the preliminary trials. Additionally, yield of microspheres and encapsulation efficiency for alginate was affected by the weight of the microparticle produced. This was due to the inability of alginate microparticles to separate from its liquid component and the weight of the microparticle produced was affected by the presence of entrapped liquid. Therefore, the higher values for encapsulation efficiency in alginate preparation was due to the increased wet weight of the microparticle produced. This result suggests that chitosan is a better microparticle to use when encapsulating both antivirals for incorporation into the shrimp feed.

A ratio of 1 part VP28 dsRNA to 3 parts rVP28 proved to be the best combination in terms of protecting the shrimp against WSSV infection. However, the combined treatment did not consistently improve the survival over dsRNA alone (significant difference was found only in trial 1) and did not improve survival over rVP28 alone (both trial 1 and 2). This could mean that addition of rVP28 improves the effect of VP28 dsRNA, but addition of dsRNA does not enhance the effect of rVP28. There was no direct comparison between the non-encapsulated and encapsulated VP28 dsRNA, rVP28 or their combination but our previous study estimated a 24–30 % increase in survival with the use of microparticle carriers and rVP28 (*manuscript in preparation*). Apart from potentially increasing survival, use of microparticle carriers could facilitate oral delivery of antiviral molecules such as dsRNA and protein without compromising their efficacy.

Conclusion

VP28 dsRNA was effective in reducing mortality due to WSSV infection in tank experiments. Based on the results of 3 trials, the best treatment was a dose of 20 µg/shrimp administered 4 times over 28 days before and during challenge for a total dose of 80 µg/shrimp. A higher dose and more frequent administration did not further increase survival. The resistance against WSSV challenge was specific to VP28 dsRNA as heterologous dsRNA gave no significant protection. The best ratio of VP28 dsRNA to rVP28 was found to be 1:3 which elicited 40–43 % protection in WSSV challenge tests. However, while addition of rVP28 significantly improved survival compared to VP28 dsRNA alone, addition of VP28 dsRNA did not significantly improve survival compared to rVP28 alone. A field efficacy evaluation of the microparticle-encapsulated VP28dsRNA and/or rVP28 by oral delivery (via feeding) in brackish water ponds is recommended.

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