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Protection against white spot syndrome virus (WSSV) infection in kuruma shrimp orally vaccinated with WSSV rVP26 and rVP28

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ABSTRACT: White spot syndrome virus (WSSV) is the causative agent of white spot disease (WSD), one of the most serious diseases affecting global shrimp farming. We compared WSSV infection induction in kuruma shrimp *Marsupenaeus japonicus* by oral, immersion, and intramuscular injection (IM) exposure methods and evaluated the oral vaccine prepared from the recombinant WSSV proteins rVP26 and rVP28. The 50% lethal doses (LD_{50}) of WSSV by oral, immersion, and IM challenges were $10^{-0.4}$, $10^{-4.4}$, and $10^{-7.7}$ g shrimp^{-1}, respectively, indicating that WSSV infection efficiency by oral challenge was significantly less than the other 2 challenge routes. However, in shrimp farms it is believed that WSSV infection is easily and commonly established by the oral route as a result of cannibalization of WSSV-infected shrimp. Kuruma shrimp vaccinated orally with WSSV rVP26 or rVP28 were challenged with WSSV by oral, immersion, and IM routes to compare protection efficacy. The relative percent survival values were 100% for oral challenge, 70 to 71% for immersion, and 34 to 61% for IM. Thus, the protection against WSSV-infection that was induced in kuruma shrimp by oral vaccination with rVP26 or rVP28 seemed equivalent to that obtained through IM vaccination.

KEY WORDS: Oral vaccination · White spot syndrome virus · WSSV · *Marsupenaeus japonicus* · Kuruma shrimp · Quasi-immune response

INTRODUCTION

White spot disease (WSD, a synonym of penaeid acute viremia, PAV) is one of the most serious diseases of cultured decapod crustaceans throughout the world (Lightner 1996, Wang et al. 1998). White spot syndrome virus (WSSV, a synonym of penaeid rod-shaped DNA virus, PRDV) (Inouye et al. 1996), the causative agent of WSD, is a member of the genus *Whispovirus* in the family *Nimaviridae* (Valk et al. 2004). WSSV is ovoid or ellipsoid to bacilliform in shape with regular symmetry (Wongteerasupaya et al. 1995). It is 120 to 150 nm in diameter and 270 to 290 nm in length, and has a thread- or flagellum-like appendage at one end (Wongteerasupaya et al. 1995). The virion consists of an inner, rod-shaped nucleocapsid with a tight-fitting capsid layer and an outer, loose-fitting, lipid-containing trilaminar envelope (Durand et al. 1997). The viral nucleocapsid contains a DNA–protein core bounded by a distinctive capsid layer and a single molecule of circular double-stranded DNA with an approximate size of 300 kbp (van Hulten et al. 2001, Yang et al. 2001). WSSV contains at least 6 major proteins: VP28 and VP19, which are associated with the envelope; VP664 and VP15, associated with the nucleocapsid; and VP24 and VP26, which are located in between the envelope and the nucleocapsid (van Hulten et al. 2000a,b, Chen et al. 2002, Leu et al. 2005, Tsai et al. 2006).

In the 1990s the kuruma shrimp *Marsupenaeus japonicus* culture industry in Japan was seriously damaged by outbreaks of WSD due to the importation of

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WSSV-contaminated kuruma shrimp seed stock originating from China (Nakano et al. 1994, Takahashi et al. 1994, 1998, Momoyama & Muroga 2005). WSSV is pathogenic to kuruma shrimp beginning at the postlarval 10 stage (PL10) (Venegas et al. 1999). The major route of WSSV infection appeared to be through vertical transmission in kuruma shrimp hatcheries, because the occurrence of WSD in postlarvae notably decreased following selection of WSSV-free broodstock (Mushiake et al. 1999). However, horizontal transmission of WSSV, both by cannibalism and through waterborne exposure, is an infection route of concern in kuruma shrimp farms (Wu et al. 2001, Momoyama & Muroga 2005). Stable seed production of specific-pathogen-free (SPF) kuruma shrimp was accomplished using countermeasures for the prevention of WSSV, such as selection of WSSV-free broodstock by PCR, disinfection of eggs with iodine, and sterilization of rearing water (Mushiake et al. 1999, Satoh et al. 2001). At shrimp farms, however, it is still difficult to prevent WSSV infection due to horizontal transmission from other crustaceans present in the farm environment and cannibalism among reared shrimp (Maeda et al. 1998, Momoyama 2003).

Recently, Venegas et al. (2000) described a 'quasi-immune response' in kuruma shrimp wherein those that naturally survived WSD were protected against subsequent WSSV challenge. Protection against WSSV infection appeared 3 wk after the primary infection and lasted 2 mo (Wu et al. 2002). Moreover, this protection toward WSSV showed a degree of specificity (Venegas et al. 2000). It is also possible to induce protection against WSSV by intramuscular (IM) injection with formalin-inactivated WSSV or with recombinant structural proteins of WSSV, rVP26 and rVP28 (Namikoshi et al. 2004). A similar degree of protection was also inducible in whiteleg shrimp *Litopenaeus vannamei*, giant tiger prawn *Penaeus monodon*, and crayfish *Procambarus clarkii* (Witteveldt et al. 2004a,b, 2006, Vaseeharan et al. 2006, Jha et al. 2006). As mentioned above, cannibalism may be one of the most important routes for the horizontal transmission of WSSV in kuruma shrimp farms; hence, the importance of oral vaccination with WSSV recombinant proteins. Recently, the effectiveness of oral vaccination with WSSV recombinant proteins in giant tiger prawns, whiteleg shrimp, and crayfish (Witteveldt et al. 2004a, 2006, Jha et al. 2006) has been reported. However, similar studies using kuruma shrimp, which require different environmental conditions for stocking and rearing (e.g. temperature) from other prawn and shrimp species, have not been reported. Thus, we investigated WSSV challenge routes for the development of an oral WSSV vaccine in kuruma shrimp, and diets containing rVP26 or rVP28 were fed to kuruma shrimp to evaluate their effectiveness as vaccines against experimental WSSV challenges by oral, immersion, and IM routes.

MATERIALS AND METHODS

Shrimp and WSSV inoculum. Kuruma shrimp (3.1 to 6.8 g) were obtained from the Kamiura Station of Stock Enhancement Technology Development Center, National Research Institute of Aquaculture, Japan and a shrimp farm with no prior history of WSD located in Miyazaki Prefecture. Shrimp were confirmed to be WSSV-free by nested PCR before being used in the experiments. The shrimp were maintained in dechlorinated, electrolyzed, flow-through seawater (24 ± 1.8°C, 33.05 ± 0.13 ppt) using double-bottomed tanks with sand beds and fed a commercial crumble diet (Shrimp feed, Juveniles P-2; Maruha) at 3% of body weight d^{-1}.

The WSSV suspension was prepared following the method reported by Nonaka et al. (1998). Briefly, muscle tissue of moribund WSD-shrimp was homogenized with 4× the volume of phosphate-buffered saline (PBS) and then centrifuged at 3000 × *g* for 10 min at 4°C. The resulting supernatant was stored at –85°C until used as a source of WSSV inoculum for the experiments.

Virulence of WSSV incoculum against shrimp. Shrimp were kept in 150 l tanks at a density of 47 to 63 shrimp m^{-2} and were challenged with WSSV by oral, immersion, or IM routes.

In the IM challenge study, the stock WSSV solution was serially diluted with PBS from 10^3 to 10^7 at 10-fold intervals. Shrimp with a mean body weight (MBW) of 6.8 g (n = 15 $group^{-1}$, 6 groups in total) were sedated by placement in 15°C seawater for 1 min and each shrimp was then intramuscularly injected with 100 µl of each inoculum or PBS (negative control).

In the immersion challenge study, shrimp with MBW of 4.4 g (n = 20 $group^{-1}$, 4 groups in total) were immersed for 1 h in 3 l of WSSV solution diluted 10^3-, 10^4-, or 10^5-fold with sterile seawater. Negative control shrimp were immersed in a 10^3-fold diluted muscle homogenate prepared from healthy shrimp. After immersion the shrimp were placed in a net, rinsed with flowing seawater for 3 min, and then returned to the rearing tanks.

In the oral challenge study, shrimp with MBW of 3.1 g (n = 15 $group^{-1}$, 5 groups in total) were fed WSD shrimp muscle at 0.25, 0.4, 0.65, or 1.02 g $shrimp^{-1}$. The maximum amount given at one feeding was kept within 15% of the MBW (≤0.5 g $shrimp^{-1}$), thus, rations exceeding 0.5 g of WSD shrimp muscle were fed to the experimental shrimp in several portions over 2 to 3 d. Control shrimp were given 1.02 g of healthy shrimp

muscle in the same manner. The WSD shrimp muscle used for the oral challenge originated from the same source as that used to prepare the WSSV homogenate utilized in the immersion and IM challenges. Following each of the 3 exposures, the test shrimp were observed for 14 d. The 50% lethal dose (LD_{50}) of the WSSV inoculum was calculated following the Behrens-Kärber method (Kärber 1931).

Preparation of shrimp diet containing rVP26 and rVP28. Recombinant WSSV proteins, rVP26 and rVP28, were prepared following the method of Namikoshi et al. (2004). Briefly, *Escherichia coli* cells, in which rVP26 or rVP28 had been induced by IPTG (isopropyl-1-1-thio-β-D-galactoside), were suspended in TE buffer (50 mM Tris–HCl and 2 mM EDTA; pH 8.0) containing 0.1% Triton X-100 and 0.1 mg ml^{-1} lysozyme and incubated at 30°C for 15 min. After sonication to eliminate viscosity, the cell suspension was washed twice by centrifugation (12 000 × *g*, 15 min) and rVP26 and rVP28 were harvested from the insoluble fraction. Proteins for the negative control group were obtained from cultured *E. coli* cells with an empty vector by the same protocol, but without IPTG-inducement. The resulting pellets containing rVP26, rVP28, or *E. coli* proteins were resuspended in PBS and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Analysis of density profiles from the digital images of the SDS-PAGE gels with ImageJ software (NIH) showed that the intensities of the prepared rVP26 and rVP28 were approximately 20 and 30%, respectively (Fig. 1). For preparation of the oral vaccine, a commercial dry diet (Maruha) was soaked with suspensions containing either the rVP26, rVP28, or *E. coli* proteins using a volume equivalent to 5% of the feed weight (w/w) and the feed then coated with 0.5% volume (w/w) of an adhesive agent (Schering-Plough Animal Health).

Oral vaccination of shrimp with rVP26 and rVP28 for WSSV challenge tests. Kuruma test shrimp (MBW = 3.7 g) were divided into 4 groups (n = 100 $group^{-1}$) and fed a commercial diet that delivered 10 µg of rVP26 or rVP28 g^{-1} of shrimp d^{-1}, 25 µg of *E. coli* proteins g^{-1} of shrimp d^{-1} (negative control 1), or PBS (negative control 2). These rations were provided for 15 d. Ten days after the final feeding, shrimp fed rVP26 or rVP28 were divided into 7 groups each (n = 13 to 15 $group^{-1}$). Replicate groups of each viral protein vaccination were exposed to WSSV by the IM, immersion, or oral routes. The 2 remaining groups of shrimp that had been vaccinated with either rVP26 or rVP28 were mock challenged with WSSV to serve as negative controls. Forty-five shrimp that were fed the diets containing *E. coli* proteins or PBS were divided into 3 groups each (n = 13 to 15 $group^{-1}$) and then challenged with WSSV by IM, immersion, or oral routes. The WSSV challenge doses were as follows: (1) IM challenge, 100 µl $shrimp^{-1}$ with 10^4-fold dilution of the virus stock solution; (2) immersion (1 h) challenge, 10^4-fold dilution ml^{-1} of the virus stock solution; and (3) oral challenge, 0.6 g of WSD shrimp muscle $shrimp^{-1}$ daily for 3 d. During the oral challenge, complete consumption of the WSD shrimp muscle was visually confirmed. The WSSV doses used in each of the 3 challenge studies were adjusted to produce 70% cumulative mortality among non-vaccinated control shrimp based on the LD_{50} data previously generated (Fig. 2).

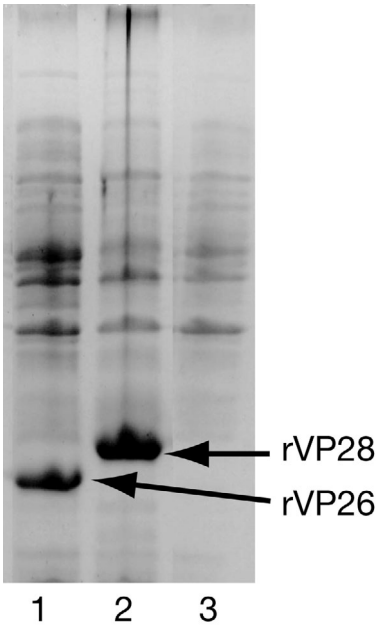

Fig. 1. SDS-PAGE analysis of expressed proteins, rVP26 and rVP28, of WSSV. Proteins in the 12% gel were stained with Coomassie brilliant blue. Lane 1: rVP26, lane 2: rVP28, lane 3: *E. coli* proteins

In the experimental infection groups, dead shrimp were removed twice daily and stored at –30°C for PCR analysis to confirm that WSSV infection was the cause of death. For the detection of WSSV by PCR, total DNA was extracted from shrimp following the method described by Nonaka et al. (1998), and 2 specific PCR primer sets were used: (1) P1 (5′-ATC ATG GCT GCT TCA CAG AC-3′) and P2 (5′-GGC TGG AGA GGA CAA GAC AT-3′) for the first-step PCR, and (2) P3 (5′-TCT TCA TCA GAT GCT ACT GC-3′) and P4 (5′-TAA CGC TAT CCA GTA TCA CG-3′) for the nested PCR (Kimura et al. 1996).

Statistical analysis. The mortalities of the experimental versus control groups were analyzed using chi-squared tests with a significance level of 1%. The relative percentage survival (RPS) values were calculated according to the method of Amend (1981).

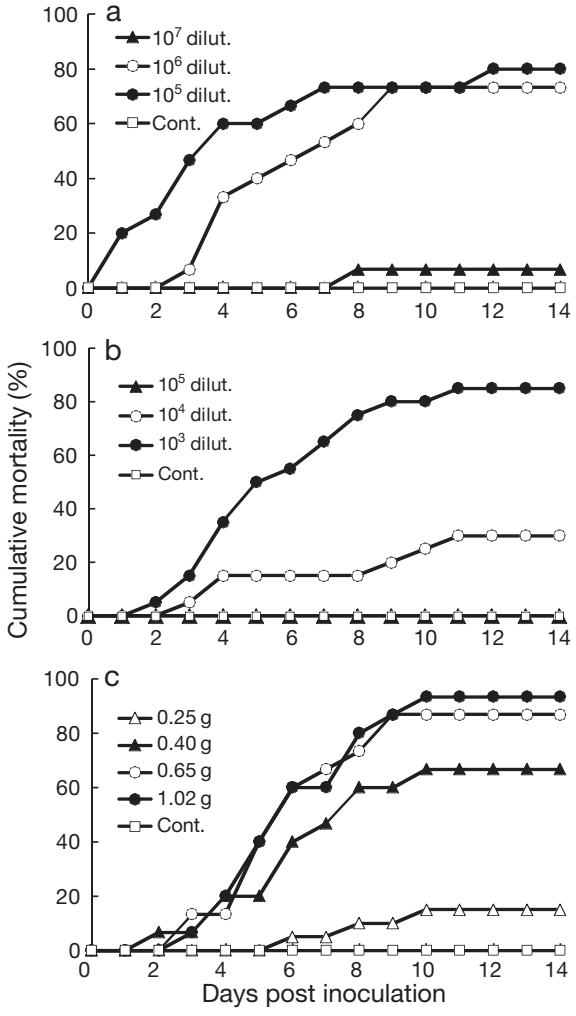

Fig. 2. *Marsupenaeus japonicus*. Cumulative mortality of kuruma shrimp after experimental WSSV challenge by 3 routes: (a) intramuscular challenge with 0.1 ml of diluted WSSV solution, (b) immersion challenge with diluted WSSV solution, and (c) oral challenge with WSD shrimp muscle. Cont.: control group

RESULTS

Virulence of WSSV inoculum against shrimp

The virulence of WSD shrimp muscle and its homogenate was assessed in kuruma shrimp using 3 challenge methods: IM, immersion, and oral (Fig. 2). No mortality was observed among the negative control groups in each of the 3 challenge studies. In the IM challenge group that received the $10^{5.0}$-fold dilution of the WSSV solution, mortality was observed at 1 d post challenge (dpc) and reached 80% at 14 dpc. Mortality in the IM group challenged with the $10^{6.0}$-diluted WSSV solution started at 3 dpc and the cumulative mortality was 73% at 14 dpc. In shrimp injected with $10^{7.0}$-diluted WSSV solution, the only death recorded was of 1 shrimp at 8 dpc and the cumulative mortality was 6.7% (Fig. 2a). The calculated LD_{50} for the IM challenge route was $10^{-7.0}$ ml shrimp^{-1} (Table 1).

For the immersion exposure study, shrimp challenged with $10^{3.0}$-and $10^{4.0}$-diluted WSSV solutions started dying at 2 or 3 dpc with cumulative mortalities of 85% and 30%, respectively. No mortality was observed among the shrimp challenged with $10^{5.0}$-diluted WSSV solution (Fig. 2b). The LD_{50} of the WSSV solution administered by immersion was $10^{-3.7}$ ml ml^{-1} (Table 1).

In the oral exposure study, shrimp were challenged with 0.25, 0.40, 0.65, and 1.02 g of WSD shrimp muscle and cumulative mortalities were 15, 67, 87, and 93%, respectively (Fig. 2c). The LD_{50} of WSD shrimp muscle administered by the oral route was 0.37 g shrimp^{-1} (Table 1).

Protective ability of oral vaccination with rVP26 and rVP28 against WSSV

After the oral administration of rVP26 and rVP28, shrimp were challenged with WSSV by oral, immersion, and IM exposure routes. The WSSV dose used in each of the 3 challenge studies was adjusted to induce 70% cumulative mortality among non-vaccinated control groups (administrated with PBS).

In the oral challenge study, shrimp vaccinated with *E. coli* proteins began dying 3 to 7 dpc with a cumulative mortality of 31%, significantly lower than the 67% cumulative mortality of the PBS (control) group ($\chi^2 = 3.59$, $p < 0.058$). In contrast, no mortality was recorded in shrimp vaccinated with rVP26 or rVP28 (Fig. 3a). In shrimp challenged by immersion exposure to WSSV, mortality started 3 to 8 dpc and the cumulative mortalities of shrimp vaccinated with rVP26, and rVP28 were 21% ($\chi^2 = 11.008$, $p < 0.001$) and 22% ($\chi^2 = 11.008$, $p < 0.002$), respectively, which were significantly lower than that of control shrimp with PBS (73%) (Fig. 3b). No significant difference

Table 1. Virulence of WSSV in kuruma shrimp challenged by intramuscular, immersion, and oral routes

Challenge route	50% of lethal dose (LD_{50})	
	Measured value[a]	Converted value (g shrimp^{-1})
Intramuscular	$10^{-7.0}$ ml shrimp^{-1}	$10^{-7.7}$
Immersion	$10^{-3.7}$ ml ml^{-1}	$10^{-4.4}$
Oral	0.37 g shrimp^{-1}	$10^{-0.4}$

[a]Measured values calculated from data shown in Fig. 2

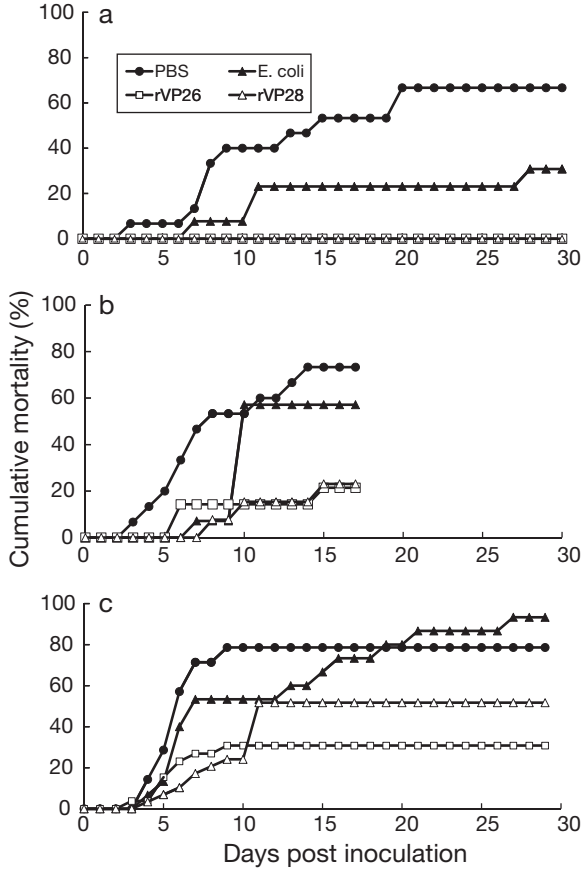

Fig. 3. *Marsupenaeus japonicus*. Cumulative mortality of kuruma shrimp vaccinated orally with WSSV rVP26 or rVP28 and challenged with WSSV by (a) oral challenge, (b) immersion challenge, and (c) intramuscular challenge

was observed in the cumulative mortalities between shrimp with *E. coli* proteins (57%) and with PBS (Fig. 3b). In shrimp challenged by IM injection, mortality was observed beginning 3 dpc and the cumulative mortalities of shrimp vaccinated with rVP26 and rVP28 were 31% ($\chi^2 = 8.34$, $p < 0.004$) and 52% ($\chi^2 = 2.85$, $p < 0.092$), respectively, which were significantly lower than that with PBS (79%). There was no significant difference in mortality between shrimp with *E. coli* proteins (93%) and with PBS (Fig. 3c). No mortality was recorded in any of the 3 mock-challenged groups. The WSSV PCR results of the orally vaccinated shrimp for the 3 challenge routes are shown in Table 2. In the non-vaccinated control groups subjected to the 3 challenge routes, WSSV was detected in all dead shrimp by PCR and more than 66.7% of the surviving shrimp by nested PCR. Of the dead shrimp that had been vaccinated with rVP26 and rVP28, between 33 and 60% were positive for WSSV by PCR and between 73.3 to 100% were positive by nested PCR. However, all of the surviving shrimp vaccinated with rVPs were negative for WSSV by PCR and nested PCR with the exception of the oral and immersion challenge survivors in which ≤10% were found to be positive by nested PCR. These collective PCR results show that the prevalence of WSSV-infection in vaccinated shrimp was significantly lower than in non-vaccinated shrimp.

The calculated RPS values of orally vaccinated shrimp with rVP26 and rVP28 are shown in Table 3. Shrimp vaccinated with rVP26 showed 100% RPS after oral challenge, 71% after immersion challenge, and 61% after IM challenge; the corresponding values for those vaccinated with rVP28 were 100%, 70%, and 34%, respectively. Taken collectively, the RPS values of the orally vaccinated shrimp were all >60% with the exception of the rVP28-vaccinated shrimp challenged with WSSV by the IM route (34% RPS). The RPS values of shrimp vaccinated with *E. coli* proteins were 54% after oral challenge, 22% after immersion, and 0% after IM. These RPS values were significantly lower than those for shrimp vaccinated with rVP26 and rVP28 (Table 3).

Table 2. *Marsupenaeus japonicus*. PCR detection rates of WSSV in orally vaccinated shrimp after experimental challenge with WSSV by oral, immersion, and intramuscular (IM) routes. Nos. in parentheses: no. of positive/no. of examined. –: no cumulative mortality, nt: not tested

Antigen	Oral challenge				Immersion challenge				IM challenge				Mock challenge	
	Dead		Survivors		Dead		Survivors		Dead		Survivors		Survivors	
	PCR	Nested PCR	PCR	Nested PCR	PCR	Nested PCR	PCR	Nested PCR	PCR	Nested PCR	PCR	Nested PCR	PCR	Nested PCR
rVP26	–	–	0% (0/30)	6.7% (2/30)	50.0% (3/6)	83.3% (5/6)	0% (0/22)	9.1% (2/22)	50.0% (4/8)	100% (8/8)	0% (0/18)	38.9% (7/18)	0% (0/12)	0% (0/12)
rVP28	–	–	0% (0/30)	10% (3/30)	33.3% (2/6)	83.3% (5/6)	0% (0/21)	0% (0/21)	60.0% (9/15)	73.3% (11/15)	0% (0/14)	35.7% (5/14)	0% (0/12)	0% (0/12)
E. coli	100% (4/4)	100% (4/4)	0% (0/9)	0% (0/9)	12.5% (1/8)	50.0% (4/8)	0% (0/6)	0% (0/6)	100% (13/13)	100% (13/13)	0% (0/1)	0% (0/1)	nt	nt
PBS	100% (10/10)	100% (10/10)	40.0% (2/5)	100% (5/5)	100% (11/11)	100% (11/11)	25.0% (1/4)	75.0% (3/4)	100% (11/11)	100% (11/11)	0% (0/3)	66.7% (2/3)	nt	nt

Table 3. *Marsupenaeus japonicus*. Protection against WSSV challenge by oral, immersion, and intramuscular (IM) routes in kuruma shrimp vaccinated orally with rVP26 and rVP28. RPS: relative percent survival. *Significantly different (1% level) from the non-vaccinated groups by χ^2 test. –: no values, nt: not tested

Antigen	Oral challenge			Immersion challenge			IM challenge			Mock challenge	
	n	Mortality (%)	RPS (%)	n	Mortality (%)	RPS (%)	n	Mortality (%)	RPS (%)	n	Mortality (%)
rVP26	30	0*	100	28	21*	71	26	31*	61	12	0
rVP28	30	0*	100	27	22*	70	29	52	34	12	0
E. coli	13	31	54	14	57	22	14	93	0	nt	nt
PBS	15	67	–	15	73	–	14	79	–	nt	nt

DISCUSSION

As a preliminary step towards the development of an oral vaccine against WSD in kuruma shrimp, the virulence of WSSV was compared using 3 different challenge routes. Measured LD_{50} values for WSSV by IM injection, immersion, and oral challenge routes were $10^{-7.0}$ ml shrimp^{-1}, $10^{-3.7}$ ml ml^{-1}, and 0.37 g shrimp^{-1}, respectively. Since the WSSV stock solution for the virulence tests was prepared from the same lot of WSD muscle as that used to challenge the vaccinated shrimp, the measured LD_{50} values were used to calculate the approximate weight of WSD shrimp muscle used per shrimp in each of the 3 exposure studies. The resulting values were $10^{-7.7}$ g shrimp^{-1} for the IM challenge, $10^{-4.4}$ g shrimp^{-1} for the immersion challenge, and $10^{-0.4}$ g shrimp^{-1} for the oral challenge study (Table 1). These results show that the quantity of WSSV-infected tissue needed to obtain an LD_{50} by the oral route was $10^{4.0}$- and $10^{7.3}$-fold greater than that needed to achieve an LD_{50} by the immersion and IM routes, respectively. Standardization of the WSSV challenge dose was performed by Escobedo-Bonilla et al. (2005, 2006), which demonstrated that 10 times the dose was needed in the oral challenge as compared to the IM challenge in order to obtain the same cumulative mortality. While it is generally considered that the quantity of WSSV-infected tissue needed to achieve an LD_{50} varies according to exposure method, shrimp species, and viral strain, we did confirm that infection efficiency of WSSV by the oral route was significantly lower than by the immersion and IM routes.

Wu et al. (2001) suggested that cannibalism is one of the most important modes of WSSV transmission, as shrimp mortality decreased significantly when cannibalism was prevented in a WSSV infection experiment. Thus, it appears that WSSV infection is easily established when shrimp cannibalize WSD shrimp even though the efficiency of virus transmission by the oral route is low. Actually, a high frequency of cannibalism was also observed during the present study, meaning that it probably influenced the cumulative mortality of the shrimp. Cannibalism was permitted and high stocking densities were utilized to better duplicate actual farm conditions in an effort to evaluate the efficacy of the WSSV oral vaccine.

Protection against WSSV infection in shrimp is inducible by IM and oral inoculation with inactivated WSSV, rVP26, and rVP28 (Namikoshi et al. 2004, Witteveldt et al. 2004a,b, 2006, Jha et al. 2006). However, the experimental WSSV infections reported in these studies were by either IM or immersion challenge with the exception of the study by Jha et al. (2006). As described above, cannibalism is one of the most important modes of WSSV transmission in shrimp farms. Therefore, the effects of rVP26 and rVP28 vaccines should be evaluated using oral WSSV challenge. In the present study, 10 d after the final oral vaccination with rVP26, rVP28, or *E. coli* proteins, kuruma shrimp were challenged with WSSV by oral, immersion, and IM routes. We believe that the WSSV doses used for the experimental challenges were reasonably high and effective for our purposes as cumulative mortalities among groups of non-vaccinated control shrimp ranged from 67 to 79% (Table 3). Under these challenge conditions, RPS values of the rVP26 and rVP28 orally vaccinated shrimp were 100% for the oral challenge route and more than 70% for immersion (Table 3). Moreover, PCR analysis demonstrated that there was a significantly higher number of PCR positive non-vaccinated shrimp versus orally vaccinated shrimp (Table 2). Thus, it was confirmed that oral vaccination of kuruma shrimp with either rVP26 or rVP28 conferred adequate protection against ingested WSSV-infected tissue and can be utilized to prevent horizontal transmission of this virus through cannibalism in shrimp farms. Notably, the RPS values after IM challenge were lower than those after oral and immersion challenges (Table 3). Namikoshi et al. (2004) showed that booster vaccination of shrimp by the IM route with rVP26, rVP28, and formalin-inactivated WSSV led to enhanced protection against WSSV. In the present study, orally vaccinated shrimp showed adequate protection against WSSV after oral challenge

even though the WSSV dose needed to achieve an infection by oral challenge was significantly higher than those needed to achieve infection by immersion and IM challenges (Table 1). Moreover, RPS values after oral challenge were also higher than those after immersion and IM challenges. These results strongly support the importance of the oral route mediated by cannibalism in the infection of shrimp with WSSV as described by Wu et al. (2001) and Momoyama & Muroga (2005). Furthermore, our findings suggest that the horizontal transmission of WSSV through cannibalism in shrimp farms can be prevented by oral vaccination with rVP26 or rVP28.

In the present study, *E. coli* proteins were used as one of negative control vaccines because the *E. coli* cells were used to generate rVP26 and rVP28, and bacterial proteins comprised part of each vaccine as shown in Fig. 1. A low level of protection against WSSV challenge was observed in the control shrimp that were orally vaccinated with *E. coli* proteins, with a 54% RPS following oral WSSV challenge and 22% by immersion challenge (Table 3). We believe this low level of WSSV protection suggests that the *E. coli* proteins might have an immunostimulatory effect on the shrimp as in previous studies (Itami et al. 1998, Chen et al. 1999, Sritunyalucksana et al. 1999).

Wu et al. (2002) reported that resistance to WSSV in shrimp that survived WSD appeared 3 wk after primary infection and persisted for about 2 mo. However, Namikoshi et al. (2004) found that the protection induced by IM injection with formalin-inactivated WSSV did not persist any longer than that induced by natural infection. The present data shows that adequate protection was induced by oral vaccination of kuruma shrimp with either rVP26 or rVP28. The onset and duration of the protection induced by oral vaccination will be an interesting topic for further research.

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