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Standardized white spot syndrome virus (WSSV) inoculation procedures for intramuscular or oral routes

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ABSTRACT: In the past, strategies to control white spot syndrome virus (WSSV) were mostly tested by infectivity trials *in vivo* using immersion or per os inoculation of undefined WSSV infectious doses, which complicated comparisons between experiments. In this study, the reproducibility of 3 defined doses (10, 30 and 90 shrimp infectious doses 50% endpoint [SID_{50}]) of WSSV was determined in 3 experiments using intramuscular (i.m.) or oral inoculation in specific pathogen-free (SPF) *Litopenaeus vannamei*. Reproducibility was determined by the time of onset of disease, cumulative mortality, and median lethal time (LT_{50}). By i.m. route, the 3 doses induced disease between 24 and 36 h post inoculation (hpi). Cumulative mortality was 100% at 84 hpi with doses of 30 and 90 SID_{50} and 108 hpi with a dose of 10 SID_{50}. The LT_{50} of the doses 10, 30 and 90 SID_{50} were 52, 51 and 49 hpi and were not significantly different ($p > 0.05$). Shrimp orally inoculated with 10, 30 or 90 SID_{50} developed disease between 24 and 36 hpi. Cumulative mortality was 100% at 108 hpi with doses of 30 and 90 SID_{50} and 120 hpi with a dose of 10 SID_{50}. The LT_{50} of 10, 30 and 90 SID_{50} were 65, 57 and 50 hpi; these were significantly different from each other ($p < 0.05$). A dose of 30 SID_{50} was selected as the standard for further WSSV challenges by i.m. or oral routes. These standardized inoculation procedures may be applied to other crustacea and WSSV strains in order to achieve comparable results among experiments.

KEY WORDS: *Litopenaeus vannamei* · WSSV · Experimental inoculation · Intramuscular route · Oral route · LT_{50} · Probit analysis

INTRODUCTION

White spot syndrome virus (WSSV) is one of the most lethal pathogens in shrimp aquaculture. First reported in Taiwan in 1992 (Chou et al. 1995), it has spread to several shrimp farming countries. Within a decade, it has become a serious threat to the shrimp culture industry throughout Asia and Latin America (Hill 2002). WSSV also infects many other crustacean species from several regions of the world (Lo et al. 1996, Chang et al. 1998, Kanchanaphum et al. 1998, Kasornchandra et al. 1998, Wang et al. 1998, Rajendran et al. 1999, Corbel et al. 2001, Hossain et al. 2001, Sahul-Hameed et al. 2003).

The WSSV virion is bacilliform, non-occluded and enveloped. It contains a circular, double-stranded DNA genome with size between 293 and 307 kilobase pairs (kbp) (van Hulten et al. 2001, Yang et al. 2001, Chen et al. 2002). Several WSSV strains have been

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identified by differences in their genomic size (Wang et al. 2000), restriction enzyme profile (Nadala & Loh 1998), deletion variants (Lan et al. 2002), or pathogenicity (Q. Wang et al. 1999).

The disease caused by this virus is characterized by the presence of white spots on the inner surface of the exoskeleton of *Penaeus monodon* and other Asian shrimp species during the acute phase. Other clinical signs include reduced feeding and locomotion, and reddish discoloration of the body (Otta et al. 1999). Mass mortalities (up to 100%) have been reported within 10 d after the onset of disease (Y.G. Wang et al. 1999).

Several approaches to reducing mortality due to WSSV have been tested using experimental challenges; these have included (1) feeding shrimp with immunostimulants to enhance the defense response (Chang et al. 1999, 2003, Huang & Song 1999, Takahashi et al. 2000, Yusoff et al. 2001, Chotigeat et al. 2004), (2) 'vaccinating' shrimp with formalin-fixed virus or recombinant WSSV-envelope proteins (Namikoshi et al. 2004, Witteveldt et al. 2004a, 2004b), (3) administering antimicrobial peptides (mytilin) (Dupuy et al. 2004) or double-stranded RNA (dsRNA) (Robalino et al. 2004), and (4) manipulating water temperature (Vidal et al. 2001, Granja et al. 2003, Guan et al. 2003, Jiravanichpaisal et al. 2004).

Other strategies with potential to combat WSSV infections include the induction of antiviral genes present in shrimp (Luo et al. 2003), the application of synthetic antiviral peptides (Yi et al. 2003), and the induction of a 'WSSV neutralizing factor' in shrimp using sublethal concentrations of WSSV (Venegas et al. 2000, Wu et al. 2002).

So far, most of the WSSV challenge tests developed to control WSS disease used different inoculation routes and undefined amounts of infectious virus. The routes of inoculation used were immersion, per os feeding of infected tissues, and intramuscular (i.m.) injection. The amount of infectious virus taken up by individual using immersion or feeding may be quite different, making it very difficult to compare results among different studies. The development of standardized WSSV inoculation procedures that yield reproducible results in terms of onset and severity of disease would significantly improve challenge tests.

One of the main requirements for a reproducible model of infection is to use a virus stock with known infectivity titer. The shrimp infectious dose 50% endpoint (SID_{50} ml^{-1}) of the Thai WSSV stock used in this study was determined by *in vivo* titration in specific pathogen free (SPF) *Litopenaeus vannamei* by i.m. and oral routes (Escobedo-Bonilla et al. 2005). Determination of the infectivity titer allows the establishment of a reproducible dose-response curve for experimental WSSV infections in shrimp.

The objectives of this study were to (1) develop standardized WSSV inoculation procedures by i.m. and oral routes and (2) characterize the mortality pattern (time of onset of disease, median lethal time [LT_{50}], and cumulative mortality) of 3 doses of a Thai WSSV stock. Thus, a reproducible dose-response relationship was established to determine an appropriate WSSV dose to be used as a standard in further experimental challenges.

MATERIALS AND METHODS

Shrimp and rearing conditions. SPF *Litopenaeus vannamei* of the Kona strain (Wyban et al. 1992) were used. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL 8 to 12; mean body weight [MBW] = 0.0013 g). Shrimp at this stage were fed *Artemia nauplii* once daily for 1 wk. Afterwards, they were fed with a crumbled commercial pelleted feed (A2 monodon high performance shrimp feed/shrimp complete grower, INVE aquaculture NV) at a rate of 2.5% MBW twice daily. Older juvenile shrimp were fed a pelleted feed at the same rate twice daily. Water temperature was 27 ± 1°C, salinity ranged between 30 and 35 g l^{-1}, total ammonia was less than 0.5 mg l^{-1}, and nitrites ranged between 0.05 and 0.15 mg^{-1}.

WSSV stock and *in vivo* infectivity titers. The WSSV stock used in this study was prepared and titrated by i.m. or oral inoculations as described previously (Escobedo-Bonilla et al. 2005). The median virus titer of infection was $10^{6.6}$ SID_{50} ml^{-1} by i.m. route and $10^{5.6}$ SID_{50} ml^{-1} by oral route.

Doses. Three doses of the WSSV stock were prepared in phosphate-buffered saline pH 7.4 (PBS) for i.m. or oral inoculation: 10, 30 and 90 SID_{50} in a volume of 50 µl.

Experimental conditions. Shrimp were acclimatized to a salinity of 15 g l^{-1} over 4 d at the ARC and then transported to the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where experiments were carried out under biosafety conditions. Shrimp were acclimatized to experimental conditions 24 h before challenge and during this time they were not fed. After inoculation, shrimp were fed daily with only 6 pellets in order to maintain water quality.

Groups of 10 shrimp were each placed in 50 l glass aquaria with glass covers and a plastic sheath to prevent virus transmission by aerosol. Artificial seawater was prepared at 15 g l^{-1} with Instant Ocean (Marine systems) in distilled water. Each aquarium was fitted with a mechanical filter (Eheim classic 2213), a water heater (Visitherm aquarium systems) and aeration. Water temperature was 27 ± 1°C, total ammonia was

between 0 and 5 mg l^{-1}, and nitrites ranged between 0 and 0.15 mg l^{-1} as monitored daily.

Intramuscular inoculation procedure. Three experiments were performed using the i.m. route. In each experiment, 3 groups of 10 shrimp (MBW = 9.40 ± 4.92 g, n = 120) were inoculated with 10, 30 or 90 SID_{50}. In addition, 3 groups of 10 shrimp were mock-inoculated with 50 µl PBS and used as controls. Shrimp were injected between the 3rd and 4th segments of the pleon. Before and after injection, this surface was wiped with 70% ethanol. These experiments were run until all the infected shrimp died. Control shrimp were sacrificed at 120 h post inoculation (hpi).

Oral inoculation procedure. Three experiments were performed using the oral route. In each experiment, 3 groups of 10 shrimp (MBW = 9.72 ± 2.24 g, n = 120) were inoculated with 1 of 3 doses (10, 30 and 90 SID_{50}). Three groups of 10 shrimp were mock-inoculated with 50 µl PBS and used as controls. Oral inoculation was performed as follows: shrimp were placed in a tray ventral side up, a flexible and slender pipette tip (no. 790004 Biozym) was introduced into the oral cavity, and the inoculum was delivered into the lumen of the foregut. These experiments were run until all the infected shrimp died. Control shrimp were sacrificed at 120 hpi.

Evaluation of WSSV infection. Inoculated shrimp were monitored every 12 h throughout the experiment. Moribund and dead shrimp were removed and processed for indirect immunofluorescence (IIF) analysis. Control shrimp were also analyzed by IIF.

Clinical signs: *Litopenaeus vannamei* rarely display white spots during WSSV infection (Nadala et al. 1998, Rodriguez et al. 2003). Empty guts and reduced response to mechanical stimulation are the first clinical signs to appear in WSSV-diseased shrimp, and are good indicators of infection and mortality. These clinical signs were used to monitor the onset of disease in shrimp inoculated by i.m. or oral routes.

Indirect immunofluorescence analysis (IIF): Shrimp were processed for the detection of WSSV-infected cells as follows: tissues from the pereon were embedded in methylcellulose (Fluka) and frozen at −20°C. Cryosections (5 to 6 µm) were made and tissues were fixed in absolute methanol at −20°C, washed with PBS, and incubated for 1 h at 37°C with 2 mg ml^{-1} of the monoclonal antibody 8B7 against VP28 (Poulos et al. 2001). Tissues were washed and incubated for 1 h at 37°C with 0.02 mg ml^{-1} of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (F-2761 Molecular Probes) in PBS, washed with PBS, rinsed in deionised water, and mounted with a solution containing glycerin and 1,4-diazobicyclo-2,2,2,-octane (DABCO). Tissue sections were analyzed by fluorescence microscopy (Leica DM RBE).

Statistical analysis. The cumulative mortality and SD of the 3 experiments performed by i.m. or oral routes were calculated for each dose. The mean cumulative mortality was analyzed by probit, which is a generalized linear model with a probit link function (Agresti 1996). After checking that no significant interactions existed between dose and time, the probit model had the form:

$$\text{Probit}(x) = \alpha + \beta(\text{time}) + \gamma(\text{dose}) \quad (1)$$

where α is the intercept, β is the rate of probability change per unit change of time (for a constant dose), and γ is the rate of probability difference for each dose (for a constant time)

The statistical software Minitab (Minitab v. 14, Minitab) was used to calculate the parameters of the regression and to determine the median lethal time (LT_{50}) or the time at which 50% of the tested organisms died (Yi et al. 2003) for each dose. Differences in the LT_{50} of doses were evaluated by the significance of dose in Eq. (1) (significance level = 0.05) using the same statistical software.

RESULTS

Intramuscular inoculation

Clinical signs and onset of disease

Shrimp inoculated with the 3 doses of WSSV by i.m. route first displayed empty guts and reduced response to mechanical stimulus between 24 and 36 hpi. The proportion of shrimp from each of the 3 doses that displayed these clinical signs is presented in Tables 1 & 2.

Table 1. Proportion of shrimp with empty guts after intramuscular (i.m.) inoculation with 3 doses of WSSV. Number of shrimp indicates totals from 3 experiments. hpi: h post inoculation

Group	Number of shrimp	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	6/30	12/30	9/22	18/20	4/6	2/2	2/2	1/1	
30 SID_{50}	30	0/30	0/30	5/30	16/29	13/22	11/15	5/5	2/2			
90 SID_{50}	30	0/30	0/30	6/30	18/30	14/26	13/17	3/4	1/1			

Table 2. Proportion of shrimp with reduced response to mechanical stimulus after i.m. inoculation with 3 doses of WSSV. Number of shrimp indicates totals from 3 experiments

Group	Number of shrimp	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	0/30	0/30	5/22	13/20	4/6	2/2	2/2	1/1	
30 SID_{50}	30	0/30	0/30	2/30	11/29	8/22	9/15	3/5	2/2			
90 SID_{50}	30	0/30	0/30	1/30	7/30	12/26	11/17	3/4	1/1			

Shrimp used as controls did not display any of these clinical signs: they remained healthy and survived throughout the experiments.

Mortality

Each of the 3 doses of WSSV inoculated by i.m. route induced 100% mortality. The first mortalities were recorded at 36 hpi with each of the 3 doses tested. The cumulative mortality reached 100% at 84 hpi in shrimp inoculated with doses 30 and 90 SID_{50}, while shrimp inoculated with 10 SID_{50} were all dead at 108 hpi (Fig. 1a).

The cumulative mortality of the 3 doses was analyzed with the probit model (Fig. 2a) and the LT_{50} of the 3 doses were compared. After challenge with doses of 10, 30 and 90 SID_{50}, LT_{50} values of 52, 50 and 49 hpi were obtained, respectively, which were not significantly different (Table 5). IIF analysis confirmed that all WSSV-inoculated shrimp became infected. Control shrimp were WSSV-negative.

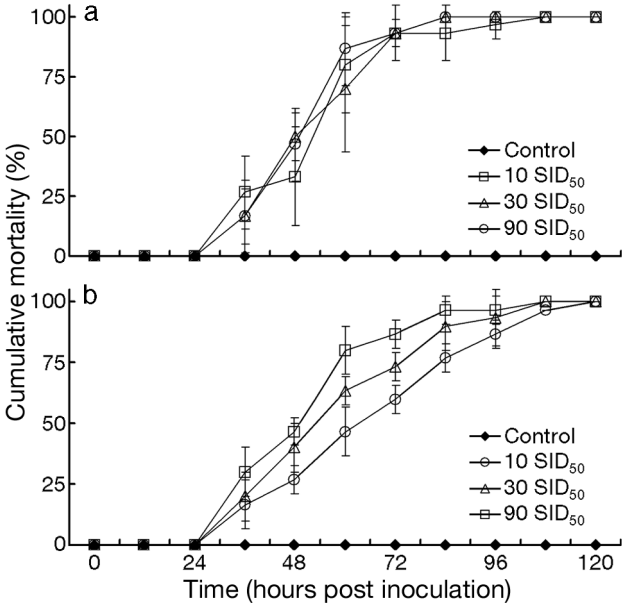

Fig. 1. Cumulative mortality (mean of 3 experiments ± SD) of shrimp inoculated with 3 doses of WSSV by (a) intramuscular (i.m.) or (b) oral routes

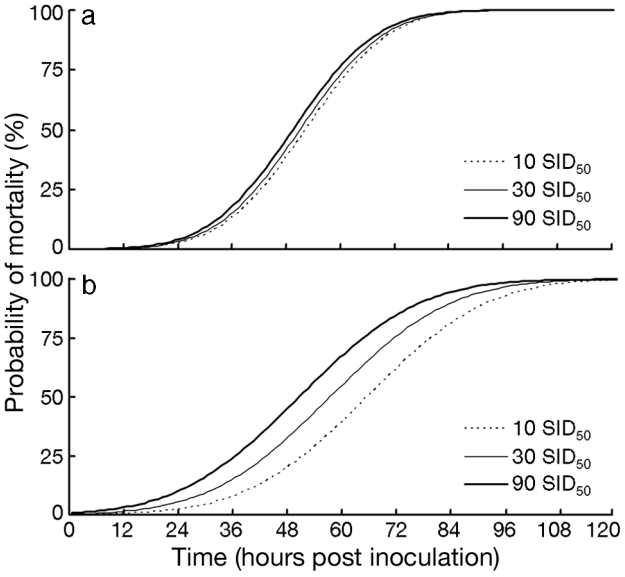

Fig. 2. Probability of mortality (probit) of the 3 doses of WSSV inoculated into shrimp by (a) i.m. or (b) oral routes

Oral inoculation

Clinical signs and onset of disease

Shrimp inoculated with any of the 3 doses of WSSV by oral route first displayed empy guts and reduced response to mechanical stimulus between 24 and 36 hpi. The proportion of shrimp from each of the 3 doses that displayed these clinical signs is presented in Tables 3 & 4. Control shrimp did not display any of these clinical signs: they remained healthy and survived throughout the experiments.

Mortality

Each of the 3 doses of WSSV inoculated by oral route induced 100% mortality. After oral inoculation, the first mortalities due to WSSV were recorded at 36 hpi for each dose. Cumulative mortality was 100% at 108 hpi in shrimp inoculated with doses 30 and 90 SID_{50}, while the cumulative mortality of shrimp inocu-

Table 3. Proportion of shrimp with empty guts after oral inoculation with 3 doses of WSSV. Number of shrimp indicates totals from 3 experiments

Group	Number of shrimp	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	7/30	11/30	13/25	15/22	10/16	8/12	6/7	4/4	1/1
30 SID_{50}	30	0/30	0/30	6/30	13/30	14/24	13/18	7/11	7/8	3/3	2/2	
90 SID_{50}	30	0/30	0/30	7/30	24/30	16/24	14/16	4/6	3/4	1/1	1/1	

Table 4. Proportion of shrimp with reduced response to mechanical stimulus after oral inoculation with 3 doses of WSSV. Number of shrimp indicates totals from 3 experiments

Group	Number of shrimp	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	2/30	4/30	8/25	12/22	9/16	6/12	6/7	4/4	1/1
30 SID_{50}	30	0/30	0/30	0/30	12/30	11/24	13/18	7/11	7/8	3/3	2/2	
90 SID_{50}	30	0/30	0/30	1/30	13/30	8/24	11/16	3/6	2/4	1/1	1/1	

lated with 10 SID_{50} was 100% at 120 hpi (Fig. 1b). Probit analysis (Fig. 2b) revealed significant differences ($p < 0.05$) in the LT_{50} of each of the 3 doses inoculated by oral route (Table 5). The LT_{50} of doses of 10, 30 and 90 SID_{50} were 65, 57 and 50 hpi, respectively. IIF analysis confirmed infection in all shrimp inoculated with WSSV. Control shrimp were WSSV-negative.

DISCUSSION

In the past, experimental challenge tests have been used to determine the pathogenicity of WSSV, and the susceptibility of different species to the virus, and to test products and strategies to control the disease (Lu et al. 1997, Lightner et al. 1998, Chang et al. 2003). In all these experiments, different viral strains, shrimp species, ages and routes of inoculation were used, which makes it difficult to compare results from different studies. Moreover, the infectivity of the virus stock is mostly undefined.

This study is the first to use defined infectious doses of WSSV to standardize experimental challenge protocols by i.m. and oral routes using SPF shrimp of similar age. Each of 3 doses of WSSV inoculated by either i.m. or oral route induced infection and mortality in all shrimp, and their mortality patterns were reproducible according to the criteria used. The clinical signs used in these experiments were useful to indicate the time of onset of disease caused by WSSV infection for each dose.

Clinical signs appeared at least 12 h before the first mortalities, and were displayed by similar proportions of shrimp whether inoculated by i.m. or oral routes. The onset of disease and the first mortalities occurred at the same time regardless of whether shrimp were inoculated i.m. or by the oral route. However, shrimp inoculated orally died between 12 and 24 hpi later than shrimp inoculated i.m. with equivalent doses. Accordingly, the LT_{50} were less for doses delivered by i.m. route compared with the LT_{50} of equivalent doses inoculated orally. The influence of the route of inoculation

Table 5. Parameters of the probit regression model of the 3 doses inoculated by i.m. or oral routes; *significant differences at $p < 0.05$

Inoculation route	Dose (SID_{50})	Time of 100% mortality (hpi)	α	β	γ (dose)	LT_{50}	LT_{50} similarity (Z, $p = 0.05$)
i.m.	10^a	108	−3.5616	0.06866	0	51.87	$c \leq b \leq a$
	30^b	84	−3.5616	0.06866	0.09214	50.53	
	90^c	84	−3.5616	0.06866	0.19966	48.96	
Oral	10^a	120	−3.1279	0.04809	0	65.04	$c^* < b^* < a^*$
	30^b	108	−3.1279	0.04809	0.393898	56.85	
	90^c	108	−3.1279	0.04809	0.721668	50.03	

on the speed of mortality produced by WSSV infection has been determined previously in *Penaeus monodon* and *Fenneropenaeus indicus.* Shrimp infected per os displayed 100% mortality 2 to 4 d later than those inoculated by i.m. route (Sahul-Hameed et al. 1998, Rajendran et al. 1999, Rajan et al. 2000). In the sergestoid shrimp *Acetes* sp., individuals inoculated by i.m. route had 100% mortality by the 3rd day post inoculation (Supamattaya et al. 1998). In contrast, mortality due to WSSV infection was reduced 5-fold when shrimp were inoculated with infected tissues per os, and shrimp mortality occurred over a period of 9 d post feeding.

As a consequence of i.m. inoculation, infectious viral particles are placed directly into the shrimp's body, which avoids any natural barrier in the shrimp to prevent pathogen entry. With this inoculation technique most of the injected infectious viral particles have a high probability of reaching susceptible cells and to initiate the infection process. In contrast, the oral inoculation places the virus in the lumen of the foregut, which represents a hostile environment. The cuticle layer lining the epithelial cells in the foregut (Icely & Nott 1992, Ceccaldi 1997, Martin & Chiu 2003) constitutes an important physical barrier that may hinder the passage of infectious WSSV particles to the epithelial cells. The pH and enzymes present in the digestive tract of the shrimp (Lovett & Felder 1990, Talbot & Demers 1993, Lemos et al. 1999, Ribeiro & Jones 2000, Gamboa-Delgado et al. 2003) may damage infectious viral particles leading to their inactivation. It is likely that only a small proportion of infectious virus inoculated orally actually infects cells, which is why it is necessary to use 10 times more virus to infect shrimp by the oral route compared with i.m. inoculation (Escobedo-Bonilla et al. 2005).

Even when the doses inoculated were increased 10 times for oral intubation, there was still a difference in the time required to produce 100% mortality between i.m. and oral inoculation of WSSV, which suggested the existence of barriers other than those alluded to — for example, the basal lamina (Mellon 1992) underlying the epithelial cells of the foregut. Once epithelial cells are infected with WSSV, the newly produced infectious virus has to break through the basal lamina to reach the underlying connective tissues in order to spread to other organs. It is possible that a critical number of epithelial cells has to be infected before infectious virus can cross the basal lamina, thus explaining the dose-dependent pattern. Once infectious WSSV particles reach the connective tissues that may be in contact with hemolymph sinuses and lacunae bathing these tissues, the infectious WSSV particles can be carried by the hemolymph circulation and spread to other target organs. Mortality of WSSV-infected shrimp probably occurs when the level of infection in target organs causes necrosis and loss of function.

Based on the cumulative mortality patterns of the 3 doses used in these experiments, a dose of 30 SID_{50} was selected as the standard for further WSSV inoculation procedures by i.m. and oral routes. Such a dose ensures infection in every inoculated shrimp, but is not so excessive as to cause acute mortality. This is a desirable feature, especially when these inoculation protocols will be applied to test the efficacy of WSSV control strategies. The oral inoculation procedure may be more relevant for testing these strategies because it mimics the natural mode of WSSV infection. Further, it allows for the testing of products that may have a synergistic effect with the natural physico-chemical barriers to viral entry.

The standardized inoculation procedures described in our study may be applied to other crustacean species and different WSSV strains. Parameters such as onset and severity of disease and LT_{50} are specific for each viral strain and set of experimental conditions used. Therefore, it will be necessary to determine these parameters under specific experimental conditions when other WSSV strains, shrimp species, and laboratory conditions are used. These standardized inoculation procedures may also be used for (1) comparison of the susceptibility of different shrimp species to WSSV, (2) determination of the virulence of different WSSV strains, and (3) evaluation of the effect of different strategies with potential to control WSSV.

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