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NOTE

In vivo titration of white spot syndrome virus (WSSV) in specific pathogen-free *Litopenaeus vannamei* by intramuscular and oral routes

C. M. Escobedo-Bonilla[1,2], M. Wille[1], V. Alday Sanz[3], P. Sorgeloos[1], M. B. Pensaert[2], H. J. Nauwynck[2,*]

[1]Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Rozier 44, 9000 Ghent, Belgium

[2]Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[3]INVE technologies, Hoogveld 93, 9200 Dendermonde, Belgium

ABSTRACT: White spot syndrome virus (WSSV) is a devastating pathogen in shrimp aquaculture. Standardized challenge procedures using a known amount of infectious virus would assist in evaluating strategies to reduce its impact. In this study, the shrimp infectious dose 50% endpoint (SID_{50} ml^{-1}) of a Thai isolate of WSSV was determined by intramuscular inoculation (i.m.) in 60 and 135 d old specific pathogen-free (SPF) *Litopenaeus vannamei* using indirect immunofluorescence (IIF) and 1-step polymerase chain reaction (PCR). Also, the lethal dose 50% endpoint (LD_{50} ml^{-1}) was determined from the proportion of dead shrimp. The median virus infection titers in 60 and 135 d old juveniles were $10^{6.8}$ and $10^{6.5}$ SID_{50} ml^{-1}, respectively. These titers were not significantly different ($p \geq 0.05$). The titration of the WSSV stock by oral intubation in 80 d old juveniles resulted in approximately 10-fold reduction in virus titer compared to i.m. inoculation. This lower titer is probably the result of physical and chemical barriers in the digestive tract of shrimp that hinder WSSV infectivity. The titers determined by infection were identical to the titers determined by mortality in all experiments using both i.m. and oral routes at 120 h post inoculation (hpi), indicating that every infected shrimp died. The determination of WSSV titers for dilutions administered by i.m. and oral routes constitutes the first step towards the standardization of challenge procedures to evaluate strategies to reduce WSSV infection.

KEY WORDS: *Litopenaeus vannamei* · WSSV · *In vivo* titration · Intramuscular inoculation · Oral inoculation

INTRODUCTION

White spot syndrome virus (WSSV) is a devastating pathogen which causes disease and mortality in shrimp aquaculture. First recorded in Taiwan in 1992 (Chou et al. 1995), it has spread to several shrimp-farming countries in Asia and Latin America (Wang et al. 2000, Hill 2002). In culture ponds, mortalities up to 100% may occur within 10 d after the onset of disease (Kasornchandra et al. 1998). In many Asian shrimp species the acute phase of disease is characterized by the presence of white spots on the inner surface of the exoskeleton (Lo et al. 1996) from which the disease name is derived. Other clinical signs include anorexia, lethargy and reddish discoloration of the body (Otta et al. 1999). WSSV is an enveloped, non-occluded bacilliform-shaped virus containing a double-stranded DNA genome of between 293 and 308 kbp (van Hulten et al.

*Corresponding author. Email: hans.nauwynck@ugent.be

2001, Yang et al. 2001). This pathogen was first grouped with the non-occluded enveloped baculoviruses (Durand et al. 1996), but gene sequencing and characterization of its major structural proteins have shown low homology with this virus family. These molecular differences supported the formation of a new virus family for WSSV: the *Nimaviridae* (Vlak et al. 2002). Several decapod crustaceans (Chang et al. 1998, Sahul-Hameed et al. 2003) and shrimp species (Wongteerasupaya et al. 1996, Chou et al. 1998, Wang et al. 1999) are susceptible to WSSV infection.

Several experiments have been carried out with WSSV to determine its pathogenicity in crustacean hosts using (1) intramuscular (i.m.) inoculation (Jiravanichpaisal et al. 2001), (2) the *per os* route by feeding WSSV-infected tissues to experimental animals (Rajendran et al. 1999, Wang et al. 1999) and (3) immersion (Chou et al. 1998, Rajan et al. 2000). In these studies, the dose of infectious virus given to each animal was not known. A standardized inoculation procedure requires 2 major components: (1) the use of animals with low genetic variability and high susceptibility to the virus, preferably free of specific pathogens and (2) a WSSV stock with a known titer of infection. Such a standardized procedure is essential (1) to compare the susceptibility of different host species and life stages to WSSV, (2) to determine the virulence of different WSSV strains, and (3) to test the efficacy of strategies aimed to control the disease. To date, no shrimp cell cultures are available for *in vitro* titration of WSSV; therefore, *in vivo* titration is the only alternative.

Only 1 previous study has determined the LD_{50} of a WSSV tissue suspension. A South Carolina isolate was inoculated i.m. in specific pathogen-free (SPF) *Litopenaeus vannamei* of 1 g mean body weight, and the virus titer was determined using probit analysis (Prior et al. 2003).

The aims of the present study were (1) to determine the shrimp infectious dose 50% endpoint (SID_{50} ml^{-1}) and the lethal dose 50% endpoint (LD_{50} ml^{-1}) of a Thai isolate of WSSV in 2 sizes of juvenile SPF *Litopenaeus vannamei* by i.m. inoculation, (2) to determine these virus titers by oral inoculation and (3) to establish the relationship between WSSV infection and shrimp mortality for the 2 routes of inoculation.

MATERIALS AND METHODS

Experimental shrimp and rearing conditions. SPF *Litopenaeus vannamei* of the Kona strain was used (Wyban et al. 1992). Batches of shrimps arrived as postlarvae (PL 8 to 12; mean body weight [MBW] = 0.0013 g) and were acclimatized and reared for a period of 36 to 50 d after arrival. Postlarvae were fed with *Artemia* nauplii once daily. Juveniles were fed with a commercial pelleted feed (A2 monodon high performance shrimp feed/shrimp complete grower, INVE aquaculture nv) at a rate of 2.5% MBW twice daily. Water temperature was kept at 27 ± 1°C, salinity between 30 and 35 g l^{-1}, total ammonia-N less than 0.5 mg l^{-1} and nitrite-N between 0.05 and 0.15 mg l^{-1}.

Experimental conditions. Shrimp were acclimatized to a salinity of 15 g l^{-1} over 4 d prior to experimental infectivity trials. Shrimp were kept individually in 10 l plastic aquaria provided with aeration and covered with acrylic plates. Shrimp were fed a restricted diet of 6 pellets divided into 2 rations a day to maintain water quality. Water temperature was between 25 and 28°C, total ammonia levels were between 0 and 5 mg l^{-1} and nitrite was between 0 and 0.15 mg l^{-1} as monitored daily. Artificial seawater at a salinity of 15 g l^{-1} was prepared with instant ocean (Marine Systems) dissolved in distilled water.

Virus and production of the WSSV stock. A Thai isolate of WSSV from naturally-infected *Penaeus monodon* was used. This virus isolate was passaged once in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al. 2001). A gill suspension from crayfish (10^{-2} in L-15 medium) was kindly donated by P. Jiravanichpaisal and K. Söderhäll (Uppsala University, Sweden). It was diluted 10^{-1} in phosphate-buffered saline (PBS) pH 7.4, and 50 µl were injected i.m. into SPF *Litopenaeus vannamei* to amplify the virus. The inoculated shrimp were collected at 48 h post inoculation (hpi) and were frozen at –70°C. Tissues from these shrimps were analyzed by indirect immunofluorescence (IIF) to confirm WSSV infection. Thawed carcasses without hepatopancreas, gut and exoskeleton were minced. A 10^{-1} suspension was made in PBS and centrifuged (3000 × *g* at 4°C for 20 min). The supernatant was centrifuged (13 000 × *g* at 4°C for 20 min), filtered (0.45 µm) and aliquoted for storage at –70°C. The total volume was 250 ml. Samples from tissues used to produce the viral stock were sent to Dr. James Brock (Moana Technologies LLC, Hawaii) for detection of the major viral pathogens of shrimp by polymerase chain reaction (PCR). PCR analysis confirmed the sole presence of WSSV DNA in the tissues.

In vivo* titration by intramuscular inoculation. *Virus infection titer in 135 d old juveniles and its correlation to the mortality titer: Five experiments were performed using shrimp of this age (MBW = 13.34 ± 4.08 g; n = 172). In 3 experiments, shrimp were inoculated i.m. with 200 µl of a 10-fold serial dilution of WSSV. Four shrimp were used per dilution. Moribund and dead shrimp were recorded, removed from the aquaria and processed for detection of WSSV infection. Surviving shrimp were sacrificed at the end of the experiments and analyzed for WSSV infection. These experiments

were terminated at 72, 96 and 168 hpi. In the other 2 experiments shrimp were inoculated i.m. with 50 µl of a 10-fold serial dilution of WSSV. Twelve shrimp were used per dilution. Moribund, dead and surviving shrimp were analyzed for WSSV infection. These experiments were terminated at 120 and 168 hpi (see Table 1).

Virus titers in 60 d old juveniles: Three experiments were performed with shrimp (MBW = 3.00 ± 1.18 g; n = 75) inoculated i.m. with 50 µl of a 10-fold serial dilution of WSSV. Five shrimp were used per dilution. Moribund and dead shrimp were processed for detection of WSSV infection. These experiments were terminated at 120 hpi (see Table 2).

The statistical comparison of the virus titers in these 2 sizes of juvenile shrimp was undertaken using the Mann-Whitney non-parametric test (Zar 1996).

In vivo* titration by oral inoculation. *Virus titers in 80 d old juvenile shrimp: The minimum size of shrimp that could be inoculated by the oral route was 6 to 7 g. The virus titer of the WSSV stock after oral inoculation was evaluated using the overall median virus titer after i.m. inoculation as the basis to define 5 doses (10^3, 10^2, 10^1, 10^0 and 10^{-1} SID_{50} [i.m.] in 50 µl). This procedure permitted a direct comparison of the infectivity of the virus stock when applied i.m. and when applied orally. Two experiments were performed in 80 d old shrimp (MBW = 7.75 ± 2.83 g; n = 50). Five shrimp per dose were intubated using a sterile flexible pipette tip (Biozym) and a magnifying glass (2.5×) to locate the mouth. Shrimp were placed with the ventral side up, the pipette tip was introduced into the oral cavity and the viral inoculum was delivered into the lumen of the oral tract. Moribund and dead shrimp were analyzed for WSSV infection.

Determination of the virus titers. The virus infection titers (SID_{50} ml^{-1}) and mortality (LD_{50} ml^{-1}) were calculated using the method of Reed & Muench (1938). Briefly, data of infected and uninfected shrimp at each dilution were recorded. Totals were calculated for infected shrimp from the lowest to the highest concentration and for uninfected shrimp in the inverse direction, so as to obtain the percentage of infected shrimp for each dilution. The 2 dilutions with the nearest percentage above (a) and below (b) 50% were used to calculate a proportional distance (50%−b)/(a−b) which was added to the $\log_{10}$ of the highest dilution below 50% in order to determine the 50% endpoint of infection (SID_{50} ml^{-1}) according to the volume inoculated.

Evaluation of WSSV infection. At the end of the experiments moribund, dead and surviving shrimp were processed to detect WSSV infection using IIF and 1-step PCR.

IIF analysis: Tissues from the pereon were embedded in methylcellulose (Fluka) and frozen at −20°C. Cryosections (5 to 6 µm) were made and fixed in absolute methanol at −20°C, washed with PBS, incubated for 1 h at 37°C with 2 mg ml^{-1} of the monoclonal antibody 8B7 specific for VP28 (Poulos et al. 2001), 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, dried and mounted. Slides were analyzed by fluorescence microscopy (Leica). Tissues of moribund shrimp infected with WSSV and uninfected shrimp were stained and used as positive and negative controls, respectively.

One-step PCR: Total DNA was extracted from shrimp muscle with lysis buffer (Intelligene). Primers F002 and R002 were used to amplify WSSV DNA, and primers F and R3 which amplify β-actin from shrimp were used as controls. The amplicon for WSSV was 306 bp, while that for b-actin was 339 bp (Dhar et al. 2001).

Extracted DNA (2 µl) was added to a PCR tube containing 48 µl of a PCR master mix (1× PCR buffer [Eurogentec], 1.5 mM $MgCl_2$, 0.3 mM of each of the respective forward and reverse primers for WSSV or β-actin, 1.6 mM dNTPs [Eurogentec], 1 U hot goldstar *Taq* polymerase [Eurogentec] total reaction volume 50 µl). One-step PCR was carried out, which included a preheating step at 95°C for 10 min followed by 35 cycles each with the following steps: denaturation (94°C for 45 s), annealing (55°C for 45 s) (72°C for 75 s) and a final extension at 72°C for 5 min. PCR products were stored at 4°C. PCR products (12 µl), negative (ultrapure water) and positive (DNA from a 10^{-2} dilution of WSSV stock) controls, as well as DNA markers (smart ladder, Eurogentec) were resolved on a 1.2% agarose gel in tris-acetate-EDTA (TAE) buffer. The gel was stained with ethidium bromide (0.02 mg ml^{-1}) and visualized by UV transillumination.

RESULTS

In vivo titration by intramuscular inoculation

Virus infection titer in 135 d old juveniles and its correlation to the mortality titer. The number of shrimp that died before termination of the experiments at 72, 96, 120 and 168 hpi and the number of dead or euthanized shrimp found positive by IIF (Fig. 1) and 1-step PCR are presented in Table 1. The virus infection titers in the experiments terminated at 72 and 96 hpi were $10^{6.0}$ and $10^{6.4}$ SID_{50} ml^{-1} respectively, and the infection titer in the experiment terminated at 168 hpi was $10^{6.5}$ SID_{50} ml^{-1}. The experiments performed with 12 shrimps $dilution^{-1}$ had virus infection titers of $10^{6.4}$ SID_{50} ml^{-1} at 120 hpi and $10^{6.6}$ SID_{50} ml^{-1} at

168 hpi. The same proportion of infected shrimp was detected by both IIF and 1-step PCR in each of these experiments.

The mortality titers were lower than the infection titers when the experiments were terminated at 72 or 96 hpi ($10^{5.7}$ and $10^{5.4}$ LD_{50} ml^{-1}, respectively), whereas the mortality titer fully matched the infection titer in the experiment terminated at 120 hpi ($10^{6.4}$ LD_{50} ml^{-1}) as well as in the 2 experiments terminated at 168 hpi ($10^{6.5}$ and $10^{6.6}$ LD_{50} ml^{-1}).

Virus titers in 60 d old juveniles. The virus infection titers in each of the 3 experiments performed on this size of juveniles were $10^{6.8}$, $10^{6.9}$ and $10^{6.5}$ SID_{50} ml^{-1} (Table 2). The same proportion of WSSV-infected shrimp was determined by IIF and 1-step PCR (Fig. 2). Likewise, the mortality titers were the same as the infection titers for each experiment.

The statistical comparison of the 3 virus titers determined at 120 and 168 hpi in 135 d old shrimp and the 3 titers determined in 60 d old juveniles inoculated by the i.m. route showed no significant differences ($p \geq 0.05$). Therefore, these 6 virus titers were used to establish the overall median virus infection titer of the WSSV stock at $10^{6.6}$ SID_{50} ml^{-1}, and the overall median virus mortality titer was $10^{6.6}$ LD_{50} ml^{-1}.

Table 1. *Litopenaeus vannamei.* Mortality (LD_{50} ml^{-1}) and infection (SID_{50} ml^{-1}) titers of the white spot syndrome virus (WSSV) stock by intramuscular inoculation in 135 d old shrimp. hpi: hours post-inoculation, IIF: indirect immunofluorescence

Expt	Termination (hpi)	Dilution	No. of shrimps	Mortaliy	Infection determined by IIF	Infection determined by PCR
1	72	10^{-5}	4	2/4	2/4	2/4
		10^{-6}	4	0/4	1/4	1/4
		10^{-7}	4	0/4	0/4	0/4
		10^{-8}	4	0/4	0/4	0/4
			Titer	$10^{5.7}$ LD_{50} ml^{-1}	$10^{6.0}$ SID_{50} ml^{-1}	$10^{6.0}$ SID_{50} ml^{-1}
2	96	10^{-2}	4	4/4	4/4	4/4
		10^{-3}	4	4/4	4/4	4/4
		10^{-4}	4	4/4	4/4	4/4
		10^{-5}	4	1/4	4/4	4/4
		10^{-6}	4	0/4	1/4	1/4
			Titer	$10^{5.4}$ LD_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}
3	120	10^{-4}	12	11/12	11/12	11/12
		10^{-5}	12	7/12	7/12	7/12
		10^{-6}	12	1/12	1/12	1/12
		10^{-7}	12	0/12	0/12	0/12
		10^{-8}	12	0/12	0/12	0/12
			Titer	$10^{6.4}$ LD_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}
4	168	10^{-5}	4	4/4	4/4	4/4
		10^{-6}	4	1/4	1/4	1/4
		10^{-7}	4	1/4	1/4	1/4
		10^{-8}	4	0/4	0/4	0/4
			Titer	$10^{6.5}$ LD_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}
5	168	10^{-4}	12	12/12	12/12	12/12
		10^{-5}	12	7/12	7/12	7/12
		10^{-6}	12	3/12	3/12	3/12
		10^{-7}	12	0/12	0/12	0/12
		10^{-8}	12	0/12	0/12	0/12
			Titer	$10^{6.6}$ LD_{50} ml^{-1}	$10^{6.6}$ SID_{50} ml^{-1}	$10^{6.6}$ SID_{50} ml^{-1}

In vivo titration by oral inoculation

Virus titers in 80 d old juvenile shrimp. The 50% endpoint of the virus titers by oral inoculation was 1.2 log_{10} and 0.7 log_{10} times higher than by i.m. inoculation (Table 3). Thus, the titers of the WSSV stock determined using the oral route of inoculation were $10^{5.6}$ SID_{50} ml^{-1} and $10^{5.6}$ LD_{50} ml^{-1}.

DISCUSSION

The *in vivo* titration of viral stocks using the 50% endpoint dilution assay is commonly used when virus titers cannot be calculated *in vitro* (Flint et al. 2000). In the present study, the virus infection and mortality titers of a WSSV stock inoculated by i.m. and oral routes were determined using this method. To our knowledge, this is the first study to describe the relationship between routes of exposure (i.m. vs. oral) and virus infectivity of a WSSV stock in *Litopenaeus vannamei.* The virus infection and mortality titers in 60 and 135 d old juveniles were not significantly different, indicating that these 2 stages of juvenile shrimp had the same susceptibility to WSSV when the virus is inoculated i.m.

In vivo titrations are important to evaluate differences in susceptibility between life stages within a host species or between related species

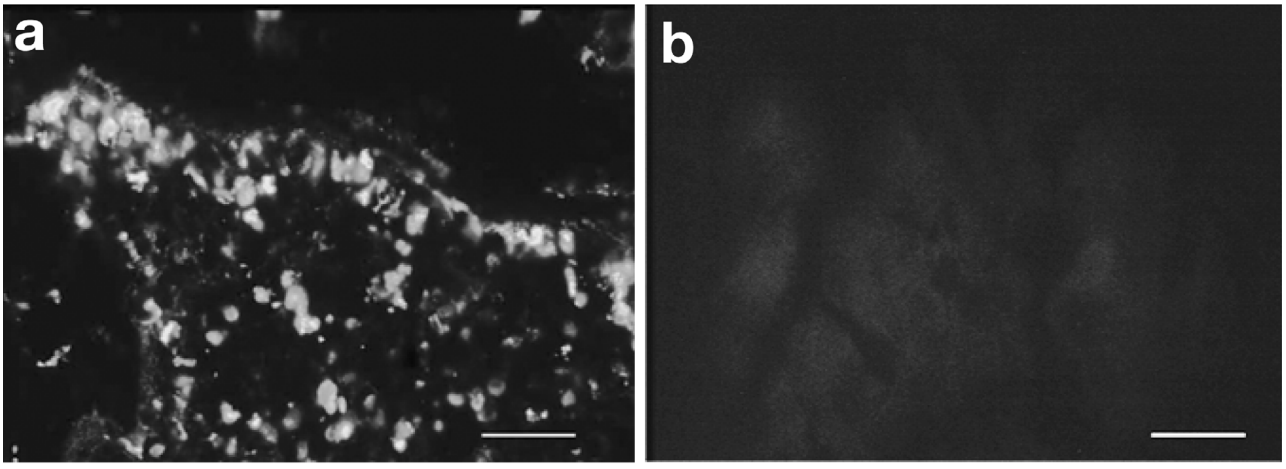

Fig. 1. *Litopenaeus vannamei*. (a) Presence or (b) absence of white spot syndrome virus (WSSV)-infected cells in tissues of (a) WSSV-infected or (b) uninfected shrimp as determined by indirect immuno-fluorescence. Scale bar = 100 µm

(Plumb & Zilberg 1999). In shrimp, there are indications that susceptibility to WSSV may differ between life stages (Pramod-Kiran et al. 2002, Yoganandhan et al. 2003), shrimp species (Lightner et al. 1998, Wang et al. 1999) and different decapods (Wang et al. 1998, Sahul-Hameed et al. 2003). However, the use of a known dose of infectious virus is critical to demonstrate these differences.

The virulence of a pathogen or its power to produce disease in a host is a measurable feature (Shapiro-Ilan et al. 2005). In order to compare the virulence of different virus strains, their infectivity (SID_{50} ml^{-1}) should be known. Every shrimp should be inoculated with the same amount of infectious virus. The inoculation techniques described in the present study may be used both to determine the infectivity of different WSSV strains and to test their virulence.

Table 2. *Litopenaeus vannamei*. Mortality (LD_{50} ml^{-1}) and infection (SID_{50} ml^{-1}) titers of the white spot syndrome virus (WSSV) stock by intramuscular inoculation in 60 d old shrimp. hpi: hours post-inoculation, IIF: indirect immunofluorescence

Expt	Termination (hpi)	Dilution	No. of shrimps	Mortality	Infection determined by IIF	Infection determined by PCR
1	120	10^{-4}	5	5/5	5/5	5/5
		10^{-5}	5	5/5	5/5	5/5
		10^{-6}	5	0/5	0/5	0/5
		10^{-7}	5	0/5	0/5	0/5
		10^{-8}	5	0/5	0/5	0/5
			Titer	$10^{6.8}$ LD_{50} ml^{-1}	$10^{6.8}$ SID_{50} ml^{-1}	$10^{6.8}$ SID_{50} ml^{-1}
2	120	10^{-4}	5	5/5	5/5	5/5
		10^{-5}	5	5/5	5/5	5/5
		10^{-6}	5	1/5	1/5	1/5
		10^{-7}	5	0/5	0/5	0/5
		10^{-8}	5	0/5	0/5	0/5
			Titer	$10^{6.9}$ LD_{50} ml^{-1}	$10^{6.9}$ SID_{50} ml^{-1}	$10^{6.9}$ SID_{50} ml^{-1}
3	120	10^{-4}	5	5/5	5/5	5/5
		10^{-5}	5	3/5	3/5	3/5
		10^{-6}	5	0/5	0/5	0/5
		10^{-7}	5	0/5	0/5	0/5
		10^{-8}	5	0/5	0/5	0/5
			Titer	$10^{6.5}$ LD_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}

The virus infection titers determined both by IIF and 1-step PCR were identical in each of the experiments performed either by intramuscular or oral inoculation. Every shrimp detected positive by PCR was equally detected by IIF. All shrimp surviving at 120 hpi or longer were not infected with WSSV as determined by these 2 methods. Although it is well established that PCR is more sensitive than IIF (Sizun et al. 1998, Walker et al. 1998), under these experimental conditions IIF was able to detect WSSV-infected cells in all shrimp detected positive by PCR. In addition, it was very convenient: cheap, easy to perform, yielding rapid results (within 4 h) and with good sensitivity. Thus, these results indicate that IIF may be considered as a suitable diagnostic tool in areas where PCR is not available.

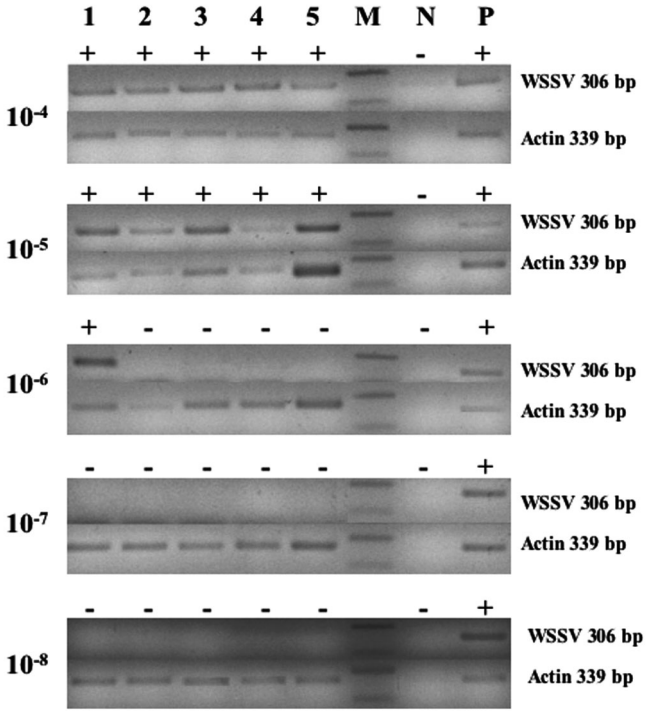

Fig. 2. Detection of white spot syndrome virus (WSSV) DNA in shrimp tissues (1-step polymerase chain reaction). Results of the second *in vivo* titration (i.m.) in 60 d old *Litopenaeus vannamei*. Lanes 1 to 5: shrimp number, M: DNA weight marker (upper = 400 bp, lower = 200 bp), N: negative-control, P: positive control. Amplification of β-actin was made as an internal control

Table 3. *Litopenaeus vannamei*. Doses of white spot syndrome virus (WSSV) (based on the median virus titer by the intramuscular, i.m., route) orally inoculated in 80 d old shrimp, and mortality (LD_{50} ml^{-1}) and infection (SID_{50} ml^{-1}) titers of the WSSV stock obtained by the oral route. hpi: hours post-inoculation, IIF: indirect immunofluorescence

Expt	Termination (hpi)	Dose (SID_{50} IM)	No. of shrimps	Mortaliy	Infection determined by IIF	PCR
1	120	10^3	5	5/5	5/5	5/5
		10^2	5	5/5	5/5	5/5
		10^1	5	2/5	2/5	2/5
		10^0	5	0/5	0/5	0/5
		10^{-1}	5	0/5	0/5	0/5
				$10^{1.2}$*	$10^{1.2}$*	$10^{1.2}$*
			Titer	$10^{5.4}$ LD_{50} ml^{-1}	$10^{5.4}$ SID_{50} ml^{-1}	$10^{5.4}$ SID_{50} ml^{-1}
2	120	10^3	5	5/5	5/5	5/5
		10^2	5	5/5	5/5	5/5
		10^1	5	3/5	3/5	3/5
		10^0	5	1/5	1/5	1/5
		10^{-1}	5	0/5	0/5	0/5
				$10^{0.7}$*	$10^{0.7}$*	$10^{0.7}$*
			Titer	$10^{5.9}$ LD_{50} ml^{-1}	$10^{5.9}$ SID_{50} ml^{-1}	$10^{5.9}$ SID_{50} ml^{-1}

*Oral inoculation required 0.7 log_{10} or 1.2 log_{10} times more infectious virus than i.m. inoculation to cause infection or death

The relationship between the virus infection and mortality titers using the Thai isolate of WSSV by the i.m. or oral route was 1:1 only in experiments which were terminated at 120 hpi or later. Thus, every shrimp that became infected with this strain of WSSV by either of these routes of inoculation died within 120 hpi. Although previous studies have determined WSSV infection and mortality of *Litopenaeus vannamei*, none has indicated a full match between these 2 parameters: All surviving shrimp analyzed by histopathology were WSSV-negative, but not all the dead shrimp (6%) were WSSV-positive (Soto & Lotz 2003) or the analysis of WSSV infection was not performed in all the survivors (Prior et al. 2003). Therefore, the present study is the first to show the identity between infection (SID_{50} ml^{-1}) and mortality (LD_{50} ml^{-1}) in shrimp inoculated by i.m. or oral routes. Moreover, the present study extends the findings of Prior et al. in the following respects: (1) the virus titers were determined by i.m. injection and oral intubation in contrast to the evaluation of the LD_{50} only by i.m. inoculation but not by immersion; (2) the WSSV strain used is different (a Thai strain vs. an American strain); (3) the infectivity of the WSSV stock was determined by the i.m. route in 2 sizes of juvenile shrimp, in contrast to only 1 shrimp size; and (4) the use of IIF to detect WSSV replication in infected cells represents a more appropriate virological technique than histopathology to determine WSSV infection.

The oral route represents the natural way of WSSV entry into shrimp through ingestion (Wu et al. 2001, Lotz & Soto 2002). Experimental infections using the oral route have been performed by feeding shrimp and other crustaceans with WSSV-infected tissues. However, this procedure cannot guarantee that every animal receives the same amount of infectious virus. The results presented here demonstrate that oral intubation makes it possible to deliver a fixed quantity of virus to all inoculated shrimp and therefore represents a solution to this problem.

Once WSSV enters the oral cavity it has to overcome a series of physical and chemical barriers in the digestive tract of the shrimp in order to reach the susceptible epithelial cells. The cuticle sheath coating the epithelium of the foregut forms an important physical barrier (Ceccaldi 1997, Martin & Chiu 2003). Digestive enzymes such as trypsin, amylases and lipases (Gamboa-Delgado et al. 2003)

form a chemical barrier. These barriers may account for the reduction of WSSV infection by approximately 1 $\log_{10}$ compared with the i.m. route of inoculation. It is possible that these barriers may be able to prevent WSSV from reaching the epithelial cells in the stomach when orally inoculated.

In conclusion, the virus titers of the WSSV stock determined *in vivo* by intramuscular and oral routes constitute the first step towards standardization of infectivity models. These models can be used for the evaluation of different strategies (immunostimulants, antivirals and vaccines) aimed at reducing the impact of WSSV disease and for comparing the virulence of WSSV strains.

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