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Effect of dose and challenge routes of *Vibrio* spp. on co-infection with white spot syndrome virus in *Penaeus vannamei*

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ABSTRACT

This study was conducted to investigate the effect of dose and challenge routes of *Vibrio* spp. on co-infection with white spot syndrome virus (WSSV) in specific pathogen-free (SPF) *Penaeus vannamei* shrimp. Juvenile shrimp were first injected with WSSV at a dose of 30 SID_{50} $shrimp^{-1}$ (SID_{50} = shrimp infectious dose with 50% endpoint) and 24 h later with 10^3, 10^4, 10^5 or 10^6 CFU $shrimp^{-1}$ of *V. campbellii*. Controls did not die during the experiment, except the ones that received 10^6 CFU $shrimp^{-1}$ (35–65%). In WSSV-inoculated shrimp, the 100% cumulative mortality were reached at 144–360 h post injection (hpi). WSSV-infected shrimp died much faster when injected with at least 10^4 CFU of *V. campbellii* with the 100% cumulative mortality reached at 48–96 hpi of virus. The density of *V. campbellii* in haemolymph of co-infected moribund shrimp collected 6 h after *V. campbellii* injection was significantly higher than that in shrimp injected with *V. campbellii* only. There was no difference in the number of WSSV-infected cells between shrimp inoculated with WSSV only, compared to dually inoculated ones. Shrimp which were first injected with WSSV and 24 h (or 48 h) later exposed to 10^6, 10^7, or 10^8 CFU ml^{-1} of *V. campbellii* by immersion did not show any accelerated mortality. When WSSV-infected shrimp were challenged with another *Vibrio* species, *V. harveyi* BB120, no accelerated mortality was noted in WSSV-infected shrimp injected with 10^6 CFU $shrimp^{-1}$ of *V. harveyi* BB120.
In conclusion, it can be stated that the synergistic effect between WSSV and *Vibrio* is influenced by the dose, species and infection route of inoculation of the *Vibrio* bacteria.

1. Introduction

Infectious diseases especially caused by bacterial and viral pathogens are serious loss factors in shrimp farming (Primavera, 1998). One of the viruses considered to be particularly problematic in shrimp culture around the world is the white spot syndrome virus (WSSV), which belongs to the genus *Whispovirus* in the family *Nimaviridae* (Mayo, 2002). WSSV is found in almost all shrimp producing countries and lethal to all commercially cultivated penaeid shrimp species (Wang et al., 2000; Sanchez-Martinez et al., 2007; Escobedo-Bonilla et al., 2008). White spot syndrome disease is characterized by the presence on the inner surface of the exoskeleton of white spots from which the name is derived (Lo et al., 1996). Other clinical signs include anorexia, lethargy and reddish discoloration of the body (Wang et al., 1999).

Amongst the bacterial pathogens, *Vibrio* species are reputed for causing vibriosis in penaeid shrimp. This important disease is known to affect hatchery-reared *Penaeus monodon* as well as juvenile shrimp in grow-out cultures and adults (Lavilla-Pitogo et al., 1990) and is mostly caused by *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. penaeicida*, *V. campbellii*. *Vibrio* spp. can act as primary pathogens in pond waters with increased *Vibrio* populations (Vandenberghe et al., 1998; Saulnier et al., 2000a) but often act as opportunistic agents in secondary infections (Saulnier et al., 2000b). Most outbreaks of shrimp vibriosis happen either in combination with physical stress factors or following primary infections with other pathogens (Sung et al., 2001). In experimental studies, shrimp exposed to ammonium stress prior to challenge, showed higher susceptibility to vibrios (Liu and Chen, 2004). It has also been indicated that a primary WSSV infection may weaken shrimp, increasing their susceptibility to bacterial infections (Selvin and Lipton, 2003). The influence of all these factors on the susceptibility to *Vibrio* could explain the highly variable mortality in shrimp, ranging from a few individual shrimp to 100% of the population.

Under field conditions, animals are often infected with more than one pathogen. Bacteria–bacteria co-infections have been demonstrated in *P. monodon* displaying red disease syndrome. After performing challenge tests with a combination of *V. parahaemolyticus* and *V. harveyi* isolated from diseased shrimp, Alapide-Tendencia and

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Dureza (1997) concluded that these bacterial strains can reproduce the syndrome in healthy shrimp. Virus–virus co-infection was reported in *P. monodon* shrimp postlarvae (PL_8–PL_{10}) in an India hatchery. These shrimp were heavily infected with monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) and WSSV (Manivannan et al., 2002). Co-infection of infectious hypodermal and haematopoietic necrosis virus (IHHNV) and WSSV in cultured *P. vannamei* was reported by Yang et al. (2006). Using histopathology and PCR, Flegel et al. (2004) found a very high prevalence of dual, triple and quadruple infections with HPV, WSSV, IHHNV and MBV in commercial shrimp ponds in Thailand. While 94% of the sampled shrimp gave a positive test for at least one of the four viruses, dual to quadruple infections accounted for 73% of the total samples.

Selvin and Lipton (2003) demonstrated the presence of a virulent strain of *V. alginolyticus* in shrimp from a pond hit by a WSSV outbreak. Although not all sampled shrimp were infected by both pathogens, it was stated that shrimp weakened by WSSV would succumb to a secondary infection by *Vibrio*. In other investigations, *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus*, *V. damsela*, *Vibrio* sp. were detected in healthy shrimp without gross signs of disease (GÓmez-Gil et al., 1998). Flegel et al. (2004) found WSSV in the shrimp without gross or histological signs of disease.

From all these data, it seems plausible that co-infections occur regularly in shrimp ponds. In a previous paper, a dual WSSV-*Vibrio* infection protocol has been described by Phuoc et al. (2008). The aim of this study was to test whether the outcome of the experimental co-infection of WSSV and *V. campbellii* is influenced by (1) the dose of *V. campbellii*, (2) the bacterial species and (3) the challenge route of the *Vibrio* component.

2. Materials and methods

2.1. Viral and bacterial stocks

2.1.1. Viral stock

A Vietnamese WSSV isolate was used in this study. This isolate has been studied before and was shown to be significantly less virulent than two other isolates from Thailand (Rahman et al., 2007a,b). The original WSSV isolate from naturally infected *P. monodon* was passaged once into crayfish (*Cherax quadricarinatus*). Crayfish gill suspension containing WSSV was received from Research Institute for Aquaculture No.2, Vietnam. The isolate was amplified in SPF *P. vannamei* juveniles. The virus stock was titrated *in vivo* by Escobedo-Bonilla et al. (2005). A dose of 30 SID_{50} was prepared in a volume of 50 µl by diluting the stock with phosphate buffered saline (PBS).

2.1.2. Bacterial stock

Two bacterial strains were used in this study. *Vibrio campbellii* (LMG21363) was obtained from the BCCM collection (http://bccm.belspo.be/about/lmg.php) which is an internationally recognized laboratory for storing strains. *Vibrio harveyi* BB120 was directly obtained from the laboratory of Bonnie Bassler (Department of Molecular Biology, Princeton University) and stored in the −80 °C from a collaborating laboratory (Laboratory for Microbial Ecology and Technology, Ghent University) who keeps the stock of strains we are working with. See Phuoc et al. (2008) for more detail on preparation of bacteria for the challenge test.

2.2. Experimental animals and conditions

Specific pathogen-free (SPF) *P. vannamei* were imported from Sy-Aqua Siam Co., Ltd. Bangkok 10110, Thailand. Animals were certified to be free of Taura Syndrome Virus (TSV), WSSV, Yellow Head Virus (YHV) and IHHNV by the Thai Department of Fisheries. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL_{8-12}). They were kept in a recirculation system at a water temperature of 28 °C, 35 g l^{-1} salinity, and pH of 7.8–8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After one week their diet was shifted to A2 monodon high performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Dendermonde, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. In this study, we applied the challenge protocol described by Rahman et al. (2008). Therefore, shrimp were acclimatized to 15 g l^{-1} salinity before being challenged. Acclimatized shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the infection experiments were performed under biosafety conditions. See Phuoc et al. (2008) for more detail on the challenge protocol.

2.3. Rifampicin-resistant Vibrio campbellii

In some experiments (2 and 3), *V. campbellii* had to be quantified by re-isolation and enumeration. To facilitate this procedure, rifampicin-resistant (RR) *V. campbellii* were used instead of rifampicin-sensitive (RS) *V. campbellii*. The method for producing (RR) *V. campbellii* was described by Phuoc et al. (2008). When bacteria cultures were growing well in the final concentration of rifampicin (100 mg l^{-1}), they were inoculated on MA plates containing 100 mg l^{-1} rifampicin for obtaining single colonies. The stock was stored in 20% glycerol at −80 °C for long term storage. An *in vivo* challenge test confirmed that the selection process had not altered the virulence of this strain (data not shown).

2.4. Immunohistochemistry and quantification of WSSV-infected cells

Shrimp samples were collected and fixed in Davidson's fixative for 36 h and kept in 50% ethanol afterwards. Samples were processed as described by Bell and Lightner (1988). Paraffin-embedded tissue sections were cut at 5 µm and placed onto Silane-coated slides (A3648, Sigma-Aldrich). Sections were deparaffinized and rehydrated. The endogenous peroxidase was blocked by incubating the slides for 30 min at room temperature in a solution of 1% sodium azide and 0.02% hydrogen peroxidase in Tris buffer pH 7.4. Sections were incubated for 1 h at 37 °C with 2 µg ml^{-1} of monoclonal antibody 8B7 (Diagxotics Inc, USA) raised against WSSV envelope protein VP28 (Poulos et al., 2001). Sections were washed in Tris buffer (pH 7.6) and incubated for 1 h at 37 °C with a 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN1001, Amersham Biosciences). Afterwards, they were washed, incubated for 30 min at room temperature with 1:200 dilution of streptavidine-biotinylated horseradish peroxidase complex (RPN1051 Amersham Biosciences) and washed again. Color was developed with 0.01% of 3, 3′-diaminobenzidine (D8001 Sigma-Aldrich). Sections were counterstained with Gill's hemaluin and washed in water, dehydrated and mounted. WSSV-infected cells were counted using light microscopy (Leica DM RBE) at a 400× magnification in five fields in gills and lymphoid organs and in two-three fields in haematopoietic tissue. These counts were converted to the number of WSSV-infected cell mm^{-2}. Both WSSV-infected and uninfected cells in stomach epithelium were counted in five fields and the average percentage (%) of infected cells was calculated.

2.5. Enumeration of bacterial density

RR *V. campbellii* were enumerated on MA with 100 mg l^{-1} rifampicin (MAR). *V. campbellii* density in the shrimp's haemolymph was determine by the method previously described by Phuoc et al. (2008).

2.6. Experimental design

2.6.1. Experiment 1: dose effect of V. campbellii on mortality of WSSV-infected P. vannamei (1st run)

This experiment was conducted to test the clinical outcome of WSSV infections combined with different doses of *V. campbellii*

Table 1
Design of experiment 1 to examine the effect of *V. campbellii* injection with different doses (10^3, 10^4, 10^5, 10^6 CFU shrimp^{-1}) on mortality of WSSV-infected *P. vannamei*.

	Treatments	WSSV injection	*V. campbellii* injection	Number of shrimp
1	WSSV	30 SID_{50}	–	6
2	VC	–	10^3 CFU shrimp^{-1}	6
3	VC	–	10^4 CFU shrimp^{-1}	6
4	VC	–	10^5 CFU shrimp^{-1}	6
5	VC	–	10^6 CFU shrimp^{-1}	6
6	WSSV + VC	30 SID_{50}	10^3 CFU shrimp^{-1}	6
7	WSSV + VC	30 SID_{50}	10^4 CFU shrimp^{-1}	6
8	WSSV + VC	30 SID_{50}	10^5 CFU shrimp^{-1}	6
9	WSSV + VC	30 SID_{50}	10^6 CFU shrimp^{-1}	6
10	Control	–	–	6

WSSV = White spot syndrome virus; VC = *Vibrio campbellii*; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; – = mock inoculation.

Table 3
Design of experiment 4 to examine the effect of immersion challenge with different doses of *V. campbellii* (10^6, 10^7, 10^8 CFU ml^{-1}) on mortality of WSSV-infected *P. vannamei*.

	Treatments	WSSV injection	*V. campbellii* immersion	Number of shrimp
1	WSSV	30 SID_{50}	–	6
2	VC	–	10^6 CFU ml^{-1}	6
3	VC	–	10^7 CFU ml^{-1}	6
4	VC	–	10^8 CFU ml^{-1}	6
5	WSSV (24 h) + VC	30 SID_{50}	10^6 CFU ml^{-1}	6
6	WSSV (24 h) + VC	30 SID_{50}	10^7 CFU ml^{-1}	6
7	WSSV (24 h) + VC	30 SID_{50}	10^8 CFU ml^{-1}	6
8	WSSV (48 h) + VC	30 SID_{50}	10^6 CFU ml^{-1}	6
9	WSSV (48 h) + VC	30 SID_{50}	10^7 CFU ml^{-1}	6
10	WSSV (48 h) + VC	30 SID_{50}	10^8 CFU ml^{-1}	6
11	Control	–	–	6

WSSV = White spot syndrome virus; VC = *Vibrio campbellii*; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; – = mock inoculation.

(Table 1). At 0 h, groups of 6 shrimp (MBW = 3.88 ± 0.60 g) were either injected with 30 SID_{50} of WSSV (treatment 1, 6, 7, 8, and 9) or mock inoculated (treatment 2, 3, 4, 5, and 10). After 24 h, shrimp were either injected with different doses of *V. campbellii* (treatment 2, 3, 4, 5, 6, 7, 8, and 9), or mock inoculated (treatment 1 and 10). After each injection procedure, shrimp were placed in their individual 10-l aquarium. Every 12 h, they were monitored for disease symptoms and moribund/dead shrimp were collected. Every five days, 75% of water was replaced with new seawater (15 g l^{-1} salinity) to minimize ammonia build-up.

2.6.2. Experiment 2: dose effect of V. campbellii on mortality of WSSV-infected P. vannamei (2nd run)

In this experiment, we aimed to repeat experiment 1. The experiment was identical to the first experiment except for a very slight difference in shrimp size (MBW = 4.59 ± 0.8 g).

2.6.2.1. Enumeration of V. campbellii. Forty two extra shrimp from treatments with only *V. campbellii* and dual injection were prepared for enumeration of *V. campbellii*. These shrimp were injected with 10^5 CFU of *V. campbellii*. This dose was chosen as it did not cause any mortality when injected alone but could lead to a very clear acceleration of mortality in co-infections with WSSV. Three shrimp from each treatment were collected at 1, 2, 3, 4, 6, 8, and 10 h post *V. campbellii* injection (hpvi).

2.6.3. Experiment 3: co-infection of P. vannamei with WSSV and V. harveyi BB120

This experiment aimed to investigate whether co-infection also occurs with another *Vibrio* strain. The experiment was set up with 6 treatments (Table 2). At 0 h, groups of six shrimp (MBW = 5.17 ± 0.94 g) were either injected with 30 SID_{50} of WSSV (treatment 1, 4, and 5) or mock inoculated (treatment 2, 3, and 6). After 24 h, all shrimp were either injected with 10^6 CFU of *V. campbellii* (treatment 2 and 4) or 10^6 CFU of *V. harveyi* BB120 (treatment 3 and 5) or mock inoculated (treatment 1 and 6). Shrimp were kept in the same conditions as described in experiment 1.

2.6.3.1. Quantification of WSSV and V. campbellii. Eighteen extra shrimp treated either with a single injection of 10^5 CFU *V. campbellii* (or WSSV) or a dual injection were sampled at 6 hpvi for quantification of WSSV and *V. campbellii*. After taking the haemolymph, shrimp were fixed in Davidson's fixative for quantification of WSSV-infected cells by IHC.

2.6.4. Experiment 4: immersion challenge of WSSV-infected P. vannamei with different doses of V. campbellii

The aim of this experiment was to test whether immersion challenge, instead of intramuscular injection, with different doses of *V. campbellii* would also result in accelerated mortality of WSSV-infected *P. vannamei*. Twenty-four or 48 h after WSSV injection, shrimp (MBW = 2.41 ± 0.65 g) were immersed in artificial sea water containing 10^6, 10^7 or 10^8 CFU ml^{-1} of *V. campbellii* (Table 3). Bacteria were added once to the tanks and remained there during the experimental period. Shrimp were kept in the same conditions as described in experiment 1.

2.6.4.1. Statistical analysis. Differences between treatments were evaluated by performing *t*-test analysis using statistical analysis software SPSS (version 13.0 for Windows). Values in percentages (WSSV-infected cells in stomach epithelium) were ArcSin-transformed to satisfy the requirement for a normal distribution.

Table 2
Design of experiment 3 to examine the synergistic effect of WSSV and *V. harveyi* BB120 injection on mortality of *P. vannamei*.

	Treatments	WSSV (injection)	VC (injection)	BB120 (injection)	Number of shrimp
1	WSSV	30 SID_{50}	–	–	6
2	VC	–	10^6 CFU shrimp^{-1}	–	6
3	BB120	–	–	10^6 CFU shrimp^{-1}	6
4	WSSV + VC	30 SID_{50}	10^6 CFU shrimp^{-1}	–	6
5	WSSV + BB120	30 SID_{50}	–	10^6 CFU shrimp^{-1}	6
6	Control	–	–	–	6

WSSV = White spot syndrome virus; VC = *Vibrio campbellii*; BB120 = *Vibrio harveyi* BB120; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; – = mock inoculation.

3. Results

3.1. Experiment 1: dose effect of V. campbellii on mortality of WSSV-infected P. vannamei (1st run)

Shrimp injected with WSSV only started to die at 48 hpi. At 156 hpi, cumulative mortality had reached 83%. No mortality was observed when shrimp were injected with 10^3, 10^4, or 10^5 CFU shrimp^{-1} of *V. campbellii* but a higher dose (10^6 CFU shrimp^{-1}) resulted in 66.7% cumulative mortality. WSSV-infected shrimp which also had been injected with bacteria typically died at earlier time points than the shrimp in the single treatments. Shrimp in dual treatments receiving bacterial doses of 10^4 CFU or more started to die at 36 hpi and cumulative mortality reached 100% at 60–96 hpi (Fig. 1). At 96 hpi, 100% mortality was obtained in dual treatments with WSSV and *V. campbellii* while only 50% was found in shrimp injected with only WSSV. Shrimp injected with WSSV and 10^3 *V. campbellii* did not display acceleration in their mortality rate.

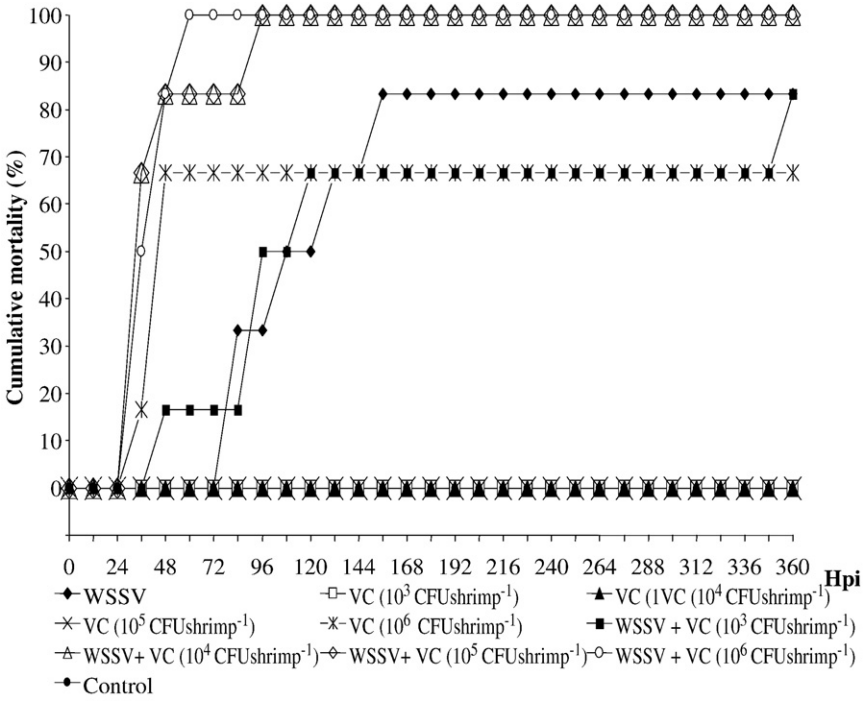

Fig. 1. Cumulative shrimp mortality (%) after challenge with WSSV and different doses of V. campbellii (1st run, each treatment is started with six shrimp).

3.2. Experiment 2: dose effect of V. campbellii on mortality of WSSV-infected P. vannamei (2nd run)

The mortality patterns in the various groups were similar to those in experiment 1 (Fig. 2). Mortality in the group with WSSV injection only started at 48 hpi and reached 100% at 204 hpi. No mortality was observed in the groups injected with 10^5 CFU or lower quantities of *V. campbellii*. Injection of 10^6 CFU of *V. campbellii* killed approximately 35% of the shrimp. In dual treatments with WSSV and *V. campbellii*, 80–100% mortality was recorded at 96 hpi, while for the shrimp injected with WSSV alone this was only 33% at that time point. Once again, injections with 10^3 CFU of *V. campbellii* did not have any effect on the mortality in co-infection with WSSV.

3.2.1. Enumeration of V. campbellii in the shrimp's haemolymph

In the treatment with *V. campbellii* only, the bacterial density was 126 ± 14 CFU ml^{-1} at 1 h after *Vibrio* injection, and decreased gradually in the following hours. In the haemolymph of dually infected shrimp, the amount of *V. campbellii* was significantly higher than that in shrimp injected with *V. campbellii* only from 3 hpvi

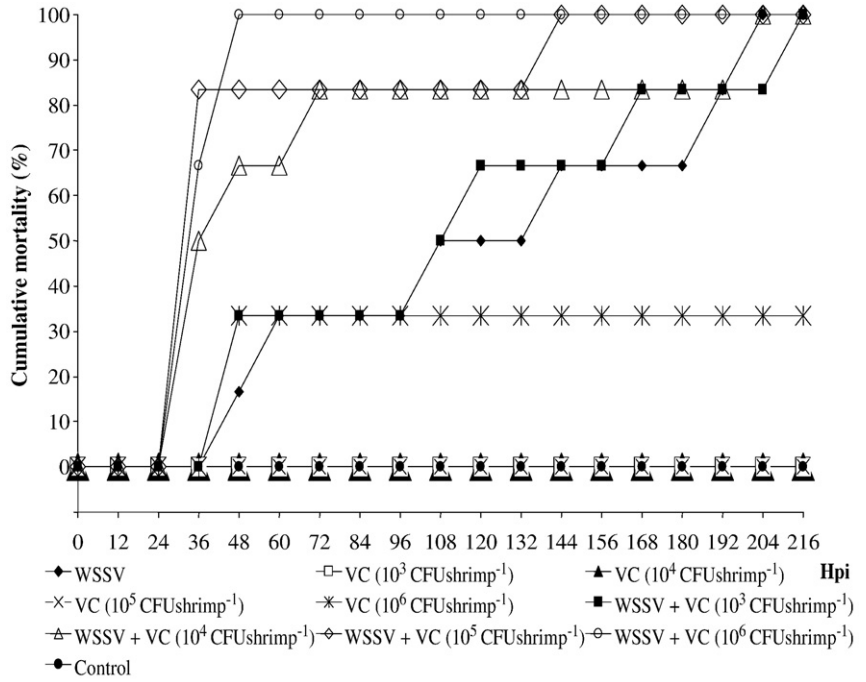

Fig. 2. Cumulative shrimp mortality (%) after challenge with WSSV and different doses of V. campbellii (2nd run, each treatment is started with six shrimp).

Table 4
Bacterial density in the haemolymph of euthanized shrimp (CFU ml^{-1} haemolymph; mean ± SD) collected at 1, 2, 3, 4, 6, 8 and 10 hpvi.

Time points	Treatments	
	V. campbellii	WSSV + *V. campbellii*
	(CFU ml^{-1} of haemolymph)	(CFU ml^{-1} of haemolymph)
1 h	126 ± 14	356 ± 204
2 h	90 ± 67	384 ± 205
3 h	83 ± 55	1037 ± 842
4 h	36 ± 26	4466 ± 5193
6 h	8 ± 13	5975 ± 6597
8 h	8 ± 13	8236 ± 8683
10 h	26 ± 45	8500 ± 4237

WSSV = White spot syndrome virus; *V. campbellii* = *Vibrio campbellii*; CFU = Colony forming unit.

Table 5
Quantification of WSSV-infected cells and *V. campbellii* (mean ± SD) in gills (G), stomach epithelium (SE), lymphoid organ (LO) and haematopoietic tissue (HP) of shrimp collected 6 h after *V. campbellii* injection (shrimp in dual treatment were moribund).

Treatments	WSSV-infected cells in organs				VC (CFU ml^{-1} of haemolymph)
	G (cells mm^{-2})	SE (%)	LO (cells mm^{-2})	HP (cells mm^{-2})	
WSSV	189 ± 130[a]	14 ± 9[a]	59 ± 72[a]	210 ± 154[a]	–
VC	–	–	–	–	231 ± 445[a]
WSSV + VC	183 ± 51[a]	15 ± 6[a]	39 ± 18[a]	143 ± 86[a]	83430 ± 66871[b]

G = Gills; SE = Stomach epithelium; HP = Haematopoietic tissue; LO = Lymphoid organ; WSSV = White spot syndrome virus; VC = *Vibrio campbellii*; CFU = Colony forming unit. Numbers of infected cells in the same tissue or CFU ml^{-1} with different superscripts were significantly different between the two treatments ($P<0.01$).

onwards (Table 4). In the dual treatment, the bacterial density increased spectacularly from 356 ± 204 CFU ml^{-1} haemolymph (1 hpvi) to 8500 ± 4237 (10 hpvi).

3.3. Experiment 3: co-infection of *P. vannamei* with WSSV and *V. harveyi* BB120

Shrimp injected with WSSV only started to die at 72 hpi. No mortality was observed when shrimp were injected with 10^6 *V. harveyi* BB120 only. As in previous experiments, dually inoculated shrimp with WSSV and *V. campbellii* died quickly after *V. campbellii* injection and reached 100% mortality at 60 hpi.

In contrast with the results obtained with *V. campbellii*, the injection of 10^6 *V. harveyi* BB120 did not accelerate mortality of WSSV-infected shrimp (Fig. 3).

3.3.1. Quantification of WSSV and *V. campbellii*

Moribund shrimp in dual treatment of this experiment were collected at 6 hpvi for quantification of WSSV and *V. campbellii*. At the same time, shrimp only treated with WSSV or *V. campbellii* were also collected. No significant difference in the number of WSSV-positive cells was found between groups which were administered both pathogens or WSSV alone ($P<0.01$). The number of WSSV-infected cells in the haematopoietic tissue (10–443 cells mm^{-2}) was higher than that in the gills (12–374 cells mm^{-2}) and the lymphoid organs (2–185 cells mm^{-2}) (Table 5). In the stomach epithelium, 1–23% of cells were infected. The number of *V. campbellii* isolated from shrimp injected with only bacteria was lower than 300 CFU ml^{-1}. In contrast, a very high density of *V. campbellii* (0.8×10^5 CFU ml^{-1}) was observed in the haemolymph of shrimp in the dual treatment (Table 5).

3.4. Experiment 4: immersion challenge of WSSV-infected *P. vannamei* with different doses of *V. campbellii*

In this experiment, mortalities were only obtained in shrimp injected with WSSV. Shrimp injected with WSSV only started to die at 48–60 hpi and reached 100% cumulative at 144–168 hpi. Immersion challenge with 10^6, 10^7 or 10^8 CFU ml^{-1} of *V. campbellii* only did not cause any mortality in *P. vannamei* juveniles (Fig. 4). Shrimp injected with WSSV and challenged by immersion 24 h or 48 h later with different doses of *V. campbellii*, did not show any significant acceleration in mortality compared to single WSSV treatment (Figs. 4 and 5) The first dead shrimp was observed at 48 h, the time at which

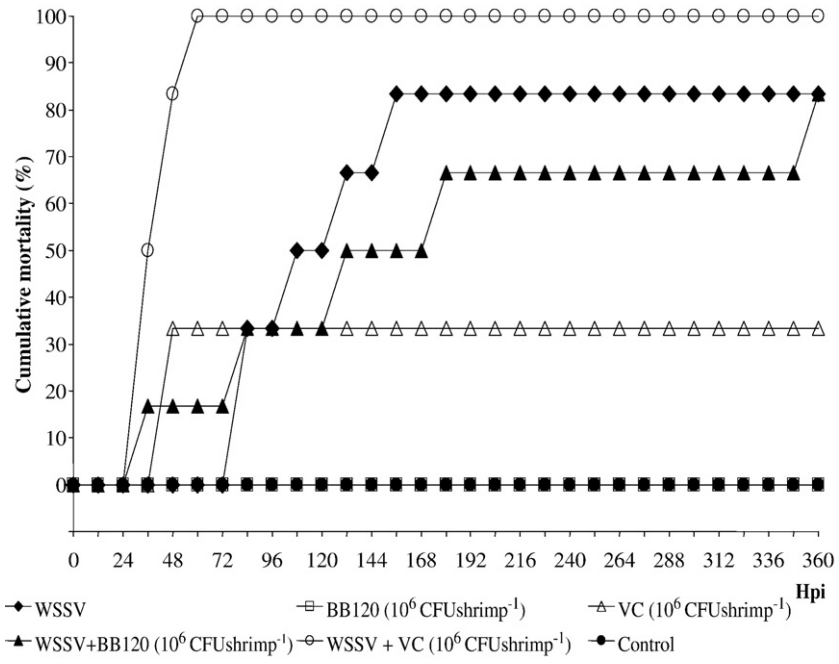

Fig. 3. Cumulative shrimp mortality (%) after challenge with WSSV and V. harveyi BB120 (each treatment is started with six shrimp).

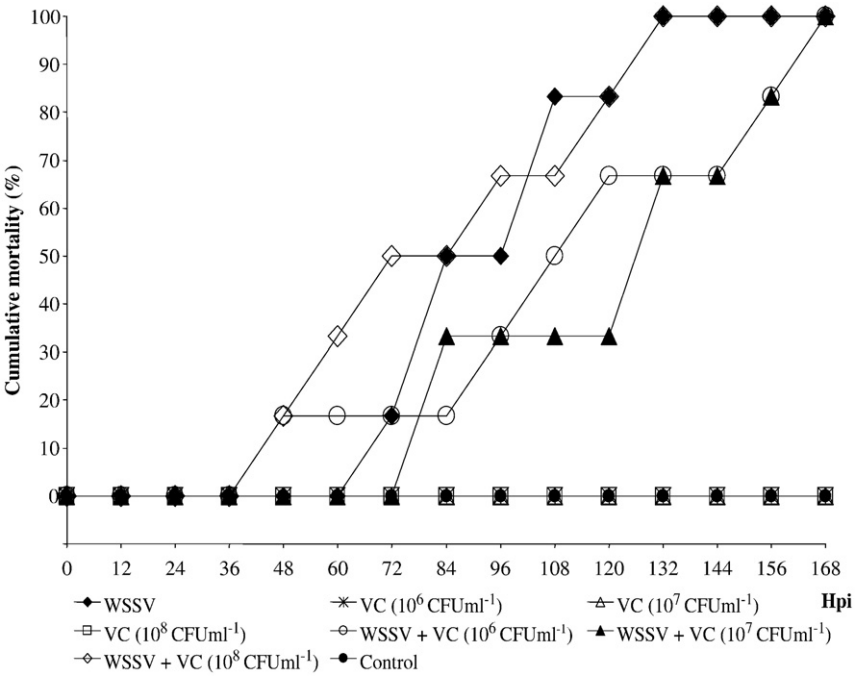

Fig. 4. Cumulative shrimp mortality (%) after immersion in V. campbellii 24 h after WSSV injection (each treatment is started with six shrimp).

WSSV-infected shrimp were challenged with 10^6 or 10^8 CFU of *V. campbellii* (Fig. 4).

4. Discussion

Previous study revealed acceleration in the mortality rate of *P. vannamei* shrimp when dually infected with WSSV and *V. campbellii* (Phuoc et al. 2008). The current study was conducted to determine the threshold dose of *V. campbellii* which can still produce accelerated mortality upon injection in WSSV-compromised shrimp. As in our previous study, shrimp injected with WSSV only started to die between 48 and 84 hpi and cumulative mortality reached 100% at 144–336 hpi. When only *V. campbellii* was injected, a dose as high as 10^6 CFU was needed to kill 30–60% of the experimental animals. A clear acceleration in the mortality rate of WSSV-infected shrimp was observed after inoculation with *V. campbellii*. Shrimp receiving these dual inoculations started to die at 36–48 hpi and cumulative mortality reached 100% at 48–96 hpi. Dual inoculations only resulted in faster mortality when at least 10^4 CFU of *V. campbellii* was administered.

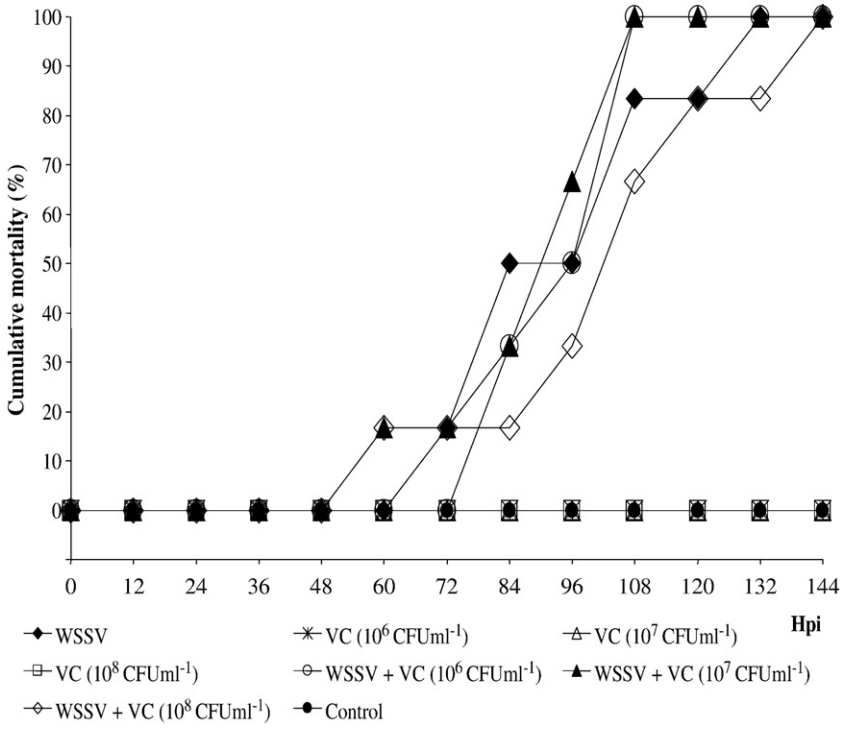

Fig. 5. Cumulative shrimp mortality (%) after immersion in V. campbellii 48 h after WSSV injection (each treatment is started with six shrimp).

In this study, the lethal *Vibrio* dose for WSSV-compromised shrimp was a 100-fold lower than for WSSV-free shrimp. Lee et al. (1999) reported an increase in mortality of grouper (*Epinephelus* sp.) when challenged with *Vibrio carchariae* by immersion or injection after being already infected with infectious pancreatic necrosis virus for two weeks. This kind of virus–bacteria interaction was also described by Pakingking et al. (2003) and Oh et al. (2006). So far, virus–virus or virus–bacteria co-infections in shrimp ponds have been described but no experimental studies on co-infections in shrimp have been published (Mohan et al., 1998; Selvin and Lipton, 2003; Umesha et al., 2006). The present study substantiates the existence of a synergistic effect on mortality of shrimp caused by dual infection under experimental conditions.

In an attempt to uncover the reason behind the accelerated mortality due to dual infection, WSSV and *V. campbellii* were quantified inside the shrimp. By immunohistochemistry, WSSV-infected cells were detected in gills, lymphoid organ, haematopoietic tissue and stomach epithelium. These organs were identified as major target organs for replication of WSSV (Chang et al., 1996) and were also previously selected by Escobedo-Bonilla et al. (2007) and Rahman et al. (2008) for enumeration of WSSV-infected cells. The quantification results showed some variation between the organs, but did not show any significant difference in the number of WSSV-infected cells between the single and dual infection treatments. As monoclonal antibodies for *V. campbellii* were not available, re-isolation was chosen as an alternative method to quantify *V. campbellii* in shrimp's haemolymph. Plating methods have been applied with success in previous studies (Mermoud et al., 1998; van de Braak et al., 2002a). Normally, shrimp possess a fast clearing mechanism to eliminate bacteria from their body. Martin et al. (1996) reported that radiolabeled *Bacillus subtilis* injected into the haemolymph of the Ridgeback shrimp *Sicyonia ingentis* were cleared rapidly as bacteria were phagocytized and degraded by haemocytes within the first hour after injection. In a similar study by van de Braak et al. (2002b), the concentration of live bacteria in the haemolymph decreased by 97% in between 5 min and 2 h after injection. The present findings are in accordance with these earlier reports. Very low *V. campbellii* densities were detected in the haemolymph of shrimp only challenged with *V. campbellii*. It appears that these shrimp had sufficient clearing capacity and managed to eliminate most of the *V. campbellii* shortly after injection. On the other hand, the density of *V. campbellii* was much more elevated in the haemolymph of euthanized co-injected shrimp than in shrimp injected with *V. campbellii* only. The amount of *V. campbellii* in the haemolymph of co-injected shrimp was already three times higher 1 h after injection and continued to increase up to 10 hpvi (Table 4). Additionally, plate countings were done from haemolymph samples of moribund shrimp collected at 6 hpvi (30 hpi of WSSV). Here, the difference was even more spectacular. It is therefore postulated that the bacterial clearing capacity of shrimp is severely compromised by a WSSV infection.

The hypothesis that WSSV undermines this mechanism is supported by the findings of Selvin and Lipton (2003) who reported that a primary WSSV infection probably weakened shrimp and made them more susceptible to bacterial infection. Moreover, Mathew et al. (2007) found significant reductions in the activities of phenoloxidase, glutathione-dependent antioxidant enzymes and antiperoxidative enzymes of WSSV-infected shrimp. We supposed that the drop in circulating haemocytes of WSSV-infected shrimp allowed the proliferation of *V. campbellii* inside shrimp since haemocytes are responsible for encapsulation and phagocytosis of bacteria. Van de Braak et al. (2002a) and Wongprasert et al. (2003) also found a significant drop in the number of circulating haemocytes of *P. monodon* shrimp after WSSV injection.

In this study, the absence of a significant difference in WSSV-infected cells between single and dually infected shrimp and the rapid proliferation of *V. campbellii* in dually infected shrimp, strongly suggested that dually infected shrimp died because of *Vibrio* proliferation.

Using the dual infection strategy, the virulence of another bacterial strain, *V. harveyi* BB120, was tested. A first test showed that the wild-type *V. harveyi* BB120 was not able to cause any mortality in single-injected shrimp at a dose of 10^6 CFU. Since the same dose in WSSV-compromised shrimp did not cause any accelerated mortality, the clinical outcome of co-injection must be strain dependent. The dual infection protocol using a weakly virulent WSSV strain, as it is used in the present study, might be an elegant tool to determine the virulence of *Vibrio* strains as secondary pathogens.

Next to the susceptibility to *V. campbellii* infection by intramuscular route, infection by immersion route was also evaluated in WSSV-compromised shrimp. No co-infection was observed when WSSV-compromised shrimp were challenged by immersion with different doses of *V. campbellii*, even with doses as high as 10^8 CFU ml^{-1}. Results were the same when *Vibrio* was added 24 or 48 h after WSSV injection. This outcome illustrates a weakness of the dual infection protocol presented in this study: both WSSV and *Vibrio* need to be injected to achieve the synergistic effect. Although this protocol might provide data on the virulence of *Vibrio* strains, it does not take into account that under natural conditions, bacteria need to overcome certain protective barriers before they can invade shrimp. The results obtained here confirm those described by Pakingking et al. (2003) who could not find any significant difference in cumulative mortality of fish between the control group and groups co-challenged with marine birnavirus (MABV-F) by injection and *V. harveyi* or *E. tarda* by immersion. In contrast, when co-infection was established by injecting virus and bacteria, cumulative mortality reached more than 90%.

The fact that immersion challenge with *Vibrio* in WSSV-compromised shrimp did not result in co-infection raises questions about the possible mechanisms involved in co-infection in the field as observed by many researchers. With the current knowledge, it is supposed that outbreaks of bacterial disease in shrimp ponds are the result of complex interactions. The status of the hosts aside, occurrence of bacterial disease is determined by two main factors: physical stressors and other pathogens which make the way. The former comprise fluctuations in the water (salinity, temperature, pH, alkalinity), deteriorating water quality, high stocking densities, injuries and cannibalism (Kautsky et al., 2000; Fegan and Clifford, 2001; Kiran et al., 2002). The latter can be a myriad of primary and secondary pathogens. Our results proved that WSSV can damage some internal tissues, but that damage is apparently not sufficient to allow *Vibrio* infection from the water. It is possible that the shrimp's immune system was not undermined enough by WSSV to prevent it from responding efficiently to the bacterial challenge. Moreover, shrimp are fully covered by cuticle except for the midgut epithelium, which seems to be refractory to WSSV infection. This possible entry route for the bacteria would then not be directly weakened by the viral replication, while the cuticular epithelia are not accessible. The absence of multiple stress factors in experimental designs could explain the difficulty to experimentally infect shrimp with *Vibrio* by immersion challenge in contrast with field conditions. Since no mortality was observed when shrimp were co-injected with WSSV and 10^3 CFU of *V. campbellii*, we hypothesise that the amount of bacteria able to penetrate into the shrimp's body remained below this threshold level.

In conclusion, the clinical outcome of WSSV and *V. campbellii* co-infection in SPF *P. vannamei* shrimp depends on the bacterial dose, species and challenge route. An intramuscular dose of 10^4 CFU $shrimp^{-1}$ of *V. campbellii* can be considered as the threshold to enhance the mortality rate of WSSV-compromised shrimp. Injection of *V. campbellii* did not cause any increase of WSSV replication, but in WSSV-compromised shrimp, the density of *V. campbellii* in the haemolymph increased spectacularly when compared to only

Vibrio-challenged animals. Immersion challenge with *V. campbellii* did not result in any mortality, not even in WSSV-infected shrimp.

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