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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*