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Enhanced immune defences in Pacific white shrimp (*Litopenaeus vannamei*) post-exposure to a vibrio vaccine

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ABSTRACT

This study was conducted to determine if exposure of shrimp, *Litopenaeus vannamei*, to a commercial anti-vibrio vaccine caused changes in antibacterial and cellular (phagocytosis) defences. Shrimp post-larvae were administered either Vibromax™ vaccine or a blank preparation. Whole body homogenates were prepared before (day 0), during (day 10) and after (day 20) vaccination and incubated with a selection of pathogenic vibrios. Homogenate from day 0 animals showed natural antibacterial activity towards *Vibrio anguillarum* which was significantly enhanced for bacteria-exposed shrimp at 10 days post-challenge. This effect of the vaccine was short-term in its duration. No antibacterial activity was observed in day 0 shrimp homogenate against *Vibrio alginolyticus* but it was significantly enhanced for both vaccinated and blank-vaccinated shrimp by day 10. No natural or inducible antibacterial activity was observed against *Vibrio harveyi* at 0, 10 or 20 days post-challenge. To determine if prior exposure of shrimp to inactivated vibrios results in elevated hemocyte phagocytic activity, juveniles were injected with either a mixture of formalin-inactivated vibrios or saline. Hemocyte monolayers made from these shrimp were overlaid with a 1:1 mix of *Bacillus subtilis* and these vibrios. Hemocytes from vibrio-exposed animals showed elevated levels of internalised vibrios compared with those from the saline injected group. These studies show selectively enhanced cellular defences of shrimp following 'vaccination'.

1. Introduction

Global aquaculture regularly produces over 2.4 million tonnes of cultured shrimp annually, worth an estimated $11 billion (FAO, 2006). Shrimp aquaculture is particularly vulnerable to viral and bacterial disease pandemics, however, with diseases such as vibriosis causing extensive mortality and production losses (Vandenberghe et al., 1999; Lightner, 2005). Several species of *Vibrio* are considered to be primary or opportunistic pathogens to shrimp (Austin, 2010) especially during the early stages of development. For example, a highly pathogenic strain of *Vibrio nigripulchritudo* has been shown to be the causative agent of a disease called Summer Syndrome that causes high mortalities in the Pacific blue shrimp, *Litopenaeus stylirostris* in New Caledonia (Walling et al., 2010). Some vibrios associated with crustaceans, such as *Vibrio vulnificus*, are also human pathogens, causing clinical symptoms including wound infection, septicaemia and gastroenteritis (Gopal et al., 2005; Austin, 2010). Disease outbreaks are therefore controlled using a range of approaches including increased biosecurity and the application of prophylactic chemicals including various antibiotics with associated deleterious environmental effects (Cabello, 2006). Because in many countries the use of antibiotics in shrimp culture has now been prohibited, there has been a search for more sustainable approaches to disease control, such as orally delivered immune stimulants (Rodríguez et al., 2007), pre- (Li et al., 2007) and pro-biotics (Castex et al., 2008; Ninawe and Selvin, 2009; Thompson et al., 2010) and putative vaccines (e.g. Teunissen et al., 1998).

Vibrio challenge studies with shrimp have shown heightened disease resistance following 'vaccination' (Teunissen et al., 1998) although whether such preparations are simply acting as immunostimulants (i.e. without specificity in relation to the immunogen) or 'true' vaccines remain unclear. To our knowledge, there is only one commercial vaccine for invertebrates (AquaVac™ Vibromax™), a multivalent bacterin from Intervet/Schering – Plough Animal Health designed to give protection to shrimp larvae from a range of pathogenic *Vibrio* species. This vaccine has recently been reported to enhance the growth and survival of post-larval Pacific white shrimp (*Litopenaeus vannamei*) and Black tiger shrimp (*Penaeus monodon*) following challenge with *Vibrio parahaemolyticus* (Wongtavatchai et al., 2010) but its potential effect on the immune system of such animals is unknown.

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The question of vaccines in invertebrates is a controversial one, however. Historically, invertebrates have been considered to lack the specific, adaptive immune system of vertebrates and in theory, are incapable of responding to vaccines in a specific or selective way (e.g. Rowley and Powell, 2007). Recent studies have challenged these views and shown a form of specific immunity in invertebrates often referred to as 'specific immune priming' (e.g. Roth and Kurtz, 2009; Roth et al., 2009). For example, elevation of phagocytic activity was found in the woodlouse, *Porcellio scaber* following exposure to different types of bacteria that was strain and species specific (Roth and Kurtz, 2009). The potential therapeutic basis of vaccines to invertebrates would therefore be negligible unless an alternate mechanism of immune specificity existed.

The current study determined the effects of Vibromax on antibacterial activity of homogenates of post-larvae of the Pacific white shrimp, *L. vannamei*, a species that dominates shrimp aquaculture (FAO, 2006). Juvenile shrimp were also challenged with the same species of vibrios as in this commercial vaccine so that changes in phagocytic activity of the hemocytes could be assessed.

2. Materials and methods

2.1. Animals

For the antibacterial activity experiments, *L. vannamei* post-larvae (0.67 g mean weight; stage PL5) were distributed randomly across 12 × 20 L white polyethylene rectangular tanks (*ca.* 25 cm × 30 cm × 40 cm) at a density of *ca.* 75 post-larvae L^{-1}. These tanks were connected to a single fully re-circulating system incorporating continuous mechanical and biological filtration, UV disinfection and temperature control. Tank inlet water was kept at 30 °C and 30 ppt salinity with minimal, low concentrations of dissolved inorganic nitrogen. For phagocytosis experiments, juvenile shrimp (11.30 ± 1.05 g, mean ± 1 SD) were held in 20 L tanks under the same environmental conditions as used for post-larvae and fed daily with a commercial diet (3% biomass d^{-1}, Dragon Feeds Supreme™ shrimp diet, Dragon Feeds, U.K.). Both post-larvae and juvenile shrimp were raised from disease free brookstock originally obtained from Bonaire in the Caribbean.

2.2. Changes in antibacterial activity of shrimp following exposure to Vibromax

A vaccine preparation (AquaVac™ Vibromax™) containing formalin-inactivated *Vibrio* spp. (*Vibrio (Listonella) anguillarum* biotypes I and II, *V. harveyi, V. parahaemolyticus* and *V. vulnificus*) or a provided 'blank' vaccine containing no bacteria but the other components in the full vaccine (Intervet/Schering-Plough Animal Health (Aquaculture), UK), were orally delivered to the shrimp post-larvae via enriched brine shrimp, *Artemia salina* (INVE Aquaculture NV), as instructed by the manufacturer. Briefly, *Artemia* cysts which had been previously chemically decapsulated were hatched on the day prior to vaccine administration in aerated 1L Imhoff cones. After 34 h, *Artemia nauplii* (instar II) were harvested and rinsed, then aerated with the appropriate concentration of vaccine or blank vaccine (containing no bacteria) for 90 min. After this ''enrichment'' period, the *Artemia* were rinsed and held at 4 °C with aeration before feeding. *Artemia* were prepared and enriched daily, and administered to the shrimp post-larvae four times per day over a 10 day treatment period. After 10 days, administered *Artemia* were not enriched with vaccine or blank preparations until the end of the study. In accordance with commercial shrimp farming practice, feeding was supplemented with recommended quantities of *Chaetoceros muellerii* and a formulated particulate diet (Frippak PL + 500, INVE Aquaculture NV). Replicate tanks contained either (i) control blank group – shrimp fed with blank vaccine-enriched brine shrimp, or (ii) shrimp fed with Vibromax-enriched brine shrimp at the manufacturer's recommended dose rate (0.046 mg L^{-1}).

Due to the small size of the shrimp post-larvae used in these experiments (*ca.* 3 mm in diameter at PL5 stage), a whole body assay was employed to investigate any potential effect of the commercial Vibromax™ vaccine on levels of antibacterial activity. Post-larvae (of mean weight 0.67 g; $n = 10$) were collected on day 0 (before Vibromax administration), followed by samples of *ca.* 10 post-larvae per replicate tank on each of days 10 and 20 post-vaccination. Post-larvae were flash frozen in liquid nitrogen and stored at −80 °C for later analysis. Freezing did not impair antibacterial potency when compared to preliminary experiments that used fresh tissue immediately in the assay (data not shown).

Post-larval samples were thawed and transferred into 1 mL sterile, ice-cold 3% NaCl solution before being homogenised and centrifuged (10,000*g*, 10 min, 4 °C). The supernatant was transferred and filter sterilised (0.22 μm) to create a sterile supernatant from shrimp tissues for immediate use in the antibacterial assay.

This antibacterial assay was based on a densitometric 96 well microplate assay. Prior to the assay, two species of bacteria found in the vaccine – *V. anguillarum* (NCIMB 829) and *V. harveyi* (NCIMB 1280), plus another *Vibrio* species not included in the Vibromax vaccine, *Vibrio alginolyticus* (NCIMB 1339) were grown in tryptic soy broth (TSB) supplemented with 2% NaCl (12 h, 25 °C). After washing twice in sterile 3% NaCL solution (1800*g*, 10 min, 4 °C) serial dilutions were made using sterile 3% NaCL and the bacterial number adjusted to 1×10^8 total bacteria mL^{-1} immediately prior to their use in the assay. Bacteria (50 μL; 0.5×10^7 bacteria) and 100 μL of either shrimp homogenate or sterile 3% NaCl were pipetted into each well of a sterile flat-bottom 96-well plate and incubated at 20 °C for 30 min with shaking. Subsequently, 50 μL of this suspension was added to another 96-well plate, 200 μL of TSB containing 2% NaCl added and incubated at 25 °C for > 14hr in a plate reader with the absorbance at 550 nm measured each hour. The values at $T = 0$ were subtracted for each well and bacterial growth in the absence of shrimp homogenate was used as a 'bacteria-only control'.

2.3. Phagocytosis experiments

To test if shrimp show elevation of hemocyte-mediated phagocytosis following exposure to bacteria, formalin-inactivated *V. anguillarum* biotype I and II, *V. harveyi, V. parahaemolyticus* and *V. vulnificus* (the same bacterial strains as in Vibromax) were prepared. Larger juvenile shrimp were used for these experiments because it is impossible to produce enough hemocytes for phagocytosis assays from post-larvae. Juvenile shrimp were injected between the abdominal segments with either 100 μL of 10^8 bacteria $animal^{-1}$ or with 100 μL of 3% NaCl solution. Experimental groups were maintained in separate tanks and re-injected after 7-days. Animals were harvested for the phagocytosis assay 7-days after the second injection (i.e. Day 14).

Bacillus subtilis (NCIMB 1048; originally isolated from the marine environment) was grown overnight in TSB (+2% NaCL) with constant agitation, centrifuged (2000*g*, 4 °C, 10 min) and washed three times in filter sterilised (0.22 μm) marine saline (0.5 M NaCL, 12 mM $CaCL_2{\cdot}2H_2O$, 11 mM KCL, 26 mM $MgCL_2{\cdot}6H_2O$, 50 mM Tris; pH 7.4). The resulting bacterial suspension was taken through 21, 26 and 27G needles to disrupt any chains of bacilli before the bacteria were formalin-inactivated (2% formaldehyde, overnight, 4 °C). The suspension was then washed extensively in sterile marine saline with a cell count performed during the final wash. The sterility of the formalin-inactivated *Vibrio* and *B. subtilis* suspensions was verified by plating on tryptic soy agar (+2% NaCL)