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Challenge of New Zealand Greenshell™ mussel *Perna canaliculus* larvae using two *Vibrio* pathogens: a hatchery study

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ABSTRACT: Bacterial diseases remain a large problem in aquaculture hatcheries. The successful design and implementation of protective measures in the hatchery depends on an understanding of the dynamics of the infection process. Developing an *in situ* experimental protocol for pathogen challenge is therefore of paramount importance. Here, we demonstrated the minimum effective pathogenic dose (MEPD) of *Vibrio splendidus* (10^5 CFU ml^{-1}) and a *Vibrio coralliilyticus/neptunius*-like isolate, *Vibrio* sp. DO1 (10^6 CFU ml^{-1}), for New Zealand Greenshell™ mussel (GSM, *Perna canaliculus*) larvae during hatchery production. In a flow-through water hatchery system, larvae given 1 to 2 h of static water exposure to these pathogen doses showed respective average cumulative mortalities of 58 and 69% on the fourth day following pathogen exposure. After the 1 to 2 h static exposure, larvae were returned to flow-through water. Larvae exposed to a dosage one order of magnitude greater than the MEPD had higher mortalities of 73 and 96% for *V. splendidus* and *Vibrio* sp. DO1 respectively. These 4 levels of mortality were significantly greater than those of the non-exposed control larvae which respectively averaged 23 and 35% in experiments involving *V. splendidus* and *Vibrio* sp. DO1. Experiments were repeated 4 times to confirm reproducibility. After pathogen exposure, pathogens were detected in the larvae and tank water of treatments with dosages of $\geq 10^5$ CFU ml^{-1} (*V. splendidus*) and 10^6 CFU ml^{-1} (*Vibrio* sp. DO1), but not in treatments with lower pathogen dosages. The challenge protocols are reproducible and provide an opportunity to assess measures for the protection of GSM larvae against infection in the hatchery environment.

KEY WORDS: Vibriosis · Challenge protocol · Greenshell™ mussel · Larviculture

INTRODUCTION

In aquaculture operations worldwide, bacterial infections during the hatchery stages persist, with vibriosis commonly being implicated (Grisez et al. 1996, Diggles et al. 2000a,b, Villamil et al. 2003). Such diseases hinder aquaculture operations and can cause massive economic losses (Prayitno & Latchford 1995). *Vibrio* spp. are the main etiological agents in shellfish hatchery diseases (Elston et al. 1981, Sugumar et al. 1998, Waechter et al. 2002, Gómez-León et al. 2005). Methods to deal with these issues have commonly employed antibiotics although alternatives, such as naturally occurring probiotics, are highly desirable due to the emergence of drug-resistant bacterial strains, human health concerns and government restrictions (Kesarcodi-Watson et al. 2008).

Two pathogens of the New Zealand Greenshell™ mussel (GSM, *Perna canaliculus*) larvae were previously demonstrated using a static bioassay in tissue culture dishes (TCD): *Vibrio splendidus* and *Vibrio* sp. DO1, a *Vibrio coralliilyticus/neptunius*-like isolate (Kesarcodi-Watson et al. 2009). However, in the hatchery production environment of GSM, conditions are markedly different from that of the TCD bioassay. Water flow is continuous, food and aeration are pro-

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vided and a milieu of ambient microflora is present. In order to understand the dynamics and facilitate effective management of microbial infections occurring at a hatchery facility, it is necessary to have an understanding of the conditions that initiate infection.

Many studies have previously demonstrated experimental infections of aquatic animals: shrimp (Roque et al. 1998, Soto-Rodríguez et al. 2006), fish (Bergh et al. 1997, Itano et al. 2006) and molluscs (Sainz et al. 1998, Estes et al. 2004, Gay et al. 2004). Currently, there is a lack of literature on infection models under hatchery conditions, although Planas et al. (2005) developed an infection model under hatchery conditions where static water rather than flow-through systems prevailed. Likewise, there are few reports on infection models in flow-through water systems. Arkush et al. (2005) described an infection model in white seabass incorporating flow-through water and intraperitoneal injection of the pathogen; however, this method of administration is not possible with GSM larvae. Additionally, both Nordmo et al. (1997) and Itano et al. (2006) utilized flow-through water systems in documenting experimental infections in Atlantic salmon and yellowtail fish respectively. In these studies, the animals were exposed to the pathogen using immersion periods of 45 and 10 min (for Atlantic salmon and yellowtail respectively) before placing them back into flow-through systems. Literature on the experimental infection of molluscs have either described adult animals (Hervio et al. 1995, Gay et al. 2004), or larvae tested under static *in vitro* conditions (Sainz et al. 1998, Sugumar et al. 1998, Estes et al. 2004).

Here we sought to examine the effect of varying levels of *Vibrio splendidus* and *Vibrio* sp. DO1 on GSM larvae under flow-through hatchery conditions, thereby developing a protocol for an experimental pathogen challenge. This information would permit future hatchery-based trials on treatments for bacterial infections.

MATERIALS AND METHODS

Experimental animals. Experiments were conducted at the Glenhaven Aquaculture Centre hatchery facility (GACL) in Glenduan, Nelson, New Zealand. GSM larvae were obtained using the following hatchery protocol: once a mussel began to spawn, it was transferred into a separate container that would hold its gametes separately prior to fertilization. Fertilization followed a sperm:egg ratio of ~10:1, in a volume of 20 l seawater at ambient temperature. Fertilization proceeded in this concentrated environment for 20 min, before fertilized eggs were transferred into a 170 l static water tank with aeration and EDTA (Sigma; final concentration 1 mg l^{-1}) (Buchanan 1998) for incubation. Incubation occurred in static water until D-veliger larvae were observed (~36 h), with food (0.5 l of *Chaetoceros calcitrans* at 15 000 cells ml^{-1}) being added to the tanks after 24 h. At this stage, larvae were screened on 45 µm nylon mesh to separate D-veliger larvae from trochophore larvae. D-veliger larvae were then placed in 2.5 l tanks at a density of 200 larvae ml^{-1} for use in the experiments (King et al. 2005). Throughout the experiments, larvae were fed with a 2:1 mix of *Chaetoceros calcitrans*:*Isochrysis galbana* to provide a final algal concentration of 40 cells $µl^{-1}$ in tanks, and water flow was continuous at 80 ml min^{-1} (providing 2 full water exchanges h^{-1} $tank^{-1}$). Incoming water was filtered to 1 µm, after a progression through sand filter, bag filter (5 µm) and cartridge filters (1 µm), and conditions were maintained within the following limits: dissolved oxygen of 100 to 107% saturation (Handy Delta, Oxy-Guard International), ammonia ≤ 0.015 mg l^{-1} NH_3-N (QuickChem 8000, Lachat Instruments), pH of 7.78 to 8.50 (M-11 PT100, Innovative Sensors) and temperature of 16.01 to 18.48°C (teflon sheathed PT100 sensor, Intech Instruments). Inflow and outflow water quality parameters were found not to differ, likely due to the continuous water flow which ensured that the tank water was replaced twice per hour.

Bacteriology. Two pathogens of GSM larvae, *Vibrio* sp. DO1 and *V. splendidus*, which were previously isolated from diseased larvae were used (KesarcodiWatson et al. 2009). Each bacterium was revived from −70°C stores in tryptone soy broth made to 2% salinity using natural seawater (TSB-2%Sea). Test isolates were streaked to ensure purity and subcultured 3 times on tryptone soy agar made to 2% salinity (TSA-2%Sea).

Each pathogen was subcultured into separate 10 ml volumes of Marine Broth 2216 (MB, Difco) and incubated at 25°C for 10 h. A volume of 0.1 ml of each culture was further subcultured into another 10 ml MB and further incubated for 10 h at 25°C. The final 10 ml volume of each culture was aseptically transferred into 500 ml MB and incubated for 20 h at 24–27°C on a gyrotory shaker (New Brunswick G10) at 150 rpm.

The cell concentration of final broth cultures was determined based on optical density at 600 nm using a spectrophotometer (PharmaSpec UV-1700, Shimadzu). Broth cultures were then centrifuged (2423 × *g*, 10 min, 15°C, Beckman J2-21M/E) and washed twice in autoclaved seawater. Supernatant-free cells were resuspended in autoclaved seawater to their original concentrations (~10^9 CFU ml^{-1}) and transported to the GACL (~10 min drive) for use in the experiments. Prior to use, 10-fold dilutions of the washed cultures were made and then surface-spread plated for enumeration on TSA-2%Sea to verify experimental concentrations.

Experimental design. Pathogens were added to Day 2, post-hatching larvae housed in 2.5 l flow-through tanks. Pathogens were added at concentrations ranging from 10^4 to 10^8 CFU ml^{-1}. For exposure to the pathogens, water flow was switched off and static conditions maintained for 1 h (*Vibrio splendidus*) and 2 h (*Vibrio* sp. DO1), after which water flow was resumed. This condition was also applied to control tanks in which no bacterial species had been added. Treatments were run in triplicate. Each day for the duration of the experiment, triplicate aliquots (250 µl, containing ~50 larvae aliquot^{-1}) were removed from the tanks and the percentage larval survival determined. On alternate days, either outflow barrier screens (nylon mesh size 40 µm) were cleaned, or full tank cleaning was performed (screens, tanks and larvae). Experimental duration was 4 d following pathogen inoculation (6 d post-hatching). Four independent experiments were carried out for each pathogen, each utilising larvae spawned from different broodstock and, hence, from different genetic stocks.

The presence or absence of the pathogens was monitored daily in both the water column and in larval samples. A 20 ml water sample (containing larvae) was taken from the tanks and passed through a 45 µm screen. The first 20 ml to pass through the screen were retained for assessing the presence of the pathogen in tank water. Larvae that were retained on the screen were rinsed 5 times with 50 ml autoclaved seawater and macerated using a Bosch homogenizer on number 2 speed for 30 s. Samples were serially diluted in sterile 2% salinity seawater before plating on selective media. The pathogens were isolated and identified as previously described by Kesarcodi-Watson et al. (2009).

Data analysis. Percent survival data were arcsine square root transformed to approximate normality. Treatment differences were analysed using ANOVA ($p = 0.05$). Post hoc comparisons of survival data were done using Tukey's HSD test. STATISTICA (StatSoft) version 7.1 was used for data analysis.

RESULTS

Dose response

The concentrations of the pathogens tested resulted in various levels of larval response (Fig. 1). In *Vibrio* sp. DO1, levels ≤10^5 CFU ml^{-1} did not cause significant mortality ($p > 0.05$) by Day 6, and 10^6 CFU ml^{-1} was the minimum dose required to initiate mortality given the 2 h static period of water flow ($p = 0.00$). *V. splendidus* caused larval mortalities after lower exposure than that required for *Vibrio* sp. DO1; 10^5 CFU ml^{-1} at 1 h static exposure caused significant larval mortality ($p = 0.00$). In both pathogens, mortality rates further increased after exposure to pathogen concentrations higher than the minimum infective dose (Fig. 1).

In all repeat experiments, larval survival was significantly lower in the pathogen doses tested (10^6/10^7 CFU ml^{-1} for *Vibrio* sp. DO1 and 10^5/10^6 CFU ml^{-1} for *V. splendidus*) than in the controls (Table 1). The average survival observed across all experiments with *Vibrio* sp. DO1 was 3.6 and 30.7% for the high and low doses of the pathogen respectively, compared with 65.2% in controls. For *V. splendidus*, mean survival across all repeat experiments was 27.3 and 42.1% for the high and low doses of the pathogen respectively, compared with 77.3% in controls. Mean larval survival for all

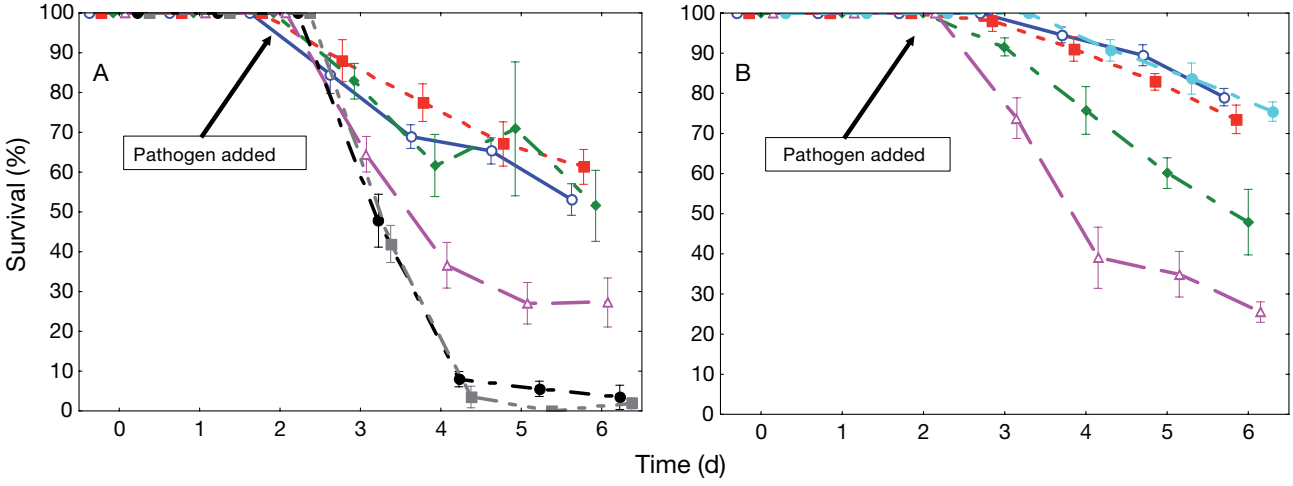

Fig. 1. *Perna canaliculus*. Time course of larval survival (%, mean ± 95% CI) upon exposure to varying levels of (a) *Vibrio* sp. DO1 and (b) *V. splendidus*. (○): Control, (●): 10^3 CFU ml^{-1}, (■): 10^4 CFU ml^{-1}, (◆): 10^5 CFU ml^{-1}, (△): 10^6 CFU ml^{-1}, (●): 10^7 CFU ml^{-1}, and (■): 10^8 CFU ml^{-1}. Concentrations are the final pathogen concentrations in tanks after pathogen addition

Table 1. *Perna canaliculus*. Percentage larval survival (±95% CIs) during 4 experiments involving pathogen challenges with *Vibrio* sp. DO1 and *V. splendidus* (Day 4 post-challenge). Values in each column with different superscripts are statistically different ($p < 0.05$)

Treatment (CFU ml^{-1})	Trial 1	Trial 2	Trial 3	Trial 4	Mean
Vibrio sp. DO1					
Control	53 ± 4^a	34 ± 7^a	90 ± 3^a	84 ± 4^a	65 ± 8^a
10^6	27 ± 6^b	8 ± 3^b	57 ± 5^b	–	31 ± 8^b
10^7	3 ± 3^c	2 ± 1^c	3 ± 3^c	7 ± 5^b	4 ± 2^c
	Trial 5	Trial 6	Trial 7	Trial 8	Mean
V. splendidus					
Control	79 ± 2^a	69 ± 6^a	77 ± 5^a	84 ± 4^a	77 ± 3^a
10^5	48 ± 8^b	21 ± 7^b	58 ± 10^b	–	42 ± 8^b
10^6	26 ± 3^c	27 ± 14^b	25 ± 5^c	32 ± 6^b	27 ± 4^b

pathogen treatments were significantly lower than that for controls.

Bacteriology

Pathogens were detected in larvae and the surrounding water throughout the course of the experiments in tanks receiving the higher pathogen dosages, i.e. $\geq 10^6$ CFU ml^{-1} for *Vibrio* sp. DO1, and $\geq 10^5$ CFU ml^{-1} for *V. splendidus*. Test pathogens were not detected in the water column and in larvae in both the control tanks and in tanks with pathogen doses of $\leq 10^5$ CFU ml^{-1} for *Vibrio* sp. DO1, and $\leq 10^4$ CFU ml^{-1} for *V. splendidus*.

DISCUSSION

This study demonstrated a reproducible infectious dose of the pathogens *Vibrio* sp. DO1 and *V. splendidus* to GSM larvae under flow-through hatchery conditions using a defined protocol. Larval survival in control treatments varied between 33.8 and 89.6% (average 71.2 ± 4.4%) across all experiments, illustrating the existence of batch variability. The variation was possibly caused by genetic differences of the larvae due to variation in broodstock, potential health differences of the cohorts, and differing environmental factors during experiments, such as extraneous bacteria. Such variation is known to exist in routine larval production at GACL, and underpinned the need to conduct 4 independent experiments. Despite the variation, doses equal to or above 10^6 CFU ml^{-1} for *Vibrio* sp. DO1 and 10^5 CFU ml^{-1} for *V. splendidus*, consistently significantly increased larval mortality relative to the controls during the 4 independent trials (Table 1), thereby confirming the reproducibility of the challenge protocol.

Previous mollusc larval infection models were conducted *in vitro*, without water exchange (Sainz et al. 1998, Sugumar et al. 1998, Estes et al. 2004). Such laboratory conditions are not representative of naturally occurring hatchery infections during flow-through conditions and favour the effect of the pathogen. Indeed, in earlier work with *Vibrio* sp. DO1 and *V. splendidus* on GSM larvae, we observed that levels as low as 10^2 CFU ml^{-1} initiated infection under *in vitro* static conditions (Kesarcodi-Watson et al. 2009). This is markedly lower than the lethal dosages required under flow-through hatchery conditions as witnessed in the present study. In a study by Sainz et al. (1998), a dose of 10^5 CFU ml^{-1} of *Vibrio alginolyticus* was required to initiate infection in catarina scallop (*Argopecten ventricosus*) larvae under static conditions.

Prior to the present study, preliminary experiments (Kesarcodi-Watson 2009) showed that infection with *Vibrio* sp. DO1 was more difficult to establish, hence, the protocol was designed to allow successful infection by both pathogens. This was achieved by turning off the water flow for 2 h of static exposure with *Vibrio* sp. DO1 rather than 1 h as with *V. splendidus*. The different abilities of the pathogens to cause mortalities may be due to their different attachment capabilities, attachment capability being a mechanism previously shown to be important in *Vibrio* sp. infections. Grisez et al. (1996) suggested that in *Vibrio anguillarum* infection of turbot larvae, the binding capabilities of *V. anguillarum* to brush border membranes of the intestinal epithelium might play an important role in its virulence. It is possible that the pathogens in the present study behave similarly as those tested by Grisez et al. (1996).

Of interest is that we occasionally noted that some of the larvae in the pathogen exposed tanks not only survived but also appeared to develop similarly as the controls over time. We consider this finding to be of considerable significance because the similarity of larval development between pathogen-exposed and non-exposed larvae suggests that the surviving pathogen-exposed larvae were resistant to these pathogens. Such resistance might have developed as a consequence of the broodstock having been exposed to the pathogens previously; both pathogens were isolated from the GACL hatchery. Occurrence of surviving larvae after the challenge protocol might allow for the selection of vibriosis resistant animals, similar to the study of Hervio et al. (1995).

This study has demonstrated procedures and effective pathogenic doses required to initiate mortalities of GSM larvae under hatchery production conditions. To

our knowledge, this is the first report where a challenge protocol has been developed using mollusc larvae in a flow-through rearing process. *In situ* procedures to alleviate the effect of these pathogens using this protocol are currently under investigation. Additionally, knowledge of the lethal dosages will complement routine monitoring of these pathogens in order to predict and consequently prevent impending larval crashes.

In conclusion, a challenge protocol has been developed using mollusc larvae in a flow-through rearing process which takes into account variability in both genetic and environmental factors that exist in the hatchery production of cultured animals. The method is simple and requires that the flow-through system be stopped only for a short period during pathogen exposure for reproducibility of the data under the hatchery production conditions of the experiments. The main aim of this study was to obtain an easily replicated protocol to initiate mortality, which could be used in the future to investigate remedial actions against pathogen effects. For this purpose, a short static period was effective. The challenge system has the potential to be adopted by others, and the bacterial concentrations and short period of static water exposure can be used as reference points. However, the pathogen concentrations required in challenges would be specific to the cultured animal and the bacterial pathogen in any given situation.

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