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# Development of a bath challenge for the marine shrimp *Penaeus vannamei* Boone, 1931

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## Abstract

Despite the major financial losses caused by vibriosis in shrimp culture, no reliable laboratory model has yet been developed for this disease. A reproducible experimental model is therefore urgently required for further study. In this study, a bath challenge technique was developed for juveniles of the marine shrimp *Penaeus vannamei*. Four different treatments were applied to the shrimp: (1) shrimps were wounded on the third abdominal segment and were immediately exposed to a bath of *Vibrio parahaemolyticus* afterwards; (2) shrimps were only wounded; (3) shrimps were only exposed to *V. parahaemolyticus*; and (4) control, shrimps were neither wounded nor challenged. The experiment was repeated four times to verify the reproducibility of the technique. All the mortalities occurred within four days and the cumulative mortalities were significantly different between treatments: varying from 37 to 52% for the first treatment, 12 to 22% for the second, 0 to 13% for the third, and 0 to 3% for the control. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Bath challenge; *Penaeus vannamei*; *Vibrio* sp.

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## 1. Introduction

Available bath challenges for vibriosis in marine shrimp do not reflect natural infections. None of the challenges have been able to reproducibly induce the clinical

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signs, pathology, and mortality associated with natural infections. To date, experimentally induced vibriosis has only been detectable by histology (de La Pena et al., 1992; Esteve and Quijada, 1993). The bacterial isolates have not been demonstrated to reproduce inside the shrimp.

The objective of this study was the development of a bath challenge method for the further study of vibriosis. A bath challenge is required for a number of reasons, such as assessing the virulence of isolates and evaluating treatments or methods of prevention. A bath challenge may help to determine which environmental factors affect the onset of vibriosis in cultured shrimp and lead to a better understanding of the relationship between environmental stressors and bacteria in the aetiology of the disease.

## 2. Materials and methods

### 2.1. Origin of the shrimp

The shrimp used in this series of trials came from a commercial hatchery in the state of Sinaloa, Mexico as postlarvae 6–7 and were grown in recirculating 500-l round black PVC tanks (Rotoplas, Guadalajara, Mexico) until they reached just over 1.0 g in weight (six to eight weeks old juveniles). The water in this system was maintained at  $27 \pm 1^\circ\text{C}$  and 35‰ salinity. The tanks were individually aerated through airstones connected to a high-volume air blower. Partial water changes were made once a week to maintain the water quality. The shrimps were initially fed *Artemia* sp. and were weaned onto a commercial diet (Rangen, Quality Feed for Aquaculture) when they were approximately postlarvae 20.

### 2.2. Experimental system

The experimental system used was a static system consisting of 24, 10-l glass tanks. Each tank was individually aerated by an airstone. The water conditions were: temperature =  $25 \pm 1^\circ\text{C}$ ; salinity = 35‰; pH  $8.0 \pm 0.1$ .

### 2.3. Origin of the *Vibrio* sp. isolate and preparation of the inoculum

The *Vibrio* sp. used for this series of experiments was isolated from the haemolymph of a diseased juvenile shrimp (*P. vannamei*) collected in a farm from the state of Sinaloa, Mexico. The shrimps from that pond presented reddish coloration, anorexia, and were lethargic. With the help of the BIOLOG system (Biolog, Hayward, USA), it was identified as *V. parahaemolyticus* (BIOLOG scoring number 3725- 2507- 3123- 7050- 0501- 4377- 6114- 3517), hereafter referred to as HL58. The Biolog system identifies bacteria according to their ability to utilise a variety of carbon sources. HL58 was preserved in Protect Bacterial Preservers (Technical Service Consultant, Heywood, Lancs, UK) at  $-70^\circ\text{C}$  and resuscitated into Tryptone Soya Broth (TSB, Difco laboratories, Detroit, USA) with 2% sodium chloride (NaCl) at the beginning of each experiment. HL58 was grown in 10 ml of TSB + NaCl at  $30^\circ\text{C}$  for 24 h and then transferred into 60 ml of TSB + NaCl for 24 h more before being used in the experiments.

#### 2.4. Wounding technique

In order to facilitate the induction of vibriosis, some of the shrimps were artificially wounded. To achieve this, the shrimps were individually netted out and a small incision (approximately 2 mm long) was made throughout the cuticle and into the muscle of the third abdominal segment by pushing a scalpel against the carapace until it penetrated.

#### 2.5. Bacteriology

The concentration of inoculum used was also monitored daily, through the plate count method (Austin, 1988). Daily *Vibrio* spp. total counts from the water of each tank were made in Thiosulphate Citrate Bile Sucrose agar (TCBS, Difco Laboratories). Because the data was not normally distributed, a rank sum test was used to compare the number of bacteria found in the treatments where bacteria were added against the treatments where bacteria were not added.

#### 2.6. TSB study

A trial was conducted in order to investigate the influence of TSB on the bath challenge. The following treatments were applied.

1. The shrimps were wounded and then exposed to TSB (wound + TSB).
2. The shrimps were wounded only (wound).
3. The shrimps were exposed to TSB only (TSB).
4. The shrimps were neither wounded nor exposed to TSB (control).

There were two replicates for each treatment and 10 shrimps per tank.

The tanks were filled with 3.0 l of UV-sterilised seawater. The following day, the 10 shrimps were introduced to each tank. They were left to adapt to this system for another two days and then the trial was initiated. On the first day, 75% of the water in each tank was replaced with UV-sterilised seawater. The 'TSB' tanks were inoculated with 1.5 ml/l of TSB with 2% NaCl. The 'wound' shrimps were removed from their tanks and wounded as described above. For the 'wound + TSB' treatment, the shrimps were removed from the tanks and the tanks were inoculated with the same volume of TSB as for the 'TSB' treatment. Then the shrimps were wounded and placed back in the tanks. Thereafter, 75% of the water in all the tanks was replaced with UV-sterilised water daily and then the 'TSB' and 'wound + TSB' tanks were inoculated again with 1.5 ml/l of TSB. The shrimps were fed daily with a maintenance diet of commercial pellet (Rangen). A record of the daily mortalities of each tank was kept for five days.

#### 2.7. Challenge study

During these series of trials, four different treatments were applied to the shrimp.

1. The shrimps were wounded and then challenged with HL58 (wound + vibrio).
2. The shrimps were wounded only (wound).
3. The shrimps were challenged with HL58 only (vibrio).

4. The shrimps were neither wounded nor challenged (control). Each treatment had six randomly allocated replicates.

### 2.8. Experimental procedure

The tanks were filled with 3.0 l of UV-sterilised seawater. The following day, 10 shrimps were introduced to each tank. They were left to adapt to this system for another two days and then the trial was initiated. On the first day of challenge, 75% of the water in each tank was replaced with UV-sterilised seawater. The 'vibrio' tanks were inoculated with 1.5 ml/l of HL58 cultured in TSB with 2% NaCl. The 'wound' shrimps were removed from their tanks and wounded as described above. For the 'wound + vibrio' treatment, the shrimps were removed from the tanks and the tanks were inoculated with the same volume of HL58 as for the 'vibrio' treatment. Then the shrimps were wounded and placed back in the tanks. Thereafter, 75% of the water in all the tanks was replaced daily with UV-sterilised water and then the 'vibrio' and 'wound + vibrio' tanks were inoculated again with 1.5 ml/l of HL58 culture. The shrimps were fed daily with a maintenance diet of commercial pellet (Rangen). A record of the daily mortalities of each tank was kept for five days. This trial was repeated four times to verify the reproducibility of the technique. In the last trial, only seven shrimps were used per tank, but still with six replicates per treatment. Since the data were not normally distributed, a Kruskal–Wallis test was performed and wherever significant differences were demonstrated, a posteriori Student–Newman–Keuls (SNK) test was applied to identify the differences.

## 3. Results

Table 1 shows the average bacterial numbers measured during the trials for the different treatments. The numbers and types of colonies grown from the water samples were different in the treatments where bacteria were added (wound + vibrio and vibrio) from the treatments where bacteria were not added (wound and control). Not only the number of bacteria present in the water was significantly higher ( $T = 155$ ,  $n = 10$ ,  $P < 0.0001$  for all the trials) in the treatments where bacteria were added, but also the types of bacteria sampled in TCBS were different. For the treatment 'wound + vibrio' and 'vibrio,' most of the colonies were green and their macroscopic features matched the ones of HL58 (4 mm in diameter of the colony, border complete, convex elevation, bright and smooth texture). Some yellow colonies also appeared, but they represented less than 20% of the total *Vibrio* sp. count. For the treatments 'wound' and 'control,' most of the bacteria sampled formed yellow colonies in TCBS.

The results of the TSB study showed very few mortalities. 'Control' and 'wound' had no mortalities, while 'TSB' and 'wound + TSB' had 5% mortalities each.

All the mortalities occurred within four days of the initial challenge. The cumulative mortalities for each trial are presented in Fig. 1. For the 'wound + vibrio' treatment, the mortalities varied from 32–52%; for 'wound,' from 12 to 22%; for 'vibrio,' from 0 to

Table 1  
Average *Vibrio* sp. counts for each treatment and experiment

Experiment	Treatment	Green colonies (CFU/ml)	Yellow colonies (CFU/ml)
1	wound + vibrio	2936000 (1430693)	0 (0)
	vibrio	4202083 (1518171)	0 (0)
	wound	0 (0)	20430.13 (4920.498)
	control	0 (0)	245008 (380202.1)
2	wound + vibrio	12191.66 (10701.48)	132 (184.038)
	vibrio	4129.717 (3267.539)	66 (45.60702)
	wound	9.733333 (104094)	170 (227.9751)
	control	0.4 (0.89442)	179.3333 (231.5006)
3	wound + vibrio	868717.3 (829054.9)	7060 (4246.528)
	vibrio	998110.8 (522123.7)	1517.2 (602.4547)
	wound	544.6 (3369455)	12749.5 (7892.303)
	control	1815.2 (2415.427)	9711.173 (429681)
4	wound + vibrio	848444.1 (435201.8)	0 (0)
	vibrio	869666.3	0 (0)
	wound	0 (0)	1969.167 (1481.027)
	control	0 (0)	619.7333 (705.770)

The number in the brackets is the standard deviation of the average of the five-day counts. Each tank was sampled daily.

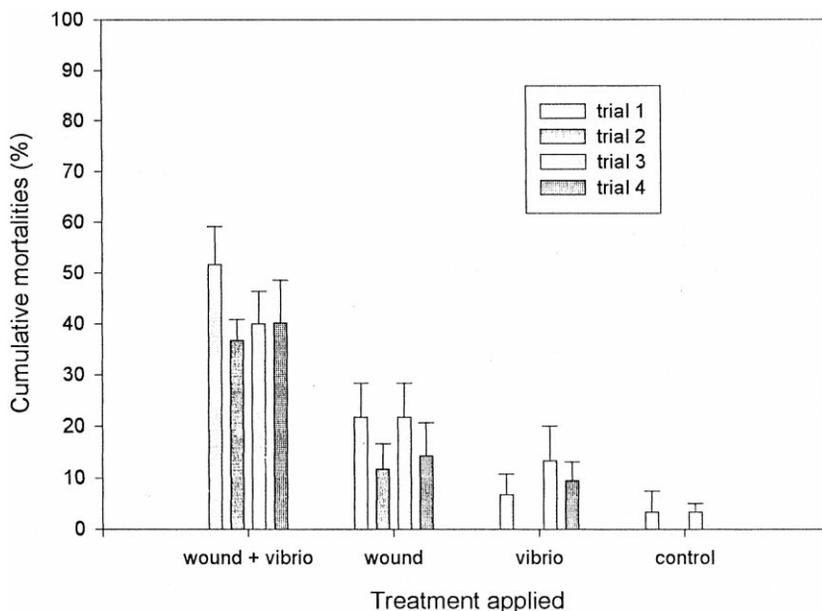


Fig. 1. Final cumulative mortalities found for each treatment during the four trials. The values are presented as percentages and the bars represent the standard error of the mean of six replicates.

13%; and for the 'control,' from 0 to 3%. The cumulative mortalities for the treatment 'wound + vibrio' were significantly different from all the other treatments in each trial ( $P < 0.001$ ).

#### 4. Discussion

The objective of this study was to develop a bath challenge technique which would mimic, as far as possible, the conditions of a natural outbreak of vibriosis in a farm situation.

The number of *Vibrio* sp. used in this challenge was  $10^5$  CFU/ml which is higher than the *Vibrio* sp. densities reported from shrimp pond water, for example,  $1.41 \times 10^3$  CFU/ml (Guerra-Flores, pers. comm., 1997). The densities of bacteria were closer to that reported for shrimp pond sediments:  $3.98 \times 10^5$  CFU/ml (Guerra-Flores, pers. comm., 1997). Shrimps spend most of the time close to the bottom of the pond and are therefore probably exposed to higher densities of bacteria than present in the rest of the water column (Tsai, 1989). From previously unpublished studies (Roque, 1995), it was concluded that a relatively high density of bacteria would be required to induce disease.

Flegel et al. (1992) speculated that shrimps were continually invaded by bacteria. If this were so, high numbers of bacteria in the water could overwhelm the shrimp haemocytes and even if only a small proportion of the total count consisted of pathogenic *Vibrio* sp., disease would result. Based on this hypothesis, the challenge model developed in this series of trials used daily inoculation of bacteria in an attempt to maintain high levels of bacteria. Bacteria were added daily, since it is difficult to maintain a high concentration of *Vibrio* spp. in small glass tank systems with insufficient organic matter to support their growth despite the addition of TSB.

As described in Section 3, for the treatments 'wound' and 'control,' most of the bacteria sampled formed yellow colonies in TCBS. Nevertheless, some green colonies were formed which could be confused with the HL58, that is probably due to the fact that the HL58 is a *V. parahaemolyticus* which is a bacterium commonly associated with shrimp. It seems probable though that the number of green colonies found is of no real significance (Table 1).

Because *Vibrio* spp. are opportunists, a stressor was considered necessary. Wounding was thought to be an appropriate stressor since physical damage has been referred as a primary risk in the development of vibriosis (Rosen, 1970; Lightner, 1978, 1983, 1988; Ruangpan and Kitao, 1991). In addition to stressing the shrimp, physical damage may provide a route of access for bacterial invasion.

In shrimps, it has been shown that wounds take approximately 16 days until they are completely sealed (Fontaine and Lightner, 1973). By 48 h, there is a haemocytic concentration at the ends of the wound. By 96 h, the epidermis has begun to migrate into the wound using the haemocytic network as a basal support, thus, at this stage, the wound is already closed although not totally repaired. These observations may explain why the mortalities ceased after four days, since after that time, there was no easy route of access from the water to the shrimp body for the bacteria. The brief duration of the mortalities may suggest that either vibriosis only occurs when the shrimps are continu-

ally invaded by bacteria and/or that the shrimps rapidly die or recover from any infection established.

During all these trials, six replicates were used because in an immersion challenge with catfish (*I. punctatus*) (Wise et al., 1993), the authors verified that there was an innate variability with a bath challenge, which limited its effectiveness to detection of only large differences in treatment effects. Wise et al. (1993) reported that a minimum of six replicates per treatment should be used when conducting a bath challenge, if the applied treatment is expected to affect mortality rates by 30%. Wise et al. (1993) also suggest that the tendency to make type II statistical errors increases as the number of replicates used per treatment decreases. This type of error is defined as failing to detect the existence of a significant treatment effect.

Water quality has frequently been proposed as a causal factor in the development of vibriosis (Lightner, 1983, 1988; Baticados, 1988). To date, there is no conclusive evidence to linking any individual water quality parameter to the onset of vibriosis. It is possible, however, that the observed mortalities in the challenge method presented here were affected by variations in the water quality. Further experimentation is planned to investigate such relationships. The stocking densities used in the experiment were also larger than commonly used in commercial culture facilities due to limited space within the experimental facilities.

The specific cause of death was not verified in these experiments; the cause of death was inferred by comparison with the controls. The dead shrimps would have been invaded by any bacteria in the water; invalidating bacterial analyses and autolyses would have rendered them unsuitable for histology. It may be possible to sample moribund animals for specific diagnoses, but this would require continuous monitoring of the tanks.

The same trial was repeated four times to ensure the reproducibility of this technique and suggested that this is a reliable bath challenge technique which may be used for a variety of studies.

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