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A new method to evaluate the effects of bacterial dosage, infection route and *Vibrio* strain in experimental challenges of *Litopenaeus vannamei*, based on the Cox proportional hazard model

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ABSTRACT

In the shrimp challenge test the *Vibrio* dosage, infection route, and strain are considered as risk factors that result in mortality. Assessment of *Vibrio*/shrimp interactions, and disease dynamics following infection by *Vibrio*, are useful techniques needed for detailed studies on the control of risk factors. In this paper we used an application of the Cox proportional hazard model to assess relative survival probability, estimate mortality risk, and construct a prognostic model to assess predictions of estimated time to death. Results indicate that infection route was the most important prognostic factor contributing to mortality in the challenge test ($\beta = 3.698$, $P < 0.000$). The shrimp infection rate following injection was found to be 40.4 times greater than that following immersion (hazard ratio (HR) = 40.4; $p = 0.000$). Our results also indicated that the HR resulting in shrimp mortality following a high dose of 10^8 cfu/shrimp was significantly greater (HR = 5.9, $P < 0.000$), than that following a baseline dosage of 10^7 cfu/shrimp. Strain *Vh* was found to be more virulent than Strain *Vp* (HR = 4.8; $P < 0.000$). The prognostic index also indicated that the infection route is the most important prognostic factor contributing to mortality in the challenge test.

1. Introduction

Pathogens may cause severe systemic infection and death, following the penetration of the epithelium and subsequent spreading throughout the infected organism [1]. Bacterial challenge tests are widely used in such research on selecting individuals, or families, that have an immune resistance to disease [2], assessing the virulence of newly isolated bacteria [3] and verifying evaluation methods or cures [2].

Based on results from shrimp challenge tests it has been hypothesized that variations in *Vibrio* infection efficacy levels are due to differences in the hazard factors (encompassing dosage, infection route, and strain) associated with such infections. Some studies have suggested that the presence of high numbers of *Vibrio* in shrimp as well as in the surrounding environment increase the risk of infection due to shrimp disease [4] and [5]. There is thus a need to monitor *Vibrio* population levels, for the purpose of controlling outbreaks of shrimp disease in aquaculture systems [6]. The nature of the infection route, which affects the efficiency of pathogen transfer and the response of the host's immune system [7], has an effect on the onset of the disease as well as host mortality [8] and [9]. Injection and immersion are common infection routes in fish pathology. In shrimp, vibriosis is often caused by a number of infections originating from multiple infection routes at the same time [10] and [5]. Selection in pathogens tends to favor a satisfactory performance in growth and reproduction and an appropriate virulence level, in terms of damage caused to the host organism [11].

A considerable amount of research has focused on the development of reliable challenge models, with the purpose of standardizing experimental protocols in such a way that all relevant controlling factors are taken into account [12] and [13]. Nevertheless, most research projects have been confined to experimental and observational studies and many studies on challenge tests have

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certain limitations, such as relatively small sample sizes [14], or the use of inadequate statistical methods when attempting to determine the major factors that influence survival and resistance to diseases [15]. For these reasons, there is a need to develop a rapid sampling method that is inexpensive and reliable in terms of reproducibility. It is hoped that the development of such a method will facilitate the development of precise guidelines and predictions relating to the control and prevention of diseases in shrimp [16]. The Cox PH model does not require assumptions concerning the distribution of survival data [9] and can thus be used to investigate the relationship between the 'death' event and a set of covariates, in the presence of censoring [17] or to test the effects of specific covariates or risk factors [18]. In this study we assessed relative survival probability and death hazards associated with fatal factors. We then constructed a prognostic model, in order to produce individualized predictions of estimated time-to-death in shrimp challenge tests.

2. Methods

2.1. Bacterial strains and culture conditions

Vibrio pathogens were obtained from the following sources: *Vibrio harveyi 2011053001*(*Vh*) from the Yellow Sea Fisheries Research Institute, Qingdao; *Vibrio parahaemolyticus FZ* (*Vp*) from Ocean University of China, Qingdao. Bacterial solutions were cultured in trypticase soy broth (TSB) containing 2% NaCl for 18 h at 30 °C and maintained, under constant shaking, at an air-to-medium ratio of 3:1, to attain a stable virulence level [5]. The cells were collected by centrifugation (5000 × g, 10 min) and pellets were washed three times in PBS. The test inoculum was prepared by dilution of bacteria in sterile seawater, to produce the required dosages. Bacterial concentrations were determined by measuring the optical density at 560 nm (OD_{560}). The bacterial suspension used in the infection challenge was prepared not long before the initiation of the challenge test.

2.2. Shrimp

Experimental shrimp were obtained from a commercial farm in Qingdao, Shandong Province and maintained, for at least 2 weeks prior to each challenge test, in a 2000-L recirculating system, at 25–28 °C and salinity of 28–32 ppt, and supplied with a standard pellet diet (Dale Commercial Diet). No signs of disease or mortalities were observed during this period. Shrimp population were detected at low *Vibrio* level and then randomly allocated (5–8 shrimps/tank) into 10-L plastic tanks, supplied with sterile seawater (30‰ salinity, at 28 °C). The body length was 7.67 ± 0.35 cm and body weight was 6.08 ± 0.70 g. No significant differences in shrimp weight were noted among different tanks for a given host species. Shrimps were fasted for 1 day prior to infection.

2.3. Challenge test

Based on preliminary experiments, in order to obtaining maximum mortality of shrimps in eight infection groups within 26 h, infection dosage of 10^7 and 10^8 cfu/ml was determined. Or a prolonged survival time (almost > 26 h in immersion groups) at a lower dose and no variance (shrimps in injection groups were all dead within 1 h) at a higher dose will decrease precision of model. The *Vibrio* doses, in immersion-route groups, were produced by dilution of bacteria in sterile seawater to 10^7 and 10^8 cfu/ml. A short time of challenge, 2 h should be preferred to prevent possible bacterial reinfection, then the shrimps were transferred to clean seawater [5]. Inoculations, by means of injections between the fifth and sixth abdominal segments, were given at doses of 10^7 and 10^8 cfu/shrimp. All shrimp were monitored after *Vibrio* infection. The total observation period was 26 h. Individual survival times—measured as the period between *Vibrio* infection and death—were always <26 h.

Cox model was first used in shrimp challenge experiment with no reference sample size. The sample size of Cox PH model in a clinical trail is commonly 5–15 times than the numbers of covariates in the experiential research. Three covariates entered in Cox PH model, while sample size, according to the experiential value was total 15–45 shrimps. The sample size of 40 shrimps was roughly selected in this study. These three covariates are binary covariates. For each specific binary covariate of interest, shrimp population was divided into two equal groups and conducted by two treatments (20 shrimps/treatment). Therefore, 8 infection groups (5 shrimp/groups) were designed for *Vibrio* infection. Two control groups (batch of uninfected shrimp, batch of shrimps injected with sterile saline solution) were conducted to reduce the impact of some specific environmental factors and injection injury on the shrimp. Detailed information on grouping situation is illustrated in Table 1.

2.4. Statistical analysis

Survival rates were calculated using the Kaplan–Meier method [18] and survival curves were compared by applying the log-rank test. Covariates, or risk factors, that significantly influenced overall survival were tested according to χ^2 and the *p*-value, in univariate analysis. Prior to the analysis of survival data, using the Cox PH model, a graphic method was used to verify that the covariates, or risk factors, satisfied the key assumption of proportional hazards (PHs) [19], i.e. that the effect of a given covariate or risk factor does not change over time. An Enter Cox regression analysis was then performed [20] to estimate the relative importance of the covariates (or risk factors) that contributed to the hazard (including the *Vibrio* dose, infection route, and *Vibrio* strain covariates). The hazard ratio (HR) was used to suggest the relative hazard (see Appendix A). A prognostic index (PI) was calculated from the Cox PH model, with all statistically-significant covariates or risk factors; with PI of the *i* th individual being described as $PI_i = \beta_1 Z_{i1} + \beta_1 Z_{i2} + \ldots + \beta_p Z_{ip}$. Hazards functions of three covariates were estimated in Cox model, which were subsequently put into formula to calculate the sample size required (N). Statistical analysis was performed using SPSS, Version 20.0 (SPSS Inc., Chicago, IL, USA) and Stata, Version 12.0 (Stata Corp., College Station, TX, USA).

Table 1
Grouping information of eight infection group.

	Numbers of cases	Treatments in infection test		
		Vibrio dose[a]	Infection route	*Vibrio* strain
Group 1	5	10^7 cfu/ml	Immersion	*Vh*
Group 2	5	10^8 cfu/ml	Immersion	*Vp*
Group 3	5	10^7 cfu/shrimp	Injection	*Vh*
Group 4	5	10^8 cfu/shrimp	Injection	*Vp*
Group 5	5	10^7 cfu/ml	Immersion	*Vp*
Group 6	5	10^8 cfu/ml	Immersion	*Vh*
Group 7	5	10^7 cfu/shrimp	Injection	*Vp*
Group 8	5	10^8 cfu/shrimp	Injection	*Vh*

[a] The dosage units in different infection routes were not unified. The dosage unit was determined in challenge tests according to selection of infection route (i.e. cfu/mL is corresponding to immersion infection, cfu/shrimp is used in injection infection). Therefore, the impact of dosage units on shrimp mortality was ascribed to infection routes.