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Research Article

SIMPLE, FAST AND RELIABLE METHOD TO DETERMINE PATHOGENIC POTENTIAL OF FISH BACTERIAL PATHOGEN

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ABSTRACT

The emergence of fish bacterial pathogens lead to large economic losses in aquaculture. Currently, the confirmation of causative agent is urgently needed. In addition, current methods for evaluation of pathogenic bacteria are laborious and time consuming. Hence, this study was conducted to establish the simple, fast and reliable method for the evaluation of pathogenic potential of bacteria. This method was developed by utilizing brine shrimp as model organism. The result showed that the method can distinguish pathogenic and non-pathogenic bacteria, and confirmed by statistical analysis. Finally, this method was proven to assess the pathogenic potential of the bacteria in simple, fast and reliable manners. Thus, the rapid assessment of pathogenic bacteria can facilitate the formulation of right prevention and treatment strategies in aquaculture in future.

Keywords: bacterial pathogen; brine shrimp; pathogenic potential, aquaculture

INTRODUCTION

Emerging bacterial diseases in fish cause substantial losses among population of fish resulting in huge economic losses in commercial aquaculture. In recent years, the number of fish bacterial pathogens is continued to increase [1]. One of the cause of this problem is the delay of confirmatory of the causative agent [2]. Besides the identification method, the pathogenicity evaluation is also needed for determination of causative agent. In addition, potential pathogenic bacterium can be pathogenic and non-pathogenic to the fish [3]. Therefore, the confirmatory of causative agent is important for development of prevention and treatment strategies of the target bacterial pathogen.

Nowadays, there is a need to have simple and rapid tests to determine pathogenicity of bacterial isolated from disease fish. Previously, most of the pathogenicity evaluation are conducted onto fish [4, 5]. This assay is laborious, time-consuming and required the special permission in some country (i.e. animal ethic guideline). Recently, brine shrimp was used as test organism to evaluate the pathogenic potential of bacteria [6, 7, 8]. However, the current methods are also time consuming and laborious. Therefore, this study is conducted to establish simple, fast and reliable method for the evaluation of pathogenic potential of bacteria.

MATERIALS AND METHODS

Vibrio harveyi VHJR7 (VHJR7) was used in this study. It was previously isolated from Asian seabass [9] and highly pathogenic to fish and shrimp [4]. VHJR7 was maintained in thiosulfate citrate bile sucrose (TCBS) agar. It was cultured in tryptic soy broth supplemented with 2% NaCl to prepare the bacterial inoculum. Dosage 10^7 CFU was used for the evaluation of bacterial pathogenicity [10]. For bacterial controls, attenuated *V. harveyi* VHJR7 (attVHJR7) and formalin killed *V. harveyi* VHJR7 (inVHJR7) were used. The attVHJR7 and inVHJR7 were prepared following method described by Hu et al. [11] and Thuy et al. [12], respectively. The authentication of attVHJR7 is confirmed by method described by Ransangan and Lal [13] and its identity was found as *Vibrio harveyi*. In order to determine the bacterial density would produce final CFU/ml of approximately 10^7, procedure described by Lai et al. [14] was followed with small modification. The overnight culture bacteria was washed twice before re-suspended in sterile natural seawater. The optical density (OD) was observed and adjust to 1.0. It was found that both VHJR7 and attVHJR7 produce desirable final value of $\sim 10^7$ CFU/ml on OD at 600 nm of 1.0. Both VHJR7 and attVHJR7 suspension can be directly used to determine its pathogenicity to brine shrimp.

Pathogenicity assessment to brine was conducted according to Svensson et al. [15]. Brine shrimp nauplii instar II (48 hours after hatched; Figure 1) were used for the test. Nauplii instar II was the most sensitive [16] and appropriate for determination of bacterial pathogenicity [7, 8].

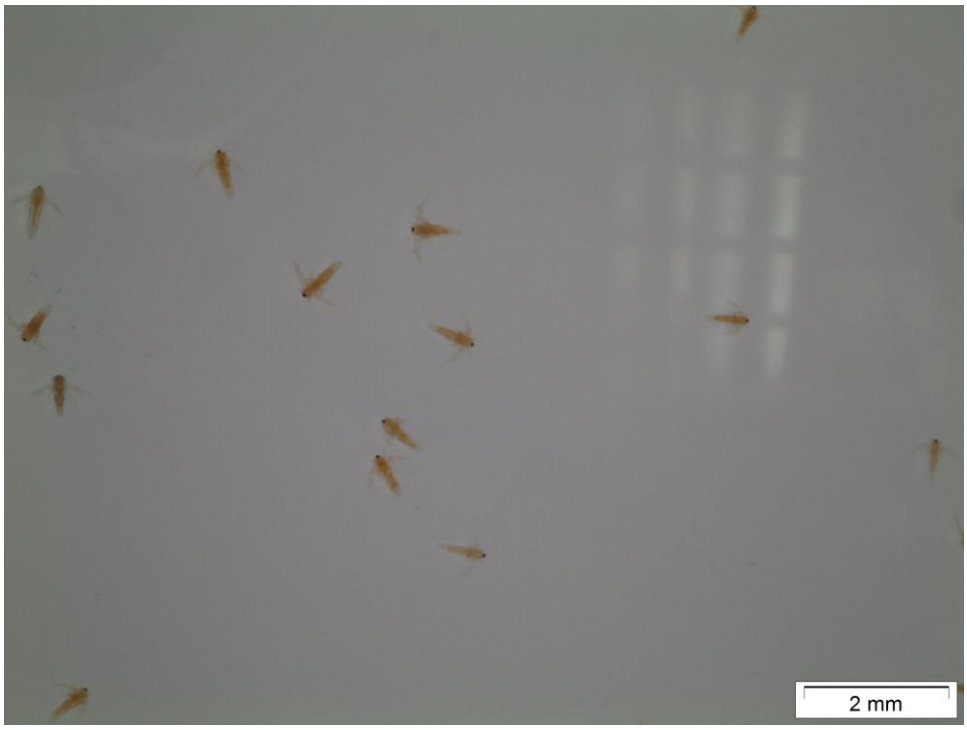

Figure 1: Instar II brine shrimp *nauplii*

To facilitate the rapid execution of this test, the final volume was set in 1.5 ml (Figure 2). About 30 nauplii (instar II) in 0.5 ml sterile natural seawater was mixed with 1.0 ml bacterial suspension (~10^7 CFU/ml). The test was conducted for 24 hours for rapid observation. All test were conducted in three independent replicates and repeated trice. The data were statistically analyzed using the t-test (two-sample assuming equal variances) of MS Office Excel (Microsoft, Redmond, WA). The result was considered significant if the *p*-value was lower than 0.05 [8].

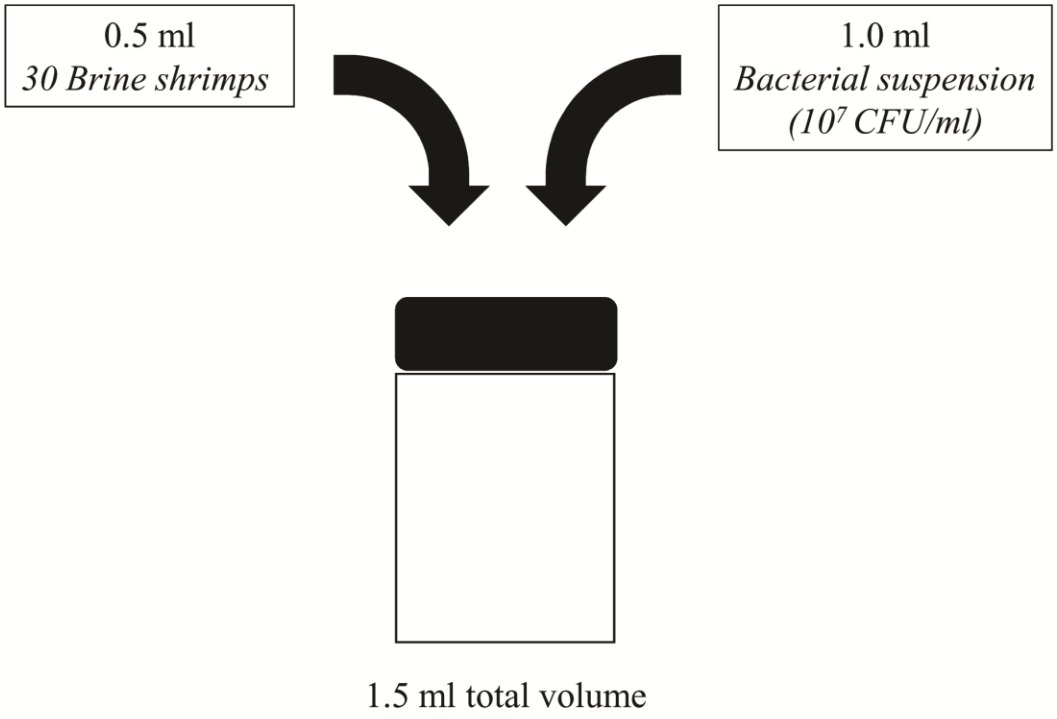

Figure 2: Schematic diagram of evaluation on bacterial pathogenic potential.

RESULTS AND DISCUSSION

VHJR7 was used to infect brine shrimp at dosage of 10^7 CFU. As bacterial control, the inactivated and attenuated VHJR7 were used. As for negative control, sterile natural seawater without bacteria was used. The mortality of the brine shrimp was counted after 24 hours. The result showed that the mortality of the brine shrimp is higher by VHJR7 (10^7 CFU) compared to the attenuated VHJR7, inactivated VHJR7 and negative control (Figure 3). Therefore, the assessment of pathogenicity of *V. harveyi* VHJR7 to brine shrimp instar II after 24 hours using 10^7 CFU was statistically significant ($P<0.05$).

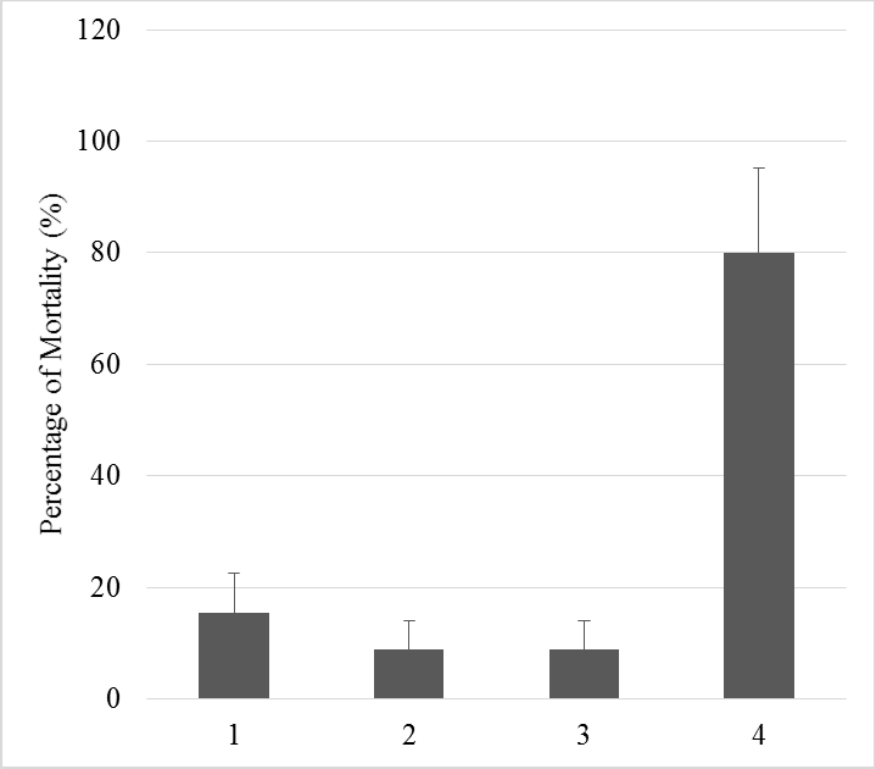

Figure 3: Percentage of mortality of brine shrimp after 24 hours incubation. 1: sterile seawater, 2: attVHJR7, 3: inVHJR7, 4: VHJR7.

This study was aimed to establish simple, fast, reliable and practical method to assess pathogenic potential of bacteria. The finding showed that method in this study can served this purpose. The simplicity of this method lies in the procedure of the challenge test. Most brine shrimp challenge assays, such as described in the Haldar et al. [7] and Lee et al. [8] require many laborious calculation in order to setup the challenge test i.e. (30 nauplii/6 ml /10^2, 10^5, 10^8 CFU/ml) and (20 nauplii/5 ml/various CFU), respectively. Here, this study only required mixture of brine shrimp and bacteria in 1.5ml for the setup of (30 nauplii/1.5 ml/10^7 CFU). In addition, this method is fast since it require only 24 hours (1 day) for the determination of pathogenic potential of bacteria. Previously, all test were conducted for at least 48 hours in order to evaluate the pathogenic potential of the bacteria [7, 8]. Moreover, the statistically significant result indicated that this method is reliable. In previous report, the pathogenic potential of bacteria was related to the mortality of brine shrimp after 24 hours. Both Haldar et al. [7] and Lee et al. [8] reported that pathogenic strains of bacteria induced more than 50% mortality to brine shrimp. In addition, *Vibrio harveyi* VHJR7 is highly pathogenic, while, attenuated bacteria is previously reported non-pathogenic. Even though both strains are still alive, they have different pathogenic potential [17]. Moreover, the mortality between attVHJR7 and inVHJR7 are not significantly different. Thus, this method can be used to differentiate pathogenic potential of bacteria.

This study is not intended to provide assessment of the virulence of the bacteria. Instead, this study is only focus in simple, rapid and reliable method to separate the pathogenic and non-pathogenic strains of bacteria. Therefore, this study wanted to propose the indicator for pathogenic potential of bacteria, which the bacteria is considered pathogenic if the mortality of brine shrimp is statistically significant compared to control, after inoculated with 10^7 CFU bacteria for a period of 24 hours. This indicator is proposed based on: 1) 10^7 CFU is commonly used in lethal study of bacteria in mice [18] and fish [10], 2) the percentage of brine shrimp mortality after 24 hours is statistically significant ($P<0.05$) when compared to control. However, the result from this method is only acceptable if the result is consistent after repeated for at least trice.

CONCLUSION

In conclusion, the method in this study is proven able to assess the pathogenic potential of bacteria. This simple, fast and reliable method may help the researchers to identify potential causative agent of bacterial diseases and used for the rapid assessment of pathogenic bacteria for the formulation of right prevention and treatment strategies.

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REFERENCES

1. Buller, N.B. Bacteria and Fungi from Fish and Other Aquatic Animals, 2nd Edition: A Practical Identification Manual. CAB International, United Kingdom, 2014, pp 919.
2. Walker, P.J. and JR Winton. Emerging viral diseases of fish and shrimp. *Veterinary Research* 2010; **41**(6), 51.
3. Travers, M.A., Le Bouffant, R., Friedman, C.S., Buzin, F., Cougard, B., Huchette, S., Koken, M. and Pailard, C. Pathogenic *Vibrio harveyi*, in contrast to non-pathogenic strains, intervenes with the p38 MAPK pathway to avoid an abalone haemocyte immune response. *Journal of Cellular Biochemistry*2009; **106**(1), 152–160.
4. Ransangan, J., Lal, T.M. and Al-Harbi, A.H. Characterization and experimental infection of *Vibrio harveyi* isolated from diseased Asian seabass (*Lates calcarifer*). *Malaysian Journal of Microbiology* 2012; **8**(2), 104–115.
5. Khouadja, S., Lamari, F. and Bakhrouf, A. Characterization of *Vibrio parahaemolyticus* isolated from farmed sea bass (*Dicentrarchus labrax*) during disease outbreaks. *International Aquatic Research* 2013; **5**, 13.
6. Austin, B., Austin, D., Southerland, R., Thompson, F. and Swings, J. Pathogenicity of vibrios to rainbow trout (*Oncothynchus mykiss*, Walbaum) and *Artemia* nauplii. *Environmental Microbiology* 2005, **7**, 1488–

1495.

7. Haldar, S., Chatterjee, S., Sugimoto, N., Das, S., Chowdhury, N., Hinenoya, A., Asakura, M. and Yamasaki, S. Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in *Artemia* model. *Microbiology.* 2011; **157**, 179–188.
8. Lee, M.N., Kim, S.K., Li, X.H. and Lee, J.H. Bacterial virulence analysis using brine shrimp as an infection model in relation to the importance of quorum sensing and proteases. *Journal of General and Applied Microbiology,* 2014; **60**, 169–174.
9. Ransangan, J. and Mustafa, S. Identification of *Vibrio harveyi* isolated from diseased asian seabass *Lates calcarifer* by use of 16S ribosomal DNA sequencing. *Journal of Aquatic Animal Health.* 2009; **21**, 150–5.
10. Carraschi, S.P., Da Cruz, C., Neto, J.G.M., de Mores, F.R., Júnior, O.D.R., Neto, A.N. and Bortoluzzi, N.L. Evaluation of experimental infection with *Aeromonas hydrophila* in pacu (*Piaractus mesoptamicus*) (Holmberg, 1887). *International Journal of Fisheries and Aquaculture* 2012; **4**(5), 81–84.
11. Hu, Y.H., Deng, T., Sun, B.G. and Sun, L. Development and efficacy of an attenuated *Vibrio harveyi* vaccine candidate with cross protectivity against *Vibrio alginolyticus*. *Fish Shellfish Immunology,* 2012; **32**, 1155–1161.
12. Thuy, N.T.T., Nguyen, D.H. and Wergeland, H.I. Specific humoral immune response and protection against *Vibrio parahaemolyticus* in orange-spotted grouper *Epinephelus coioides*. *International Journal of Aquatic Science*2013: **4**(1), 24–35.
13. Ransangan, J. and Lal, M.M.T. Simultaneous detection of *Photobacteriumdamselae*, *Vibrio alginolyticus*, *Vibrio harveyi* and *Vibrio parahemolyticus* using multiplex PCR amplification method. *International Research Journal of Biological Sciences*2013; **2**(12), 79–84.
14. Lai, H.C., Ng, T.H., Ando, M., Lee, C.T., Chen, I.T., Chuang, J.C., Mavichak, R., Chang, S.H., Yeh, M.D., Chiang, Y.A., Takeyama, H., Hamaguchi, H., Lo, C.F., Aoki, T. and Wang, H.C. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. *Fish Shellfish Immunology.* 2015; **47**, 1006–1014.
15. Svensson, B.M., Mathiasson, L., Mårtensson, L. and Bergström, S. *Artemia salina* as test organism for assessment of acute toxicity of leachate water from landfills. *Environ. Monitoring Assessment*. 2005; **102**, 309–321.
16. Sanchez-Fortún, S., Sanz, F., Barahona, M.V. Acute toxicity of several organophosphorus insecticides and protection by cholinergic antagonists and 2-PAM on *Artemia salina* larvae. *Archives of Environmental Contamination and Toxicology* 1996; **31**, 391–8.
17. DeFranco, A., Locksley, R.M. and Robertson, M. Immunity: The Immune Response in Infectious and Inflammatory Disease. New Science Press Ltd, Singapore, 2007, pp. 387.
18. Fritz, D.L., Vogel, P., Brown, D.R., Deshazer, D., and Waag D.M. Mouse model of sublethal and lethal intraperitoneal glanders (*Burkholderia mallei*). *Veterinary Pathology* 2000; **31**, 626–636.