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Detection of *vibrio harveyi* using iavh primer in shrimp fry infected under different immersion time

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Detection of *vibrio harveyi* using iavh primer in shrimp fry infected under different immersion time

I A K Kadriah and Nurbaya

Research Institute for Coastal Aquaculture, Maros, Indonesia

E-mail: inceayu@gmail.com

Abstract. Methods for the early detection of the pathogenic luminous Vibrio bacteria would be beneficial through enabling timely (early) implementation of preventative measures to reduce shrimp mortality due to vibriosis. This study aimed to measure the sensitivity of the IAVh haemolysin primer in detecting pathogenic *Vibrio harveyi* in tiger shrimp (*Penaeus monodon*) fry. This research was conducted in the Fish Health and Environment Laboratory at the Research Institute for Coastal Aquaculture (BPPBAP) in Maros, South Sulawesi, Indonesia. In this study, tiger shrimp fry were infected with the pathogenic V. harveyi bacteria through an immersion method at concentrations of 105, 106, and 107CFU/mL. Samples were collected after immersion times of 6 hours, 12 hours, and 24 hours. The presence of the haemolysin gene was detected through the PCR (Polymerase Chain Reaction) technique using the IAVh primer set. Electrophoresis results detected the presence of pathogenic V. harveyi bacteria after 6 hours of exposure, although the DNA bands produced were still thin. After 12 hours exposure, the DNA bands of pathogenic V. harveyi bacteria were clearly visible. However, after 24 hours exposure, the presence of the haemolysin gene of pathogenic V. harveyi bacteria was no longer detected. Similar results were obtained for all bacterial concentrations tested. It is thought that the 24 hour results are due to a decline in the population of V. harveyi bacteria living within the shrimp body and shrimp culture media. These findings indicate that it is important to implement rapid detection methods early on in cases of bacterial infection in shrimp (0 – 12 hours), since it was difficult to detect the presence of pathogenic bacteria in the shrimp body tissues more than 12 hours post infection.

1. Introduction

Disease is the main inhibiting factor in the success and sustainability of aquaculture since it may hamper the growth of cultured organisms and even result in death. One disease which often attacks penaeid shrimp is vibriosis caused by bacteria of the Genus *Vibrio*. Shrimp infected with this disease appear luminescent in the dark like fire-flies, so that vibriosis is often referred to as fire-fly disease. In shrimp culture, vibriosis can cause a considerable decrease in production. Some species of *Vibrio* bacteria often present in both hatchery and grow-out ponds affected by vibriosis are *V. harveyi* [1, 2, 3, 4], *V. Parahaemolyticus* [5], *V. alginolyticus* and *V. campbelli* [6] These species of bacteria are widely distributed in the aquatic environment and are known to be the main cause of fire-fly disease in both marine and brackish-water organisms. These species can not only act as an initial cause of disease, but can also often act as opportunistic agents causing secondary infection [7].

The development of an early detection method would have significant benefits, since it could be used in efforts to prevent outbreaks of vibriosis disease in the field, both in hatcheries and shrimp grow-out ponds. Preventive measures should be implemented before the bacterial colonies reach

quorum. Research conducted by [8] concluded that the ability of *Vibrio* bacteria to employ quorum sensing was greatly affected by the density of the bacterial population in nature. A population density of 10^4 CFU/ml was the minimum density facilitating *Vibrio* bacteria to perform cellular communication both within species (interspecies) and with other pathogenic *Vibrio* species (intraspecies) [8]. Such a rapid detection method would also require the ability to analyze samples collected from the field without performing bacterial isolation processes beforehand.

Before the emergence of PCR based molecular methods, the diagnosis of disease mostly depended on the culture of pathogenic organisms in a specific media or cell, phenotypic analysis or identification of the serological characteristics of pathogenic organism, and histological examination of pathogens in host tissue. Molecular methods can be a solution in the study of organisms that cannot yet be cultured in vitro or where the method to grow the pathogenic organism is not yet available. Molecular methods also have the potential to further increase detection sensitivity. Significant improvement in the specificity of molecular detection method has been shown through the ability to detect genetic polymorphisms or mutations that distinguish organisms and to identify pathogens as well as providing ways to test the relationship between genotypic and phenotypic characteristics [9].

This study aimed to measure the sensitivity of IAVh haemolysin primer in detecting pathogenic *Vibrio harveyi* at different bacterial concentrations and after different exposure times. This study is expected to be useful in the development of an early warning detection system for vibriosis in aquaculture, especially penaeid shrimp culture.

2. Methods

2.1. Culture of pathogenic bacteria

Vibrio harveyi to be used in the trials were grown on TCBSA (Thiosulfate Citrate BileSalt Sucrose Agar) media for 24 hours. Bacteria grown on TCBSA media were inoculated into Nutrient Broth (NB) liquid culture media with 1 inoculating loop. Sub cultures were made, after using a shaker at 150 rpm for 24 hours, by transferring about 1 mL into 100 mL of new liquid NB media (main culture)[10]. Bacteria were grown on this main culture media for 4 hours before being used to infect the experimental animals (Fig. 1A.). Before being used to infect the experimental animals, the bacterial cultures were centrifuged (6000 rpm for 10 minutes) to separate the bacteria cultured from the NB media. After this initial centrifuging, 500 µL of bacterial culture was placed in each Eppendorf tube and centrifuged 3 times, removing the supernatant, until a precipitate (pellet) was obtained. Finally the pellet was washed using SS (Saline Solution).

2.2. Artificial infection with pathogenic Vibrio harveyi

At this stage, shrimp fry of post-larvae (PL) size 10 – 12 were collected from the Research Institute for Coastal Aquaculture in Takalar and brought to the BPPBAP tiger shrimp hatchery facility. Once the bacterial cultures had been obtained, the larvae were artificially infected through immersion. Experimental tanks used in the immersion process were 3 aquaria with a capacity of 22 litres each. Each aquarium was filled with 10 litres of sterilised seawater at 28 ppt salinity. The seawater was sterilized by adding 150 mg/L of calcium hypochlorite overnight and then neutralized using 75 mg/L of sodium thiosulfate. Each aquarium was stocked with 300 shrimp fry. In this study, the bacterial concentrations used to infect the shrimp fry (Fig. 1B) were 10^5, 10^6, and 10^7 CFU/mL, with exposure times of 6 hours, 12 hours, and 24 hours. There were thus 9 treatments; with 3 replicates per treatment, giving a total of 27 experimental units.

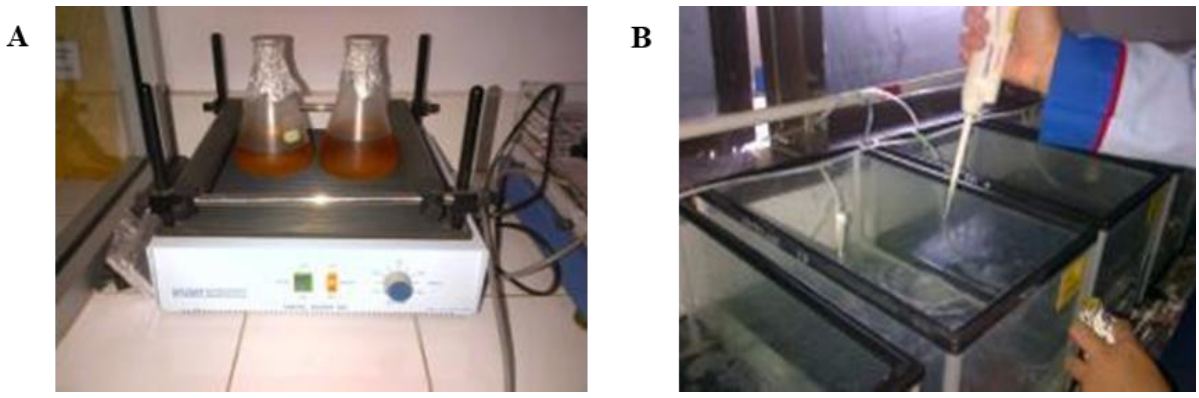

Figure 1. A. Pathogenic bacterial culture process; B. Bacterial infection of tiger shrimp post larvae

2.3. DNA Extraction

Isolation of genomic DNA used the DTAB-CTAB (Dodecyl Trimethyl Ammonium Bromide/Cetyl Trimethyl Ammonium Bromide) method (Fig. 2A.). This process was begun by weighing a 30 mg sample, which was then mashed using a mortar and pestle before adding 600µL DTAB Solution and mixing it with the mortar and pestle. The mix was the removed from the mortar, homogenized for 20 seconds, incubated on Thermoblock Biometra (TB) for 5 minutes at temperature of 75ºC, chilled at room temperature, and further re-homogenized and precipitated. Chloroform (700 µL) was added and the sample homogenized for 20 seconds before being centrifuged for 5 minutes at 12,000 rpm. The clear liquid at the top was taken and placed in a 2 mL Eppendorf tube, to which 100 µL of CTAB solution and 900 µL ddH2O were then added. The solution was homogenized, incubated for 5 minutes at 12,000 rpm, and then chilled at room temperature before being further centrifuged for 10 minutes at 12,000 rpm. The supernatant was then removed and 150 µL of Dissolve Solution was added. The solution was then incubated for 5 minutes at a temperature of 75ºC, chilled at room temperature, and centrifuged for 5 minutes at 12,000 rpm. The supernatant was taken and placed in an Eppendorf tube to which 300 µL of cold 95% Ethanol was then added, after which the solution was mixed using vortex mixer before being centrifuged for 5 minutes at 12,000 rpm. Supernatant added with 95% ethanol was slowly removed, then 200 µL of 70% ethanol of was added and the mix was centrifuged again for 5 minutes at 12,000 rpm. The ethanol was then removed and the Eppendorf tube was placed upside down on tissue paper for 2 hours for the DNA pellet to dry. After 2 hours drying time, 200 µL of TE Buffer was added and the sample was stored at a temperature of 20ºC.

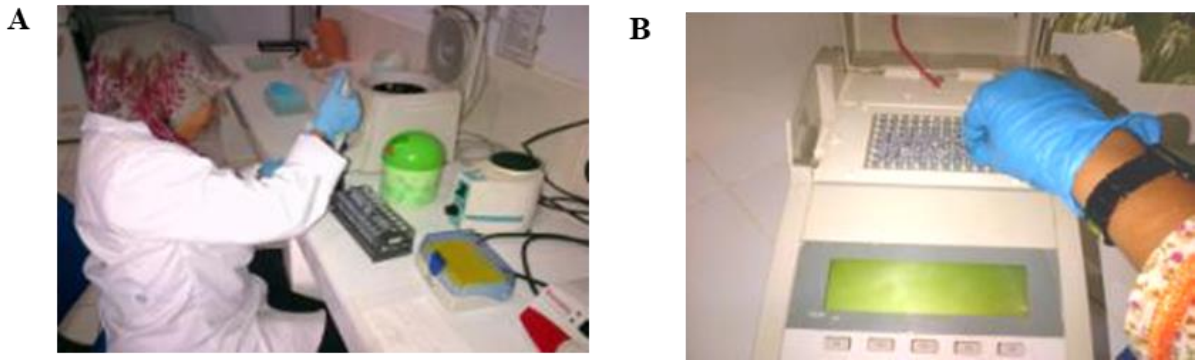

Figure 2. A. The process of genomic DNA extraction; B. The process of DNA amplification using a PCR machine

2.4. DNA amplification and visualization of PCR product

After completing the extraction of genomic DNA from the shrimp samples, Polymerase Chain Reaction (PCR) was performed using IAVh specific marker primer set as a detector for the presence of

the haemolysin gene in the sample (Fig. 2B). The PCR process was begun by preparing a Master Mix solution. This solution was made by mixing 10X Dream Taq Buffer (containing 5 µL $MgCl_2$20 mM), 5 µL dNTP Mix, and 0,25 µL DreamTaq DNA Polymerase. After this, 1 µL each of IAVh forward and reverse primers [11] were added and then ddH_2O was added until a total volume of 49 µL was reached. The solution was homogenized, and then 1 µL of DNA template was added. The PCR program for the haemolysin primer was set for 25 cycles with a denaturation temperature of 94^0C for 1 minute, annealing at 63^0C for 1 minute and 30 seconds, elongation at 68^0C for 1 minute and 30 seconds and also a final extra elongation at 72^0C for 10 minutes. This PCR process was repeated twice (re-PCR) to increase the sensitivity of the detection result.

Electrophoresis on 2% agarose gel was used to observe and document the PCR product. Running conditions of the minigel electrophoresis were 150 V and 70 A, with a run time of 30 minutes. Tris-Borate-EDTA (TBE) buffer was used. The PCR product volume was 3 µL with 2 µL loading dye. The DNA ladder volume was 5 µL without the addition of loading dye since the DNA ladder used already contained loading dye. A stock bacterial collection of *V. harveyi* was used as a positive control [11]. The presence of a band at 151bp base length (haemolysin gene indicator) during the visualization of the electrophoresis gel under ultraviolet light indicated the contamination of pathogenic luminous *Vibrio* bacteria in that shrimp sample.

3. Results and Discussion

Based on the electrophoresis results, the application of the specific marker test, namely the use of the IAVhF1 and IAVhR1 primer pair to detect haemolysin gene indicating the presence of pathogenic *Vibrio* bacteria, revealed differences between the exposure time treatments. For the 6 hour exposure treatment, the presence of the haemolysin gene was detectable, but thin. After 12 hours of exposure, the DNA bands from the pathogenic bacteria were clearly visible (Figure 3). Similar results were obtained for all concentrations tested, however, there was difference in the thickness of the DNA band for the 10^5 CFU/mL treatment compared to the 10^6 CFU/mL and 10^7 CFU/mL treatments.

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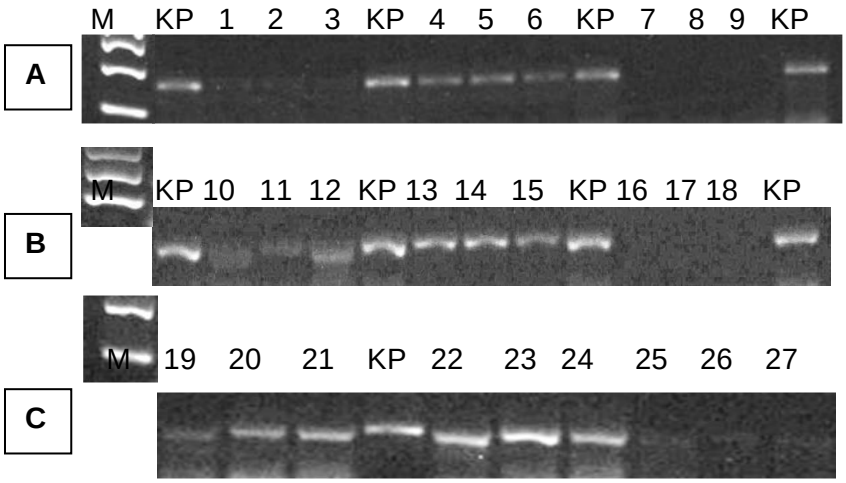

Figure 3. Electrophoresis of PCR products; M = DNA ladder; KP = positive control. A. 10^5 CFU/ml concentration; line 1-3, 6 hour exposure; line 4–6, 12 hour exposure; line 7–9, 24 hour exposure. B. 10^6 CFU/ml concentration: line 10-16, 6 hour exposure; line 13–15, 12 hour exposure; line 16–18, 24 hour exposure. C. 10^7 CFU/ml: line 19-21, 6 hour exposure; line 22–24: 12 hour exposure; line 25–27: 24 hour exposure.

After 24 hours of exposure, the haemolysin gene from *Vibrio* bacteria was no longer able to be detected. It is considered likely that this was due to a decrease in the population of *Vibrio* bacteria

present within the culture media and living within the body of the shrimp larvae. The results obtained in this study can be explained by observing the data on the bacterial population isolated from experimental media during a previous experiment [10]. The growth curve of bacterial concentration in the shrimp culture media (Figure 4) shows a decrease over time in the bacterial population [10].

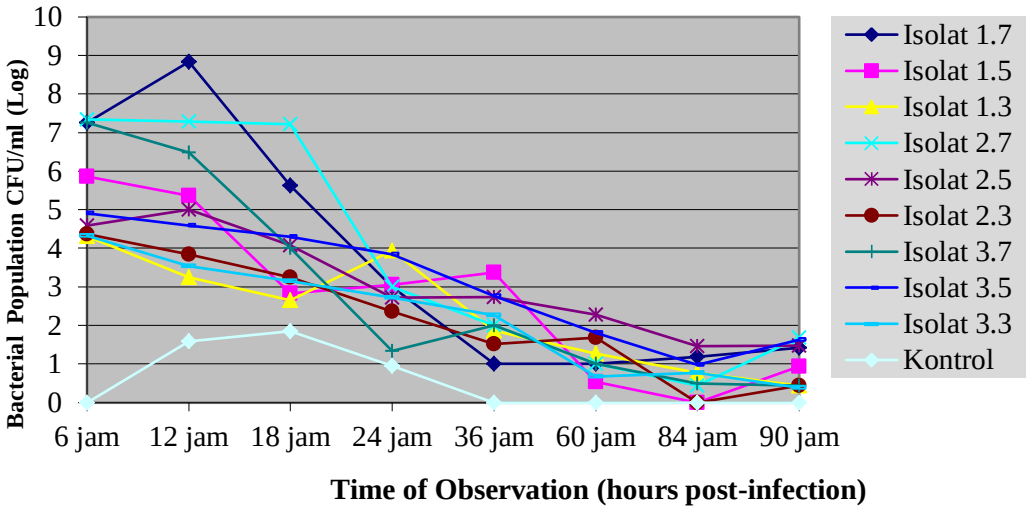

Figure 4. Growth curve of bacteria in shrimp culture water media, adapted from [10]

Figure 4 shows that the highest bacterial population levels were found at 6 and 12 hours post infection, and were obtained from a treatment with infection by *V. harveyi* isolate 1 at an initial concentration of 10^7 CFU/ml. The bacterial population had already begun to decrease by 6 hours post infection in the other treatments, with lower initial concentrations. The decreasing in the pathogenicity level of bacteria after 12 hours of treatment is thought to be related to the declining bacterial population in the culture media. It is known from pathogenicity tests using the immersion method that pathogenicity is greatly affected by the bacterial population surviving in the culture media.

The challenge test performed on PL 10–12 tiger shrimp fry was expected to be representative of infection occurring in nature and in a hatchery environment. The attack of *V. harveyi* in nature is commonly not in the form of single direct attack, since infection is frequently preceded by a shock due to changes in water quality of the shrimp culture media or the attack of other pathogens such as viruses [12]. For one decade, strains of *V. harveyi* bacteria have been reported as the main pathogen causing high mortality in the shrimp culture industry worldwide [2, 3, 13]. The emergence of disease is mainly due to poor environmental management, resulting in an accumulation of disease vectors around shrimp culture locations. Several parasites like *Epistylis* and *Zoothamnium sp.,* when present in relatively high numbers, can cause shrimp to have difficulty in moving and lose appetite, and can also interfere with the moulting process. Under such conditions, opportunistic bacteria such as *Vibrio* sp. will tend to thrive and act as secondary agents in the process leading to tiger shrimp mortality [14]. One of the genes involved in the pathogenic process of *Vibrio* bacteria is the haemolysin gene. *Haemolysin* is a gene involved in the process of cell membrane destruction (haemolysis) and has been found in several species of *Vibrio* bacteria [15]

The results of this study indicate that it is important to perform early detection at the beginning of bacterial infection in shrimp (3 – 12 hours post-infection) since it was difficult to detect the presence of pathogenic bacteria in the shrimp body at 12 hours post infection. Good primer design is essential for the success of PCR reactions; an ideal primer has a good balance between specificity and sensitivity, where sensitivity reflects the minimum amount of target template DNA needed to enable detection in the PCR product. In this study, the pathogenic bacterial detection used a haemolysin primer pair which has been tested for its specificity and sensitivity [11]. The absence of a DNA band in samples at 24 hours post infection does not necessarily mean that there were no pathogenic bacteria

within the shrimp body. It is possible that pathogenic bacteria were present, albeit in low numbers; however the tools used were not able to detect their presence at the concentrations then present. This condition reinforces the importance of performing examinations at the beginning of disease attack.

4. Conclusion

As we know, the application of an early detection method which is able to quickly detect contamination by pathogenic luminous *Vibrio* will be very helpful for treatment as well as timely and early prevention to decrease shrimp mortality. The results of this study showed that the sensitivity of haemolysin primer only enabled the detection of the presence of *V. harveyi* in shrimp fry during a time interval of 3 – 12 hours post-infection.

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