SPECIFICITY OF *Haemolysin* AND *Gyrase* GENE MARKER FOR RAPID DETECTION OF VIBRIOSIS ON PENAEID SHRIMP

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ABSTRACT

Pathogenic *Vibrio* spp. have been implicated as being one of the major causes of the disease problems in penaeid shrimp culture. For many pathogens, the outcome of the interaction between host and bacterium is strongly affected by the bacterial population size. Efforts for rapid molecular detection is to isolate specific genes which was owned by the pathogenic luminescent *Vibrio* and used it as the marker molecular in the rapid diagnosis of the disease. *Haemolysin* and *gyrase* gene known as one of the specific genes possessed pathogenic bacteria including *Vibrio*. The objectives of this study were to determine the specificity of designed specific primer from local isolate. Specificity test was performed for *haemolysin* primer (IAVhF1 and IAVhR1), *gyrase* primer (IAGyrF1 and IAGyr2) and commercial detection kit. The specificity test comparing the PCR result of pathogenic *Vibrio* DNA template as positive control, and DNA template from non pathogenic luminescent *Vibrio*, as well as pathogenic non *Vibrios*. The result showed that at annealing temperature of 60°C, *haemolysin* primer was more specific in detecting pathogenic *Vibrio* for penaeid shrimp than that of *gyrase* primer and commercial detection kit.

KEYWORDS: **vibriosis, *haemolysin*, *gyrase*, specific primer, commercial detection kit**

INTRODUCTION

Research Background

Vibrio species are known to cause serious diseases in the aquatic organisms such as penaeid shrimps tiger prawn (Lightner & Redman, 1998), several fish species and molluscs (Austin & Zhang, 2006), and also for corals (Ben-Haim *et al.*, 2003). Some of the luminescent vibrios, which include *Vibrio cholerae* (biotype albensis), *V. fischeri*, *V. harveyi*, *V. logei*, *V. splendidus*, *V. mediterranei* (Farmer & Hickman-Brenner, 1992), *V. orientalis* (Yang *et al.*, 1983), *Photobacterium leiognathi*, and *P. phosphoreum* have been implicated principally with disease outbreaks in shrimp larviculture facilities (Lavilla-Pitogo *et al.*, 1990), and to a lesser degree in grow-out ponds (Lavilla-Pitogo *et al.*, 1998). Luminescent vibrio are known as agent of vibriosis that infected many species of shrimp (Baticados *et al.*, 1990; Karunasagar *et al.*, 1994; Moriarty, 1998; Zhang & Austin, 2000).

Vibriosis in shrimp culture can lead to a serious decrease in shrimp production. Rapid detection method for luminescent pathogenic *Vibrio* will be very useful for early detection of vibriosis which avoid shrimp mass mortality. As it is known that the ability of the bacteria to infect its host is also influenced by the density of bacteria in culture media. So, if the presence of bacteria can be detected before

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the number reached quorum, prevention efforts can be made to inhibit the growth of pathogenic bacteria in culture media.

The identification of vibrios isolated from the aquaculture environment has been imprecise and is labour-intensive, requiring many biochemical, and/or physiological tests (Vandenberghe *et al.*, 2003). When dealing with pathogens, that require lengthy culture prior to detection by other methods, PCR may greatly increase the speed of diagnosis. Amplification of pathogen nucleic acid can also achieve significant increases in specificity and sensitivity of detection, either directly or in conjunction with other techniques (Cunningham, 2002). In general, molecular methods combine higher discriminatory power and reproducibility than phenotypic tests (Cano-Gomez *et al.*, 2009).

A molecular approach to develop detection procedures for bacterial pathogens involves targeting one of the genes encoding a virulence factor (Conejero & Hedreyda, 2004). One such gene implicated in the virulence of *Vibrio* species encodes for haemolysin. Haemolysin are responsible for the disruption of the erythrocyte membrane or hemolysis and genes encoding for haemolysin that have been reported to be present in several members of the genus including *V. harveyi* (Hirono *et al.*, 1996; Nishibuchi & Kaper, 1995; Nishibuchi *et al.*, 1990; Zhang *et al.*, 2001), *V. parahaemolyticus* (Bej *et al.*, 1999) and *V. vulnificus* cytolysin gene (Lee *et al.*, 1998).

Gyrase genes can be used as a fluorescent marker of pathogenic bacteria *Vibrio* bases 363 bp in length (Thaithongnum *et al.*, 2006). GyrB gene encodes the subunit B protein of DNA gyrase (topoisomerase type II). The enzyme DNA gyrase regulate superkoiling double-stranded DNA. GyrB gene is necessary for the replication of DNA in which genes play a role in the formation of proteins that encode enzymes gyrase. Pathogenic bacteria have a Gyr-B gene structure specific than other bacteria (Thaithongnum *et al.*, 2006). If the gyrB gene destroyed, supercoiling of double-stranded DNA that is controlled by the enzyme *gyrase* not be able to take place.

The practical application of specific gene marker in diagnostic testing does require significant attention to develop of suitable methodologies and validation to ensure greatest possible specificity and sensitivity which are obtained. This method is more accurate and faster than expected is also less expensive than the use of kit that still imported.

Objective

The objective of this study was to determine the specificity of *haemolysin* and *gyrase* gene marker designed from local isolate comparing with to commercial detection kit for rapid detection of vibriosis in penaeid shrimp culture.

MATERIAL AND METHOD

Bacteria Isolates

There were eighteen isolates used in specificity test with PCR. Four isolates *V. harveyi* (P-275, By-1, M-120, T-672, and T-673), one isolate *V. parahaemolyticus* from diseased gouper (6iL), Two isolates *V. campbelli* (Nb-2 dan M-5575), one isolate *Vibrio* sp. (1 us), two isolates *V. natriegens* (4 us and 2 us), four isolates pathogenic non-*Vibrio* bacteria (*A. hydrophila*, *S. iniae*, *Salmonella* sp., *E. tarda*, and *E. coli*) and two isolates from diseased juvenile from Barru hatchery. Bacteria isolates were collected from diseased Penaeid shrimp larvae in hatchery, from pond water, and pond sediment. Isolates non-*Vibrio* bacteria was collected from diseased fish. All isolates bacteria were cultured in Tio Citrate Bile Sucrose Agar (TCBSA) and Tryptic Soy Agar (TSA, Sigma Aldrich) media.

PCR Amplification

Specificity test was performed for *haemolysin* primers (IAVhF1 and IAVhR1) and *gyrase* primers (IAGyrF1 and IAGyrR2). The specificity test was proposed to compare the PCR result of pathogenic *Vibrio* DNA template as positive control, and DNA template from non pathogenic luminescent *Vibrio*, as well as pathogenic non-*Vibrios*. To test the specificity of the primer compare to the commercial detection kits used genomic DNA samples of pathogenic luminescent bacteria *V. harveyi* and *V. parahaemolyticus* as a positive control for comparison with non-pathogenic *Vibrio* (*Vibrio* sp., *V. natriegens*) and the genome of pathogenic luminous *Vibrio* bacteria derived from grouper (*V. parahaemolyticus*) and as negative controls are milliQ distilled water. The PCR procedure was as follows. The PCR mixture consisted of one tube of *Ready To Go* (RTG, GE Healthcare UK Limited Little Chalfont

Table 1. Experimental bacteria isolates employed in this study

No	Isolates code	Species	No	Species
1	P-275	*V. harveyi*	14	*A. hydrophila*
2	By-1	*V. harveyi*	15	*S. iniae*
3	M-120	*V. harveyi*	16	*E. tarda*
4	T-109	*V. parahaemolyticus*	17	*Salmonella* sp.
5	T-168	*V. parahaemolyticus*	18	*E. coli*
6	T-672	*V. harveyi*		
7	T-673	*V. harveyi*		
8	6 iL (from diseased grouper)	*V. parahaemolyticus*		
9	Nb-2	*V. campbelli*		
10	M-5575	*V. campbelli*		
11	4 us	*V. natriegens*		
12	2 us	*V. natriegens*		
13	1 us	*Vibrio* sp.		

Buckinghamshire, UK) PCR kit added with 20 µL aquadest milliQ, one µL each forward and reverse primer and 3 µL template DNA.

The amplification condition of the pathogenic luminescent *Vibrio* haemolysin gene were 25 cycles at 94°C for 1 min., 60°C for 1 min., and 68°C for 1 min., and 30 seconds, and then an extra extension step of 72°C for 10 min. The amplification condition of the pathogenic luminescent *Vibrio gyrase* gene were 25 cycles at 94°C for 1 min., 60°C for 1 min., and 72°C for 30 seconds, and then an extra extension step of 72°C for 10 min.

Amplification protocol of the commercial detection kit were 1x PCR PreMix (22.5 µL) added with 0.5 µL Taq Enzyme and 2 µL template. Final volume was 25 µL and spin down for 15 second. The amplification condition in PCR machine were 35 cycles at 94°C for 5 min. (predenaturation), 94°C for 1 min. (denaturation), 55°C for 30 second (annealing), and 72°C for 45 second and 30 seconds, and then an extra extension step of 72°C for 5 min.

Data Analysis

Data were analyzed by descriptive analysis by comparing the DNA bands provided by PCR visualized on electrophoresis gel. DNA bands on the amplification results of the PCR marker of the presence of haemolysin gene was 151 bp in length bases and 197 bp of the presence of *gyrase* gene. The presence of DNA bands other than the length of 151 bp and 197 bp indicates that used non-specific marker.

RESULT AND DISCUSSION

Specificity Test of *Haemolysin* and *Gyrase* Gene Marker

Haemolysin and *gyrase* gene can be used as markers for the rapid detection of bacterial pathogens. In the process of detection by PCR is usually more effective to use a single primer pair, therefore be compared to the specificity of the primary *haemolysin* and *gyrase*. The specificity of the *haemolysin* primers (IAVhF1 and IAVhR1) that were designed in the previous study for the amplification of luminescent pathogenic *Vibrio haemolysin* gene was investigated by using DNA templates from a wide range of *Vibrios* and non-*Vibrio* bacteria. The specificity of *haemolysin* primers was compared to *gyrase* primers (IAGyrF1 and IAGyrR2) shown in Figure 1. Results showed that the expected 151-bp fragment to detect *haemolysin* gene could only be observed in the PCR profile of *V. harveyi*, *V. campbelli*, and *V. parahaemolyticus* from diseased tiger shrimp type (Figure 1). The expected size fragments were not observed from the PCR profiles of other Vibrionaceae type including *V. natriegens* and *Vibrio* sp. Profiles from PCR using DNA templates from *V. parahaemolyticus* collected from diseased grouper did not exhibit the 151 bp amplified fragment either (Figure 1) using *haemolysin*

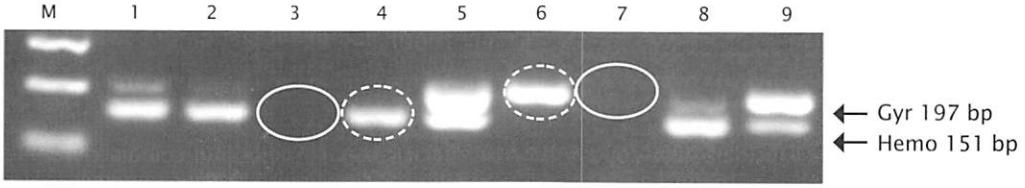

Figure 1. PCR result of *haemolysin* (Pair 1) and *gyrase* (Pair 2) primer with DNA templat (Lines 1-9) *V. harveyi* (P-275), *V. parahaemolyticus* (T-168), *V. natriegens* (4us), *V. campbelli* (Nb-2), *V. parahaemolyticus* (T-109), *V. parahaemolyticus* (Grouper fish), *Vibrio* sp. (1us), *V. harveyi* (By-1), *V. parahaemolyticus* (Gb-3); M is a 100 bp DNA *ladder*

primers. This indicates a difference in the composition of bases in the *haemolysin* gene owned by pathogenic bacteria that infects penaeid shrimp with *haemolysin* genes from pathogenic bacteria infecting grouper. The same result also showed from PCR using DNA templates from non-Vibrio bacteria including *A. hydrophila*, *S. iniae*, *Salmonella* sp. and *E. coli* (Figure 2; line 1-4).

The specificity of the *gyrase* primers (IAGyrF1 and IAGyrR2) observed from PCR product showed that *gyrase* primers could be amplified DNA from pathogenic *V. parahaemolyticus* from diseased Gouper (Figure 1; Line 6 and Figure 2; Table 2). This result showed that *gyrase* primers was not specific to detect penaeid shrimp's pathogenic *Vibrio* but also pathogenic *Vibrio* from diseased fish. Results of DNA amplification of non pathogenic *Vibrio* sample were compared with samples of luminous pathogenic *Vibrio* DNA as a positive control. There was no visible DNA bands on the electrophoresis results for DNA samples from non-Vibrio pathogenic bacteria (*A. hydrophila*, *S. iniae*, *Salmonella* sp., and *E. coli*). Primer specificity is very important to avoid mistakes in the diagnosis of disease outbreaks in the field (Conejero & Hedreyda, 2004).

Amplification of genes encoding virulence determinants appears to be a promising approach for the detection of pathogenic luminescent *Vibrio*. The PCR protocol specifically amplifying the 151-bp fragment in pathogenic luminescent *Vibrio* may facilitate the rapid and accurate detection of pathogenic *Vibrio* from infected penaeid shrimp species. This will allow early diagnosis of pathogenic *Vibrio* infection for effective disease prevention and surveillance.

Specificity Test of Commercial Detection Kit

On the results of PCR using a commercial kit there was a visible DNA band 197 bp in length (according to the owner's manual kit) for DNA samples from non-pathogenic *Vibrio*

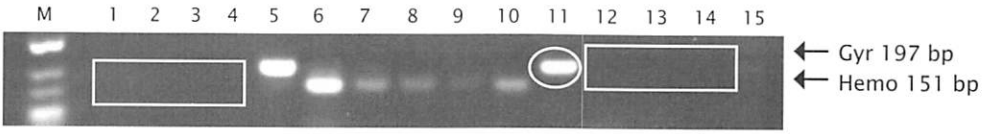

Figure 2. PCR profiles using *haemolysin* (IAVhF1 & IAVhR1) and *gyrase* (IAGyrF1 dan IAGyrR1) primers and DNA templates from pathogenic and non-pathogenic *Vibrio* and non-*Vibrio* pathogenic bacteria; Lines 1-4 (*A. hydrophila*, *S. iniae*, *Salmonella* sp., and *E. coli*; Line 5 *V. campbelli* (Nb-2); Line 6-10 (*V. harveyi* code: By-1, P-275, M-120, T-672, and T-673); Line 11 *V. parahaemolyticus* (diseased Grouper); Line 12-14 *V. natriegens* and *Vibrio* sp.; Line 15 and 16 DNA template from disease shrimp's larvae (BWil); M is a 50 bp low range DNA *ladder*

Tabel 2. The result of specificity test of *haemolysin* and *gyrase* primer showed in Figure 1 and Figure 2

No	Isolates code	Species	Primer	
			Haemolysin	*Gyrase*
1	P-275	*V. harveyi*	+	+
2	By-1	*V. harveyi*	+	+
3	M-120	*V. harveyi*	+	+
4	T-672	*V. harveyi*	+	+
5	T-673	*V. harveyi*	+	+
6	**6 iL**	***V. parahaemolyticus***	-	+
7	Nb-2	***V. campbelli***	+	-
8	2 us	*V. natriegens*	-	-
9	1 us	*Vibrio* sp.	-	-
10	Ah	*A. hydrophila*	-	-
11	Si	*S. iniae*	-	-
12	Sm	*Salmonella* sp.	-	-
13	*E. coli*	*E. coli*	-	-
14	BWil		-	+
15	BWil		-	+

Appendix:

+ = There were a visible DNA bands on the electrophoresis results

- = no visible DNA bands on the electrophoresis results

bacteria and *V. parahaemolyticus* from diseased grouper (Figure 3; Table 3). Compared with the results of the specificity test of PCR using *haemolysin* primer design results did not reveal any DNA bands on the length of 151 bp from the non-pathogenic bacterium *Vibrio* samples and *V. parahaemolyticus* from diseased grouper (Figure 3).

These results lead to the conclusion that the *haemolysin* primer design results was more specific for detecting luminous pathogenic *Vibrio* of penaeid shrimp compared to commercial kit. Although commercial detection KIT is more sensitive for the detection of DNA from pure cultures of bacteria but not sensitive for the detection of pathogenic bac-

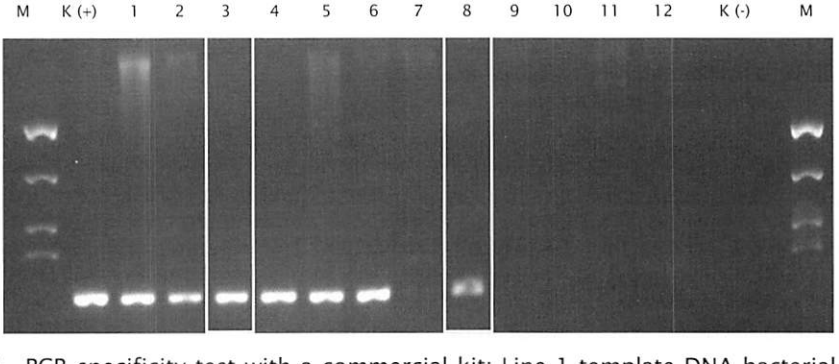

Figure 3. PCR specificity test with a commercial kit; Line 1 template DNA bacterial from *V. campbelli*; Lines 2-5 each bacterial DNA template *V. parahaemolyticus*, which is the template DNA; Line 3 *V. parahaemolyticus* from grouper; Lines 6-12 template DNA from bacterial isolates *V. harveyi*, *V. natriegens*, *Vibrio* sp., *Salmonella* sp., *S. iniae*, *A. hydrophila*, and *E. coli*; M is a marker and K (+) is the positive control *V. harveyi*

Table 3. The result of specificity test of commercial detection kit showed in Figure 3

Kind of sample	Commercial detection kit	Designed primer
V. campbelli	+	+
V. parahaemolyticus	+	+
***V. parahaemolyticus* (diseased grouper)**	+	-
V. parahaemolyticus	+	+
V. parahaemolyticus	+	+
V. harveyi	+	+
V. natriegens (Biofloc)	-	-
***Vibrio* sp. (Biofloc)**	+	-
Salmonella sp.	-	-
Streptococcus iniae	-	-
Aeromonas hydrophila	-	-
E. coli	-	-

Appendix:

+ = There were a visible DNA bands on the electrophoresis results

- = no visible DNA bands on the electrophoresis results

teria DNA from diseased shrimp organs.

Haemolysins act on erythrocytes membranes thus lysing the cells which leads to the freeing up of the iron-binding proteins namely haemoglobin, transferrin, and lactoferrin. This iron can then be picked up by various siderophores, and is subsequently taken up through receptors in the cell membrane (Zhang & Austin, 2005). Genes indicating *haemolysin* were observed from some species of *Vibrios*: *V. harveyi* (Nishibuchi *et al.*, 1990; Nishibuchi & Kaper, 1995; Hirono *et al.*, 1996; Zhang *et al.*, 2001), *V. parahaemolyticus* (Bej *et al.*, 1999), and *V. vulnificus* cytolysin gene (Lee *et al.*, 1998).

Nucleic acid technology provides an opportunity to detect pathogens directly, by making genetic material as a target and as a development of the detection methods of culturing, serological or histological. Molecular markers are pieces of genetic material that is easily identified in the laboratory that can be used to separate cells, individuals, populations or species.

A great primer design is essential to the success of the PCR reaction. The ideal primer has a balance between specificity and sensitivity. Specificity can be judged from the frequency of occurrence of misprime (error attachment) primer on where it should not. While sensitivity is how minimum detection limit as the number of PCR products with the acquisition of the theoretical value that should be achieved. The specificity test results showed that the *haemolysin* and *gyrase* gene owned by *Vibrio* can be used as a specific molecular markers but the result showed that at annealing temperature of 60°C, *haemolysin* primer was more specific in detecting pathogenic *Vibrio* for penaeid shrimp than that of *gyrase* primer. Annealing temperature differences in the process will affect the specificity of PCR primers. The temperature 55°C used is basically a compromise. Amplification would be more efficient if performed at a lower temperature (37°C), but it usually will happen misspriming the primer in the wrong place. At higher temperatures, the specificity of the amplification reaction will increase, but the overall efficiency will decrease (Yuwono, 2005).

CONCLUSION

The result showed that at annealing temperature of 60°C, *haemolysin* primer was more specific in detecting pathogenic *Vibrio* for penaeid shrimp than that of *gyrase* primer.

Haemolysin primer can detect pathogenic luminous *Vibrio* directly from penaeid shrimp tissues and distinguish it from non-pathogenic *Vibrio* and non-*Vibrio* pathogens. Primer designed can detect pathogenic luminous *Vibrio* on penaeid shrimp more specific than commercial kits.

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