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# Detection of Luminous *Vibrio harveyi* in Penaeid Shrimp Through Nested PCR Using Haemolysin Gene Primer

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**Whiteleg shrimp (*Litopenaeus vannamei*) is one of the most important aquaculture commodity in Indonesia. However, the luminous disease primarily caused by *Vibrio harveyi* bacteria still becomes an obstacle in penaeid shrimp farming, especially in shrimp hatchery. This study was aimed to identify the presence of *V. harveyi* in *L. vannamei* through nested PCR using haemolysin gene primer. First, initial primers were designed using *V. harveyi* VIB 391 haemolysin gene sequence (accession number: DQ640264), flanking the position 133 to 756. This primer pairs were used to identify haemolysin gene in both *V. harveyi* MR5339 and *V. harveyi* 275 strain. Sequencing results from each sample showed 99% similarity with haemolysin gene sequence in Genebank. Furthermore, the sequence of *V. harveyi* MR5339 haemolysin gene was used to design the nested PCR primers. The first primer pairs of nested PCR have successfully amplified the haemolysin gene fragment of all *V. harveyi* strains samples from position 52 to 405. The second primer pairs of nested PCR have amplified position 204 to 405 where it can detect all of *V. harveyi* strains used as sample sources in this study. The application of nested PCR technique in this study was able to identify *V. harveyi* strains at serial dilution of cells density as low as $10^0$ cfu/mL, which is equal to a single cell or at DNA concentration up to $10^1$ fg/µL.**

Keywords: *Vibrio harveyi, Litopenaeus vannamei,* haemolysin, DNA, nested PCR, primer

## INTRODUCTION

*Litopenaeus vannamei* also known as whiteleg shrimp is one of the most important aquaculture commodity in Indonesia. The luminous Vibrio bacterial disease still becomes problem in whiteleg shrimp hatcheries in Indonesia (Felix *et al*. 2011). Species of Vibrio that frequently leads to mass death especially to shrimp larval is *Vibrio harveyi* (Teo *et al.* 2000). Vibriosis leads to quick and massive shrimp death (Austin & Zhang 2006).

Detection of luminous Vibriosis in shrimp is generally conducted through observation of clinical symptoms of the shrimp's body and glowing in the dark, conventional isolation of the bacteria which cause the disease, or through physiological and biochemical testing which normally would take several days. During that time, the only action that can be carried out is water flushing (Lightner 1996). The availability of molecular diagnostic techniques are necessary to perform appropriate and rapid detection for this disease. Currently, there are diagnostic kits available for detecting *V. harveyi,* however, those kits mostly applied to non-local strains sequence. In this study, we offer an application of luminous *V. harveyi* local strain sequence as sources for primers design. Haemolysin is an exotoxin produced by luminous Vibrio bacteria that lyses the host's blood cells membrane (Zhang & Austin 2005). Haemolysin is encoded by haemolysin gene where the sequences can be accessed in the GeneBank. Kadriah *et al.* (2013) showed that haemolysin gene resulted a better specificity and sensitivity than *toxR* and *gyrB* genes as molecular marker to detect *V. harveyi.* Nested PCR primers designed from this study was able to detect *V. harveyi* at $10^0$ cfu/mL cells density and at DNA concentration of $10^1$ pg/µL.

Application of polymerase chain reaction (PCR) allows early detection of disease caused by bacteria, including Vibriosis in shrimp. PCR technique is applied to amplify a region of DNA between two predetermined sites. Oligonucleotides complementary to these sites serve as primers for synthesis of copies of the DNA between the sites.

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Each cycle of PCR doubles the number of copies of the amplified DNA until a large quantity has been made (Sambrook & Russel 2001). Nested PCR is a modification of the single step PCR reaction, in which it uses two successive runs of PCR. The first PCR product served as the template for the second PCR. The advantage of nested PCR is to minimize error by using 2 pairs of primers which also means increasing the PCR sensitivity (Siebert *et al.* 1995). Therefore, by designing a more spesific and sensitive nested PCR primers to amplify the haemolysin gene of *V. harveyi*, the presence of luminous Vibriosis disease in penaeid shrimp farming will be able to be detected earlier. The aim of this study was to identify *V. harveyi* in penaeid shrimp through haemolysin gene using nested PCR technique.

## MATERIALS AND METHODS

**Bacterial Isolates.** *V. harveyi* MR5339 was collected from Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University. *V. harveyi* 275*, V. parahaemolyticus,* and *V. campbelli* were collected from Institute for Brackishwater Aquaculture Research and Development, Ministry of Marine Affairs and Fisheries Indonesia at Maros South Sulawesi. *Salmonella* sp.*, Edwardsiella tarda, Aeromonas hydrophila,* and *Streptococcus iniae* were collected from Fish Quarantine Laboratory; Agency of Fish Quarantine, Quality Control and Safety of Fishery Product; and Ministry of Marine Affairs and Fisheries, Indonesia. *V. harveyi* and *V. parahaemolyticus* were grown using Seawater Complete (SWC) medium, while *V. campbelli*, *Salmonella* sp.*, E. tarda, A. hydrophila*, and *S. iniae* were grown in Luria Bertani (LB) medium.

**Primers.** Initial primers were designed from the complete sequence of *V. harveyi* VIB391 haemolysin gene (Genebank ID DQ640264) using Bioedit ver. 7.0.9.0 program (Hall 1999) and resulted in the flanking the nucleotides from position of 133 to 756 of the gene. The primer pairs were designed to test the presence of haemolysin gene in another two *V. harveyi* strains, i.e *V. harveyi* MR5339 and *V. harveyi* 275.

Specific nested PCR primers were designed based on the direct sequencing result of *V. harveyi* MR5339 haemolysin gene which have been successfully amplified by the initial primers. Sequence obtained from the sequencing result was first analyzed by ClustalW along with other previous haemolysin genes sequence. The first primer pairs of nested PCR were designed to amplify the *V. harveyi* MR5339 haemolysin gene fragment from position 52 to 405 and the second primer pairs were designed to amplify position 204 to 405.

**Optimization Tests of the Nested PCR Primers.** Optimization tests of the nested PCR primers were done to analyze whether these primers are appropriate for the early detection of vibriosis caused by *V. harveyi*. The first optimization was performed for sensitivity test. PCR sensitivity is determined by the lowest concentration of *V. harveyi* cells or DNA concentration that can be detected. This step was performed by conducting a nested PCR that used various templates of extracted DNA resulted from the serial dilution of *V. harveyi* cells from the densities of $10^0$ up to $10^5$ cfu/mL as well as from the genomic DNA at concentrations of $10^1$ fg/µL up to $10^2$ ng/µL. PCR products were then run on 1% (w/v) agarose gel to observe the presence of the amplicons. Amplicon from the first amplification using initial primers at concentration of $10^2$ ng/µL was used as the positive control and deionized distilled water ($dH_2O$) was used as the negative control.

The second optimization was to verify the primer spesificity. PCR spesificity is determined by the ability of the primer in amplifying the haemolysin gene in *V. harveyi* isolate. The spesificity of the nested PCR primers were evaluated against non *V. harveyi* bacteria, i.e. *V. parahaemolyticus* and *V. campbelli* as comparators in the same *Vibrio* genus; and *Salmonella* sp., *E. tarda*, *A. hydrophila,* and *S. iniae* as comparators for non *Vibrio* genus. Each sample were amplified with the nested PCR primers, also with serial dilutions from $10^0$ cfu/mL to $10^5$ cfu/mL and from $10^1$ fg/µL to $10^2$ ng/µL. Amplicon from the first amplification using initial primers at concentration of $10^2$ ng/µL was used as the positive control and deionized distilled water ($dH_2O$) was used as the negative control.

**PCR Using Initial Primers.** The genomic bacterial DNA extraction was based on Suryati *et al.* (2013) with modification in addition of 3 to 5% PVP (polyvinyl pyrrolidone). PCR was performed to genomic DNA of *V. harveyi* MR5339 and *V. harveyi* 275 using initial primer pairs that were expected to amplify 624 bp DNA fragment representing the haemolysin gene fragment from the whole genome. Applied Biosystems PCR machine was used for PCR amplification. Eighteen µL PCR reaction mixture was prepared for each 2 µL DNA samples. The PCR consisted of a pre-PCR at 95 $^oC$ for 5 min, followed by 35 cycles succesive runs of denaturation at 94 $^oC$ for 1 min, annealing at 55 $^oC$ for 1 min and extension at 72 $^oC$ for 1 min, then ended with post-PCR at 72 $^oC$ for 5 min and holded at 20 $^oC$ for 20 min or less.

Ten µL of PCR results were run in 1% (w/v) agarose gel dissolved in 1 times volume of TAE buffer (pH 8) at 100V for 30 min. After separation, gel was stained with 0.5 mg/L ethidium bromide solution and washed with deionized distilled water ($dH_2O$) for 10 min. DNA fragments in the gel were visualized in UV trans-illuminator and the image was captured by the gel documentation system (Biorad).

**Nested PCR.** Nested PCR was performed to several bacterial strains using two pairs of nested PCR primer. Eighteen µL PCR reaction mixture was prepared for each 2 µL DNA samples. The first PCR conditions were consisted of a pre-PCR 95 °C for 5 min, followed by 35 cycles successive runs of denaturation at 94 °C for 30 sec, annealing at 63 °C for 45 sec and extension at 72 °C for 30 sec, then ended with post-PCR at 72 °C for 2 min. Two µL of the first PCR amplification product was used as template for the second PCR process. The second PCR conditions were consisted of a pre-PCR 95 °C for 5 min, followed by 35 cycles successive runs of denaturation at 94 °C for 30 sec, annealing at 64 °C for 20 sec and extension at 72 °C for 15 sec. Amplicons from the first *V. harveyi* amplification using initial primers at concentration of $10^2$ ng/µL were used as the positive control and deionized distilled water ($dH_2O$) was used as the negative control.

**Surveillance Test.** Surveillance test was performed by direct DNA extraction of *L. vannamei* post larvae stadia that have been experimentally infected at various cells concentrations of *V. harveyi.* Each sample of *V. harveyi* was grown in SWC solid medium containing rifampicin 50 mg/L to simplify the verification (Widanarni *et al.* 2004). Post larvae *L. vannamei* were acclimatized for 24 h before being infected for 2 h in *V. harveyi* cells suspension in seawater concentrations ranging from $10^0$ to $10^5$ cfu/mL. Seawater then replaced with sterilized seawater. During the experimental infection, post larvae were fed with nauplii *Artemia* sp. Experimental infection of *V. harveyi* in post larvae *L. vannamei* was performed for 5 d with daily observation. The genomic DNA extraction was performed from live and dead larvae (Robertson *et al.* 1998). This method was done according to CTAB protocol modified with PVP addition as described by Suryati *et al.* (2013). The extracted DNA were then used as templates of nested PCR. Ten µL of PCR products were then run and visualized by the same method as described above. For the dead post larvae, the sample sources were grown on SWC medium containing 50 mg/L of rifampicin prior to DNA extraction. Amplicons from the first *V. harveyi* amplification using initial primers at concentration of $10^2$ ng/µL were used as the positive control and deionized distilled water ($dH_2O$), sterilized seawater and DNA extracted from the uninfected post larvae *L. vannamei* were used as the negative control.

## RESULTS

**PCR Using Initial Primers and Sequencing of Haemolysin Gene Fragment.** Products obtained from PCR amplification of *V. harveyi* MR5339 and *V. harveyi* 275 haemolysin gene using initial primers resulted in the expected length, i.e. 624 bp (Figure 1). Furthermore, the sequence comparison with other haemolysin gene sequences available in the Genebank revealed that both haemolysin gene sequences have 99% similarity to the sequence of *V. harveyi* strain VH34 (accession number: EU827170). It means that the amplification of *V. harveyi* haemolysin gene is successful.

**Optimization Tests of the Nested PCR Primer Design.** The designated nested PCR primers were tested for the specificity among other *Vibrio* and non *Vibrio* bacteria, and for the sensitivity among several range of cells or DNA concentrations. Results of gel electrophoresis from serially diluted cells of *V. harveyi* MR5339 and *V. harveyi* 275 i.e. from $10^5$ cfu/mL to $10^0$ cfu/mL and extracted DNA from concentration of $10^1$ fg/µL to $10^2$ ng/µL (sensitivity tests) are visualized on Figure 2 and Figure 3.

The designated nested PCR primers confirmed the sensitivity of detection up to cell density as low as $10^0$ cfu/mL, which result from the serial dilution test (equal to a single cell) (Figure 2a,b). The nested PCR also showed to be able to detect the *V. harveyi* MR5339 DNA concentration as low as $10^1$ fg/µL (Figure 2c,d). The same sensitivity tests was also confirmed using different template of *V. harveyi* 275 strain (Figure 3).

The specificity test of nested PCR primers was performed at various strains of other (non luminous) *Vibrio* sp., as well as non *Vibrio* bacteria; from cell density of $10^0$ cfu/mL up to $10^5$ cfu/mL and extracted DNA concentration of $10^1$ fg/µL to $10^2$ ng/µL. Our results showed that no amplicon were shown from other *Vibrio* bacteria (*V. campbelli* and *V. parahaemolyticus*) nor in non *Vibrio* bacteria

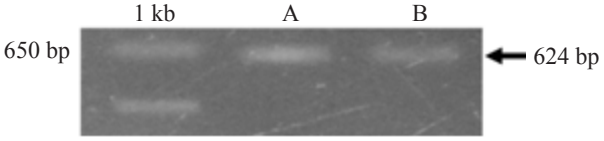


Figure 1. Visualization of PCR product using initial primers for the extracted DNA of *V. harveyi* MR5339 (A) and *V. harveyi* 275 (B) with 1 kb DNA marker from Invitrogen.

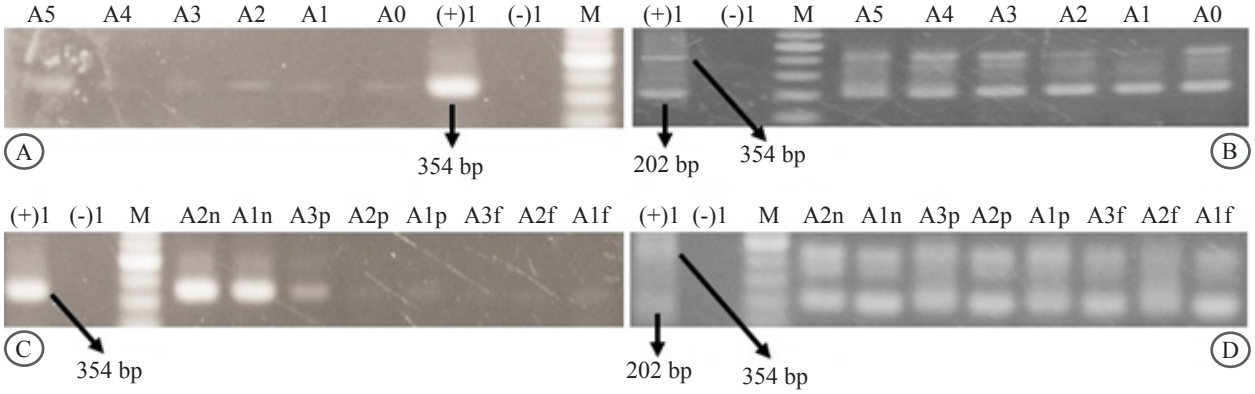


Figure 2. Sensitivity test of nested PCR primers using *V. harveyi* MR5339 serial dilution of DNA as the template. First PCR controls: (+)1 = *V. harveyi* MR5339 at $10^2$ ng/µL concentration as positive control, (-)1 = $ddH_2O$ as negative control. Second PCR controls: (+)1 = positive control from first PCR product, (-) 1 = negative control from first PCR product. M = 60 ng marker 100 bp from Jena Bioscience. (A) PCR product of first nested PCR primers using $10^5$ cfu/mL to $10^0$ cfu/mL template (A5-A0); (B) PCR product of first nested PCR primers using $10^2$ ng/µL to $10^1$ fg/µL template (A2n = $10^2$ ng/µL DNA, A1n = $10^1$ ng/µL DNA, A3p = $10^3$ pg/µL DNA, A2p = $10^2$ pg/µL DNA, A1p = $10^1$ pg/µL DNA, A3f = $10^3$ fg/µL DNA, A2f = $10^2$ fg/µL DNA, A1f = $10^1$ fg/µL DNA); (C) PCR product of second nested PCR primers using first PCR product as template (A5-A0 = $10^5$ cfu/mL to $10^0$ cfu/mL of first PCR product); (D) PCR product of second nested PCR primers using each first PCR product as template (A2n to A1f = $10^2$ ng/µL to $10^1$ fg/µL of first PCR product).

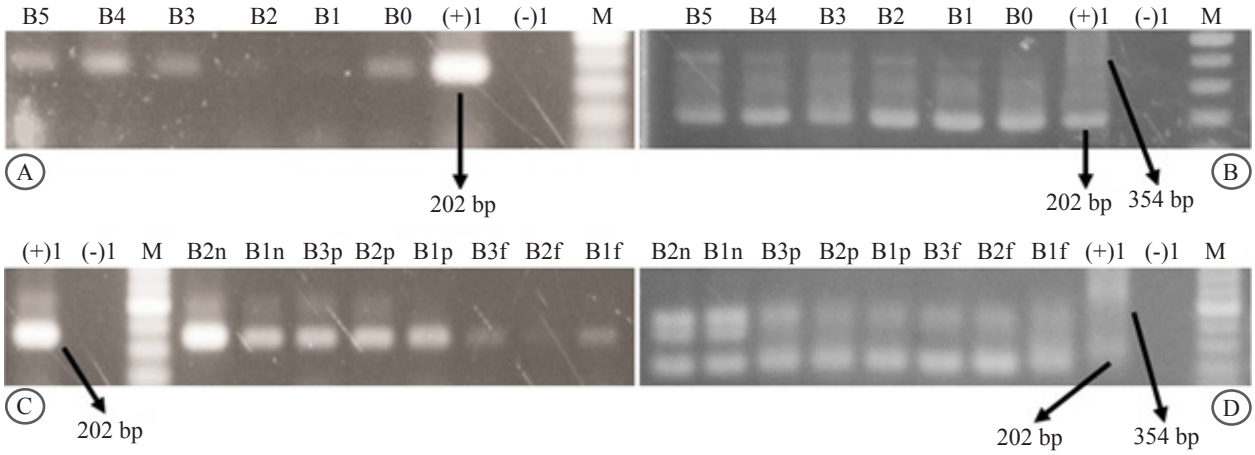


Figure 3. Sensitivity test of nested PCR primers using *V. harveyi* 275 serial dilution of DNA as the template. First PCR controls: (+)1 = *V. harveyi* MR5339 at concentration $10^2$ ng/µL as positive control, (-)1 = $ddH_2O$ as negative control. Second PCR controls: (+)1 = positive control from PCR I product, (-)1 = negative control from PCR I product. M = 60 ng marker 100 bp from Jena Bioscience. (A) PCR product of first nested PCR primers using $10^5$ cfu/mL to $10^0$ cfu/mL template (B5-B0); (B) PCR product of second nested PCR primers using first PCR product as template (B5-B0 = $10^5$ cfu/mL to $10^0$ cfu/mL of first PCR product); (C) PCR product of first nested PCR primers using $10^2$ ng/µL to $10^1$ fg/µL template (B2n = $10^2$ ng/µL DNA, B1n = $10^1$ ng/µL DNA, B3p = $10^3$ pg/µL DNA, B2p = $10^2$ pg/µL DNA, B1p = $10^1$ pg/µL DNA, B3f = $10^3$ fg/µL DNA, B2f = $10^2$ fg/µL DNA, B1f = $10^1$ fg/µL DNA); (D) PCR product of second nested PCR primers using each first PCR product as template (B2n to B1f = $10^2$ ng/µL to $10^1$ fg/µL of first PCR product).

(*S. iniae*, *Salmonella* sp., *A. hydrophila,* and *E. tarda*) (Figure 4 & 5).

**Surveillance Test.** Our results showed that the second PCR can amplified all samples, although sample a, b, f, g (Figure 6) and h (Figure 7) where at first nested PCR were not detected. The verification results on the dead shrimp by growing the colonies on SWC medium containing 50 mg/L rifampicin confirmed *V. harveyi* infection. The colonies grown were 1.28 x $10^{10}$ cfu/g *V. harveyi* on sample a, 2.36 x $10^{11}$ cfu/g on sample d, and 1.73 x $10^{11}$ cfu/g on sample e.

## DISCUSSION

Product amplified by first nested PCR primers resulted in 354 bp band. The presence of two bands that appear on the second nested PCR were possibly because of adjacent of the annealing temperature, i.e. 63 °C for first PCR and 64 °C for second PCR, thus the first PCR primers that might retrieved from the first run of PCR also contributed in the amplification of the second PCR templates. According to Sambrook and Russell (2001), the adjacent annealing temperature of two primers PCR will allow them to amplify

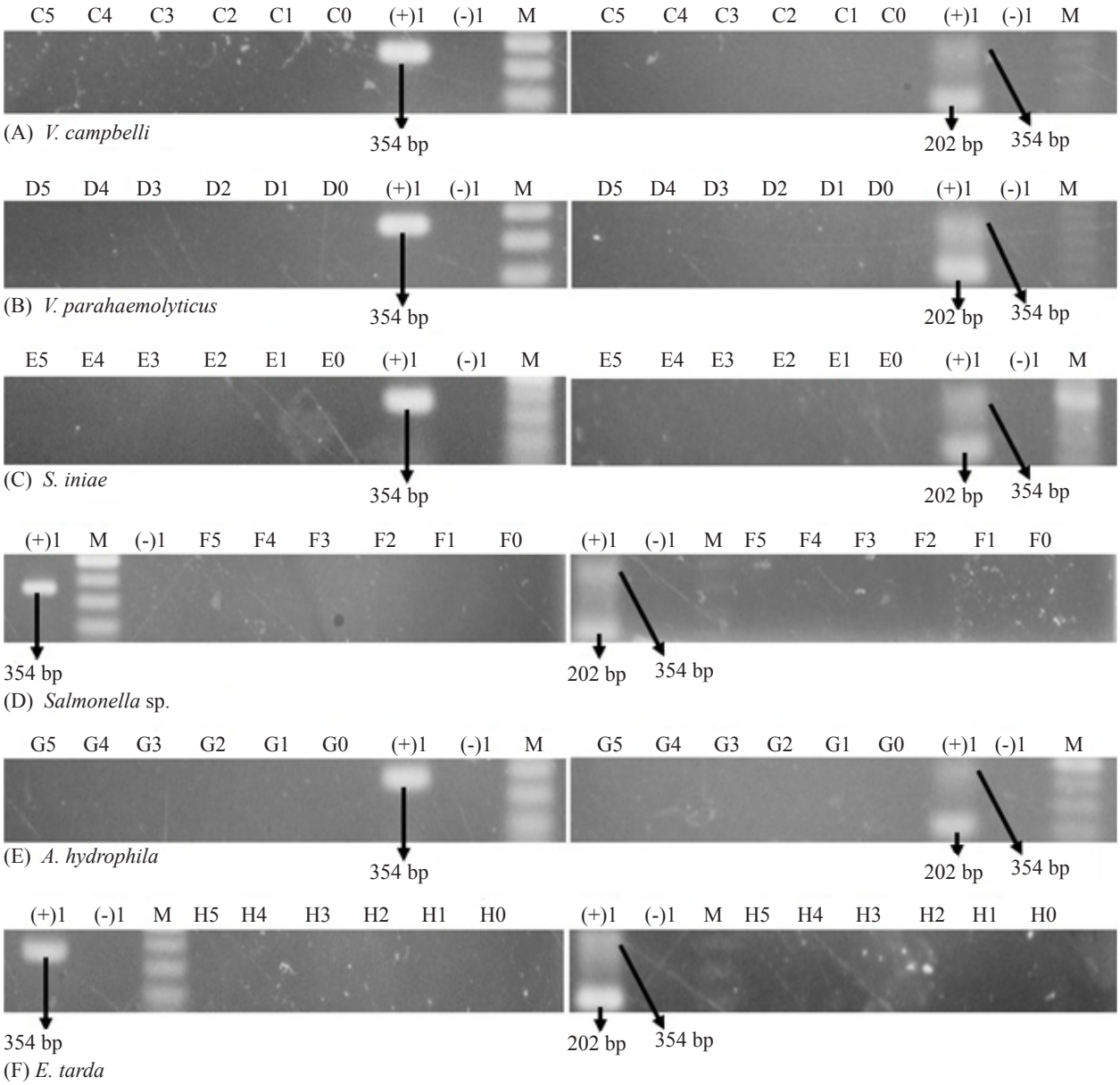


Figure 4. Specificity test results at $10^5$ cfu/mL to $10^0$ cfu/mL density. PCR I controls: (+) 1 = positive control elution results of *V. harveyi* MR5339 at concentration $10^2$ ng/µL, (-) 1 = negative control $ddH_2O$. PCR II controls: (+) 1 = positive control from PCR I product, (-) 1 = negative control from PCR I product. M = marker 100 bp from Jena Bioscience concentration 60 ng. (A) Specificity test results of *V. campbelli* (PCR I: C5 to C0 = extracted DNA from $10^5$ cfu/mL to $10^0$ cfu/mL density. PCR II: C5 to C0 = PCR results using each PCR I product). (B) Specificity test results of *V. parahaemolyticus* (PCR I: D5 to DO = extracted DNA from $10^5$ cfu/mL to $10^0$ cfu/mL density. PCR II: D5 to D0 = PCR results using each PCR I product). (C) Specificity test results of *S. iniae* (PCR I: E5 to E0 = extracted DNA from $10^5$ cfu/mL to $10^0$ cfu/mL density. PCR II: E5 to E0 = PCR results using each PCR I product). (D) Specificity test results of *Salmonella* sp. (PCR I: F5 to F0 = extracted DNA from $10^5$ cfu/mL to $10^0$ cfu/mL density. PCR II: F5 to F0 = PCR results using each PCR I product). (E) Specificity test results of *A. hydrophila* (PCR I: G5 to G0 = extracted DNA from $10^5$ cfu/mL to $10^0$ cfu/mL density. PCR II: G5 to G0 = PCR results using each PCR I product). (F) Specificity test results of *E. tarda* (PCR I: H5 to H0 = extracted DNA from $10^5$ cfu/mL to $10^0$ cfu/mL density. PCR II: H5 to H0 = PCR results using each PCR I product).

simultaneously. According to positive control, this two band do not have any influence on the final conclusion. Therefore, this result can be applied for the detection of *V. harveyi* based on PCR technique. The designated nested PCR primers were proved to have sensitivity up to $10^0$ cfu/mL in detecting both of *V. harveyi* pathogenic strain. Nested PCR using designated primers also allowed to detect both of *V. harveyi* at genomic DNA concentration as low as $10^1$ fg/µL. The result of this study, confirmed that this method is more sensitive compared to previous study done by Kadriah *et al*. (2013). According to the statement of Lee *et al.* (1995), DNA concentration of 2.6 fg is equal to DNA of a single cell of *V. parahaemolyticus*, which is in the same genus with *V. harveyi.*

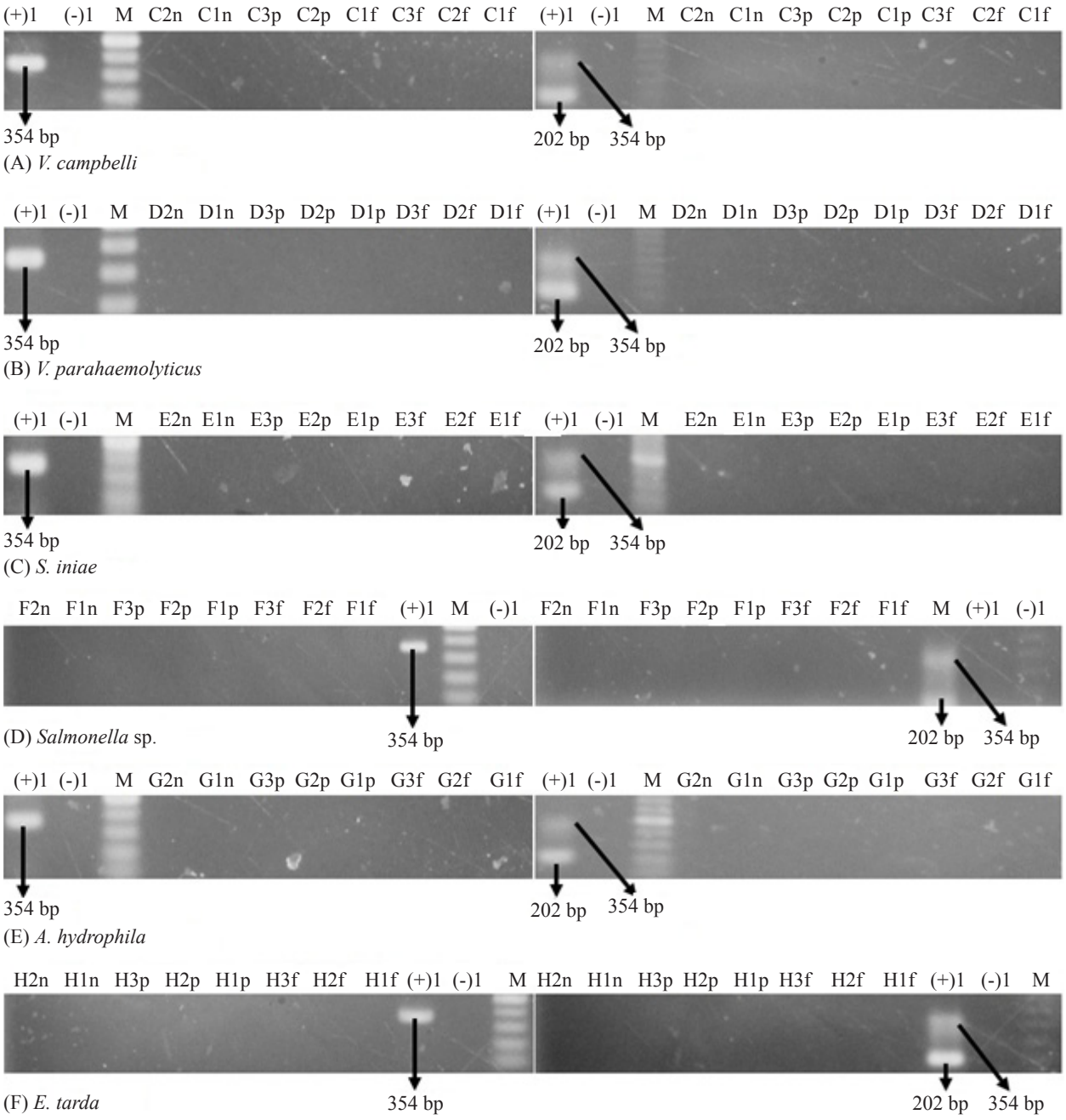


Figure 5. Specificity test results at $10^1$ fg/µL to $10^2$ ng/µL DNA extracts. PCR I controls: (+) 1 = positive control elution results of *V. harveyi* MR5339 at concentration $10^2$ ng/µL , (-) 1 = negative control dd$H_2O$. PCR II controls: (+) 1 = positive control from PCR I product, (-) 1 = negative control from PCR I product. M = marker 100 bp from Jena Bioscience concentration 60 ng. (A) Specificity test results of *V. campbelli* (PCR I: C1n to C1f = extracted DNA from $10^2$ ng/µL to $10^1$ fg/µL. PCR II: C1n to C1f = PCR results using each PCR I product). (B) Specificity test results of *V. parahaemolyticus* (PCR I: D1n to D1f = extracted DNA from $10^2$ ng/µL to $10^1$ fg/µL. PCR II: D1n to D1f = PCR results using each PCR I product). (C) Specificity test results of S. iniae (PCR I: E1n to E1f = extracted DNA from $10^2$ ng/µL to $10^1$ fg/µL. PCR II: E1n to E1f = PCR results using each PCR I product). (D) Specificity test results of *Salmonella* sp. (PCR I: F1n to F1f = extracted DNA from $10^2$ ng/µL to $10^1$ fg/µL. PCR II: F1n to F1f = PCR results using each PCR I product). (E) Specificity test results of *A. hydrophila* (PCR I: G1n to G1f = extracted DNA from $10^2$ ng/µL to $10^1$ fg/µL. PCR II: G1n to G1f = PCR results using each PCR I product). (F) Specificity test results of *E. tarda* (PCR I: H1n to H1f = extracted DNA from $10^2$ ng/µL to $10^1$ fg/µL. PCR II: H1n to H1f = PCR results using each PCR I product).

Results from the surveillance test, showed that the second PCR was able to amplify all samples although samples a, b, f, g (Figure 6) and h (Figure 7) at first nested PCR resulted in negative detection. This may be due to a very low DNA amplification on the first PCR which was not possible to be visualized (Sambrook & Russell 2001). Thus, this result confirmed that nested PCR technique is more sensitive to the single step PCR (Siebert *et al*. 1995). The dead post larvae, experimentally infected

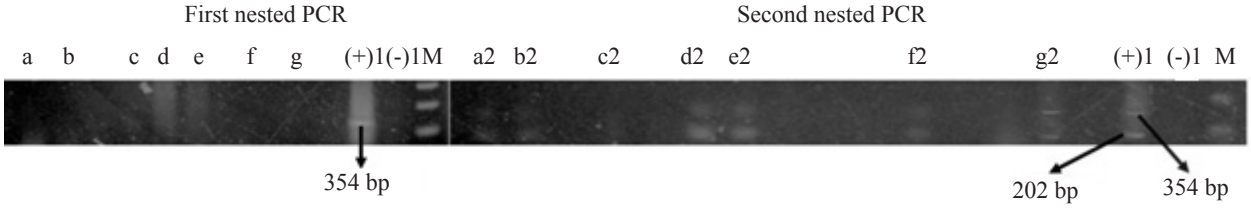


Figure 6. Surveillance test results for. PCR I: a. Sample day 3 extracted DNA of dead shrimp suspected *V. harveyi* MR5339 infected at $10^1$ cfu/mL. b. Sample day 2 extracted DNA of dead shrimp suspected *V. harveyi* MR5339 infected at $10^5$ cfu/mL. c. Sample day 3 extracted DNA of dead shrimp suspected *V. harveyi* MR5339 infected at $10^2$ cfu/mL. d. Sample day 4 extracted DNA of dead shrimp suspected *V. harveyi* MR5339 infected at $10^2$ cfu/mL. e. Sample day 3 extracted DNA of dead shrimp suspected *V. harveyi* 275 infected at $10^1$ cfu/mL. f. Sample day 3 extracted DNA of live shrimp suspected *V. harveyi* 275 infected at $10^1$ cfu/mL. g. Sample day 3 extracted DNA of live shrimp suspected *V. harveyi* 275 infected at $10^2$ cfu/mL, (+) 1 = positive control elution results of *V. harveyi* MR5339 at concentration $10^2$ ng/µL, (-) 1 = negative control $ddH_2O$. PCR II: PCR results using each PCR I product. M: marker 1 kb from Fermentas concentration 60 ng.

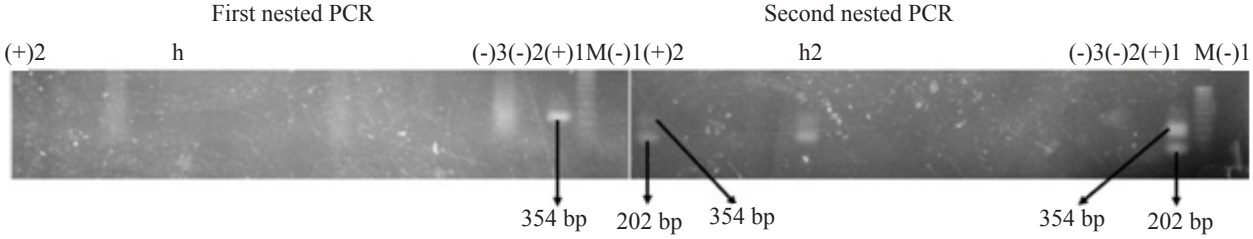


Figure 7. Surveillance test results for sample h. PCR I: (+) 2 = positive control of *V. harveyi* MR5339 at $10^2$ ng/µL extracted DNA. h=sample day 1 extracted DNA of live shrimp suspected *V. harveyi* 275 infected at $10^4$ cfu/mL, (-) 3 = negative control uninfected shrimp. (-) 2 = negative control of sterile seawater before infection, (+) 1 = positive control elution results of *V. harveyi* MR5339 at concentration $10^2$ ng/µL, (-) 1 = negative control $ddH_2O$. PCR II: PCR results using each PCR I product. M: marker 1 kb from Fermentas concentration 60 ng.

by pathogenic *V. harveyi* MR5339 at initial cells concentration of $10^1$ cfu/mL, still showed positive amplification/detection after 3 days post infection. Finally, we concluded that our designated nested PCR primers can be used as a detection kit for local isolate of luminous *V. harveyi.*

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