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RAPID DETECTION OF *Streptococcus iniae* IN RED TILAPIA TISSUE (*Oreochromis* sp.) BY POLYMERASE CHAIN REACTION

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ABSTRACT

Streptococcus iniae, as a cause of Streptococcosis outbreak of cultured tilapia, is a hemolytic and Gram-positive bacterium. In this study, a one-step PCR method was developed for the detection of S. iniae from fish kidney. The oligonucleotide primers, assigned as LOX-1 and LOX-2, are designed from lactate oxidase gene of S. iniae. Using extracted DNA from bacterial cells or head kidney of S. iniae infected fish, the PCR reaction yields a 870 bp fragment specific to the S. iniae. Primers LOX-1 and LOX-2 did not amplify DNA from other common bacteria in cultured fish (Streptococcus agalactiae, Aeromonas hydrophila, Edwardsiella ictaluri, and Vibrio harveyi). The detection limit of this primer pair is 10^2 CFU/mL of S. iniae. The improved one-step PCR method provides a diagnostic tool for: (i) the detection of S. iniae directly from infected fish tissue; (ii) and the identification of S. iniae from bacteria cells.

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1 INTRODUCTION

Streptococcosis is a septicemia disease that affects both captive and wild populations of freshwater and marine fish species throughout the world, causing many significant problems (Kitao, 1993; Austin and Austin, 1999). More than 50% of mortality was recorded in outbreak fish farms (Yanong and Francis-Floyd, 2002). Several fish species have been found susceptible with *Streptococcosis* and reported in various countries, including rainbow trout (Eldar *et al.*, 1995), Nile tilapia (Shoemaker *et al.,* 2001), hybrid Tilapia (Perera *et al.,* 1994), European sea bass (Colorni *et al.,* 2002), and barramundi (Bromage *et al.,* 1999). Causative agents associated with fish *Streptococcosis* worldwide are *Lactococcus garvieae, Streptococcus agalactiae, S. dysgalactiae, S. phocae,* and *S. iniae* (Nomoto *et al*., 2004; Toranzo *et al*., 2005).

For tilapia, especially red tilapia (*Oreochromis* sp.) cultured in Vietnam, *S. agalactiae* and *S. iniae* are the major causes of *Streptococcosis*, which was first reported in 2004 in cage culture of red tilapia in An Giang province (Thy and Oanh, 2011). Recently, the disease has also expanded to Vinh Long and Tien Giang provinces. *Streptococcosis* often occurs in rainy seasons leading to serious consequences (Thy and Oanh, 2011). It is difficult to differentiate diagnosis of *Streptococcosis* caused by *S. agalactiae* and *S. iniae.* This is because the clinical signs of *S. agalactiae* infected tilapia are similar to those infected by *S. iniae* with particular signs such as hemorrhage, exophthalmia, lethargy, and anorexia (Toranzo *et al*., 2005). At present, the

method used for the detection and identification of *S. iniae* is often based on the microbiological methods. However, these agar-based methods for the isolation and biochemical tests for the identification of the pathogens are time consuming, laborious, and easy to be mistakenly identified.

Molecular diagnostic techniques such as the polymerase chain reaction (PCR) evaluates more precisely and accurately for bacterial identification at the species level. The PCR method is often used to detect and identify many bacterial pathogens because of its sensitive and specific level in comparison with other conventional diagnostic methods for the detection of *Streptococcosis*. PCR assays have been widely used for the detection of *S. iniae* by amplifying the 16S rRNA gene (Zlotkin *et al*., 1998; Ahmed, 2011), the chaperon in HSP60 (Goh *et al.*, 1998), the 16S - 23S rRNA gene intragenic spacer region (Berridge *et al*., 1998), and the lactate oxidase gene (*lctO*) (Mata *et al*., 2004). Therefore, the goal of this research was to develop a PCR method that amplifies the local isolates of *S. iniae* with a high degree of sensitivity and specificity. The second aim of this study was to develop a PCR method for the detection of *S. iniae* directly from red tilapia's tissue.

2 MATERIALS AND METHODS

2.1 Materials

S. iniae original isolated from diseased climbing perch, was cultured in Brain Heart Infusion Agar (BHIA) within 48 hours at 28°C, and used for DNA extraction. *Streptococcus agalactiae, Aeromonas hydrophila, Edwardsiella ictaluri,* and *Vibrio harveyi* isolated from different hosts (Table 1) were used to determine of PCR specificity. The bacteria tested were from the culture collection of Aquatic Pathology Department, College of Aquaculture and Fisheries, Can Tho University. Head kidney was dissected from red tilapia in the challenge experiment.

Table 1: Bacterial strains used in the study

Bacterial species	Collection code	Source of isolation
Streptococcus iniae	R36	Climbing perch
Streptococcus agalactiae	Sa1	Red tilapia
Aeromonas hydrophila	Ae1	Striped catfish
Edwardsiella ictaluri	E16	Striped catfish
Vibrio harveyi	BL9	Black tiger shrimp

2.2 Methods

2.2.1 DNA extraction

(i) Total DNA from fish kidney was extracted using Chelex extraction procedure (Buller, 2004). Briefly, kidney sample was homogenized in Chelex-100 resin (Sigma-Aldrich, Hamburg, Germany), followed by heating at 56°C for 10 minutes. Then, 200 µL of 0.1% Triton-X-100 was added and the sample was boiled for 10 minutes. The sample was then left to cool on ice before being centrifuged at 12,000 rpm for 3 minutes. DNA dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA) was stored at 4°C.

(ii) Genomic DNA of bacteria was extracted by boiling methods (Bartie et al., 2006). The colonies were picked and resuspended in 500 µL of TE buffer, boiled for 10 minutes at 100°C and centrifuged 10,000 rpm in 10 minutes. DNA was dissolved in TE buffer and stored at 4°C for further analysis.

2.2.2 Polymerase chain reaction

Amplification of the 870 bp product was performed using primers LOX-1 and LOX-2 on extracted DNA. The primer sequences and expected size of amplicon for PCR assay are described in Table 2 (Mata *et al.*, 2004). The PCR amplification of lactate oxidase gene (*lctO*) was performed in 25 µL total reaction mixture, containing 1X PCR buffer, 2 mM $MgCl_2$, 0.24 mM dNTPs, 10 pmol of each primer LOX-1 and LOX-2, 1.5U *Taq* DNA polymerase (Promega), and 1 µL DNA template. The PCR thermal cycling conditions was performed in a thermocycler (Applied Biosystem, USA) at initial denaturation 95°C for 5 minutes, followed by 30 cycles of denaturation 95°C for 1 minute, annealing at 52°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. Following this, 10 µL of the PCR products was analyzed by electrophoresis on 1-2% agarose gel containing ethidium bromide at a final concentration of 0.5 µg/mL. A 1 kb DNA ladder (Invitrogen, Carlsbad, CA) was used as a marker. The agarose gel was examined and photographed using Geldoc (Biorad, USA).

Table 2: Primer sequences and predicted size of amplified products of target DNA

Primer	Oligonucleotide sequences	Size of amplicon
LOX - 1	5'-AAAGGGAAATCGCAAGTGCC-3'	870 bp
LOX - 2	5'-ATATCTGATTGGGCCGTCTAA-3'	

2.2.3 PCR optimization

The PCR optimizations were performed by the following parameters as primer concentration (5 and 10pmol), Taq DNA polymerase concentration (1.0, 0.75 and 0.5U) and a number of thermal cycles (30 and 35 cycles).

2.2.4 Sensitivity and specificity testing

To determine the sensitivity of the PCR method, 10-fold serial dilutions of bacterial cell solution containing the *S. iniae* were used as DNA templates for PCR assay. The concentration of bacteria cells ranged from 10^1 to 10^8 CFU/mL.

To check the specificity of the primer pair (LOX-1 and LOX-2), extracted DNA from four different bacteria species (*Streptococcus agalactiae, Aeromonas hydrophila, Edwardsiella ictaluri, Vibrio harveyi*) was used as templates for PCR amplification.

2.2.5 Application of the improved PCR for the detection of S. iniae from infected red tilapia

Red tilapia (15-20 g/fish) was challenged by injection method with 0.1 mL of 10^8 CFU/mL *S. iniae* suspension. The fish was maintained in a 60-liter plastic bucket (10 fish/bucket). Kidney of moribund fish was dissected and frozen at -80°C for DNA extraction. DNA extraction was conducted using the Chelex extraction procedure (Buller, 2004) and the extracted DNA was amplified with the improved one-step PCR procedure.

3 RESULTS AND DISCUSSION

3.1 Optimization of PCR for detection of *Streptococcus iniae*

All four optimizations showed the PCR products at the expected size of approximately 870 bp which belongs to *S. iniae* (Mata *et al.,* 2004) (Fig. 1). In the 1st optimization, reducing Taq DNA polymerase concentration (from 1.5U to 1.0U/reaction) gave good result (Fig. 1A). For the 2nd optimization, the bright PCR band was also achieved in the case of reducing half of primer concentration (from 10pm to 5pm) (Fig. 1B). In the 3rd optimization, PCR result was not affected by using Taq polymerase of 0.75 U/reaction, primers concentration of 5 pmol and increasing number of thermal cycles from 30 to 35 cycles. The PCR product was also made up and still clearly observed on the agarose gel (Fig. 1C). In the 4th optimization with a reduction of Taq polymerase from 0.75U to 0.5 U/reaction, PCR result was still at the expected size but less bright than the 3rd one (Fig. 1D). Although the number of thermal cycles was higher, the concentration of Taq polymerase might not be enough to yield a good PCR product.

The PCR amplification was successfully performed in 25 µL reaction volumes with reaction mixtures of the 3rd optimization, containing 1 µL DNA template, 1X PCR buffer, 5 pm of each primer (LOX-1 and LOX-2), 0.24 mM of each dNTPs, 0.75 U of DNA polymerase and 2mM $MgCl_2$. The reaction condition consists of a initial denaturation step of 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 7 minutes.

According to Thanh (2006) and Duong (1998), the specificity and effectiveness of PCR assay are directly affected by the concentration of Taq polymerase and primers. High Taq polymerase concentration (above 4 U/100 µL) can generate nonspecific products and may reduce the yield of the desired product (Saiki, 1989). High primer concentration leads up to nonspecific products (Binh and Thi, 2009). On the contrary, the low concentration of primers makes up unclear PCR fragments (Henegariu *et al*., 1997). Therefore, the optimizations were conducted at different concentrations of Taq and primer. For instance, Long (2013) showed that 5 pm of primers did not yield an expected PCR product. However, in this PCR assay, 5 pm of primer concentration clearly amplified an expected PCR product.

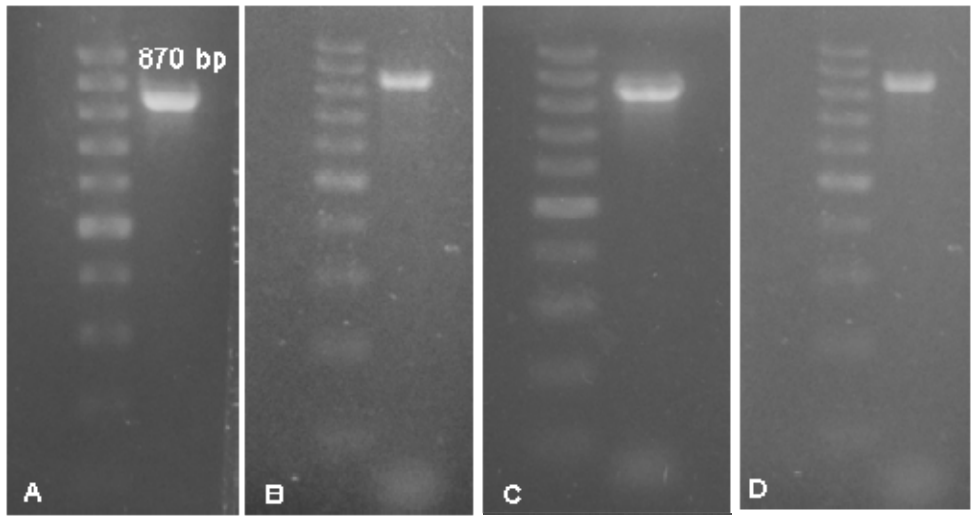

Fig. 1: PCR results showed predicted size of DNA bands from 4 optimizations, 870bp with (A) 1st optimization with Taq polymerase at 1.0 U; (B): 2nd optimization with LOX-1 and LOX-2 – 5pm; (C): 3rd optimization with LOX-1 and LOX-2 – 5pm, Taq 0.75 U, 35 cycles; (D): 4th optimization with LOX-1 and LOX-2 – 5pm, Taq 0.5 U, 35 cycles

3.2 Sensitivity and specificity of PCR for Streptococcus iniae

For the sensitivity of the assay, the improved PCR successfully amplified the *lctO* gene in the reaction contained at least 10^2 CFU/mL. The detection limit of the PCR method described here using extracted DNA (10^1 – 10^8 CFU/mL) as the template revealed by agarose gel visualization that as few as 100 copies of template DNA (lane 8) could be detected using this one-step PCR method. Higher concentration of DNA template did not inhibit PCR reaction and gave a bright band on 1.5% agarose gel (Fig. 2).

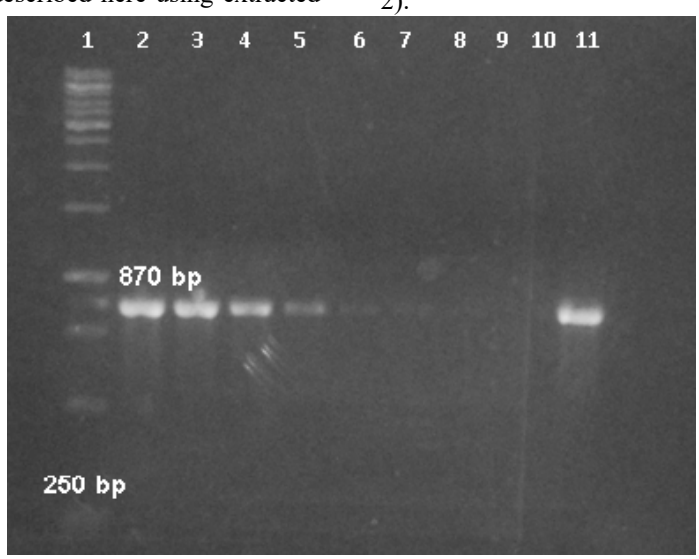

Fig. 2: PCR results of the sensitivity test with PCR bands of 870 bp. Lane 1: 1kb ladder; lane 2-9: PCR product of extracted DNA from 10^8 to 10^1 CFU/ml; lane 10: negative control; lane 11: positive control

Comparing of our finding with other studies, the detection limit of the improved PCR is higher than the 10^6 CFU/g of fish tissue for *S. iniae* and *S. agalactiae* (Rodkhum *et al*., 2012) and the 10^4 CFU/g of fish kidney for *S. agalactiae* (Long, 2013). Therefore, the sensitivity of the improved PCR assay is high enough to detect the bacterial target in diseased fish.

To ensure the specificity of primers LOX-1 and LOX-2, extracted DNA from four other bacteria that infect fish including *S. agalactiae*, *A.hydrophila*, *E. ictaluri* and *V. harveyi* were used as a template for the PCR reaction. No amplification was obtained with DNA extracted from these fish bacteria (Fig. 3). On the contrary, the specific amplification of the expected 870 bp fragment was only observed with *S. iniae* positive sample. The results showed the specificity of primer LOX-1 and LOX-2. The result is in accordance with those obtained by Mata *et al* (2004). In Mata *et al* 's study, the primer sets LOX-1 and LOX-2: (i) gave a band at both annealing temperatures (55°C and 60°C) with a single and specific amplification product of 870 bp with only *S. iniae* isolate; (ii) did not amplify any PCR products from other *Streptococcus* species (*Streptococcus difficilis; Streptococcus parauberis*).

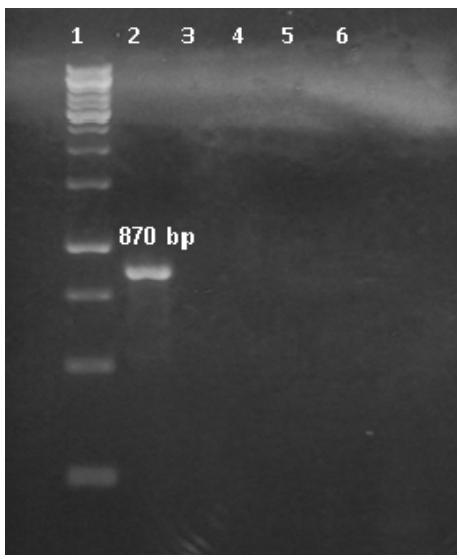

Fig. 3: PCR result of the specificity test. Lane 1: 1kb ladder; lane 2: *Streptococcus iniae*; lane 3: *Aeromonas hydrophila*; lane 4: *Edwardsiella ictaluri*; lane 5: *Streptococcus agalactiae*; lane 6: *Vibrio harveyi*

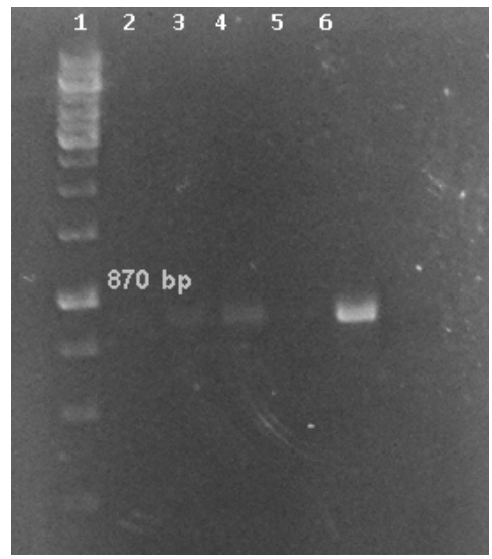

Fig. 4: PCR result from head kidney of infected fish. Lane 1: 1kb ladder; lane 2 to 4: DNA expected fragment size of three injected tissue; lane 5: negative control; lane 6: positive control

3.3 Application of the improved PCR for the detection of *S. iniae* in infected red tilapia

Amplification of bacterial targeted DNA in infected tissue was successful yielded in three infected fish samples as shown in Figure 4. All infected fish kidney yielded the predicted size amplicons of the bacteria at 870bp. Using the improved one-step PCR procedure for the detection of *S. iniae*, a serious bacterial pathogen in tilapia, was shown to be effective even in clinical specimens.

The diagnostic sensitivity of the PCR also showed the effectiveness of the Chelex extraction procedure from infected kidney samples. A similar result was also recorded on study of Lahav *et al.* (2004). In their findings, they concluded that brain, kidney or liver tissues are appropriate organs for the detection of *S. iniae* infected in rainbow trout. This is because *Streptococcus* is overwhelmed in those tissues (Lahav *et al.,* 2004).

4 CONCLUSION

In conclusion, the PCR procedure described here using primer set LOX-1 and LOX-2 provides great sensitivity and high specificity for the detection of *S. iniae* in red tilapia tissues. Therefore, the improved one-step PCR procedure provides a diagnostic tool for: (i) the detection of *S. iniae* directly from infected fish tissue; (ii) and the identification of *S. iniae* from bacteria cells.

5 RECOMMENDATION

For the next trial, PCR method for the detection of *S. iniae* can be tested from blood sample of red tilapia (*Oreochromis* sp.).

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