Streptococcus agalactiae INFECTION ON TILAPIA (*Oreochromis niloticus*) IN CIRATA RESERVOIR, WEST JAVA

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ABSTRACT

Streptococcosis is one of bacterial diseases in the culture of Tilapia, *Oreochromis niloticus* and has caused significant economic losses. *Streptococcus iniae*, is known as pathogen to marine and freshwater fishes whereas *Streptococcus agalactiae* is known as pathogen to Tilapia. The isolation and characterization of four isolates of *S. agalactiae*, were described from an infected Tilapia from Cirata Reservoir, West Java, in July 2008. Conventional and rapid identification systems were used to determine isolates of *S. agalactiae* from brain and kidney tissues. In this paper, we have characterized *S. agalactiae* and this was the first isolation of this bacteria from fish. The isolates were gram positive, catalase-negative, oxidase-negative, β haemolytic cocci colonies on blood agar. All of the of isolates were biochemically characterized with the API 20 Strep System (bioMerieux). Bacterial chromosomal DNA used in PCR assay was extracted by heating method. The forward primer is Sdi 61: 5'-AGGAAACCTGCCATTTGCG-3' and the reverse primer is Sdi 252: 5'-CAATCTATTTCTAGATCGTGG-3' with gene target 16S intergenic spacer and it has 192 bp in length. These primers were designed by Alpha DNA (Montreal, Quebec). The biochemical patterns of four isolates were rather different although almost all traits were similar with the exception of pyroglutamic acid (pyra) and L-arginin (ADH), for which we observed negative and positive reaction in this study. Therefore, some of the biochemical characteristics of the four isolates did not fit 100% with the typical patterns of *S. agalactiae*. However, the PCR result showed that this PCR assay is an effective tool for rapid and specific detection of *S. agalactiae*, the main pathogens involved in warm-water streptococcosis, obtained from pure culture of naturally infected fish. Therefore, it could be a useful alternative for culture-based routine diagnosis of warm-water streptococcal infections in fish.

KEYWORDS: *Streptococcus agalactiae*, **Tilapia, biochemical characteristic, PCR assay**

INTRODUCTION

Streptococcal infections, which have increased in number during the last decade as a consequence of the intensification of aquaculture, are responsible for significant economic losses in fish farm. The main pathogenic species responsible in Indonesia for these streptococcal infections are *Streptococcus iniae* and *Streptococcus agalactiae*. Water temperature is considered as predisposing factor for the onset of the disease caused by these pathogens. Thus, outbreaks associated with infections by *S. iniae* and *S. agalactiae* usually occur at water temperatures above 15°C and are termed warm-water streptococcosis. Fish

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infected by warm-water streptococcosis exhibit very similar symptoms and clinical signs regardless of the etiological agents and therefore a definitive diagnosis of the etiological agent has to be based on the microbiological analysis of infected fish. Warm-water streptococcosis-associated pathogens can be identified by culture-based methods and biochemical tests. Nevertheless, biochemical identification of some of these bacteria can be difficult using commercial identification systems, because some are not included in the databases of currently available commercial systems. The polymerase chain reaction (PCR) is currently being developed to replace the conventional method of identification of streptococcosis.

The aim of the present study was to identify streptococcosis species by amplification of *S. agalactiae* DNA sequence with species-specific primer Sdi 61 AGGAAACCTGCCATTTGCG and Sdi 252 CAATCTATTTCTAGATCGTGG. Such an approach is needed for rapid diagnosis of streptococcosis causing disease in fish. Screening of breeder fish stocks with the developed PCR methodology, followed by the elimination of infected stocks, would provide an efficient strategy to control fish infected with streptococcosis.

MATERIALS AND METHODS

Fish

Observations were made on fish ranging in size from 10 to 40 cm. The fish were collected from tilapia farm at Cirata Reservoir in West Java.

Microbiology

Tissue samples from brain, kidney, liver and spleen were inoculated into Brain Heart Infusion Agar (BHIA) and incubated at 25°C for 48 hours. The predominant types of colonies were subcultured and subjected to biochemical and physiological tests including catalase production, haemolytic activity in blood agar, API 20 STREP System, antibiotic susceptibility on Muller-Hinton agar, and tolerance of 6.5% NaCl.

Bacterial DNA Isolation

The sample of bacterial DNA used in PCR was extracted using boiling method. Bacterial DNA was obtained from cells by touching the colonies placed them on blood agar medium with sterile ose needle. Bacterial colonies (5-10 colonies) were suspended in 400 µL of RNAse free water. The sample was boiled for 10 minutes at 98°C and centrifuged at 8.000 x G for 10 minutes. The supernatant was used for PCR amplification.

PCR Amplification

A set of oligonucleotide primers was designed to amplify 192 base pairs from gene target 16S intergenic spacer of *S. agalactiae* (species-specific primers) (Mata *et al*., 2004).

These primers were Sdi 61: 5'-AGGAAACCTGCCATTTGCG-3' and Sdi 252: 5'-CAATCTATTTCTAGATCGTGG-3' (Alpha DNA Montreal, Quebec). The process of PCR amplification was performed in 25 µL tube containing: 12.5 µL master mix GoTaq®Green (Promega, Madison WI USA), 8.5 µL nuclease free water, 1 µL primers (reverse dan forward) each and 2 µL DNA template. The amplification cycle at thermal cycler T personal (Biometra) consisted of 2 minutes denaturation at 94°C followed by 25 cycles of denaturation at 92°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 90 seconds. Finally, the last elongation was done at 72°C for 5 minutes. As negative control (no template DNA) was RNAse free water.

Electrophoresis

The amplification result was detected by electrophoresis of each amplicon 10 µl at 1.5% gel agarose in buffer 1x Tris-acetate-EDTA (220 V for 25 minutes). Coloring of DNA was done on ethidium bromide solution (0.5 µg per 100 mL TAE buffer) for 15 minutes and the result was documented with Polaroid camera.

RESULTS

Results indicated that the colonies type on Brain Heart Infusion Agar and blood agar isolated from brain fluid were pale yellowish and smooth. They produced β-haemolysis in blood agar after 48 hours incubation at 25°C. The colonies have the same phenotypic patterns except VP test negative for *S. iniae.* These colonies were resistant to ampicilline and clindamycine but was sensitive to erythromycine, gentamycine, cephalothin, tetracycline, methicilline and chloramphenicol. The result of phenotypic characteristics of *S. agalactiae* using API 20 Strep System is presented in Table 1. Figure 1 is the

biochemical result of *S. agalactiae* using API 20 Strep System.

A pathological anatomy study was made to observe signs of infection of *Streptococcus* sp. in cultured Tilapia from West Java. The external signs were exophtalmia and dermal haemorrhage. The internal signs were dropsy, hiperrhemia liver to pale colored liver, hepatomegaly and splenomegaly. On histopathological examination, granulomas were found in liver and spleen. Almost all cells of the organs showed degeneration to cells necrosis .

Table 1. Phenotypic characteristics of *S. agalactiae* compared to *S. iniae*

Characters	*S. iniae*	*S. agalactiae*
Gram	+	+
Motility	-	-
Catalase	-	-
Hemolytic Type	β	α/β
Grow in 6,5% NaCl	-	+
Voges Proskauer	-	-
Hidrolysis from:		
Hipurat	-	+
Esculin	-	-
Pyrrolidonyl arylamidase (PYRA)	+	-
α-Galactosidase	-	-
β-Galactosidase	-	+
β-Glucuronidase	-	+
Alkaline phosphatase (PAL)	-	+
Acid from:		
Ribose	+	+
Arabinose	+	+
Mannitol	+	+
Sorbitol	-	+
Lactose	-	+
Trehalose	+	+
Inulin	-	-
Raffinose	-	-
Amidon	+	+
Glycogen	+	+
Arginine Dihydrolase	-	-
Resistancy/Sensitivity to:		
Erythromycine (15)	S	S
Gentamycine (10)	S	S
Cephalothin (30)	S	S
Tetracycline (30)	S	S
Ampiciline (10)	R	R
Methicilline (5)	S	S
Clindamycine (2)	R	R
Chloramphenicol (30)	S	S

Remarks: R, resistant; S, sensitive; +/- could be positive or negative depending on the method

Figure 1. The biochemical result of *S. agalactiae* using API 20 Strep System (1) Negative *S. agalactiae*; (2) Positive *S. agalactiae*

Agar gel electrophoresis of PCR products from the type strain of *S. agalactiae* (the second colony) with these primers were Sdi 61: 5'-AGGAAACCTGCCATTTGCG-3' and Sdi 252: 5'-CAATCTATTTCTAGATCGTGG-3' and consistenly yielded a single amplicon of approximately 192 bp.

DISCUSSION

Elliot *et al.* (1990) reported that *S. agalactiae* isolated from frogs were negative reactivity in fermentation tests of lactose and trehalose, whereas isolates from cows, mice and humans showed positive reactivity in those two fermentation tests. Moreover, Berridge *et al.* (2001) reported that *S. agalactiae* isolated from fish showed positive reactivity in these two fermentation tests. *S. agalactiae* from Cirata Reservoir showed positive reactivity on lactose and trehalose (Table 1). This result showed that there are genetic relation among fish, cows, mice, and humans. Further research to know whether these hosts act as reservoirs of one another's pathogenic linkages is needed.

Early diagnosis in the presence of infection in ponds is important for effective control of the disease. Diagnosis is difficult because of the normally subclinical expression of the pathogen. Current methods in diagnosing *S. agalactiae* are based on the biochemical characteristics of the organism. Isolation of this pathogen, particularly in a case of mixed infection, is considered as time consuming and sometimes leads to misdiagnosis. Compared to the time-consuming and costly procedures currently used to diagnose *S. agalactiae*, the PCR-based method presented here is highly sensitive and requires only a single reaction followed by product analysis. Four samples that were assumed as *S. agalactiae* according to biochemical reaction and API 20 STREP System were identified using the PCR. Figure 2 shows amplification products with *S.*

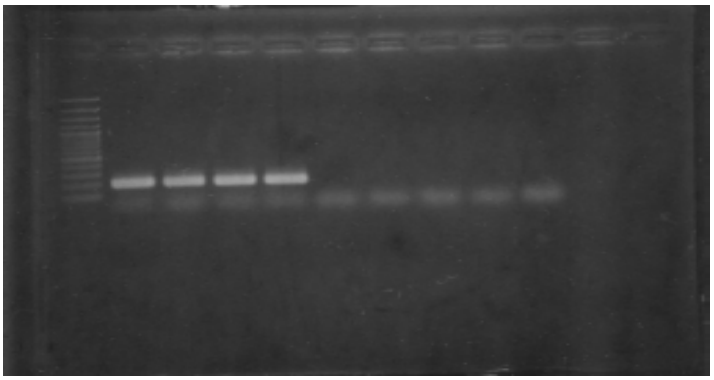

Figure 2. Amplification products obtained using PCR assay developed for *S. agalactiae* (192 bp) lanes 2-5; lane 1 100 bp DNA ladder

agalactiae using the Sdi 61 and Sdi 252 primer pairs.

Since the results of API 20 STREP System of *S. agalactiae* was almost the same with *S. iniae*, primer combination of LOX-1/LOX-2 were tried to detect whether the isolates were *S. iniae*, because of the high genetic relation between *S. iniae* and *S. agalactiae* (Vandamme *et al.,* 1997; Berridge *et al.,* 2001). Moreover, the presence of a homologous lactate oxidase-encoding gene in *S. iniae* and some other phylogenetically related bacteria was examined previously (Gibello *et al.,* 1999), but the result showed no PCR amplification products that were observed using the primer sets LOX-1/LOX-2. Therefore, these isolates were not considered as *S. iniae*. The primer combination of Sdi 62/Sdi 252 gave a single amplification product of 192 bp, which was specific for *S. agalactiae*.

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