SEQUENCE ANALYSIS OF *Streptococcus agalactiae,* A PATHOGEN CAUSING STREPTOCOCCOSIS IN TILAPIA (*Oreochromis niloticus*)

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ABSTRACT

Pathogen identification based on biochemical properties can barely differentiate *Streptococcus iniae* and *S. agalactiae*. Beside that, this technique is also limited by the length of time required to complete the assays. Therefore, rapid diagnosis is necessary to initiate prompt therapeutic and prophylactic measures in order to limit any potential economic losses caused by such pathogens. The aim of the present study was to identify *Streptococcosis* species using amplification of *S. agalactiae* DNA sequence with species-specific primer Sdi 61 AGGAAACCTGCCATTTGCG and Sdi 252 CAATCTATTTCTAGATCGTGG and perform phylogenetic analysis based on DNA nucleotide sequence data. The sequencing of PCR products was performed at BPPT Puspiptek Serpong by using the respective PCR primers, Big Dye Terminator Chemistry and AmpliTaq-FS DNA polymerase. The sequencing reactions were run on the ABI Prism version 3103 – Avant Genetic Analyzer (USA) and the result was read by Sequence Navigator program (Applied Biosystem). Alignment multiple analysis was done based on the data from Genebank with BLASTN (http://blast.ncbi.nlm.nih.gov/blast.cgi) on the nucleotide level. Neighbor-joining phylogenetic trees were generated with Genetyx programme version 7 with UPGMA and MEGA software version 4.0. The result revealed that the isolates from brain, eye, and kidney of diseased Tilapia were infected by *S. agalactiae* and it has 99% similarity with Genebank. It has close relationship with *S. agalactiae* at genebank with UPGMA method. These isolates showed high variation in the first sequence which is similar to *S. iniae*. The information of *S. agalactiae* genomes suggests that gene acquisition, duplication, and re-assortment have played an important role in genetic diversity and evolution of *S. agalactiae*. Screening of breeder fish stocks with the developed PCR methodology, followed by elimination of infected stocks, would provide an efficient strategy to control fish infected by *streptococcosis*.

KEYWORDS: ***Streptococcus agalactiae*****, tilapia, sequence analysis**

INTRODUCTION

Tilapia infected by Streptococcosis (*S. iniae* and *S. agalactiae*) exhibits very similar symptoms and clinical signs regardless of the etiologic agents (Kusuda & Salati,1999), therefore a definitive diagnosis of the etiological agent was based on the microbiological characteristics of bacteria isolated from diseased fish (Mata *et al*., 2004). Recently, molecular methods based on DNA probes or PCR have overcome the problems associated with cul-

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ture-based techniques, enabling the detection of microorganisms directly from clinical samples without isolation (Gonzalez *et al.*, 2004). Moreover, Gonzales *et al.* (2004) stated that molecular techniques have been the most effective methods in diagnosing pathogens because they are more specific and sensitive than other conventional or commercial assays. The detection of pathogens using PCR and continuing sequence analysis is faster and cost-effective. 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 and perform phylogenetic analysis based on DNA nucleotide sequence data.

MATERIALS AND METHODS

S. agalactiae isolates

Isolates containing suspected *S. agalactiae* was obtained from Cirata Reservoir, West Java, and Wadas Lintang reservoir, Central Java Province. As part of this program, *S. agalactiae* strains were isolated from diseased Tilapia (*Oreochromis niloticus*). The isolates were stored at –20°C in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, Md.) with 15% glycerol.

Bacterial DNA isolation

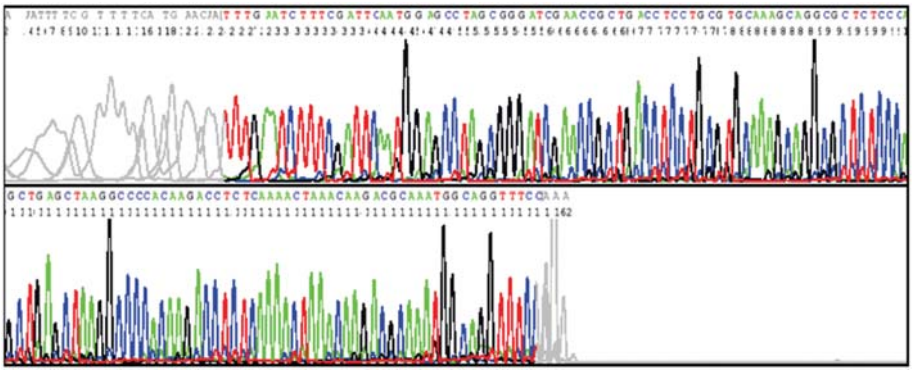
The samples of bacterial DNA used in PCR was extracted using boiling method. Bacterial aDNA was obtained from cells by touching the colonies and grown on blood agar medium with sterile ose. Bacterial colonies (5-10 colonies) were suspended in 400 µl of RNAse free water. The sample was boiled for 10 min at 98°C and centrifuged at 8.000 x g for 10 min. The supernatant was then 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* (spesies-spesific primers) (Mata *et al.*, 2004). These primers were designated as 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 and forward) each and 2 µL DNA templates. The amplification cycle at thermal cycler T personal (Biometra) consisted of 2 min denaturation at 94°C followed by 25 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 90 s. Finally, the last elongation was done at 72°C for 5 min. RNAse free water was used as negative control (no DNA template).

PCR and DNA sequencing

Cell lysates for PCR were prepared using lysozyme and proteinase K as described previously. Primers Sdi 61: 5'-AGGAAACCTGCCATTTGCG-3' and Sdi 252: 5'-CAATCTATTTCTAGATCGTGG-3' (Alpha DNA Montreal, Quebec) were used from known sequences (GenBank accession number AF064441.1) to amplify the 192-bp fragment. PCR conditions consisted of an initial 5-min denaturation step at 95°C, 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and a final 10-min extension cycle at 72°C. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Inc., Chatsworth, Calif.). Following gel electrophoresis, DNA was quantified by using the LabImage gel analysis software (version 2.62; Kapelan, Saale, Germany) to compare the band intensities of PCR products to marker band intensities of known concentrations (pGEM marker; Promega, Madison, Wis.). The sequencing of PCR products was performed at BPPT Puspiptek Serpong by using the respective PCR primers, Big Dye Terminator chemistry, and AmpliTaq-FS DNA polymerase; the sequencing reactions were run on the ABI 3700 automated sequencer (Perkin-Elmer Biosystems, Foster City, Calif.). DNA sequence data obtained with both forward and reverse primers were proofread and assembled with the Seqman software program (DNAStar, Madison, Wis.) to yield the final 16 S rRNA sequences used for analyses. 16 S rRNA sequences were aligned using BIO software and multiple aligment analysis was used to assign it with sequences data from Genebank with BLASTN (http://blst.ncbi.nlm.nih.gov/blast.cgi) at nucleotide level. Neighbor-joining phylogenetic trees were generated with MEGA software version 4.0 and Genetyx software version 7 with UPGMA method.



CGAAACTGAGAGGTCTTGTGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCA
GGAGGTCAGCGGTTCGATCCCGCTAGGCTCCATTGAATCGAAAGATTCAAATTGTTCATTG
AAAATTGAATATCTATATCAAATTCCACGATCTAGAAATAGTAGACAAGTGAGCTAAGGCCC
CACAAGACCTCTCAAAACTAAACAAGACGCAAATGGTAAAGTTTAC
TAAATTTTCGTTTTCATGAACAATTTGAATCTTTCGATTCAATGGAGCCTAGCGGGATCGAA
CCGCTGACCTCCTGCGTGCAAAGCAGGCGCTCTCCCAGCTGAGCTAAGGCCCCACAAGA
CCTCTCAAAACTAAACAAGACGCAAATGGCAGGTTTCCAAA

Figure 1. Chromatogram (top) and sequencing result of 16S rRNA of *S. agalactiae* (down)

RESULT AND DISCUSSION

Sequence Similarity

Phylogenetic analysis of the 16 S rRNA gene sequence clearly revealed that *S. agalactiae* is a member of the pyogenic group of the genus *Streptococcus*. The closest relative is *S. difficilis*, with which it shares almost identical sequence (99%). *S. agalactiae* and *S. difficilis* constitute a cluster that was separate from the other *Streptococcus* species.

The multiple alignment sequence data of 16S rRNA was similar to those of some species of S. *agalactiae* especially *S. difficilis* available at Genebank (Figure 2). The number of nucleotide between *S. agalactiae* and *S agalactiae* from Genebank is 192 bp. Kawamura *et al.* (2004) found that *S. agalactiae* has high genetic similarity with *S. difficilis*. Based on their research, it was proposed that *S. difficilis* is a later synonym of *S. agalactiae*.

However, different sequence was found in *S. agalactiae* and *S. iniae,* showed in Figure 3. Multiple alignments between *S. agalactiae* and *S. iniae* described a high variation for the first sequence.

Comparative phylogenetic tree was constructed from 16 S rRNA gene sequences. Distances were calculated by using the neighbor-joining method. *S. agalactiae* from this research were closely related, with very little sequence divergence with *S. agalactiae* from the Genebank. Elliot *et al.* (1990) showed that strains isolated from humans, mice and fish shared identical whole cell protein profiles. These reports strongly suggest that the majority of strains of group B streptococci isolated from different hosts belong to a single cluster.

The broad spectrum of *S. agalactiae* disease manifestations reflects a complex interplay between the host's innate and adaptive immune systems and bacterial virulence factors. On the other hand, *S. agalactiae* is part of normal flora, and thus an opportunistic pathogen. Therefore, the role of host and environment is very important for the occurrence of streptococcosis disease. *S. agalactiae* virulence traits mainly include: (a) factors that inhibits immunological clearance; (b) adhesion or invasion that enables penetration of epithelial and endothelial cellular barriers to reach the bloodstream and deeper tissues; (c) toxins that directly injure or disrupt host tissue components.

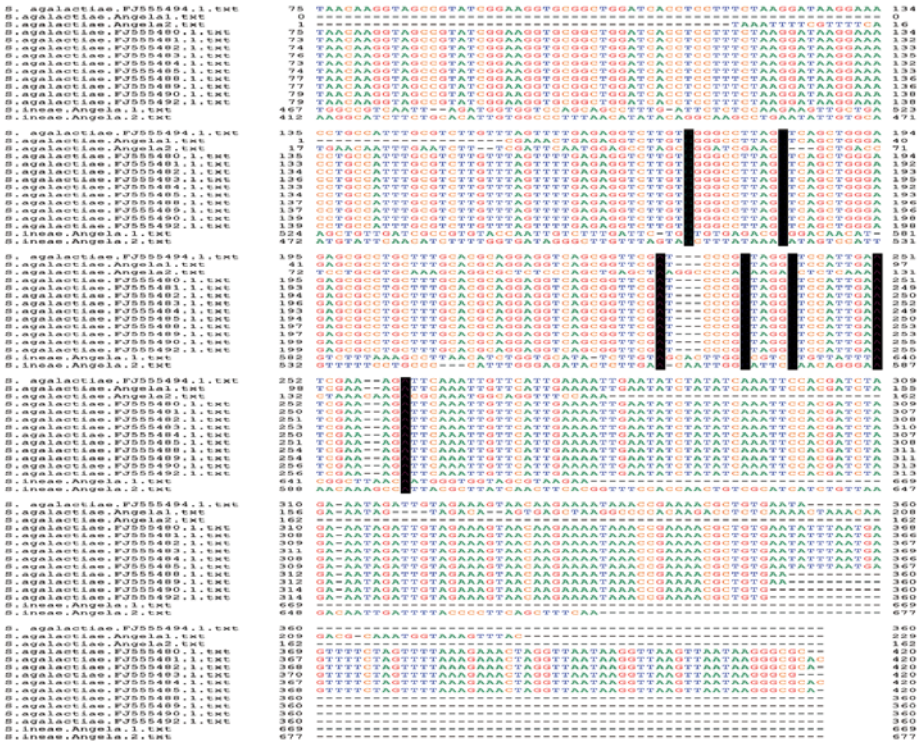


Figure 2. Multiple alignments of 192 bp fragment from 16S rRNA *S. agalactiae* and. *S. iniae* and *S. agalactiae* from Genebank

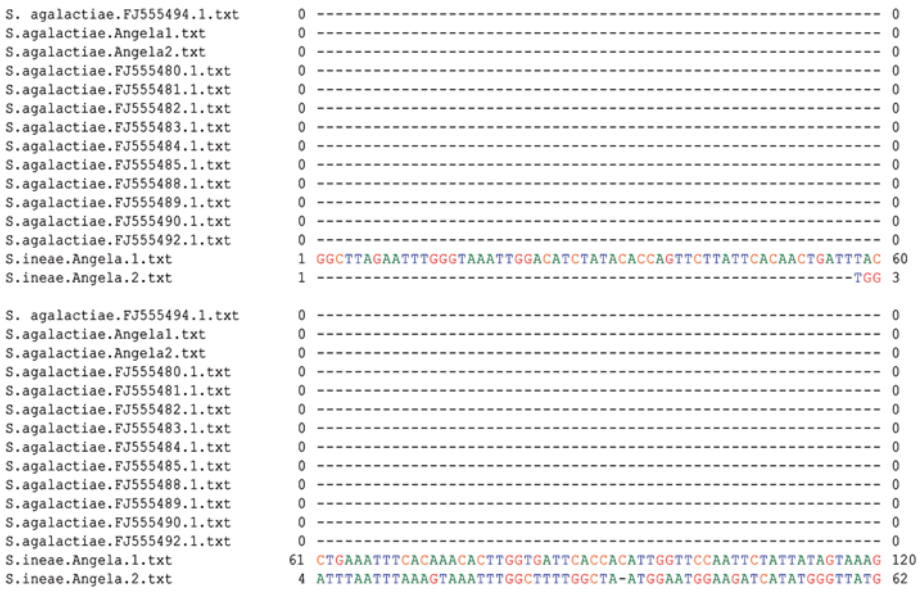


Figure 3. Multiple alignments of *S. agalactiae* and *S. iniae*. The dot means the nucleotide is identical with the genetic sequence above

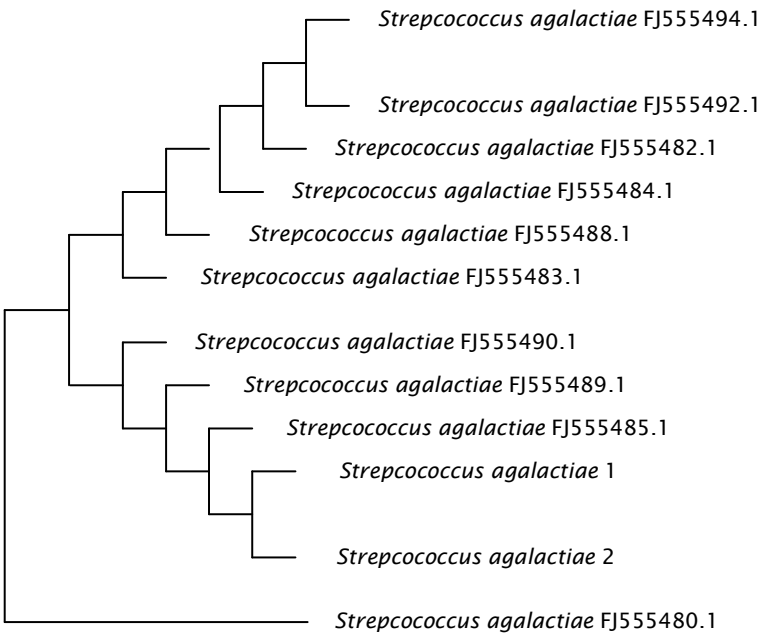


Figure 4. Dendrogram UPGMA based on nucleotide 16S rRNA *S. agalactiae* and 192 bp *S. agalactiae* from Genebank

Capsular serotyping has been the classic method used in descriptive epidemiology of *S. agalactiae*. Based on capsular expression, *S. agalactiae* are sub-classified into nine serotypes, Ia, Ib, and II - VIII. In 2002, two complete genome sequences of *S. agalactiae* were released for a serotype III strain and a serotype V strain (Tettelin *et al*., 2002). The genome sequences of additional six *S. agalactiae* invasive strains were reported in 2005, which include serotype Ia, Ib, II, III, and V (Tettelin *et al*., 2002). The *S. agalactiae* genome is around 2 Mb with low G/C content of about 36%, and is predicted to comprise more than 2,100 protein-encoding genes. Of these encoding genes, there have been 14-15 pathogenicity island-like regions identified, which are dispersed around the genome. These "islands" contain encoding genes and putative virulence genes, mostly predicted surface proteins. The "islands" may have an important role in acquisition of virulence factors and in genetic diversification of the species (Tettelin *et al*., 2002).

Importantly, *S. agalactiae* appears to have a rather large flexible gene pool, and an average of 33 new strain-specific genes is predicted to be identified when a new *S. agalactiae* strain is sequenced (Tettelin *et al*., 2002). The information of *S. agalactiae* genomes suggests that gene acquisition, duplication, and reassortment have played an important role in genetic diversity and evolution of *S. agalactiae*.

Considering the data presented here and data from other researchers, we conclude that the isolates from Cirata and Wadas Lintang, Java Province are *S. agalactiae* and distinguishable from *S. iniae* particularly from genetical aspects.

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