Phylogeny and Histopathology of *Streptococcus iniae* from Indonesia

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Abstract: *Streptococcus iniae* has been detected in several regions in Indonesia, such as Bali, Jambi and Papua. Outbreak of meningoencephalitis and septicemia was still often found in Papua. The aim of study was to compare phenotype, genotype and histopathology of *S. iniae* from Papua. Bacteria was identified using morphological and biochemical tests. It was also extracted, amplified and sequenced to see genotypic characters. The primer used were 5'-AGAGTTTGATCCTGGCT-3' (24F) and 5'-AAGGGAGGTGATCCAGCCGCA-3' (1540R) in 16S rRNA region. Result of sequencing was analyzed by the neighbor joining and maximum parsimony methods. The identification result of isolate from Papua was *S. iniae*. The amplification result was a sharp band—1,500 bp band in 16S rRNA region. The phylogenetic tree showed that isolate from Papua was closely related to *S. iniae* strains CMS005 from Guangxi China. Bacterial clumps of *S. iniae* was firstly found in the blood vessel of liver at the 3rd day after infection and then caused the inflammation to spleen, heart, brain, kidney and gut at the 7th day post infection.

Key words: Phenotype, genotype, phylogeny.

1. Introduction

Streptococcus iniae is not only one of the major causative agent of streptococcosis in aquaculture industry but also an important zoonotic bacterial disease causing morbidity and mortality in humans. The emergence of disease has occurred in a range of aquatic animals [1]. The disease has been reported in almost all continents and has caused significant losses in several commercial fish species [2]. The estimated annual impact of disease outbreaks by *S. iniae* in aquaculture sector of some countries was reported to be 100 million USD [3].

S. iniae was first isolated from multifocal subcutaneous abscesses in captive Amazon freshwater dolphins—*Iniae geoffrensis* [4]. In fishes, *S. iniae* infections were characterized by meningoencephalitis and septicemia that generally

induce high morbidity and mortality rates [1]. Clinical signs of *S. iniae* infection in fish include loss of orientation, lethargy, ulcers, exophthalmia and culminate in a fatal meningoencephalitis [1]. *S. iniae* can cause serious zoonotic infections in humans who are injured while handling death fish [5]. To date, this disease has been found in Indonesia farming of tilapia (*Oreochromis niloticus*) in Lubuk Linggau, Southern Sumatra during 2002-2003 [6] as well as in Lake Maninjau in 2010 [7]. *S. iniae* has been detected in several regions in Indonesia, such as Bali, Jambi and Papua [8]. Outbreak of meningoencephalitis and septicemia is still often found in Papua. Thus, no detailed description of *S. iniae* infection was reported until now. The aim of study was to describe the phenotypic and genotypic characteristic of *S. iniae* on the basis of 16S rDNA sequences and histopathological changes.

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2. Materials and Methods

2.1 Bacterial Isolation and Identification

Streak of brain and kidney of tilapia with clinical signs exophthalmia and abdominal distention, was isolated onto 5% sheep blood agar and incubated at 28 °C for 72 h. The colonies obtained were tested by hemolysis, gram staining, catalase and oxidase production, then continue to identify the isolate using several bacterial cultures, such as TSA 6.5% NaCl, Bile esculin, Simon citrate, Methyl red and Voges-Proskauer (MR/VP), lysin decarboxylase, urea, motility indole ornithine (MIO), McConkey, glucose, lactose, maltose, sucrose, xylose, arabinose and mannitol [1]. All identification was compared with isolated of *S. iniae* (ATCC 29177). The classification method used parameters as described in bacterial fish pathogen [2].

2.2 Molecular Studies

Isolates of *S. iniae* from Papua and *S. iniae* (ATCC 29177) were extracted using DNA Extraction Kit (Qiagen). The primers used were 5'-AGAGTTTGATCCTGGCT-3' (24F) and 5'-AAGGGAGGTGATCCAGCCGCA-3' (1540R) in 16S rRNA region. A reaction matrix of 25 µL contained 12.5 µL master mix (Roche, Switzerland), 10×10^{-12} mol concentration of each primer, 6.5 µL sterile ddH₂O and 4 µL DNA template. Typical cycling parameters were: 5 min primary denaturation at 95 °C, 30 s denaturation at 92 °C, 90 s annealing at 52 °C, 60 s extension at 72 °C for 30 cycles and then 5 min final extension step at 72 °C. The polymerase chain reaction (PCR) products were then electrophoresed in 1% gel agarose. Purification and sequencing of PCR product were done in first base Singapore.

2.3 Alignment and Phylogeny Analysis

Sequence results were compared to 16S rRNA sequences that are available in the GenBank/EMBL/DBJ databases using the Basic

Local Alignment Search Tool (BLAST) algorithm [9]. Multiple sequence alignment of representative sequences was carried out by using the CLUSTAL X program (version 1.8) [10]. Other sequences of the following *streptococcal* species are available in the National Center for Biotechnology Information (NCBI) database: *S. iniae* strains WZMH110819 from Wenzhou, China; *S. iniae* strains SK10-S from Sentani, Papua; *S. iniae* strains SF2 from Iran; *S. iniae* strains SF1 from China; *S. iniae* strains SCCF5L from China; *S. iniae* from Japan; *S. iniae* from Israel; *S. iniae* strains ISET0901 from Israel; *S. iniae* strains DGX01 from China; *S. iniae* strains CMS005 from Guangxi, China; *S. iniae* strains I1 from Japan; and *Streptococcus hongkongensis* strains HKU30, *Streptococcus thermophilus*, *Staphylococcus aureus* subsp. *aureus* strains CN1 and isolates of *S. iniae* from Papua. The genetic distance matrix was obtained using Kimura's two parameter model [11] and an evolutionary tree was created using the neighbor joining and maximum parsimony methods [12] with MEGA 6 [13]. Bootstrap values from 1,000 replicates are displayed as percentages.

2.4 Pathogenicity Test

Thirty of tilapias were injected intraperitoneally with $0.1 \text{ mL} \times 10^6$ CFU/fish. Five fish were killed at 3, 5, 7, 9 and 11 days post infection to examine the histopathological changes. Tissue samples were fixed in 10% solution of neutral buffered formalin and then stained with haematoxylin and eosin (H&E).

3. Results and Discussion

3.1 Phenotypic Result

Affected fish were loss of equilibrium, erratic swimming which is either spiralling or spinning just below the surface of water, darkening, exophthalmus, petechiae on the operculum and fins. At autopsy, ascites, hemorrhage of the internal organs and enlarged spleens were found.

In this study, the isolate was β -haemolytic, gram

positive, catalase negative and oxidase negative. The isolate showed negative reaction in voges proskauer and positive reaction in esculin. The isolate grew on MacConkey agar at 37 °C, but did not grow on 6.5% NaCl. The isolate had a negative reaction for lactose, inositol, arabinose, dulcitol, sorbitol, raffinose and inulin. Positive reactions were observed in glucose, maltose, mannitol and mannose. This result was similar to identification made in Ref. [2] and to strain ATCC 29177.

The identification results of isolate were biochemically and physiologically similar to *S. iniae*. The taxonomic position of many aquatic isolates is still controversial [2]. Standard bacteriological methods were not adequate for identification of the fish pathogen *S. iniae*. Moreover, the *S. iniae* could not be identified by most commercial bacterial identification [4], because it was not listed in the databases of the most commonly used rapid or automated identification systems, including the Rapid Strep Strip, Vitex systems, API 20E Strep, Rapid Strep 32, API CH 50 or the ATB expression systems [14-16].

3.2 Phylogeny of *S. iniae* from Papua

Phylogenetic tree of *S. iniae* using neighbour-joining

(Fig. 1) showed that it was clustered to other *S. iniae* from several countries, except for isolate of *S. hongkongensis* HKU30 and *S. thermophilus*.

Phylogenetic tree of *S. iniae* using maximum parsimony (Fig. 2) showed that *S. iniae* from Papua was grouped in the same clade with *S. iniae* CMS005 Guangxi China and *S. iniae* DGX01 China.

Homology search of the 16S rRNA sequence (1,500 bp) of strain *S. iniae* from Sentani (GenBank/EMBL/DDBJ databases) showed 84% similarity with strain *S. iniae* from Papua using maximum parsimony and 100% similarity using neighbour-joining.

Then, *S. iniae* from Papua was closest to *S. iniae* CMS005 Guangxi China, despite the low validity (19% similarity using maximum parsimony analysis).

Specific PCR primer sequences have been developed as a useful alternative approach for the accurate and rapid identification of *S. iniae*, such as the 16S rRNA gene [17]. Genotypic identification was useful in the identification of carrier fish [18]. In this study, 84% similarity using maximum parsimony was shown in the sequences of the 16S rRNA gene between the *S. iniae* isolate (Papua, Indonesia) and the *S. iniae* CMS005 (Guangxi China).

Fig. 1 Phylogenetic tree of *S. iniae* from Papua using neighbour-joining analysis.

Fig. 2 Phylogenetic tree of *S. iniae* from Papua using maximum parsimony analysis.

Fig. 3 Increasing of renal melanomacrophage (a), necrosis (arrow) of epithelial gut (b), bacterial clumps (arrow) in the blood vessel of liver (c), congestion and inflammation in the spleen (d).

Scale bar: 20 μm.

3.3 Clinical Signs

Fish began to show clinical signs and morbidity at three days post infection with *S. iniae*. Fish showed signs of disorientation, anorexic, erratic swimming and listless circling at the water surface. Some of the fish had exophthalmia. Macroscopic lesions at three days post infection were darkness of skin, external hemorrhages especially around the base of the

pectoral fins and in the opercula. These observations were in accordance with the report in other studies; darkening of the skin and lethargy were the first signs observed in infected fish. Moribund and dead fish presented external hemorrhages especially around the base of the pectoral fins and over the internal organs [19]. Clinical signs of streptococcal infections varied between species. Darkening of the skin pigment has

Fig. 4 Brain membrane was haemorrhage (a), myocarditis (b), bacterial clumps within renal macrophage (c), erosion (arrow) and necrosis of mucosal gut (d).

Scale bar: 20 μ m.

been reported in various fish species infected with *Streptococcus* sp. [20-22].

3.4 Histopathological Changes

Histopathological changes were found in several organs including spleen, heart, brain, liver, ren and gut. It was found the increasing of renal melanomacrophage, necrosis of epithelial gut, bacterial clumps in the blood vessel of liver and congestion and inflammation in the spleen at three days post infection (Fig. 3). It was more severe to find haemorrhage of meningitis, myocarditis, bacterial clumps within renal macrophage, erosion and necrosis of mucosal gut at seven days post infection (Fig. 4).

S. iniae infection in fish is systemic infection of the liver, heart and brain. The histopathology examination found the infiltration of large numbers of increasing melanomacrophage in infected areas, such as the ren and spleen. Multifocal infiltration of macrophage cells in the kidney and spleen as the principal lesions, was also reported by other authors [23-25]. These findings can be correlated with the clinical findings of lethargy and loss of orientation [24]. Necrosis was similar to

observation in tilapia infected with *Streptococcus* sp. [19]. Externally, *Streptococcus* sp. infection can cause lesions, congestions, hemorrhages, exophthalmos, corneal opacity, intra-ocular and periorbital hemorrhages. Due to the high capability of spleen and posterior kidney to trap bacteria, *Streptococci* can be commonly found in those organs in conjunction to tissue necrosis [26].

4. Conclusions

In this study, isolates from Papua were closely related to Chinese isolate (DGX01, Guangxi region). Besides, bacterial clumps of *S. iniae* was firstly found in the blood vessel of liver at the 3rd day after infection and caused the inflammation to spleen, heart, brain, kidney and gut.

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