Isolation and characterisation of the *Enterococcus faecalis* strain isolated from red tilapia (*Oreochromis* hybrid) in Indonesia: A preliminary report

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Abstract

The number of fishes were conducted a series of bacteriological examinations to confirm the clinical symptoms that appeared and led to streptococcal infection. Results of the external examination from two moribund red tilapia found the hemorrhagic traces in some parts of the body such as the cranial area near the mouth, eyes, operculum, and some body parts and erosion on the tail, pectoral, and dorsal fin. Internally, no pathological changes were found in the brain and visceral organs. The morphological, biochemical, and molecular examination using the 16S rRNA universal gene and sequencing analysis of PCR products from two moribund red tilapia resulted in both isolates are *Enterococcus faecalis* bacteria with low virulence. Antibiotics susceptibility test indicated that the isolates were resistant to ampicillin and penicillin. Based on the results of the comprehensive bacterial examination, we concluded that *E. faecalis* were found as the cause of streptococcosis, infecting and causing mild lesions in red tilapia. To our knowledge, this finding is the first report of isolation and characterisation of the *E. faecalis* strain isolated from red tilapia in Indonesia.

Keywords: Antimicrobial resistance, *Enterococcus faecalis*, *Oreochromis* hybrid, Red tilapia, Streptococcosis

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Introduction

Streptococcosis is a bacterial disease which found worldwide, infecting numerous species of fish. The disease causes severe mortalities of up to 50% in 3 to 7 days hence affecting severe economic losses (Yanong and France-Floyd, 2002; Austin and Austin, 2012). Al-Harbi (1996) reported *Streptococcus* sp. with a concentration of 1.2×10^8 CFU mL^{-1} caused 80 % cumulative mortality in red tilapia. Clinical signs have been various, determined by the high level of virulence in infecting streptococcal or enterococcal bacteria. Clinical signs include lethargy; swimming abnormalities; opacity and hemorrhagic in the eye; hemorrhagic patches on the operculum, around the mouth, anus, and fins; as well as dark pigmentation in the whole body (Siti-Zahrah *et al*., 2008; Amal and Zamri-Saad, 2011; Anshary *et al*., 2014).

The most common causal agent of streptococcosis is *Streptococcus* sp., (e.g. *Streptococcus agalactiae*, *S. iniae*, and *S. dysagalactiae*). *Lactococcus garvieae* was also found to be the cause of this infection (Netto *et al*., 2011; Taukhid and Purwaningsih, 2011; Anshary *et al*., 2014). However, several other types of streptococcal bacteria are also probable to be the cause of streptococcosis, which have been reported in several types of fish, including *Enterococcus faecalis* (Khafagy *et al*., 2009; Rahman *et al*., 2017), *E. faecium*, *L. lactis*, and *S. mutans* (Toranzo *et al*., 2005; Austin and Austin, 2012). High and low-temperature factors are believed to affect the type of isolated bacteria. Most cases of streptococcosis that occur in warm waters are due to *Lactococcus garvieae*, *S. iniae*, *S. agalactiae*, and *S. paraubelis*. Whereas, the traceable cases in cold waters are usually due to *Vagococcus salmoninarum* and *S. phocae* (Toranzo *et al*., 2005; Austin and Austin, 2012). This finding reveals that the diversity of isolated bacterial species has been reported as causative agents from streptococcosis which remains debatable. In Indonesia, the report of streptococcosis is commonly dominated by *Streptococcus agalactiae* and *S. iniae* (Taukhid and Purwaningsih, 2011). This fact has led to the effort such as isolation and identification of other species that have not been reported causing streptococcosis in Indonesia.

This study aims to explore and identify the bacteria of streptococcosis caused by *E. faecalis* by referring between the appearance of clinical signs and examination of morphological, biochemical phenotypic characterisation, and molecular analysis from the colonies of isolated *E. faecalis*. Antibiotics susceptibility test was also conducted to explore the ability of *E. faecalis* field isolates, which were obtained from its potential against several classes of antibiotics. Such test was performed to provide additional information used as an evaluation material for antibiotics application.

Materials and methods

Sampling and Anatomical Pathology Examination

Ten snow white cichlid fish (*Pseudotropheus socolofii*) from aquaculture centre for ornamental fish in Bekasi, Indonesia; one barred bichir fish

(*Polypterus delhezi*) from freshwater aquarium conservation in Jakarta, Indonesia; one carp fish (*Cyprinus carpio*), one gouramis fish (*Osphronemus goramy*), and two red tilapia fish (*Oreochromis* hybrid) from different aquaculture centres in Bogor, Indonesia were explored as samples. Samples are taken based on clinical symptoms that appeared and led to streptococcosis. Anatomical pathology examination was performed by evaluating all external parts of the fish's body and necropsy on large fish to evaluate the condition of the visceral organs and brain. Small fish (ten snow white cichlid) were externally examined and made whole body homogenates on sterile brain-heart infusion (BHI) broth as samples for bacterial culture.

Isolation and identification of bacteria

Whole body homogenates (small fish), blood, brain, eyes, and liver (large fish) samples were cultured on 5 % sheep blood agar media (Oxoid®, Basingstoke, Hants, United Kingdom) and were incubated at 28°C for 24 hours. Colonies were grown and characterized as streptococci or enterococci bacteria by the morphological identification, Gram staining, catalase and oxydase tests then were purified by re-culturing on 5% sheep blood agar (Oxoid®, Basingstoke, Hants, United Kingdom). The pure colony was then continued with biochemical phenotypic characterisation using the Vitek® 2 System (bioMérieux, Inc., Hazelwood, MO, USA).

The morphological identification of bacterial cell surface was examined by using the soft-agar (SA) and salt aggregation test (SAT) technique. The SA technique was performed by dipping ± 0.5 cm needle öse into bacteria suspension (cells concentration was matched with McFarland's tube 4 standard or 10^9 CFU mL^{-1}) and inoculated on the 10 mL of 0.15 % brain heart infusion (BHI) soft-agar media (Himedia®, Nashik, India). The procedure was continued by homogenizing the vortex. The media was incubated at 35 °C for 18–24 hours to observe its growth pattern (Wibawan and Lämmler, 1990). Salt aggregation test was conducted by mixing one drop of bacteria suspension (10^9 CFU mL^{-1}) and one drop of different molar concentrations of ammonium sulfate solution (0.3 M, 0.6 M, 1.2 M, 1.8 M, 2.0 M, 2.4 M, 2.8 M, and 3.0 M), respectively. The aggregation was further homogenized at the sterile object glasses by the toothpick and was added with ± 50 µL of safranine for aggregation staining (modification of Nayar *et al*., 2013). Negative control employed one drop of sterile phosphate buffer saline and one drop of ammonium sulfate solution with ± 50 µL of safranine. Positive result is characterized by the presence of grains such as sand.

Preparation of DNA template

Deoxyribonucleic acid (DNA) extraction of pure colony from the suspected streptococci or enterococci bacteria was conducted by the boiling method for molecular identification; while the characterisation was conducted by using the polymerase chain reaction (PCR). The pure colony was taken as much as one öse to be made a suspension in 100 µL nuclease-free water (NFW) in the

Eppendorf tube; which was later homogenized by using a vortex for 5 minutes. The suspension was heated up in a water bath at 95 °C for 10 minutes to be homogenized by using a vortex for 5 minutes. This step was repeated for three times. The suspension was centrifuged (1500 g; 3 minutes) to separate the supernatant and pellets. The supernatant was collected as a DNA template for bacterial samples and was stored at –20 °C until the PCR process was conducted.

Polymerase chain reaction

The polymerase chain reaction (PCR) method applied the 16S rRNA universal gene for bacterial identification with forward primer of 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer of 1492R 5'-GGTTACCTTGTTACGACTT-3'.

Amplification of 16S rRNA gene was managed by using GoTaq Green PCR Kit (Promega, Madison, WI USA) with a total reaction volume of 60 µL mixture consisting of 30 µL mix PCR, 6 µL 1 mM of each primer, and 18 µL sample DNA template. The PCR reaction was performed by using the BioRad T100™ Thermal Cycler following the parameters such as: initial denaturation of 95°C for 3 minutes, 35 cycles with denaturation of 95 °C for 30 seconds, annealing 50 °C for 30 seconds, elongation of 72°C for 1 minute, and final elongation of 72°C for 5 minutes. PCR products were analyzed in 0.8 % agarose gel (Thermo Scientific, (EU) Lithuania) in 1 % Tris-acetate-EDTA (TAE) buffer and were stained with 0.5 µg mL^{-1} ethidium bromide. Amplification products were visualized with UV transilluminator (Pacific Image Electronics Co., Ltd., Taiwan). DNA Ladder (Promega, Madison, WI USA) of 1 kb was used as a standard molecular weight.

Sequencing and reconstruction of phylogenetic tree analysis

Sequencing was performed by sending the 16S rRNA PCR products to the 1st BASE utilizing Sanger dideoxy sequencing technology. Results of the sequencing and reconstruction of phylogenetic tree were analyzed with the MEGA X software. Sequencing analysis results were compared with Basic Local Alignment Search Tool (BLAST) database program, managed by the National Center for Biotechnology Information (NCBI) (2006) (http://www.ncbi.nlm.nih.gov). Database which was taken and used in phylogenetic tree reconstruction included the database of *Enterococcus faecalis* and *Enterococcus* sp. isolated from fish, aquatic environment, or isolates related to the fish-food-chain and its possible predators, such as birds. DNA sequences were also analyzed for the nucleotide composition and distance. The reconstruction of phylogenetic tree analysis was performed by using the neighbor-joining method with bootstrap replicates of 1000.

Antibiotics susceptibility test

Antibiotics susceptibility test was conducted by using the disk diffusion-Kirby Bauer method on Müeller-Hinton Agar (Oxoid®, Basingstoke, Hants, United Kingdom). Bacterial cell concentrations were equaled with McFarland 0.5 standard. Seven types of antibiotic disks were engaged in this study, including:

ampicillin (10 µg), oxytetracycline (30 µg), tetracycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 µg), and ciprofloxacin (5 µg) (Oxoid®, Basingstoke, Hants, United Kingdom). The examination was conducted with Duplo testing. The interpretation of the inhibition zone diameter referred to the Clinical Laboratory Standards Institute (CLSI) (2018).

Results

Anatomical pathology examination

Based on external examination and clinical symptoms on various types of fish obtained the following results. Ten snow white cichlid (*Pseudotropheus socolofi*) showed abnormality in swimming movements. One barred bichir fish (*Polypterus delhezi*), one carp fish (*Cyprinus carpio*), and one gouramis fish (*Osphronemus goramy*) showed opacity in the eye (data not shown). Two red tilapia fish (*Oreochromis* hybrid) showed traces of brownish hemorrhage on the surface of the body, especially in the eyes, cranial areas around the mouth, and operculum. Erosion in the tail, pectoral fin, and dorsal fin are also found in both red tilapia. The results of necropsy only showed a change in the color of the liver to yellow in one barred bichir fish *Polypterus delhezi* (data not shown). The consistency of the liver in the two red tilapia were slightly fragile but did not showed other pathological changes. The brain and other visceral organs in the two red tilapia also did not showed any significant pathological changes (Fig. 1).

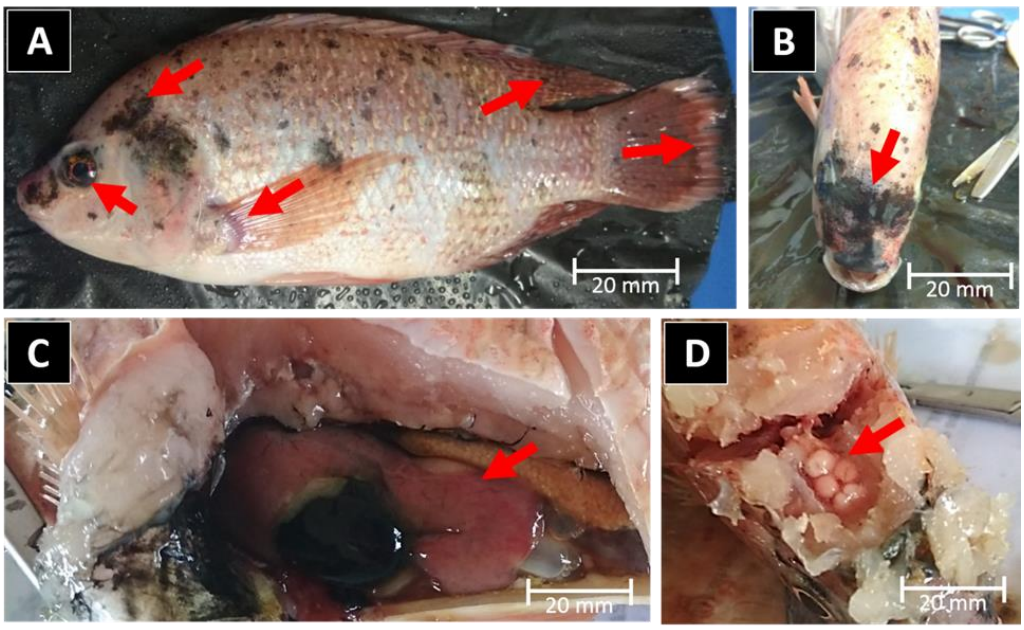

Figure 1: Results of external examination in red tilapia (*Oreochromis* hybrid) suspected streptococcosis. A = Pathology lesions indicated hemorrhagic traces in eyes and operculum and erosion in the tail, pectoral fins, and dorsal fin (arrow). B = Hemorrhagic traces were depicted in the cranial area around the mouth (arrow), without any pathology lesions in visceral organs, especially in liver (C) and brain (D) (arrow).

Bacterial isolation and identification

Based on the results of culturing on 5% sheep blood agar, we obtained the colonies with morphology grayish, round, smooth, γ-hemolysis, and small-size (0.5–1 mm) which were only isolated from the liver in the two moribund red tilapia (isolate code: 7INB and 8INB). Culture results from other fish samples did not showed the presence of colonies that led to streptococcal or enterococcal bacteria. Gram staining indicated the violet-streptococci cells bacteria. Based on the results of biochemistry phenotypic characterisation using the Vitek® 2 System (bioMérieux, Inc., Hazelwood, MO, USA) in both of two isolates, approximately 99% probabilities were obtained leading to the *Enterococcus faecalis* species with an excellent level of confidence. The biochemical phenotypic characterisation profile of the two isolates (7INB and 8INB) indicated relatively similar result on the tested parameters with only a difference in one test parameter of 0/129 *Vibrio*-static compound resistance (Table 1). Results of the morphological characteristic of cell surface indicated that both isolates of *Enterococcus faecalis* were in compact form on the SA media and had been positively aggregated in the lowest molar concentration salt solution (0.3 M) (Supplementary document).

Sequencing and reconstruction of phylogenetic tree

The PCR amplification by using the 16S rRNA gene indicated both 7INB and 8INB bands in 1396 bp and 1472 bp, respectively (Supplementary document). Based on the BLAST NCBI database program, we obtained the sequences of the 16S rRNA gene of *Enterococcus faecalis* which were isolated from the liver of two red tilapia from Bogor, Indonesia having similarities with the isolate of *E. faecalis* strain TY1 (accession no. CP031027) (Park *et al*., 2011) isolated from flatfish tissue origin on the Southern sea, South Korea. The results from BLAST analysis of 7INB indicated query coverage of 99%, E-value 0.0% and a maximum identity of 96.86% toward the isolate of *E. faecalis* strain TY1. Whereas the 8INB indicated query coverage of 99%, E-value 0.0%, and a maximum identity of 97.06%.

Both samples were found to be in different branches. However, the clusters were close to each other (Fig. 2). Analysis of the nucleotide composition between 7INB and 8INB did not present significant difference (Supplementary document). This finding is in line with the results from the analysis of nucleotide distance among 7INB, 8INB, and *E. faecalis* strain of TY1. The nucleotide distance of *E. faecalis* Strain isolated from red tilapia in this study had homology ranging from 97–100 % for all sequenced data. The nucleotide distance between 7INB isolates and *E. faecalis* strain TY1 indicated a difference of 0%; meanwhile the nucleotide distance between isolates 8INB and *E. faecalis* strain TY1 indicated a difference of 3%, and the nucleotide distance between 7INB isolates and 8INB isolates indicated a difference of 3% (Supplementary document). Two sequences of the 16S rRNA gene of this study had been deposited in GenBank under accession no. MT105346 (isolate code 7INB) and MT105348 (isolate code 8INB) (Fig. 3).

Table 1: Results of biochemical phenotypic characterisation using Vitek® 2 System (bioMérieux, Inc., Hazelwood, MO, USA) for *Enterococcus faecalis* strain isolated from red tilapia in Bogor, Indonesia

Parameter	Mnemonic	*E. faecalis* isolate 7INB	*E. faecalis* isolate 8INB
D-Amygdalin	AMY	+	+
Phosphatidylinositol Phospholipase C	PIPLC	-	-
D-Xylose	dXYL	-	-
Arginine Dihydrolase 1	ADH1	-	-
Beta-Galactosidase	BGAL	-	-
Alpha-Glucosidase	AGLU	+	+
Ala-Phe-Pro Arylamidase	APPA	-	-
Cyclodextrin	CDEX	-	-
L-Aspartate Arylamidase	AspA	+	+
Beta Galactopyranosidase	BGAR	-	-
Alpha-Mannosidase	AMAN	-	-
Phosphatase	PHOS	-	-
Leucine Arylamidase	LeuA	-	-
L-Proline Arylamidase	ProA	-	-
Beta Glucuronidase	BGURr	-	-
Alpha-Galactosidase	AGAL	-	-
L-Pyrrolidonyl-Arylamidase	PyrA	+	+
Beta-Glucuronidase	BGUR	-	-
Alanine Arylamidase	AlaA	+	+
Tyrosine Arylamidase	TyrA	+	+
D-Sorbitol	dSOR	+	+
Urease	URE	-	-
Polymixin B Resistance	POLYB	+	+
D-Galactose	dGAL	+	+
D-Ribose	dRIB	+	+
L-Lactate Alkalinization	ILATk	-	-
Lactose	LAC	-	-
N-Acetyl-D-Glucosamine	NAG	+	+
D-Maltose	dMAL	+	+
Bacitracin Resistance	BACI	+	+
Novobiocin Resistance	NOVO	+	+
Growth in 6.5% NaCl	NC6.5	+	+
D-Mannitol	dMAN	+	+
D-Mannose	dMNE	+	+
Methyl-B-D-Glucopyranoside	MBdG	+	+
Pullulan	PUL	-	-
D-Raffinose	dRAF	-	-
O/129 Resistance (Comp.Vibrio.)[a]	O129R	+	-
Salicin	SAL	+	+
Saccharose/Sucrose	SAC	+	+
D-Trehalose	dTRE	+	+
Arginine Dihydrolase 2	ADH2S	-	-
Optochin Resistance	OPTO	+	+

[a]The difference of biochemical phenotypic characterisation between *E. faecalis* 7INB and 8INB.

Antibiotics susceptibility test

Antibiotics susceptibility profile of *Enterococcus faecalis* had been resistant to ampicillin and penicillin in both isolates and intermediate against ciprofloxacin in 7INB and resistant to 8INB. Intermediate results of erythromycin were depicted by the two isolates of *Enterococcus faecalis*. Gentamicin, tetracycline, and oxytetracycline were still regarded as sensitive results in both isolates (Table 2, Fig. 4).

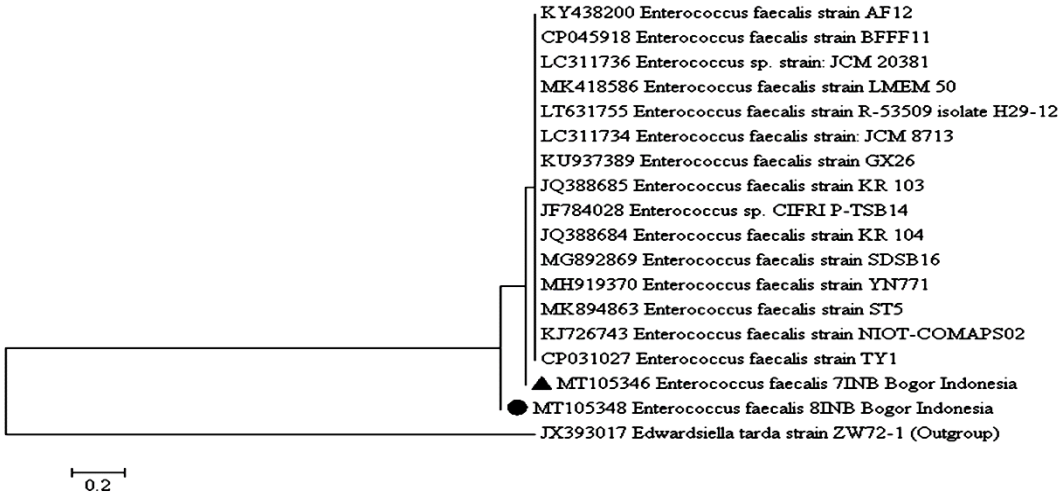

Figure 2: Phylogenetic tree reconstruction by utilizing the neighbor-joining method in MEGA X with bootstrap replicates of 1000. Results indicated *Enterococcus faecalis* 7INB (▲) and *E. faecalis* 8INB (●) isolated from red tilapia, Bogor, Indonesia in different clusters, but significantly close with *E. faecalis* strain TY1 isolated from the flatfish origin on Southern sea, South Korea.

A

TGCAAGTCGAACGCTTCTTTCCTCCCGAGTGCTTGCACTCAATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCA
TCAGAGGGGGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTTCGGGTGTCGC
TGATGGATGGACCCGCGGCTTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGCTGAGAGGTGATCGGCCACCTGG
GATGAGACCGGCCCAGATCCTACGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAG
AAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTA
GCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCAT
TAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTATTGTTAGTTGCCATCTTTATTGGGCACTACAG
ACTGCCGGAAAACCGGAGAAGGTGGGGATGACAAATCATCATGCCCTTATCTGGGCTACACACGTGCTAAATGGGAATACAACACGCTACCG
CGAGGTATGCAAATCTCTTAAAGCTTTCTCAGTTCGGATGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGC
CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGC
CAGCCGCCTAAGGGGATGG

B

GGGGAGTGCGGGTGCTATACATGCAAGTCGAACGCTTCTTTCCTCCCGAGTGCTTGCACTCAATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACAC
GTGGGTAACCTACCCATCAGAGGGGGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTTC
GGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCG
GCCACACTGGGACTGAGAACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGGCAACGCCGC
GTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGGAAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAA
GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTG
AAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTA
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GAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCACGGAGCGCAACCCTTATGGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCCGTGACAAACCGAGGGAAGG
TGGGATGAACGTCAAATCATACATGGCCTTATGACTTGGCTTACACGTGCTTCATGATGGGAAGTTCACGGATCCGTGAGACCGCGGAGCTATGCAAATC
TCTAGACGCTTCTCTCCTGTCGAATTGTAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGATTAG
GTGT

Figure 3: Sequences of the 16S rRNA gene *Enterococcus faecalis* isolated from red tilapia in Bogor, Indonesia. (A) Isolate code 7INB. (B) Isolate code 8INB.

Table 2: Inhibition zone (mm) for tested antibiotics

Antibiotics	Isolate code: 7INB	Isolate code: 8INB
Gentamycin (10 µg)	10.00 ± 0.00 (S)	10.00 ± 0.00 (S)
Ampicillin (10 µg)	11.00 ± 0.00 (R)	10.00 ± 0.00 (R)
Tetracycline (30 µg)	27.50 ± 2.12 (S)	19.50 ± 0.71 (S)
Penicillin (10 µg)	9.50 ± 0.71 (R)	9.50 ± 0.71 (R)
Ciprofloxacin (5 µg)	18.00 ± 0.00 (I)	12.00 ± 2.83 (R)
Oxytetracycline (30 µg)	19.50 ± 0.71 (S)	20.00 ± 0.00 (S)
Eritromycin (15 µg)	20.50 ± 2.12 (I)	18.00 ± 0.00 (I)

S = Susceptible; I = Intermediate ; R = Resistance. Disk diameter 6.0 mm. Data were presented as Mean ± SD (n=2)

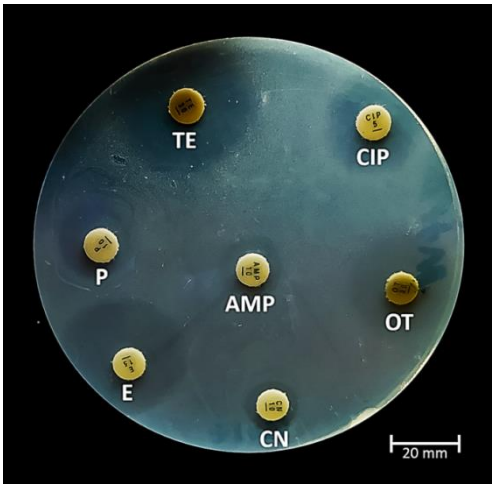

Figure 4: Antibiotics susceptibility profile of *Enterococcus faecalis* isolated from two red tilapia's liver organ in Bogor, Indonesia. AMP= Ampicillin. P= Penicillin. CIP= Ciprofloxacin. E= Erythromycin. CN= Gentamycin. TE= Tetracycline. OT = Oxytetracycline.

Discussion

Based on the results of external anatomical pathology examination which include: abnormality in swimming movement, opacity in the eye, lesions of hemorrhagic traces in the area of the eye, mouth, operculum, and most of the body and erosion on the tail, dorsal, and pectoral fins, we obtained clinical symptoms as the main guideline for directing diagnosis in streptococcosis (Siti-Zahrah *et al*., 2008; Amal and Zamri-Saad, 2011; Anshary *et al*., 2014). Cases of streptococcosis generally indicated lesions in the visceral organs, such us granulomas in the liver, kidneys, and brain (Irianto, 2005). Other diagnosed lesions included pale liver, dark red spleen, serosanguineous fluid in the coelomic cavity, and pathognomonic symptoms of exophthalmic with hyperemia (pop-eye) (Noga, 2010). Streptococcal infection caused by *Streptococcus agalactiae*, *S. iniae*, and *S. dysagalactiae* subsp. *dysagalactiae* in a pathogenicity trial demonstrated similar clinical symptoms, including: exophthalmia, hemorrhage at the base of the fin, the presence of subcutaneous abscesses in the caudal peduncle area, and erratic swimming behavior. The three species of streptococcal bacteria were also found to cause co-infection in one host (Cai *et al*., 2016). In another report, *S. iniae* has been suspected to cause spontaneous infection in the form of acute septicemia triggering sudden death in the catfish channel (*Ictalurus punctatus*) (Chen *et al*., 2011).

Different result in biochemistry parameter test, 0/219 *Vibrio*-static compound resistance, can occurs because this property is obtained and transmitted through the conjugate process between different bacterial species due to association with plasmids so as to show differences between bacterial strains according to environmental conditions (Matsusita *et al*., 1984). This is reasonable because 7INB and 8INB isolates originated from different aquaculture centres in Bogor, Indonesia. This finding is suspected because the 7INB isolate had interacted with *Vibrio* sp. bacteria which has the ability to resist 0/129 *Vibrio*-static compound in the same pool or water environment. However, this matter needs further study to reveal the origin of the 0/129 Vibrio-static resistant property possessed by *Enterococcus faecalis* with isolate code 7INB. The results of the biochemical phenotypic characterisation clarified that both isolates from bacteria species such as *Enterococcus faecalis* were successfully isolated for purification.

The virulence level of *Enterococcus faecalis* in tilapia is very diverse, ranging from high, moderate, low, to avirulent (Rahman *et al*., 2017). Anatomical pathology examination results in cases of streptococcosis caused by *E. faecalis* in Lake Temsah, Ismailia Governorate, Egypt demonstrated lesion such as hemorrhagic in dorsal and caudal fins, ulcer hemorrhagic in the caudal peduncle, ulcer in the head region, enlargement and hemorrhagic in the liver and kidney organs, and the liver turned pale (Khafagy *et al*., 2009). A study of *Enterococcus faecalis* had been conducted to observe the pathogenicity of these bacteria by making an artificial infection in *Oreochromis nicotilus*. The findings of the study navigated the clinical symptoms which initially appeared at 24 hour post-infection followed by the death at 72 hours post-infection. The main clinical symptoms were visible in the form of the opacity of the cornea, uni- or bi-lateral exophthalmia, erosion on the tail, hemorrhagic on the pelvic fins, and signs of asphyxiation (Rahman *et al*., 2017).

The absence of pathology lesions in the examination of brain and visceral organs is suggested due to the presence of low or moderate virulence level from the bacteria causing streptococcosis in two of moribund red tilapia in this study. The low virulence level could be proven by the results of the morphological of cell surface study by employing SA and SAT technique. The compact form of two isolates of *E. faecalis* colonies on the SA media indicated that both antigens are non-capsulated and protein domination on its surface. The abundance of protein on the bacterial cell surface indicated that the antigens were hydrophobic and were aggregated with salt particles. This bacteria will be retained and cannot grow lengthwise on the SA media to become diffuse form (comet-like) (Wibawan and Lämmler, 1991; Harlina, 1999). The infection level which is inflicted in the host body can relate to grown-pattern on the SA media. The protein antigen can only cause infection locally due to the virulence factor, such as surface protein (adherence), only for adhesion in some particular areas (Yumoto *et al*., 2019). Meanwhile, carbohydrate antigen has been suspected to cause infection systemically due to the capsular polysaccharide for invasive process, suggested to protect the antigen from the phagocytosis (Craig and Scherf, 2003).

E. faecalis produces exotoxin, such as cytolysin, as another virulence factor. Cytolysin can lyse the human, horse, dog, rabbit, and mice erythrocytes. However, it cannot lyse sheep and geese erythrocytes (Todd, 1934), causing *E. faecalis* colony to appear as γ-hemolysis if cultured on the 5 % sheep blood agar media. An individual's immune system is expected to affect the level of tissue damage and clinical signs as presented in the infected fish.

The pathogenesis of *Enterococcus faecalis* infection can be preceded by an adhesion process intermediated by a virulence factor in the form of adherence (Guzmàn *et al*., 1989). Colonization of the host tissues in *E. faecalis* infection is caused by the presence of protein the surface of the *Esp* which contributes to make the colony and persistent towards the

bacteria (Shankar *et al*., 2001). The eye infection is due to the contribution of protein gelatinase and serine protease caused by the *fsr* regulatory gene triggering both enzymes produced by *E. faecalis* bacteria (Engelbert *et al*., 2004). There are three types of *fsr* gene owned by *E. faecalis*, e.g. *fsrA*, *fsrB*, and *fsrC*, found in the upstream part of the gelatinase coding gene and downstream of the serine protease coding gene (Qin *et al*., 2000).

There are many species of fish reported to be streptococcal infections from various environmental and geographical temperature conditions, such as: channel catfish *Ictalurus punctatus* (Chen *et al*., 2011), red-tail black shark *Epalzeorhynchos*, rainbow shark *E. erythrurus* (Russo *et al*., 2006), golden pompano *Trachinotus ovatus* (Cai *et al*., 2016), rabbitfish *Siganus canaliculatus* (Yuasa *et al*., 1999), rainbow trout *Oncorhynchus mykiss* (Pourgholam *et al*., 2011), and other fish species. This condition indicates streptococcosis with broad tropism towards its host. The variety of causative agents causing streptococcosis in fish, should make diagnosis easier. However, determining the causal bacteria associated with streptococcosis becomes quite difficult since it ignores other causative agents that would be found in the sample. This finding leads to the confirmation bias because most reported cases of streptococcosis in Indonesia are dominated by bacteria from the species such as *Streptococcus agalactiae*, *S. iniae*, and *Lactococcus garvieae*. Ecological and geographical factors probe that a variety of streptococcal bacteria is very diverse confusing the taxonomy of species which cause uncertainty and easily changing streptococcosis in fish (Noga, 2010).

Molecular approachment by using universal primer the 16S rRNA gene for bacteria depicted the two isolates of *Enterococcus faecalis* having similarity with an isolate of *E. faecalis* strain TY1 (accession no. CP031027) which was isolated from tissues of flatfish on Southern sea, South Korea (Park *et al*., 2011). Percentage of query coverage between the two isolates with *E. faecalis* strain TY1 (99%) demonstrated high nucleotides segments that are consistent with the database of an isolate of *E. faecalis* strain TY1 in BLAST System (National Center for Biotechnology Information, 2006). E(xpect)-value (E-value) which is low to reach 0.0% also indicated a high level of homology between 7INB and 8INB with E. *faecalis* strain TY1 (Claverie, 2003), supported by the results of nucleotide composition and distance analysis (Supplementary document).

Phylogenetic tree reconstruction of the 7INB, 8INB, and *E. faecalis* strain TY1 indicated that they are on different clusters despite joining close. These findings postulated the phylogenetic originated from the 8INB as the ancestor of 7INB and *E. faecalis* strain TY1 from flatfish origin in South Korea. Bangladesh origin isolate looks very far from its kinship (Akter *et al*., 2020), when compared with the *E. faecalis* strain BFFF11 (accession no. CP045918) which was also isolated from the case of streptococcosis in tilapia from Gazipur District. However, such findings appeared due to activities, such as trade, conducted by humans who can move

bacteria with a considerable distance to adapt according to the environment and their habitat or due to the natural factors in the form of a hydrological cycle connecting the waters of Indonesia with South Korea, such as the current in the high seas. Bacteria causing streptococcosis thrives as a career in wild fish on the ocean to enable the connection between these geographical distances (Zlotkin *et al*., 1998).

Antibiotics susceptibility profile revealed that the two isolates of *E. faecalis* had been resistant to the ampicillin and penicillin. The intermediate results of ciprofloxacin were presented by 7INB isolate; while the resistant results were depicted by 8INB isolate. Intermediate results of erythromycin were indicated by both field isolates. Gentamicin, tetracycline, and oxytetracycline depicted the results of susceptibiliy toward the two isolates. These findings are in accordance with previous studies conducted in Egypt on streptococcal and enterococcal isolates from tilapia (*Oreochromis nicotilus*) at aquaculture sites in the Nile River, Egypt. In that study, approximately 17% isolates were resistant to ampicillin, 67% were resistant to erythromycin, 100% were resistant to tetracycline, and 17% were resistant to ciprofloxacin. However, there were no *E. faecalis* isolates which was resistant to penicillin and gentamicin (Osman *et al*., 2017). The findings of another study of *E. faecalis* isolated from tilapia and catfish in Bangladesh clarifies that there was resistance to ampicillin, erythromycin, and penicillin-G. Gentamicin marked the inhibition zones (Rahman *et al*., 2017). The finding of resistance to β-lactam antibiotics, ampicillin and penicillin, in both *E. faecalis* field isolates in this study emphasized that the common use of these two types of antibiotics is applied by the community in aquaculture activities as well as intermediate findings on ciprofloxacin and erythromycin. The relationship between the use of antibiotics and the incidence of resistance has a positive correlation where the high level of antibiotic use will increase the occurrence of antibiotic resistance (Asai *et al*., 2005). The findings are still susceptible to gentamicin because gentamicin is excluded as an antibiotic used in aquaculture activities in Indonesia. The susceptible findings on the type of tetracycline and oxytetracycline indicated anomaly conditions, due to its restricted use, solely permitted by the Ministry of Maritime Affairs and Fisheries of the Republic of Indonesia; however, it had a tendency presenting sensitivity to field isolates. The community role towards resistance or sensitivity of an antibiotic is suggested to contribute the discovery of this anomaly condition.

We concluded that *Enterococcus faecalis* were found as the cause of streptococcosis infecting and causing mild lesions in red tilapia in Bogor, Indonesia. This study novelty is probed by the finding section, as the first report for isolation and characterisation of the *E. faecalis* strain isolated from red tilapia in Indonesia. This study could add information to the causative agents varying from the incidence of streptococcosis infecting the red tilapia in Indonesia. This study is expected to provide further evaluation in

determining the results of the diagnosis and development of vaccines as an attempt to prevent streptococcosis, especially in tilapia commodities. Antibiotic susceptibility profiles present resistance to β-lactam, indicating sensitivity to tetracycline. Thus, the community tends to use the β-lactam group higher than antibiotics from the tetracycline group. Erythromycin presenting intermediate results conclude that there is a suspicion of excessive use of this antibiotic in the field. Antibiotic susceptibility profile is expected to provide additional information in the treatment and proper selection of antibiotics in the field of aquaculture activities.

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