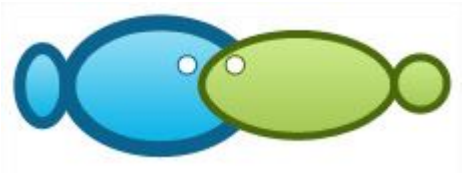

Characterization of *Streptococcus agalactiae* bacterium isolated from tilapia (*Oreochromis niloticus*) culture in Indonesia

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Abstract. *Streptococcus agalactiae* is the main pathogen that causes mortality and failure of tilapia culture in Indonesia. This research aimed to investigate the phenotype, susceptibility to antibiotics, and genotype of *S. agalactiae* bacteria from several regions in Indonesia. Characterization was carried out in morphology, physics, biochemistry, susceptibility to antibiotics, and molecular analysis. Bacterial samples were collected from tilapia during streptococcosis outbreaks with clinical signs as follow: melanosis, unilateral/bilateral exophthalmos, hemorrhage around eyes, corneal opacity, C-shape spinal curvature, erratic, whirling, and hemorrhage at the base of the fin. The results showed that all isolates were Gram-positive cocci, catalase negative, oxidase negative, grew on 6.5% NaCl, 40% bile salt, and did not significantly differ in growth at 37°C. Two biotypes of *S. agalactiae* isolates related to the outbreaks in tilapia culture in Indonesia were identified: β-haemolytics collected from Papua, Jambi and South Borneo and non-haemolytics from Java and Gorontalo. Molecular and BLAST tests at NCBI showed that all isolates were identified with *S. agalactiae* bacteria with 97-99% identity. Phylogenetic trees indicating that seven *S. agalactiae* isolates formed a clade with one comparable bacterium and three isolates forming other clusters.

Key Words: phenotypic, genotypic, Indonesia, *Streptococcus agalactiae*, tilapia.

Introduction. Tilapia (*Oreochromis niloticus*) is the future freshwater fish for aquaculture, as the development of hybridization and genetic engineering techniques enable this fish to be cultured in freshwater, brackish and marine environment, and it is productive, adaptive and has rapid growth in the tropics compared to the subtropical region (Yue et al 2016). The major producers of tilapia are several Asian countries including China, Indonesia, Philippines, Thailand, and South American countries which are Honduras, Ecuador, and Costa Rica (Nakharuthai et al 2016). Tilapia became an important commodity in Indonesia due to the total global production of 6,510,700 tons in 2017 in which Indonesia's contribution was 18.43% (Fitzsimmons 2018). Sumatera region is the largest producer of tilapia (47.8%), followed by Java (31.15%) and Sulawesi (11.53%) (DGA 2016). The increasing demand for animal protein makes intensive culture systems necessary, yet this culture may increase the risk of disease due to poor water quality, high stocking density, rapid changes in culture conditions, and these factors lead to increased stress and induce a number of agent causes of diseases mainly from streptococcal bacteria (Nakharuthai et al 2016). *Streptococcus agalactiae* is identified as the main cause of mortality in tilapia culture worldwide (Delannoy et al 2016; Kannika et al 2017). This bacterium has an impact on failure and have resulted in exceptionally large economic losses in the aquaculture industry of tilapia in several countries including Brazil (Mian et al 2009; Tavares et al 2016), Malaysia (Amal et al 2015), Thailand (Suanyuk et al 2008; Kayansamruaj et al 2014), China (Chen et al 2012), Egypt (Abu-Elala et al 2016) and Saudi Arabia (Al Harbi 2016).

Pathological studies of streptococcosis infection in tilapia culture in Lake Cirata were identified from the *S. agalactiae* bacteria as the causative agent (Lusiastuti et al 2014). Streptococcosis in Indonesia is also found in Sumatera, West Java, Central Java, Borneo, Sulawesi, and Papua. Clinical signs in fish infected with streptococcosis are almost the same which showed meningoencephalitis, hemorrhage around the anus and base of the fin, anorexia, unilateral/bilateral exophthalmos, hemorrhage of eyes, corneal opacity, abdominal swelling, C-shape spinal curvature, erratic, whirling, and hemorrhage at the base of the fin (Evans et al 2002; Li et al 2014).

Tilapia infected by *S. agalactiae* bacterium was identified in two different clusters based on phenotypic characteristics, biotype 1 (β-haemolytic) and biotype 2 (non-haemolytic). The biotype 1 bacterium infected the fish in the juvenile phase, while biotype 2 attacked the fish in the grow-out stage and was more virulent compared to biotype 1 (Sheehan et al 2009). The study of *S. agalactiae* in Indonesia showed that infections occurred in tilapia aquaculture center in Java were caused by non-haemolytic bacteria (Lusiastuti et al 2014), while streptococcosis infection in tilapia culture in other major islands in Indonesia has not been identified. A large number of cases of tilapia deaths with the symptoms of streptococcosis occurred outside Java, yet research into the exact cause of death had not been carried out. Cultivation conducted in Sumatera, Borneo, Sulawesi and Papua with an intensive system proved to be a contributor to more than 68% of national tilapia production (DGA 2016); therefore, research was needed to determine the type of bacteria causing streptococcosis outside Java. This research aimed to investigate the phenotype, susceptibility to antibiotics, and genotype of *S. agalactiae* from several regions in Indonesia, so that it can be used as a reference both for the prevention and manufacture of anti streptococcosis vaccines on tilapia.

Material and Method. This research was conducted in the microbiology and biomolecular laboratory of Fish Disease Control Research Station (IRP2I) Depok Indonesia from August 2017 to February 2018. This research collected *S. agalactiae* from various major island areas in Indonesia (Table 1). Bacteria obtained from tilapia with streptococcosis clinical signs were then cultured on brain heart infusion agar (BHIA; Oxoid Ltd, UK) and incubated for 24-48 hours at 28ºC. Bacterial identification was carried out by observing cell morphology, Gram staining, catalase test, motility test in semi-solid media, and oxidase test based on the Berge's Manual of Determinative Bacteriology (Holt et al 1994). Confirmation tests were also conducted by using API 20 Strep System commercial kit (bioMérieux Industry, Hazelwood, USA). Haemolytic activity testing was performed by growing bacteria in Blood Agar Base media with 5% (v/v) sheep blood, incubated for 18-24 hours at 37ºC.

Growth kinetic tests were measured to determine the rate of bacterial growth. The test was conducted by growing bacteria in the brain heart infusion media (BHI) and then incubated in 50 rpm orbital stuart incubator for 24 hours at 28ºC. Observations were made with a span of 4 hours until the bacteria entered the decline phase. Bacteria were in BHI media as long as the logarithm phase became the initial stock, and serial dilution was carried out. Then, the bacteria were cultured in BHIA and calculated using the total plate count method.

Table 1

Isolates of bacterial origin

Code	*Isolate*	*Origin*	*Size of tilapia (g)*
A	NP104O	Lake Sentani, Papua	200-250
B	NP105O	Lake Sentani, Papua	200-250
C	S01-196-16	Karang Intan, South Borneo	200-225
D	NB002O	Bogor, East Java	10-15
E	$N_{14}G$	Lake Cirata, East Java	12-15
F	NK1	Klaten, Central Java	30-50
G	NT010	Tasikmalaya, East Java	225-250
H	N_2O	Lake Cirata, East Java	12-15
I	SG01-16	Muaro, Jambi	150-200
J	NMbO	Lake Limboto, Gorontalo	150-200

Antimicrobial susceptibility. Antimicrobial susceptibility was analysed according to Kirby-Bauer (Bauer et al 1966). Ten strains of *S. agalactiae* were cultured on BHIA and incubated for 24 h on 30^{o}C, then were harvested and diluted to a turbidity equivalent to a MacFarland No. 0.5 standard solution, then spread onto triplicate Mueller-Hinton Agar (Oxoid Ltd, UK). All antimicrobials (Oxoid Ltd, UK) used were: chloramphenicol (C - 30 µg), chepalothin (KF - 30 µg), tetracycline (TE - 30 µg), methicillin (MET - 5 µg), enrofloxacin (ENR - 5 µg) and rifampicin (RD - 5 µg). Antimicrobial discs were inserted into bacterial inoculation and were incubated for 24-48h at 37^{o}C, and the inhibition zone diameter was calculated.

Identification of S. agalactiae. Colony of bacteria was suspended into 200 µL TE buffer (10 mmol L^{-1} Tris-HCl, one mmol L^{-1} EDTA; pH 8.0). Once homogenized, the suspension was heated in thermo mixer 98^{o}C for 10 min, and centrifugated on 14.000 rpm for 10 minutes. DNA concentration was measured by NanoDrop^{TM} Spectrophotometer (Thermo Scientific, USA).

Genotype identification of *S. agalactiae* was performed using method in Lusiastuti et al (2013) by modification using primer (Macrogen, Korea) sequence agal I 5'ATAAGAGTAATTAACACATGTTAG-3' (forward) and agal II 5'ACTTCGGGTGTTACAAAC-3' (reverse) with amplification target 1250 bp. The PCR amplification process was conducted in 25 µL reaction mix per sample: 12.5 µL master mix GoTaq®Green (Promega, Madison WI USA), 8.5 µL nuclease free water, one µL primer (reverse and forward), and two µL DNA template. Cycling conditions were 95ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1 min, followed by a final extension at 72ºC for 7 min. Amplicons were visualized by agarose gel electrophoresis using 12 µL amplicon in 1.5 % gel agarose on 12 volt in 1x Tris-acetate-electrophoresis buffer (TAE) [(0.04 mol L^{-1} Tris, 0.001 mol L^{-1} EDTA; pH 7.8)] for 15 minutes containing 100 bp DNA ladder (MBI Fermentas).

Sequencing and phylogenetic analysis. The PCR product was sequenced by 1st BASE DNA Sequencing Malaysia. The sequences generated were compared with GenBank database in National Center for Biotechnology Information using the Basic Local Alignment Search Tool (BLAST) by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates using MEGA7 software.

Challenge test of fish. Tilapia (*O. niloticus*) Nirwana III strains (17.4±1.4 g) were randomly distributed into ten groups across of 20 fish each. Fish were injected with β-haemolytic and non-haemolytic *S. agalactiae* with doses of 10^{11} and 10^{8} CFU fish^{-1}, respectively. The experimental procedures and treatment of tilapia in this study have been approved by the Indonesia local authorities (Animal Care and Use Committee, Bogor Agricultural University). The mortality of infected fish was recorded daily for two weeks, and the mean time to death (MTD) was calculated as described by Nitimulyo et al (2005).

Data analysis. Data growth kinetics test at 37ºC were subjected to one-way analysis of variance at significant test 5% using statistic software Minitab Ver.17. The other data were analyzed descriptively.

Results and Discussion. The morphology of *S. agalactiae* bacterial cells was Gram positive cocci with different characteristics. The diameters of non-haemolytic and β-haemolytic type cell colonies were around 0.591-0.748 µm and 0.787-1.231 µm respectively. Both types of bacteria could grow well at 28°C, and were not significantly different when the growth test is carried out at 37°C. The biochemical characteristics of all isolates showed non-motile, β-haemolytic/non-haemolytic, catalase and oxidase negative, fermentative positive, did not grow on media sulphide indole motility (SIM), grew on 6.5% NaCl media and bile salt media 40%. The characteristics in the confirmation test used API 20 STREP were different, and β-haemolytic bacteria

hydrolyzed more sugar than the non-haemolytic type. Test of hemolysis isolate activity in blood agar media showed a difference in hemolysis of blood (Figure 1). β-haemolytic bacteria had different characteristics when cultured on BHIA media i.e. they had thick colonies, more transparent, slimy and easily harvested, while non-haemolytic bacteria tended to be rather thin, yellowish, sticky and difficult to harvest. The characteristics of *S. agalactiae* were shown on Table 2.

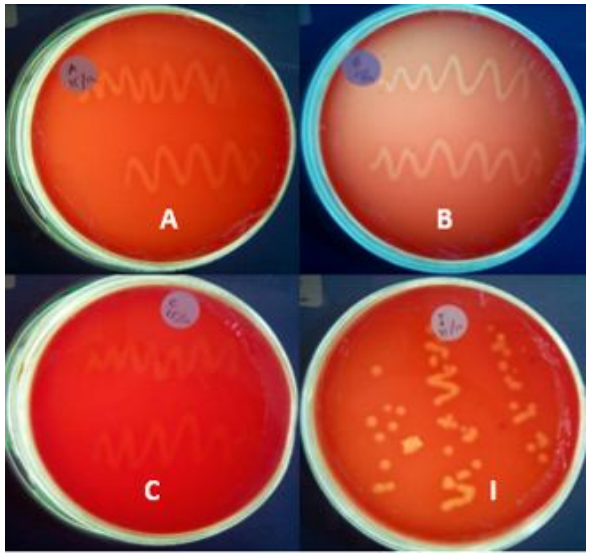

Figure 1. β-haemolytic type of *S. agalactiae*
(A: NP104O; B: NP105O; C: S01-196-16; and I: SG01-16).

Based on their morphological, biochemical and physical characteristics, bacterial isolates isolated from tilapia outbreaks were confirmed as *S. agalactiae* bacteria like previous research (Evans et al 2002; Al-Harbi 2016). Ten characterized isolates showed that 60% of the bacteria belonged to biotype 2 category from Java and Gorontalo, and the remaining 40% were biotype 1 from Sumatera, Borneo and Papua. *S. agalactiae* β-haemolytic was characterized by a lysis zone on blood agar media which could be used as an indicator of whether or not a bacterium was virulent. The brighter the lysis zone, the more virulent the bacterium.

S. agalactiae had varying cell colonies sizes in both β-haemolytic and non-haemolytic types, measuring by 0.6-1.2 µm (Pereira Ude et al 2013) and 0.5-2.0 µm (Ye et al 2011) in diameter. The diameter size of the β-haemolytic bacterial colonies was greater than that of non-haemolytic, possibly related to different environmental and virulence factors. Aquatic environments with intensive cultivation systems tended to have an impact on the size of larger colonies. Bacteria with larger cell colonies caused bacteria to be more virulent in tilapia.

In the period of 2009-2015, the streptococcosis outbreaks in tilapia were identified as a non-haemolytic type of *S. agalactiae*, and there were no reported β-haemolytic bacteria in Indonesia. β-haemolytic bacteria were associated with the serotype Ia, found mostly in tilapia culture in Thailand (Kayansamruaj et al 2015) and mostly spread in several Asian countries, while non-haemolytic bacteria were associated with serotype Ib which was reported to occur throughout the world and infected many fish species (Delannoy et al 2016). Several studies had revealed that non-haemolytic were more virulent (Sheehan et al 2009; Al Harbi 2016) than β-haemolytic bacteria. Characterization of *S. agalactiae* bacteria, carried out in in Malaysia (Laith et al 2017), Thailand (Kayansamruaj et al 2015), and China (Guo et al 2014), indicated that β-haemolytic bacteria were more dangerous and caused deaths in tilapia culture.

Table 2

Biochemical characteristics of bacterial isolates from tilapia in Indonesia

Characteristics	*Strains of Streptococcus agalactiae*										
	A	B	C	D	E	F	G	H	I	J	AH2*
Cell morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase	-	-	-	-	-	-	-	-	-	-	-
Gram	+	+	+	+	+	+	+	+	+	+	+
Oxidative/fermentative	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-
Growth in sulphide-indole-motility (SIM)	-	-	-	-	-	-	-	-	-	-	NT
Growth in bile salt 40%	+	+	+	+	+	+	+	+	+	+	NT
Growth in NaCl 6.5%	+	+	+	+	+	+	+	+	+	+	-
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	NT
D-Mannitol	-	-	-	-	-	-	-	-	-	-	NT
Aesculin	-	-	-	-	-	-	-	-	-	-	-
Haemolysis (5% sheep blood agar)	+	+	+	-	-	-	-	-	+	-	-

(-): negative reaction; (+): positive reaction; NT: no tested; (*) Al-Harbi (2016).

Bacterial growth was divided into four phases i.e. the lag phase, logarithmic (exponential) phase, stationary phase, and death phase. Bacterial adaptation to the new environment occurred in the lag phase. Varied bacterial adaptation was influenced by media, pH, temperature, and number of inoculant cells (Hardi 2011).

The phase lag started from 0-8th hour. The logarithmic phase generally started from the 12th to the 20th hour. The stationary phase started at the 24th and 28th hour then decreased at the 32nd hour. β-haemolytic bacteria growth of 10^{12} CFU mL^{-1} was faster compared to non-haemolytic 10^{9} CFU mL^{-1}, with the peak phase at the 24th hour. The number of new generations per time/second that grew from the 12th, 20th and 24th hour was $1.94x10^{7}$, $3.23x10^{7}$, $7.16x10^{7}$ and $1.5x10^{6}$, $8.1x10^{6}$, $2.26x10^{7}$ CFU seconds^{-1} respectively in *S. agalactiae* bacteria of both β-haemolytic and non-haemolytic types.

Delannoy's research results showed that the growth of β-haemolytic was faster than that of non-haemolytic bacteria (Delannoy et al 2013). Both types of bacteria could grow at 37°C for an incubation period of 24 to 48 hours. *S. agalactiae* can grow at a temperature of 5 to 45°C, yet the optimum growth occurred at 30°C with pH 7 (Laith et al 2017). There were differences in the growth pace of β-haemolytic and non-haemolytic bacteria when incubated at 28ºC, yet in general, they experienced a decrease phase in the 28th hour which was probably due to the lack of nutrients and an increased accumulation of toxin in the media inhibiting the bacterial growth.

The antimicrobial susceptibility test results of *S. agalactiae* bacteria to antibiotics were shown in Table 3. All dominant *S. agalactiae* strains were resistant to the antibiotics tested except for cephalothin antibiotics categorized to intermediate to 80% bacteria. Antibiotic resistance to *S. agalactiae* infection was a serious problem in tilapia cultivation. In this research, almost all bacteria were resistant to tetracycline, methicillin, enrofloxacin, and rifampicin. Similar results reported by Hardi (2011) in *S. agalactiae* isolated from tilapia showed resistance to tetracycline and methicillin. Tetracyclines are a wide spectrum group of aminoglycoside antibiotics which form a binding process for receptors found on the 30S ribosomal subunit of bacteria and prevent the binding of aminoacyl-tRNA from the ribosome site A; hence, they inhibit the protein translation. Methicillin is a group of bactericidal antibiotics used to inhibit bacterial cell wall synthesis, usually used for Gram positive bacteria that have formed resistance to antibiotics from β-lactam group.

Table 3

Antimicrobial susceptibility test of *S. agalactiae*

Antimicrobial agent	*Strains of Streptococcus agalactiae*									
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Cloramphenicol/C-30	R	S	R	I	S	R	S	I	R	R
Cephalothin/KF-30	I	I	I	I	R	R	I	I	I	I
Tetracycline/TE-30	R	R	R	R	I	R	I	I	R	I
Methicillin/MET-5	R	R	R	R	R	R	I	I	R	S
Enrofloxacin/ENR-5	R	R	R	R	R	R	S	I	R	I
Rifampicin/RD-5	R	R	R	R	R	R	I	R	R	S

R: resistant; S: sensitive; I: intermediate.

Various studies have been conducted and the presence of isolates is associated with environmental conditions, including high water temperatures, high ammonia levels, low dissolved oxygen levels and high stocking density (Suanyuk et al 2008; Mian et al 2009), and is likely to affect the virulence factors of *S. agalactiae* in tilapia (Evans et al 2015). The occurrence of *S. agalactiae* outbreaks in tilapia culture might be due to the error in managerial implementation, high stocking density, and low water quality parameters such as elevated water temperature, high levels of ammonia and nitrite, and low levels of dissolved oxygen in water (Al-Harbi 2016). Temperature, clarity, and pH of the water showed a significant correlation to the attack of *S. agalactiae* on fish cultured in lakes and in rivers (Amal et al 2015).

Ten bacterial isolates were sequenced with agal I and agal II universal primers, and all isolates were confirmed as *S. agalactiae*. BLAST results obtained 97-99% of *S. agalactiae* identity strain (Genbank accession number MF.113267.1). Phylogenetic trees showed that seven isolates formed clades with *S. agalactiae* accession number of KM209201.1, and second clade NT01O, S01-196-16, NMbO with *S. agalactiae* accession numbers of MF113267.1 and KM209200.1 (Figure 2).

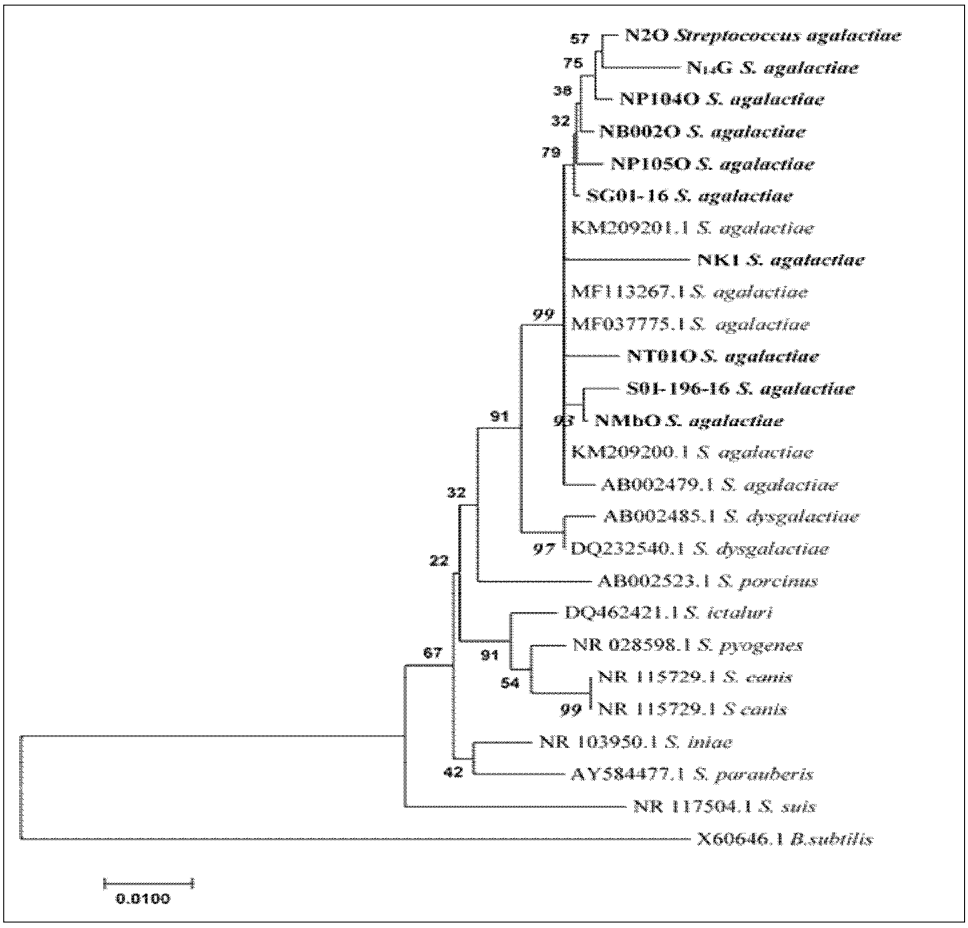

Figure 2. The phylogenetic tree showing of *Streptococcus agalactiae* from the present study based on 16S rRNA gene sequences. Neighbor-joining tree was constructed using MEGA7 maximum composite likelihood method and 1000 bootstrap replicates.

The biotypes of bacterial variations found in Indonesia were different in accordance with the geographical conditions, and the possibility of these differences was influenced by different regional characteristics (Figure 3). Sumatera, Borneo and Papua are dominated by peatlands with high organic matter, and the pH is acidic (Margono et al 2014). The condition was different in the case of Limboto lake, a karst mountainous area causing the pH to be alkaline. The results showed that the genetic diversity of *S. agalactiae* in Thailand analyzed by Random Amplified Polymorphic DNA (RAPD) was influenced by geographical factors (Kayansamruaj et al 2014), as well as serotype and virulence (Kannika et al 2017).

The MTD results showed that β-haemolytic *S. agalactiae* SG01-16 caused fish mortality within 9.30 hours, whereas non-haemolytic $N_{14}G$ strains were around 165.65 hours post-injection. In contrast, non-haemolytic NMbO isolates caused fish mortality within 207.16 hours. Clinical signs observed in infected fish were melanosis, anorexia, swimming abnormalities, curved body shape, corneal opacity, and hemorrhage; however, unilateral/bilateral exophthalmos symptoms were not detected. Challenging tests of β-haemolytic *S. agalactiae* showed acute infection resulting in rapid death on fish. In contrast, non-haemolytic bacteria showed chronic infection causing slower mortality and

all clinical signs of streptococcosis occurred. Previous reports in challenging tests of *Vibrio alginolyticus* in groupers showed that MTD was at 12-28 hours and was categorized as acute bacterial infection (Murdjani 2002). The death pattern due to *S. agalactiae* infection in tilapia tended to be chronic, and mostly mortality occurred within a span of 10 days (Li et al 2014).

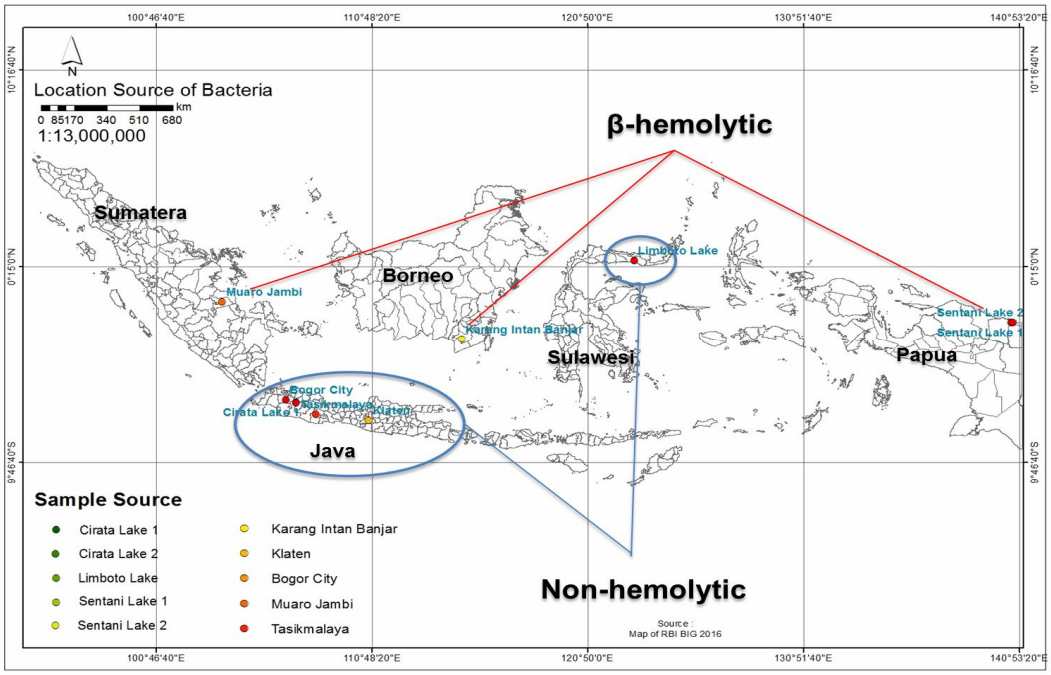

Figure 3. Distribution of *S. agalactiae* biotype from various major islands in Indonesia.

Streptococcosis infections caused by non-haemolytic *S. agalactiae* were firstly reported in Lake Cirata in West Java which attacked tilapia weighing 12-15 g fish^{-1}. Meanwhile, similar cases occurred in Lake Limboto in Gorontalo province in 2016 in which infected tilapia had a weight of about 200 g fish^{-1}. β-haemolytic bacteria were found to infect harvested-sized tilapia and potential breeders with weights above 200 g fish^{-1}. Sheehan et al (2009) reported that biotype 1 bacteria attacked fish in the juvenile phase. The attack pattern of *S. agalactiae* in Indonesia had undergone changes, both types of bacteria attacked tilapia culture in various ages and stages.

Streptococcosis is a major problem in tilapia culture caused by the *Streptococcus* sp. bacteria species of *S. agalactiae, S. iniae, S. parauberis, S. difficile, S. shiloi,* and *S. dysgalactiae* (Evans et al 2006; Costa et al 2014; Pradeep et al 2016). The case of tilapia deaths in several aquacuture centers in Indonesia was generally caused by infections of *S. agalactiae* of 85% and *S. iniae* of 15% (Taukhid & Purwaningsih 2011). Characterization is important as a reference for preventing streptococcosis infection through vaccination. Commercial vaccines from non-haemolytic *S. agalactiae* bacteria have been produced, yet are still unable to give protection against haemolytic bacterial strains (Delannoy et al 2013).

Conclusions. Based on the morphology, physics, biochemical and molecular test, it was confirmed that the *S. agalactiae* bacteria is the cause of streptococcosis in tilapia. There are two types of β-haemolytic and non-haemolytic bacterias, which, based on the phylogenetic trees, devided into two large groups. The research regarding the characterization of *S. agalactiae* β-haemolytic and non-haemolytic which are isolated from the tilapia in Indonesia is the first time being done and it is hoped to be able to be used as a reference for the development of a vaccine streptococcosis prevention.

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