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Aeromonas hydrophila AHL 0905-2 and *Streptococcus agalactiae* N14G as Combined Vaccine Candidates for Nile Tilapia

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ABSTRACT

In Indonesia, the Nile tilapia (*Oreochromis niloticus*) is the most widely farmed and available fish for consumption. Production loss due to bacterial infection by *Aeromonas hydrophila* and *Streptococcus agalactiae* is the main problem in tilapia cultivation. This study aimed to determine the occurrence of concurrent infection of *Aeromonas hydrophila* AHL 0905-2 and *Streptococcus agalactiae* N14G in Nile tilapia based on biochemical and molecular characteristics. From the results of biochemical assay and sequence analysis of the 16S rRNA fragment, *Aeromonas hydrophila* and *Streptococcus agalactiae* were confirmed. Genes for aerolysin (417 bp), nuclease (504 bp), lipase (155 bp), and serine protease (211 bp) were found in *Aeromonas hydrophila* AHL 0905-2, while *Streptococcus agalactiae* N14G was determined as a 1b serotype group that had genes for CPS L (688 bp), CPSG (621 bp), and CPS J (272 bp). The confirmation in tilapia of *Aeromonas hydrophila* and *Streptococcus agalactiae* by PCR and sequencing is important for enabling the detection of these organisms and also for the development of a combined vaccine to tackle co-infection.

1. Introduction

Indonesia is the second-largest producer of Nile tilapia (*Oreochromis niloticus*) in the world (after China) (FAO 2017). The species has high economic value and is suitable for aquaculture because of its tolerance to handling, fast growth, tolerance of a wide range of environmental conditions, such as pH, temperature and salinity, and its high marketability (Hassanien *et al.* 2004). However, tilapia is susceptible to many diseases caused by single or multiple microbial pathogen infections that can cause high mortality rates in cultivation (Dong *et al.* 2015). Motile Aeromonas Septicemia (MAS) caused by *A. hydrophila* and streptococcosis caused by *S. agalactiae* are bacterial diseases threatening the survival of these fish through co-infection.

Streptococcus agalactiae or Lancefield Group B Streptococcus (GBS) is a Gram-positive bacterium commonly causing streptococcosis in tilapia. Worldwide, this bacterial pathogen is regarded as the most serious disease for tilapia, and in the last decade has become the major infection in these fish (Garcia *et al.* 2008; Al Harbi 2016). The bacterium can also infect other fresh and seawater fish (Garcia *et al.* 2008; Bowater *et al.* 2012; Al Harbi 2016). The main entry point of of *S. agalactiae* infection is the gastrointestinal epithelium, leading to fibrinous pericarditis and peritonitis, with hemorrhages around the brain, retrobulbar regions, and intestines resulting in severe consequences (Iregui *et al.* 2015).

Aeromonas hydrophila is known worldwide as causing septicemia disease that affects numerous species of freshwater and marine fish. It is also considered as the most significant disease affecting fish farming (Aoki 1999). Congestion and hemorrhage of the abdominal wall and the base of the fins, together with scale erosion of various parts of the body, are the marked clinical signs observed (Asaad 2008). The infection caused by this bacteria is also recorded as causing severe congestion of internal organs with the accumulation of ascetic fluid in the abdominal cavity, swelling of the kidney and spleen, features on the surface of the body and gills, ulcers, abscesses, exopthalmias, and bloated stomach (Austin and Austin 1993). In Indonesia, MAS is an economic hazard causing severe losses in cultured freshwater fish species, including *O. niloticus*, common carp, and catfish. However, increasing water changing,

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elevation of aquatic column highs, and cessation of feeding can help in reducing the deleterious effects of outbreaks, although not solving the problem completely.

Co-infection occurs when hosts are infected by two or more different pathogens, either simultaneously or as secondary infections, so that two or more infectious agents are active together in the same host (Kotob *et al.* 2016). In this study, *Aeromonas hydrophila* AHL 0905-2 and *Streptococcus agalactiae* N14G are characterized based on biochemical characteristics and hemolytic ability. Therefore, this study aims to determine the identification of concurrent infection of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G in Nile tilapia through molecular PCR and sequencing methods.

2. Materials and Methods

2.1. Koch's Postulates Test

The *A. hydrophila* (AHL 0905-2) and *S. agalactiae* (N14G) bacterial isolates were obtained and analyzed using Koch's postulates (PK). An overnight culture of the isolates was prepared in a bacterial suspension (log-phase growth) through the following process: a single colony of each bacterial isolate was inoculated in 10 ml of Tryptic Soy Broth (TSB) and Brain Heart Infusion Broth (BHIB) (for *A. hydrophila* and *S. agalactiae*) at 28°C for 24 and 48 h, respectively. Afterwards, the bacterial suspension was adjusted to OD 600 nm at 0.55 to 0.60 for both isolates, respectively, before applying the PK process. A total of ten healthy fish (mean weight, 10 cm ± 10 g) were then each intraperitoneally injected with 1×10^6 CFU/ml and 1×10^8 CFU/ml of *A. hydrophila* (AHL 0905-2) and *S. agalactiae* (N14G), respectively.

2.2. Bacterial Isolation

In this study, two media, namely Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) (Oxoid Ltd, UK), were used in the culture of *A. hydrophila* and *S. agalactiae* infections, respectively. The targeted organs of diseased fishes were aseptically obtained by inserting a sterile loop into the target tissue and streaking them directly onto the different media. The plates were incubated at 28°C for 24 (TSA) and 48 (BHIA) hours, respectively.

2.3. Biochemical Characteristics

In this study, biochemical assays were also performed in order to identify culturable isolates of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G. All isolates were subjected to primary tests, including hemolysis ability, Gram staining, oxidation/fermentation (O-F), motility, oxidase, catalase, API 20 E, and API 20 Strep. The bacterial isolates were cultured at 28°C for 4 and 24 hours.

2.4. DNA Extraction from Colony Bacterial Isolates

The genomic DNA of pure bacterial isolates was extracted by the boiling method, as previously described by Arias *et al.* (2004), with modification. After bacterial isolates were cultured for 18–20 hours in agar media they were collected from 1 to 2 colonies by sterile loop. Afterwards, cell pellets were suspended in 500 µl nuclease-free water and boiled for 10 mins, before being immediately cooled down in ice. After centrifuging at 1,200 rpm for 10 mins, supernatant containing DNA templates was obtained and used in the PCR assay.

2.5. Specific PCR Assays

2.5.1. Detection of *A. hydrophila* AHL 0905-2

PCR detection assays of *A. hydrophila* AHL 0905-2 were performed using DNA templates extracted from isolated bacterial colonies grown in the TSA medium (Oxoid Ltd, UK). Specific primers (Table 1) targeting virulence genes of *A. hydrophila,* as described by Nam and Joh (2007) with Novita *et al.* (2018), were also used in this study. The PCR mixtures contained 12.5 µl master mix (GoTaq®Green, Promega, USA), 10 pmol of each primer, and 2 µl of DNA template in a final volume of 25 µl. Amplification was carried out in a thermocycler (MJ Research) through denaturation at 94°C for 2 mins accompanied by 30 cycles of amplification at 95°C for 1 min. Afterwards, annealing at 55°C for 1 min was conducted, with ordinal and final extensions both carried out at 72°C for 1 min. The genomic DNA of the isolate was further used as a template and with nuclease-free water for both positive and negative controls, respectively. Also, the amplified products of *A. hydrophila* were visualized by GelDoc, after being electrophoresed with 1.5% agarose gel in TAE 1x and stained with cybersafe (Promega).

2.5.2. Detection of *S. agalactiae* N14G

Investigation of *S. agalactiae* N14G infection was carried out using serotyping primers (Table 1) (modification of Imperi *et al.* 2010) with the bacteria being cultured in BHIA medium. PCR assay was performed in the same way as described for *A. hydrophila* detection. The thermocycling conditions applied were 95°C for 5 mins accompanied by 35 cycles at 94°C for 1 min. Afterwards, annealing and extension were carried out at 50°C and 72°C for 1 min each, with final extension then conducted at 72°C for

Table 1. Oligonucleotide primers used in PCR assays for *A. hydrophila* and *S. agalactiae*

Gene	Primers	Sequence (5'--->3')	Band PCR (bp)
A. hydrophila			
16S rDNA	16S-rDNA-F	AGAGTTTGATCATGGCTCAG	1,502
	16S-rDNA-R	GGTTACCTTGTTACGACTT	
Nuclease	Nuc-F	CAGGATCTGAACCGCCTCTATCAGG	504
	Nuc-R	GTCCCAAGCTTCGAACAGTTTACGC	
Aerolysin	Aero-F	GAGCGAGAAGGTGACCACCAAGAAC	417
	Aero-R	TTCCAGTCCCACCACTTCACTTCAC	
Serine	Ser-F	ACGGAGTGCGTTCTTCCTACTCCAG	211
Protease	Ser-R	CCGTTCATCACACCGTTGTAGTCG	
	Lip-F	GACCCCCTACCTGAACCTGAGCTAC	155
Lipase	Lip-R	AGTGACCCAGGAAGTGCAC CTTGAG	
S. agalactiae			
cps L	cps L-F	CAATCCTAAGTATTTTCGGTTCATT	688
	cps L-R	TAGGAACATGTTCATTAACATAGC	
cps J	cps J-F	GCAATTCTTAACAGAATATTCAGTTG	621
	cps J-R	GCGTTTCTTTATCACATACTCTTG	
cps G	cps G-F	ACATGAACAGCAGTTCAACCGT	272
	cps G-R	ATGCTCTCCAAACTGTTCTTGT	

7 mins. The DNA template extracted from the isolate of *S. agalactiae* was also used with nuclease-free water for positive and negative controls, respectively. Finally, amplified products were electrophoresed and visualized using GelDoc.

2.6. 16S rDNA Amplification and DNA Sequencing

The two sets of universal primer targeting prokaryotic 16S rDNA used in this study (Table 2) included F/R (Nam and Joh *et al.* 2007) for isolates of *Aeromonas* spp. and *S. agalactiae* (Agal I F1 and Agal II R) (Yildirim 2002; Lusiastuti *et al.* 2013). The PCR mixtures contained 10 pMol of each primer pair, 12.5 µl master mix (GoTaq®Green, Promega, USA), and 2 µl of DNA template in a final volume of 25 µl. Amplification was carried out in the MJ Research thermocycler through denaturation at 94°C for 2 mins accompanied by 30 and 25 cycles of amplification at 95°C for 1 min. Afterwards, annealing at 55°C and 50°C for 1 min was carried out, with initial and final extension also conducted for both at 72°C for 1 min, with amplified products of ~1.5 kb and 1.2 kb, respectively. The unpurified DNA amplicons were submitted for DNA sequencing to 1st BASE Pte Ltd, Singapore, with homology search being carried out using nucleotide BLAST from the GenBank database of the National Center for Biotechnology Information (NCBI). In this study, multiple sequence alignments of the 16S rRNA gene sequences of the bacterial isolates and their closed taxa were retrieved from GenBank using the Clustal W method. A phylogenetic tree was constructed through the use of the neighbor-joining method of MEGA version X software (Tamura *et al.* 2011).

Table 2. 16SrDNA of *A. hydrophila* and *S. agalactiae*

Primer pair	thermocycler condition
	95°C for 5 min
A. hydrophila (16 Sr DNA): 1,502 Bp	94°C for 1 min
(Nam and Joh *et al.* 2007)	55°C for 1 min
16S-rDNA-F: AGAGTTTGATCATGGCTCAG	72°C for 1 min
16S-rDNA-R: GGTTACCTTGTTACGACTT	72°C for 7 min
	30 cycles
S. agalactiae (Agal I, II): 1,250 bp	95°C for 5 min
(Yildirim 2002; Lusiastuti *et al.* 2013)	94°C for 1 min
agal I- 5'-ATAAGAGTAATTAACACATGTT AG-3'	50°C for 1 min
agal II-5'-ACTTCGGGTGTTACAAA C-3'	72°C for 1 min
	72°C for 7 min
	35 cycles

3. Results

3.1. Koch's Postulates Test

The Koch's postulates of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G bacteria, with the aim of conducted to verify abaility to couse disease of these bacteria in nile tilapia (see Figure 1). After 24 hours of bacterial infection, clinical symptoms occurred on the surface of the body, as the belly of the fish turned white and hemorrhagic extensions became evident on body surfaces, the base of the caudal fin, and the operculum. Changes in internal pathology, such as swelling of the liver and spleen and bleeding in stomach also tended to occur. The occurrence of these symptoms indicated the presence of *A. hydrophila* AHL 0905-2 infection. However, this bacteria was likely to be isolated from the kidney and liver. The symptoms of infection with

Figure 1. (A) Co-infection by *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G in tilapia after postulate assays, with hemorrhagic extensions on body surface leading to swelling of the liver, (B) spleen and bleeding in stomach, (C) necrotic gills, swollen gall bladder, hemorrhagic liver, protruding eyes, bloated stomach, bleeding in the eyes, hemorrhages of the gill and base of tail, black appearing on body surface

S. agalactiae N14G included disorientation, swimming in circles on the surface, protruding, bloating of the stomach, bleeding in the eye, hemorrhages of the gills and base of tail, body surfaces becoming black, and chaotic fast-swimming activity. Also, the internal organs exhibited damage to the kidneys, liver, spleen, and intestines. *S. agalactiae* N14G was isolated from the brain.

3.2. Bacterial Isolation and Biochemical Characterization

Re-isolation of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G after Koch's postulate assay in tilapia was carried out by re-culturing in TSA and BHIA for screening. The results obtained for characteristics and identification of these bacterial species by biochemical characterization are shown in Table 3. Based on biochemical characterization, *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G were identified as Gram-negative and Gram-positive, respectively. Also, *A. hydrophila* AHL 0905-2 was positive for short rod, motility, and API 20 E tests. The results for API 20 E showed positive for esculin hydrolysis, Voges-Proskauer test, growth at 37°C, galactosidase, arginine dihydrolase, arabinose, glucose, mannitol, hemolysis, H_2S production, indole, gelatin hydrolysis, aesculin hydrolysis, development of KCN, selisin, and sucrose. The biochemical characterization of *S. agalactiae* N14G was positive for coccus form, fermentative, esculin hydrolase, d-mannitol acid, and growth at 37°C. The API 20 STREP test results further showed that *S. agalactiae* N14G was a non-hemolytic bacterium with the ability to hydrolyze sugars from sodium pyruvate, hippuric acid, arginine dihydrolase, ribose, sorbitol, lactose, trehalose, and amidon. The ability of this bacterium to hydrolyze sugar indicates its tendency to survive in the host's body by utilizing the nutrients present in the fish.

3.3. PCR Assays of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G

Based on gene virulence and serotyping, confirmations of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G by PCR were also conducted. Virulence factor gene detection of *A. hydrophila* AHL 0905-2 was carried out using specific primers, such as aerolysin (417 bp), nuclease (504 bp), lipase (155 bp), and serine protease (211 bp) (Figure 2). *A. hydrophila* AHL 0905-2 was positive with 16S rDNA, as PCR bands resulted

Table 3. Morphology and biochemical characterization *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G d: variable reaction, (+): positive, (-): negative, F: fermentative

Biochemical characterization API 20 E	*A. hydrophila* AHL 0905-2	Biochemical characterization API 20 Strep	*S. agalactiae* N14G
Form	Short rod	Form	Coccus
Motility	+	Motility	-
Gram	-	Gram	+
Esculin hydrolisys	+	oxicidase	-
Voges-Proskauer	+	O/F	F
Growth at 37°C	+	catalase	-
Diffusible brown pigment	-	Bile salt	-
B-galactosidase	+	NACl 6.5%	-
Arginine dihydrolase	+	Esculin hydrolase	+
Arabinosa	+	D- Mannitol acid	+
Glucose	+	Growth at 37°C	+
Inositol	-	Hemolysis	-
Manitol	+	Sodium piruvate	+
Hemolysis	+	hipuric acid	+
Lysine decarboxylase	d	Escullin ferric citrate	-
Ornithine decarboxylase	-	Pyroglutamic acid-β-naphtylamide	-
Simmons citrate	d	α-Galactosidase	-
H_2S production	+	β-Glucuronidase	-
Urease	-	β-Galactosidase	-
Indole	+	Alkaline Phosphatase	-
Gelatine hydrolysis	+	L-Leucine-β-naphtylamide	-
Aesculin hydrolysis	+	L-Arginin	+
Growth in KCN	+	D-Ribose	+
Selisin	+	L-arabinose	+
Sorbitol	+	D-mannitol	-
Sucrose	+	D-Sorbitol	+
L-rhamnose	-	D-Lactose	+
D-sucrose	+	D-trehalose	+
D-melibiose	-	Inuline	-
Amygdalin	+	D-Raffinose	-
L-arabinose	-	Amidon	+
Oxidase	-	Glycogen	-

d: variable reaction, (+): positive, (-): negative, F: Fermentative

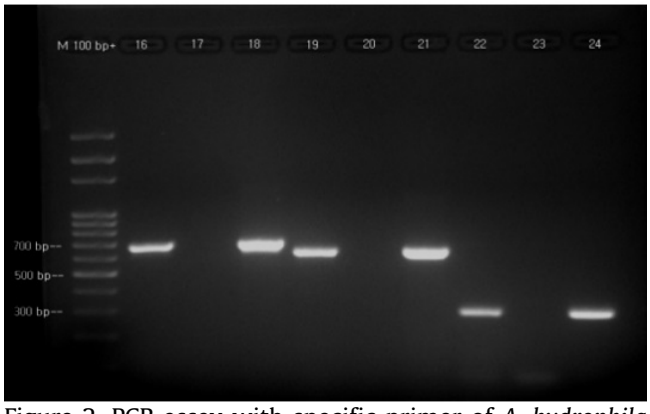

Figure 2. PCR assay with specific primer of *A. hydrophila* AHL 0905-2, M: Marker 100 bp, 1: *A. hydrophila* AHL 0905-2, 2: (-)ve control, 3: (+)ve control 16s rDNA (1,502 bp), 4: *A. hydrophila* AHL 0905-2, 5: (-)ve control, 6: (+)ve control nuclease (504 bp), 7: *A. hydrophila* AHL 0905-2, 8: (-)ve control, 9: (+)ve control aerolysin (417 bp), 10: *A. hydrophila* AHL 0905-2, 11: (-)ve control, 12: (+)ve control serine protease (211 bp), 13: *A. hydrophila* AHL 0905-2, 14: (-)ve control, 15: (+)ve control lipase (155 bp)

of 1,502 bp. Also, the 16S rDNA gene sequence was observed in almost all types of bacteria and used as a source of information for the microorganisms.

Three primer pairs were set up to discriminate the serotypes of *S. agalactiae* N14G with amplicon patterns through the use of PCR assay. The primer set was designed from *S. agalactiae* serotype group 1b based on the standard amplicon patterns described previously by Table 1. This primer created amplicons of CPS L (688 bp), CPS J (621 bp), and CPS G (272 bp) (Figure 3). The molecular serotyping of *S. agalactiae* N14G indicated that according to biochemical assay it was a non-hemolytic bacterium. Therefore, *S. agalactiae* N14G could be used in the development of a combined vaccine in tilapia.

3.4. Sequence Results of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G

The genomic DNA of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G isolated and extracted from the isolates was then used as a template for the

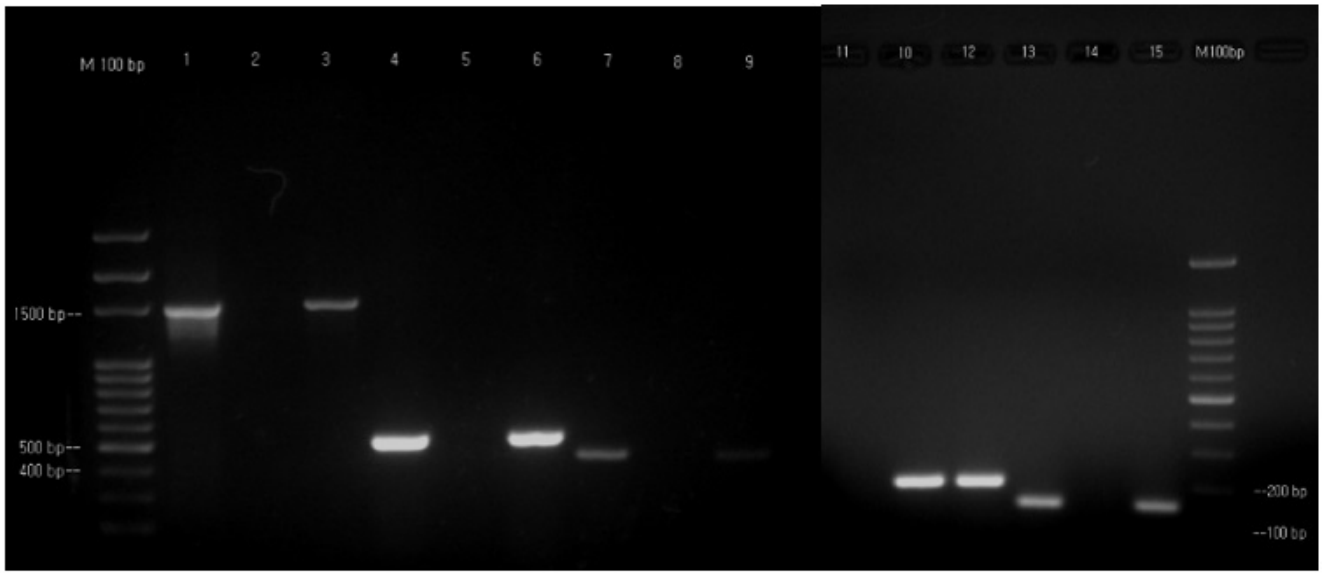

Figure 3. PCR assay of *S. agalactiae* based on serotyping in group 1b. M: Marker 100 bp; 16: *S. agalactiae* N14G; 17: (-)ve control; 18: (+)ve control CPS L (688 bp); 19: *S. agalactiae* N14G; 20: (-)ve control; 21: (+)ve control CPS J (621 bp); 22: *S. agalactiae* N14G; 23: (-)ve control; 24: (+)ve control CPS G (272 bp)

detection of 16S rRNA and serotyping using the PCR method. This amplification process was expected to amplify the usual 16S rRNA gene, which measured up to 1,500 bp in length. The results of the amplification were obtained through electrophoresis with agarose and visualized with GelDoc to identify species of bacteria. Afterwards, amplification was carried out by the sequencing process in order to confirm *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G. The results of identification via BLAST are shown in Figure 4.

Based on the BLAST findings, the nucleotide sequences of *A. hydrophila* AHL 0905-2 showed 100% similarity with *A. hydrophila* strain F_28 (MG428737.1). Also, the virulence genes of this bacteria had 97.84%, 99.48%, 99.42%, and 99.48% similarities with *A. hydrophila* subsp. strains WCHAH045096 (CP028568.2), PB80AH1 (AY442276.1), AH-1 (AY841795.1), and AHLIP7 (AB237179.1) for nuclease, aerolysin, serine protease, and lipase, respectively. Profile nucleotide sequences used serotyping for molecular identification results, as *S. agalactiae* N14G obtained by homologous levels had 98.39%, 98.64%, 96.75%, and 98.93% similarities with *Streptococcus agalactiae* strains QMA0271 (CP029632.1), YZ1605 (CP026082.1), NCTC8187 (LT671984.1), and (MT626756.1) for CPS L, CPS G, and AGAL12, respectively.

Also, sequence alignment of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G from the database Gene Bank was carried out using the MEGA X program. In order to obtain a phylogenetic tree, the NJ program on Clustal W software was used with a level of 1000x bootstrap, with results as shown in Figure 4. The phylogenetic tree shows the relationships between both of these species based on the different molecular characteristics of t strains within the same species.

Figure 4 confirms the relationship level of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G. Therefore, the isolates already existing from the morphological and biochemical test results were shown to be *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G.

4. Discussion

Co-infection of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G in tilapia often occurs and causes economic losses for fish farmers resulting from slow fish growth, longer maintenance time, high feed conversion, low stocking density, and higher mortality rates. Conventional characterization of these two bacteria by biochemical tests has often been inconsistent and weak in determining bacterial species discriminatively, with molecular detection proving to be more accurate. Also, it is known that, based on the surface of the polysaccharide antigen, *S. agalactiae* has several serotypes, namely Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX, with some having protein antigens such as C, R, and X (Gravekamp *et al.* 1999; Imperi *et al.* 2010). Hitherto, in order to determine *S. agalactiae*, capsular molecular serotyping with sequencing provided an accurate method of typing

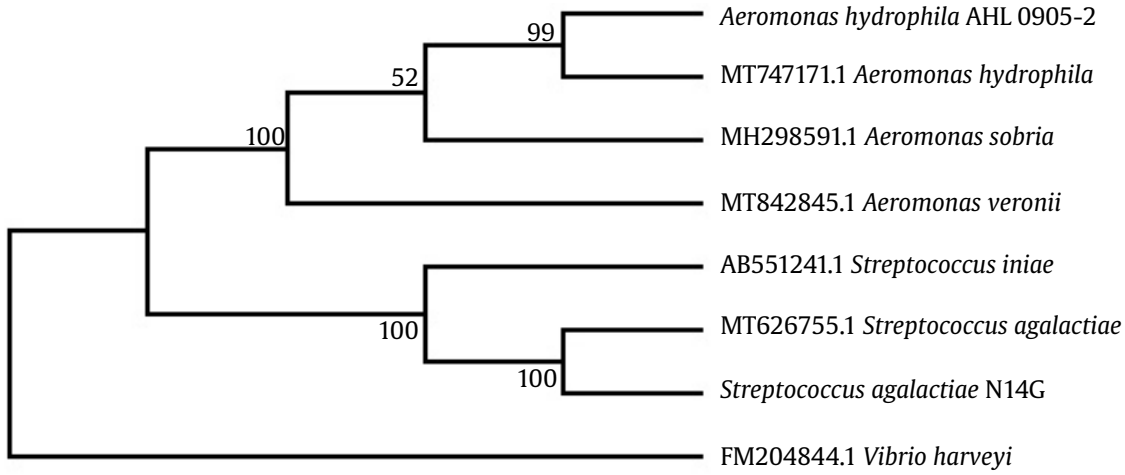

Figure 4. Philogeny tree with bootstrap 1000x of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G using the MEGA X software with NJ program on Clustal W

(Jones *et al.* 2003; Carvalho *et al.* 2017; Kapatai *et al.* 2017). This was due to the fact that capsular serotypes were more important for vaccine formulation, and capsular polysaccharides (CPS), which are highly immunogenic, provide the best form of protection against infections (Eldar *et al.* 1995; Berkley *et al.* 2016). Also, the serotypes Ia and Ib of *S. agalactiae* are often discovered in aquatic animals.

Furthermore, with *A. hydrophila* AHL 0905-2 also having many strains, this bacteria is another pathogen causing disease. As reported by Dong *et al.* (2015, 2017), other pathogens causing diseases apart from co-infection by *A. hydrophila* and *S. agalactiae* are Iridovirus and TiLV, with symptoms and target organs mimicking the main internal and external clinical signs of naturally infected tilapia. However, the genetic diversity of *A. hydrophila* bacteria is very high. Several studies of the diversity of virulence factor genes in *A. hydrophila* were often associated with the degree of pathogenicity and toxins produced (Wang *et al.* 2003). The infectious pathogenicity of *A. hydrophila* was due to the production of several virulence factors, such as proteases, hemolysins, aerolysins, and cytolytic enterotoxins, which cause disease in fish and humans (Kingombe *et al.* 2010; Hu *et al.* 2012). The secretion of extracellular hemolysin and cytolytic enterotoxin by these bacteria is an important factor causing certain lytic activities in host cells (Watanabe *et al.* 2004; Uma *et al.* 2010). Additionally, aerolysin (aerA) is one of the virulence markers used to identify the pathogenicity of *Aeromonas* sp. (Zhang *et al.* 2013).

Based on the results of molecular analysis, *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G have been identified by virulence genes and their serotypes. Both of these bacteria are also used in the development of a combined vaccine and for ascertaining the nature of both bacteria. Also, the two isolates used together in a combination vaccine provide synergy that increases the potency of the drug to suppress the attacks of both pathogens, providing better protection.

To overcome co-infection of these bacteria, antibiotics are often used, giving rise to bacterial resistance and also influencing the environment. A more effective and safe disease control alternative is therefore needed, through developing an efficient vaccine for preventing bacterial attack by *A. hydrophila* and *S. agalactiae* in tilapia. The co-infection of these two pathogens in the culturing of tilapia in Indonesia is an important consideration in developing knowledge about the MAS and streptococcus diseases causing economic losses in tilapia aquaculture.

In conclussion, co-infection of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G was confirmed as the virulent strains. Information relating to the identification of *A. hydrophila* AHL 0905-2 and *S. agalactiae* N14G is important for the development of a combined vaccine as one of the ways of preventing co-infection by these pathogenic diseases in Nile tilapia.

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