Streptococcosis on Nile tilapia (*Oreochromis niloticus*) in Indonesian freshwater aquaculture

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ABSTRACT

This review aimed to provide the current status of streptococcosis in freshwater aquaculture in Indonesia. So far, this disease has been mostly reported in tilapia aquaculture, and there have been no serious reports of this disease in other species of freshwater aquaculture. The disease is mostly encountered during the dry season or the transition period from the dry season to the rainy season or vice versa. Initially, the disease was mostly reported in tilapia larger than 250 grams, but in its development, it was also reported in smaller fish. The Ministry of Marine Affairs and Fisheries (MMAF) has classified this disease as a major disease in tilapia in Indonesia since 2014. Based on the characteristics of *Streptococcus agalactiae* isolates collected from diseased tilapia from various regions in Indonesia, two biotypes were found consisting of β-hemolytic *S. agalactiae* (biotype I) and non-hemolytic *S. agalactiae* (biotype II). Biotype I cause disease with acute mortality patterns, while biotype II causes disease with chronic and persistent mortality patterns. Based on susceptibility to *S. agalactiae* during artificial infection testing, it is known that all tilapia strains that have been released to the public for aquaculture purposes are susceptible to infection with the bacteria; although each strain has a different level of susceptibility. Fish health management strategies that are based on preventive measures and relevant to sustainable aquaculture programs should be based on ecological-biological approaches such as the implementation of biosecurity systems, the use of vaccines for endemic diseases, probiotics, essential herbs, and the development of more disease-resistant populations.

Keywords: health management, streptococcosis, *Streptococcus agalactiae*, tilapia

ABSTRAK

Tulisan ini bertujuan untuk menyajikan status terkini tentang penyakit streptococcosis pada perikanan budidaya air tawar di Indonesia. Sejauh ini, penyakit ini lebih banyak dilaporkan terjadi pada budidaya ikan nila, dan belum ada laporan serius akibat penyakit ini pada jenis ikan budidaya air tawar lainnya. Penyakit ini lebih banyak ditemukan pada musim kemarau atau masa peralihan dari musim kemarau ke musim hujan atau sebaliknya. Pada mulanya penyakit ini lebih banyak dilaporkan pada ikan nila yang berukuran lebih dari 250 gram, namun dalam perkembangannya ditemukan pula pada ikan berukuran kecil. Kementerian Kelautan dan Perikanan (KKP) sejak tahun 2014 telah mengklasifikasikan penyakit ini sebagai penyakit utama pada ikan nila di Indonesia. Berdasarkan karakteristik isolat *Streptococcus agalactiae* yang diisolasi dari ikan nila sakit dari berbagai daerah di Indonesia, ditemukan dua biotipe yang terdiri dari *S. agalactiae* β-hemolitik (biotipe I) dan *S. agalactiae* non-hemolitik (biotipe II). Biotipe I menyebabkan penyakit dengan kematian berpola akut; sedangakan biotipe II menyebabkan penyakit dengan kematian berpola kronis dan persisten. Berdasarkan kerentanan terhadap *S. agalactiae* pada saat pengujian infeksi buatan, diketahui bahwa semua strain ikan nila yang telah di-release ke masyarakat untuk tujuan budidaya, memiliki kerentanan terhadap infeksi bakteri tersebut; meskipun masing-masing strain memiliki tingkat kerentanan pada level yang berbeda. Strategi pengelolaan kesehatan ikan yang didasarkan pada tindakan pencegahan dan relevan dengan program perikanan budidaya berkelanjutan, seyogyanya didasarkan pada pendekatan ekologi-biologi seperti penerapan sistem biosekuriti, penggunaan vaksin untuk penyakit endemik, probiotik, herbal esensial, dan pengembangan populasi yang lebih tahan terhadap penyakit.

Kata kunci: ikan nila, kesehatan ikan, streptococcosis, *Streptococcus agalactiae*

Introduction

Tilapia (*Oreochromis niloticus*) is known as a fast-growing farmed fish species, relatively easy cultivation techniques, and able to adapt to a wide range of ecological zones. The fish species is also the belle of freshwater aquaculture, providing employment, improving welfare, contributing significantly to reducing stunting, and supplying animal protein for all levels of society (Caruso et al., 2013; Sukenda et al., 2014; Taukhid et al., 2014; Taukhid et al., 2021). Indonesia is the second largest tilapia producer in the world after China, with national production in 2019 reaching 1,506,156 tons with a value of 34.7 trillion rupiah (MMAF, 2021).

It was initially thought that tilapia were more resistant to infectious diseases compared to other farmed fish species; however, recently it has been shown that tilapia are also susceptible to many pathogenic agents (Klessius et al., 2008; Amal et al., 2011). In accordance with the growing and intensive farming of tilapia, the occurrence of disease has become one of the obstacles and needs to be taken seriously in the development of the species. The main diseases during the production cycle of tilapia in Indonesia are due to infection with Streptococcus bacteria (Streptococcosis) and *Aeromonas* (Motile Aeromonas Septicemia/MAS) or co-infection by both genus of bacteria. Streptococcosis is host species specific to the fish species, while MAS is a cross-species disease of freshwater fish that is always involved and aggravates cases of infectious diseases initiated by other types of pathogens (Taukhid, 2018; Taukhid et al., 2021).

Streptococcosis in Indonesian freshwater aquaculture is only reported to infect tilapia and is even recognized as a major disease in this species. The results of active and passive surveillance that were conducted from 2008 to 2010 indicated that two species have been identified, namely *Streptococcus agalactiae* (85%) and *S. iniae* (15%) (Taukhid & Purwaningsih, 2011; Taukhid et al., 2014). The disease in tilapia culture is suspected to have occurred since the early 2000s, especially in tilapia farmed in floating net cages (KJA) in a number of reservoirs. Quantitative data related to streptococcosis in tilapia production centers cultured in Java, Sumatra, South Kalimantan and North Sulawesi cannot be explored comprehensively. However, based on reports of disease incidences with clinical symptoms and mortality patterns, it was generally referred to streptococcosis cases with an estimated mortality rate of ± 30% (Taukhid & Purwaningsih, 2011; Taukhid et al., 2014).

It was noted that in many cases, the disease does not show any obvious clinical symptoms other than persistent mortality reaching ≈ 30% of the total population, predominantly in fish > 250 grams (Taukhid & Purwaningsih, 2011; Taukhid et al., 2014). In 2010, Research Institute for Freshwater Aquaculture and Fisheries Extension (RIFAFE) successfully isolated *S. agalactiae* from fry-sized tilapia (<5 grams) sampled from Bogor area, with specific clinical symptoms of the darkened body and protruding eyes (exophthalmos) with acute mortality over several days to 2 weeks. Subsequently, similar episodes occurred in tilapia fry in other areas, particularly in West Java. This indicates that streptococcosis can affect all tilapia stadia and has the potential to become an endemic disease in tilapia culture. Cumulatively, the economic loss due to the disease in Indonesia is estimated to be around 15 billion rupiah annually (FAO, 2015).

Notably, in Indonesia, there have not been any reports of serious streptococcosis occurrences in freshwater cultured fish (consumption and ornamental) other than among tilapia. Hernandez et al. (2009) confirmed that transmission and infection of *S. agalactiae* in tilapia only occurred within this species; not in other species of fish even though they were living together in the same ecosystem. Indirectly, this affirmation corroborates reports and findings that have occurred among tilapia culture in Indonesia.

Based on the phenotype characteristics of the collected *S. agalactiae* isolates derived from diseased tilapia taken from different areas (Sumatra, Java, Kalimantan, Sulawesi, and Papua), two biotypes were found, comprising β-hemolytic *S. agalactiae* (biotype I) and non-hemolytic *S. agalactiae* (biotype II). Moreover, as deduced from artificial infection studies, *S. agalactiae* biotype I cause disease with acute mortality patterns, whereas *S. agalactiae* biotype II causes disease with chronic and persistent mortality patterns (Suhermanto et al., 2019). This information indicates that the distribution of the disease has spread widely in Indonesia, although there are differences in the dominance of biotype.

In intensive aquaculture, the governance paradigm of fish health management must be directed towards effective, environmentally friendly, and sustainable precautions. The strategy of fish health management does not fully rely on the use of chemicals/antibiotics which have a negative impact on the fish, the aquatic environment, and consumers (Taukhid et al., 2021). Alternative strategies based on preventive and future-relevant measures is ecological-biological approaches such as the implement-

tation of biosecurity systems, the use of vaccines for potential endemic diseases, probiotics, essential herbs, and the development of more disease-resistant populations.

This review aims to provide the current status of streptococcosis on nile tilapia in freshwater aquaculture in Indonesia. Information was elaborated from the results of studies conducted by researchers, academics, technical implementation units under the Directorate General of Aquaculture, as well as disease case reports from district and provincial fisheries offices.

Etiological agent

Streptococcosis or "meningoencephalitis and panophthalmitis syndrome" is a disease in tilapia aquaculture with mortality rates ranging from 30 - 80% (Evans et al., 2004). Taukhid & Purwaningsih (2011) noted that streptococcosis cases in tilapia aquaculture with common symptoms such as weakness, darken body color, loss of appetite, loss of balance, uni/bilateral exophthalmia with the pale cornea, external bleeding and wounds; generally caused by infection of *Streptococcus agalactiae* (85%) and S. iniae (15%). Furthermore, it was reported that laboratorically, *S. agalactiae* infection in tilapia is considered acute, while S. iniae infection is more chronic. This indicated that *S. agalactiae* has more potential as a more serious cause of streptococcosis in tilapia aquaculture.

Collections of *S. agalactiae* isolated from diseased tilapia by the RIFAFE from a number of tilapia farms across Java since 2008 are predominantly non-hemolytic biotypes and belong to serotype Ib (Taukhid & Purwaningsih, 2011; Taukhid et al., 2014). On the other hand*, S. agalactiae* isolated from diseased tilapia from South Sumatra, Jambi, South Kalimantan, and Papua (Sentani Lake) were predominantly β-hemolytic biotypes (Anshary et al., 2014; Suhermanto et al., 2019). Moreover, as deduced from artificial infection studies, *S. agalactiae* biotype I cause disease with acute mortality patterns, whereas *S. agalactiae* biotype II causes disease with chronic and persistent mortality patterns (Suhermanto et al., 2019). The β-hemolytic type *S. agalactiae* has the capability to hydrolyze more sugars including arabinose, sorbitol, lactose, trehalose, and starch; while non-hemolytic ones generally only hydrolyze a few sugars, i. e. hippuric acid and arginine, and produce acetoin (Hardi et al., 2011).

The taxonomic hierarchy of *S. agalactiae* according to the Integrated Taxonomic Information System (ITIS) (2021) is as follows.

Kingdom: Bacteria
  Subkingdom: Posibacteria
    Phylum: Firmicutes corrig
      Class: Bacilli
        Order: Lactobacillales
          Family: Streptococcaceae
            Genus: Streptococcus
              Species: *Streptococcus agalactiae*
                (Lehmann & Neuman, 1896)

Suhermanto et al. (2019) noted that the variation of *S. agalactiae* biotypes collected from Indonesian areas was strongly influenced by geographical conditions having specific characteristics; tilapia cultured in peat-lands with high organic matter and acidic pH were dominated by hemolytic *S. agalactiae* biotypes, whereas in Java areas there were predominantly non-hemolytic *S. agalactiae* biotypes. Similar finding that geographical conditions affected the genetic diversity of *S. agalactiae* in Thailand analyzed by Random Amplified Polymorphic DNA (RAPD), and also affected to the serotype and virulence (Kayansamruaj et al., 2014).

Based on morphological, physical, and biochemical assays (Taukhid & Purwaningsih, 2011; Hardi et al., 2011; Taukhid et al., 2014; Suhermanto et al., 2019) and confirmatory molecular diagnosis (Nafiqoh et al., 2022), it was concluded that *S. agalactiae* is the predominant cause of streptococcosis on tilapia culture in Indonesia, and based on phylogenetic tree analysis of a number of isolates that have been characterized two biotypes were confirmed, i. e. *S. agalactiae* β-hemolytic and non-hemolytic (Suhermanto et al., 2019).

Susceptible hosts

Streptococcosis outbreak in tilapia farming are suspected to have occurred since the early 2000s, especially in tilapia farmed in floating net cages (KJA) in a number of reservoirs. Persistent mortality began to occur in tilapia >250 grams during the dry season or the transition period from the dry season to the rainy season or vice versa. Based on the results of the national fish disease monitoring system (SSMPI) activities carried out by the technical implementation unit under the Directorate General of Aquaculture (DGA) and the fisheries service during the period 2014 - 2018, there have been no reports of streptococcosis cases in freshwater aquaculture fish (consumption and ornamental) other than tilapia (DGA, 2019). This information strengthens the suspicion that the disease is so far considered as host species-specific, although based on our experience, *S. agalactiae* has also been isolated

from carp (Cyprinus carpio) and giant gourami (Osphronemus goramy), in a co-infection format with other species of bacteria such as Aeromonas hydrophila and Mycobacterium fortuitum. No specific clinical symptoms were suggestive of streptococcus disease found in any of the two fish species. Therefore, we strongly suspect that the presence of these bacteria is due to both fish species being raised in polyculture with tilapia exposed to *S. agalactiae* (RIFAFE Testing Laboratory, 2019).

Field trial on host susceptibility by cohabitation, had no effect on other fish species (white cachama, Piaractus brachypomus) that inhabited within the same container as tilapia affected by streptococcosis. Isolating *S. agalactiae* from white cachama which consumes carcasses of tilapia affected by the disease was unsuccessful, neither was it isolated from other fish species inhabiting the same aquatic environment as the diseased tilapia population (Hernández et al., 2009). Indirectly, this affirmation corroborates reports and findings that have occurred among tilapia culture in Indonesia.

So far, Ministry of Marine Affairs and Fisheries (MMAF) has released more than 15 strains of improved tilapia for aquaculture purposes: JICA in 2004, in 2006 (GESIT & Nirwana), Jatimbulan in 2008, in 2009 (BEST & Larasati), in 2012 (Nirwana II, Sultana, Srikandi, Anjani, Merah Nilasa, Pandu male & Kunti female), Salina in 2014, and Nirwana III in 2016 (DJA, 2016). The specific excellence of each particular strain is predominantly focused on its growth rate, biological efficiency/food conversion ratio (FCR), fecundity, and tolerability to varying environmental conditions.

Susceptibility evaluation of *S. agalactiae* (N14G) infections in a controllable study of 5 tilapia strains (BEST, Nirwana, Srikandi, GIFT, and Red Tilapia) by artificial infection at 50% lethal dose (LD50) demonstrated that there were significant differences in susceptibility among the five strains (Taukhid, 2014). Febrianti et al. (2015) conducted research on 4 tilapia strains (Sultana, red NIFI, Srikandi, and Nila Aureus) against *S. agalactiae* (N14G) infection experimentally, and showed differences in the susceptibility of the four strains; cumulative mortality at the end of the observation period was Nila Aureus (72%), Sultana (50%), Srikandi (36%), Red NIFI (24%), and control (0%). Looking at the analysis of susceptibility to *S. agalactiae* infection, it is strongly suspected that all of the released tilapia strains are susceptible to the bacterial infection, although each strain has a different level of vulnerability.

Streptococcosis which is caused by non-hemolytic biotype *S. agalactiae* infection was firstly reported in 2008 among the 100 - 250 grams tilapia cultured in Cirata, Saguling, and Jatiluhur Reservoirs - West Java. The secondary information collected from the field (Fisheries Extension Officers and farmers) indicated that the disease was more prevalent in tilapia > 250 grams with persistent mortality (3-5 fish/KJA/day) for several weeks up to the end of the culture period with an overall mortality rate of ± 30%. Despite the moderate mortality rate associated with the disease, it ultimately impacts feed conversion ratio (FCR) and production decline, as the onset of disease and mortality begins when more than 50% of production inputs (feed) have been invested. Therefore, in 2014 MMAF categorized streptococcosis as one of the endemic bacterial diseases on tilapia in Indonesia (DGA, 2014), with an estimated loss of 15 billion rupiah/year (FAO, 2015).

In 2010, RIFAFE successfully isolated *S. agalactiae* from fry-sized tilapia (<5 grams) sampled from Bogor area, with specific clinical symptoms of the darkened body and protruding eyes (exophthalmos) with acute mortality over several days to 2 weeks; subsequently, similar episodes occurred in tilapia fry in other areas including Subang, Cianjur, Sukabumi and Pandeglang (RIFAFE Testing Laboratory, 2019). This finding indicates that streptococcosis can affect all stadia of tilapia and has the potential to become an endemic disease in tilapia farming in Indonesia.

Distribution and transmission

We did not find any valid evidence regarding the timing of the first discovery of the streptococcal infecting tilapia in Indonesia, including the possible status of the bacterial species, whether as an endemic or exotic pathogenic agent. However, based on the claims of tilapia farmers in West Java, cases of disease with clinical symptoms that indicate streptococcosis have been found since the early 2000s, especially during the transitional season.

Globally, Amal & Zamri-Saad (2011) stated that the distribution of streptoccocosis in fish caused by infection with Streptococcus spp. bacteria have been reported in at least 15 countries on 5 continents (Asia, America, Africa, Europe, and Australia). Wendover et al. (2018) collected more than 1,000 bacterial isolates from 74 tilapia farming sites across 14 countries. Over half were Streptococcus spp. (*S. agalactiae* and S. iniae) comprising 26% of the isolates were confirmed as *S. agalactiae* biotype I and 56%

were confirmed as *S. agalactiae* biotype II; while the remaining (18%) were S. iniae. Subsequent studies showed that there are 2 clusters/biotypes of *S. agalactiae* which have different biochemical and phenotypic properties, they are *S. agalactiae* biotype I (β-hemolytic) and *S. agalactiae* biotype II (non-hemolytic). Non-hemolytic *S. agalactiae* is the biotype with more widely distributed globally (Asia and Latin America), whereas β-hemolytic *S. agalactiae* is confined to the Asian region with an acute mortality pattern and is typically associated with a more elevated water temperature.

The cases and distribution of streptococcosis observations among tilapia in Indonesia resemble the findings of Wendover et al. (2018) who reported that 82% of streptococcosis in tilapia in Asia and Latin America was caused by *S. agalactiae* infection, and the rest was S. inae (18%). (Taukhid & Purwaningsih, 2011; Taukhid et al., 2014) reported that on the basis on data for the period 2008 - 2010, confirmed cases of streptococcosis in tilapia culture centers were *S. agalactiae* (85%) and S. iniae (15%). Furthermore, it was noted that *S. agalactiae* has more potential to cause more serious disease in tilapia culture compared to S. iniae. Similar reports on tilapia farming in the Asian region also have similar conclusions with regard to clinical symptoms and mortality rates due to *S. agalactiae* infection on tilapia farmed in floating net cages (Zamri-Saad et al., 2014).

The introduced fish which are exposed to *S. agalactiae* is a prominent source of entry and spread of the bacteria into the aquaculture environment. When the bacteria leave the body along with feces and enter the aquaculture environment, they will eventually contaminate the healthy fish. Artificial infection by cohabiting infected with healthy fish could transmit the bacteria to healthy fish. The horizontal transmission mechanism is the most prevalent transmission of this disease, and the findings of a study have been conducted revealed no vertical transmission of the disease (Hernández et al., 2009; Jiménez et al., 2011).

Based on information/references (natural and artificial infection by cohabitation), as well as reports and findings of disease cases occurring in fish farmers, it is assumed that the transmission mechanism of streptococcosis caused by *S. agalactiae* infection in tilapia occurs horizontally (from infected fish to healthy fish), and no evidence could be traced that the transmission mechanism of this species of bacteria occurs vertically.

Triggering factors

The presence of a single pathogen (*Streptococcus* spp.) in the aquaculture environment is not enough to cause disease, there are still accompanying factors or risk factors that affect the physiological functions of fish so that they become more susceptible to pathogen infection; and in general, these risk factors are better known as stress conditions as a mediator of disease emergence (Amal & Zamri-Saad, 2011; Zamri-Saad et al., 2014; Amal et al., 2015). Stress can be caused by physical, chemical, or biological factors; both internal and external; and even stressful conditions are often caused by these factors simultaneously, resulting in multiplicative negative impacts.

The most common stress conditions associated with streptococcosis cases include: high water temperature or significant temperature fluctuations between day and night and last for a long period, high salinity and pH above 8, low dissolved oxygen (DO) concentration, poor water quality (with high ammonium or nitrite concentrations), high stocking densities per unit-area and handling/management that does not pay attention to fish welfare aspects (Zamri-Saad et al., 2014; Amal et al., 2015). Temperatures above 31°C are known to predispose tilapia to streptococcosis (Kayansamruaj et al., 2014). Oxygen is a major limiting factor for fish health; metabolism, growth, and disease resistance are depressed when DO drops below 1 mg/L for prolonged periods (Amal et al., 2015).

As informed by tilapia farmers in floating net cages from a number of tilapia farms across West Java (Jatiluhur, Cirata, and Saguling reservoirs), the prevalence of streptococcosis cases was higher during the transition period. Persistent mortality began to occur in tilapia >250 grams, particularly during the dry season or the transition period from the dry season to the rainy season or vice versa. During these periods, the water temperature is relatively high during the day (30oC - 33oC) and relatively low at night (22oC - 24oC). As the volume of water in the reservoir decreases, the sun's heat energy will have a direct impact on elevating the water temperature; conversely at night, as there are no clouds, the heat energy held in the water will be easily released to the atmosphere. These conditions will naturally result in high water temperature fluctuations between day and night (8 - 9oC over 24 hours) and are thought to contribute to the fish's compromised immune system; making it more susceptible to pathogen infection, in this instance *S. agalactiae*.
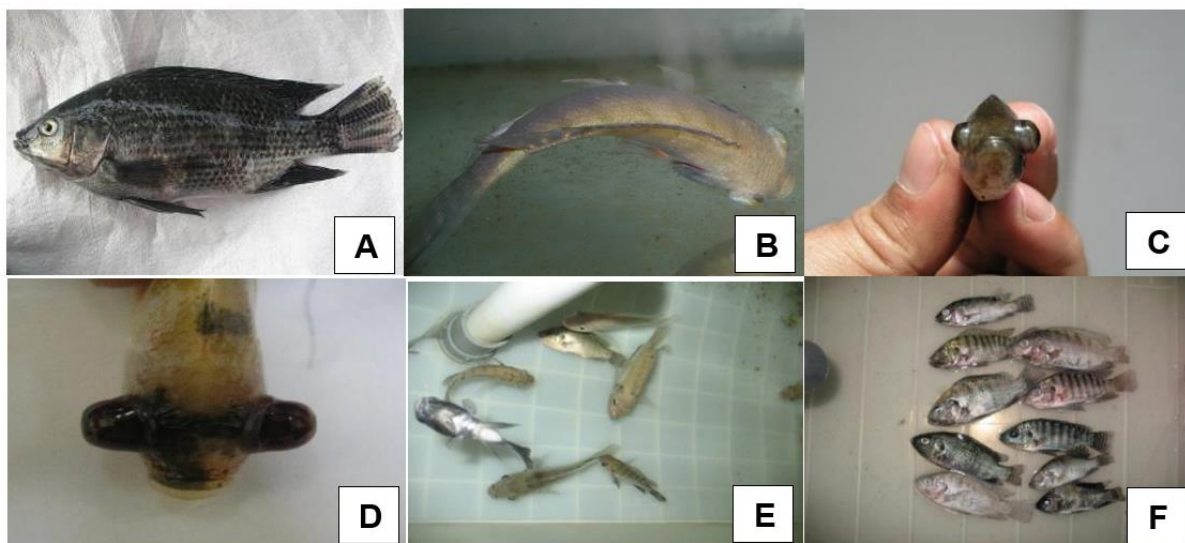
Diagnosis

In this paper, streptococcal diagnosis refers to the classification of infectious disease diagnosis in fish developed by Bondad-Reantaso *et al.* (2001). According to the diagnosis classification developed in the guidebook, it is grouped into 3 (three) levels, which are categorized as diagnosis level I, II, and III.

Diagnosis Level I, the diagnosis was based on clinical symptoms, fish health performance, and environmental conditions of aquaculture. The following summarizes the variety of clinical symptoms as indicators of abnormalities associated with visually observable streptococcosis: darker and/or paler body color (melanosis), bulging eyes (uni/bi-lateral exophthalmos), corneal opacity, accumulation of blood/hemorrhage in the abdominal cavity (dropsy/ascites), hemorrhages in the eyes, gills and the base of the fins (hemorrhages), body shape resembling the letter "C"/deformation of the spine (C-shape), flaccid swim bladder, black/brown liver, swelling of the spleen, kidneys and other internal organs. Symptoms thought to be "typical" of this disease are disorders of the brain and eyes, so is often termed "meningoencephalitis and panophthalmitis syndrome" (Taukhid & Purwaningsih, 2011; Hardi *et al.*, 2011; Yanong & Francis-floyd, 2013; Kayansamruaj *et al.*, 2014; Iregui *et al.*, 2014; Taukhid *et al.*, 2014; Suhermanto *et al.*, 2019;

Taukhid *et al.*, 2021; Sarjito *et al.*, 2021). Some of the clinical signs of tilapia infected by *S. agalactiae* are shown in Figure 1.

Observable indicators of fish health performance include a drop in appetite (anorexia), and unbalanced/erratic swimming movements as a result of brain and nerve dysfunction, although in some other cases of streptococcosis there are no obvious symptoms of illness, although persistent mortality occurred. Based on pathological-anatomical observations, it has been strongly suspected that septicemia infection mainly affects the brain and nervous system (Salvador *et al.*, 2005; Taukhid & Purwaningsih, 2011; Yanong & Francis-floyd, 2013; Iregui *et al.*, 2014; Suhermanto *et al.*, 2019; Taukhid *et al.*, 2021; Sarjito *et al.*, 2021).

Diagnosis Level II, the diagnosis was based on laboratory analysis, primarily to confirm the presence of bacteria and their associated effects. Diagnosis procedures could be accomplished by isolation, and characterized by biochemistry, histopathology, immunohistochemistry (IHC), Koch's postulate, etc. Simple and reasonably faster methods to identify the presence of the targeted bacteria are Gram staining of smears of targeted tissues/organs such as kidney and liver, or histopathological preparations of brain/eye through IHC testing. Hernández *et al.* (2009) and Iregui *et al.* (2014) suggested that the IHC analysis for diagnosing *S. agalactiae* on fish tissue utilizing polyclonal antibodies prepared in

Figure 1. Clinical signs (Level I Diagnosis) of tilapia (Oreochromis niloticus) infected by *Streptococcus agalactiae*: darker and/or paler body color (A), body shape deformation/rotating swimming movements (B), protruding eyes/pop eyes/uni-lateral exophthalmos (C), bi-lateral exophthalmos (D), undirected swimming movements and loss of balance (E), mass mortality with clinical symptoms of bulging eyes (F). Source: Taukhid and Purwaningsih (2011).

rabbits showed improved sensitivity and specificity compared to bio-chemical characterization assays.

Characterization of pure isolates of bacteria taken from targeted organs of diseased tilapia suspected of being infected with the bacteria, showing general symptoms as described in level I diagnosis. Cultures of isolates were then planted onto media (TSA, BHIA, NA, blood agar), and incubated at 28-33 ºC for 24-72 hours. The identification method and schematic performed in our laboratory are based on a number of characteristics including the colony and bacterial cell form, Gram's, motility, catalase, oxidative-fermentative, hemolytic activity, protein and carbohydrate antigen composition, sugar fermentation, and other biochemical reactions (RIFAFE Testing Laboratory, 2019). Some of the physical and biochemical tests in characterizing *Streptococcus* spp. bacteria in our laboratory are shown in Figure 2.

Generally, the major characteristics of *S. agalactiae* bacteria are as follows: Pinpoint colony morphology, round or ovoid bacterial cells with a diameter range of 0.5-2.0 µM. Bacterial cells are paired or form chains when grown in liquid media, Gram-positive, non-motile, and non-spore-forming. Catalase negative, and on blood agar media for biotype I (hemolytic) shows a greenish (α-hemolytic) or transparent (β-hemolytic) color. It is facultatively anaerobic and produces lactic acid in the absence of gas. Commonly, the key characteristics of *S. agalactiae* were also mentioned by other authors (Austin & Austin, 1996; Salvador *et al.*, 2005; Taukhid & Purwaningsih, 2011; Hardi *et al.*, 2011; Rodkhum *et al.*, 2011; Yanong & Francis-floyd, 2013; Kayansamruaj *et al.*, 2014; Iregui *et al.*, 2014; Suhermanto *et al.*, 2019; Sarjito *et al.*, 2021).

Comparative diagnosis may be performed by using a commercial kit (API 20 Strep System, bio-Mérieux Industry, Hazelwood, USA). In our laboratory, testing for hemolytic activity was conducted by growing the bacteria on a Blood Agar Base medium supplemented with 5% sheep blood and then incubating at 37ºC for 18-24 hours.
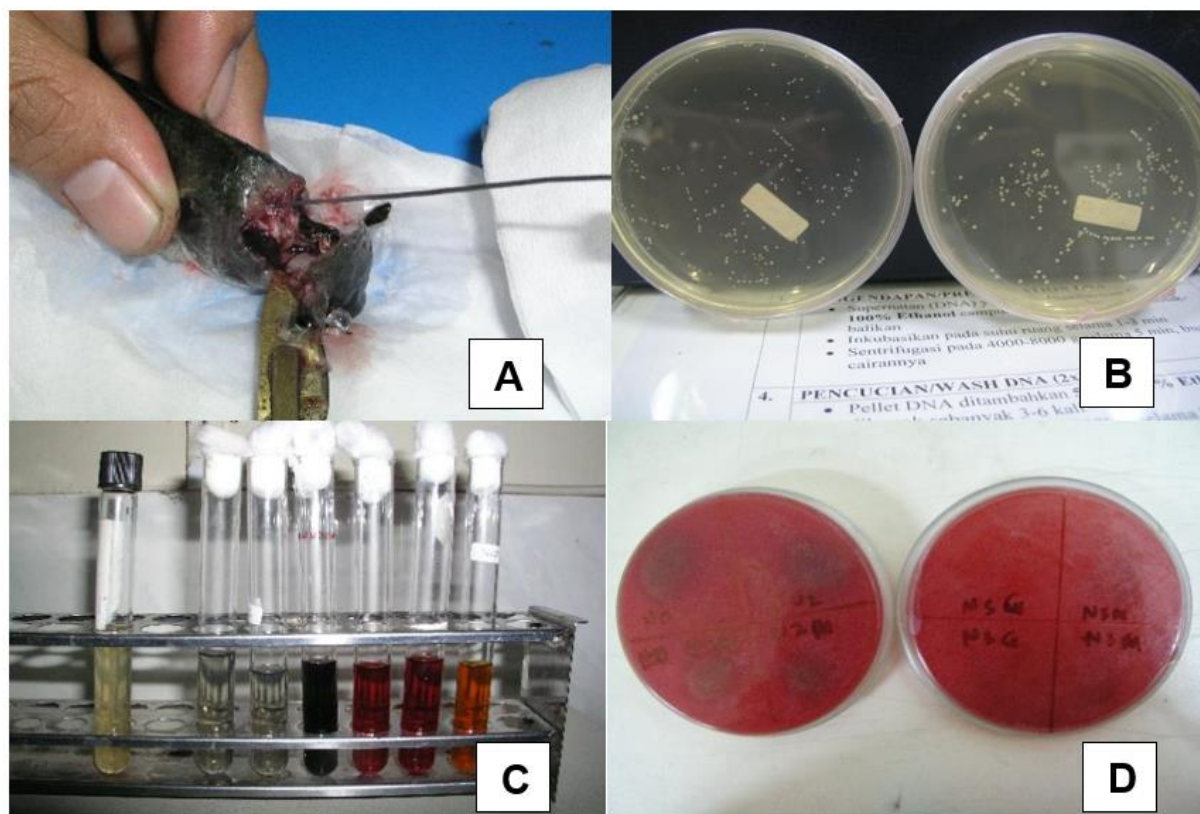
Figure 2. A number of diagnosis level II assays for the presence of *Streptococcus agalactiae* infections on tilapia (*Oreochromis niloticus*) exhibiting symptoms of streptococcosis. Isolation of bacteria from targeted organs onto agar media (A), colony appearance of suspected *Streptococcus* spp. bacterial isolates (B), sugar assay (C), and an assay on blood agar media (D). Source: Taukhid and Purwaningsih (2011).

Diagnosis Level III, the diagnosis can be used as confirmatory/definitive diagnosis (if needed) performed molecularity (Polymerase Chain Reaction/PCR and/or DNA sequencing with 16 and 23S rRNA genes) of pure cultures of *S. agalactiae* bacteria. Molecular detection of *S. agalactiae* bacteria has been widely practiced, among others by (Jiménez *et al.*, 2011; Rodkhum *et al.*, 2011; Angel *et al.*, 2014; Kayansamruaj *et al.*, 2014; Nafiqoh *et al.*, 2022). Detecting genes to the molecular level, not only knows the species of bacteria to the species level; but can also determine the genetic clustering of the bacteria isolates.

Control

In aquaculture, fish health management should be part of standardized procedures at all phases of the production process, starting from broodstock management (hatchery and nursery units), water quality management, and feeding management. Fish health management based on preventive and future-relevant measures can be performed by integrating ecological-biological-chemical approaches such as the implementation of biosecurity systems, the use of vaccines for potential endemic diseases, essential herbs, and the development of more disease-resistant populations.

Biosecurity in aquaculture is preventing and/or protecting cultured fish species from all potentially infectious pathogenic agents (viruses, bacteria, fungi, and parasites). The biosecurity system can only be optimally applied to culture areas/units that can be controlled, either physically, chemically, or biologically. Designing an effective biosecurity program requires an understanding of the culture system being implemented, the general concept of fish disease risk analysis, the eco-biology of disease-causing pathogens, and the eco-biology of the fish species being cultured.

The application of the biosecurity system concept in tilapia culture in Indonesia has not yet been fully implemented. This is because the spawning system is generally still carried out in bulk in the broodstock pond, and there is practically no individual screening process for candidates for spawning. Fertilized eggs are collected manually from the mouth of the parent fish, and then hatched in a more controlled egg-hatching facility. Some hatcheries have implemented strict biosecurity systems, but most others are still weak in implementing biosecurity procedures. The above conditions lead to difficulties in the process of tracing the health and disease status (streptococcosis) of the fish fry

population produced. Moreover, tilapia farming in Indonesia is typically practiced in relatively uncontrolled aquaculture environments, such as rivers and reservoirs/lakes, where the transmission of infectious diseases is more difficult to control.

Vaccination, prevention of potential and endemic fish diseases by vaccination is considered to be an effective, efficient, and safe approach for both the environment and consumers. Fish vaccination in many countries has been proven to contribute significantly to increasing aquaculture production, reducing the use of chemicals/antibiotics, and the resistance of some pathogens to chemicals/antibiotics. The successful vaccination program in aquaculture is believed to have impacts in terms of (1) decreasing the mortality rate of farmed fish due to potential pathogen infections, (2) decreasing the use of antibiotics in fish farming, and (3) reducing the resistance a number of pathogens to antibiotics (Chideroli *et al.*, 2017).

Vaccination is a widely accepted and effective method to control *S. agalactiae* infection and prevent mass mortality on tilapia farming (Liu *et al.*, 2016). Research and development of vaccines to control *S. agalactiae* infecting tilapia in Indonesia have been initiated by many research & development agencies and universities, including in different vaccine preparations, doses, and applications. Based on the documentation traced, the development of fish vaccines intended to prevent streptococcosis in tilapia culture in Indonesia has started since the 2010s (Taukhid & Purwaningsih, 2011; Dwinanti *et al.*, 2014; Taukhid *et al.*, 2014; Nisaa *et al.*, 2016; Firdausi *et al.*, 2018; Sukenda *et al.*, 2018; Reynalta *et al.*, 2019; Rahmi *et al.*, 2021; Taukhid *et al.*, 2021) with various results after being subjected to challenge against targeted bacteria.

Taukhid & Purwaningsih (2011) screened *Streptococcus* spp. isolates as antigen candidates in vaccine development, and their effectiveness to prevent streptococcosis in tilapia. Based on the screening protocol, the N4M isolate was used as an antigen source in vaccine development, and its efficacy was evaluated accordingly. The highest level of antibody titer and survival rate of vaccinated fish was attained from formalin killed vaccine compared to the others (heat killed and sonicated vaccine).

Briefly, in the following years, a comprehensive study was carried out concerning the potential for developing anti-streptococcosis vaccines, for example Dwinanti *et al.* (2014) conducted an analysis the ECP (extracellular product) toxicity and immunogenicity of 2 (two)

isolates of non-hemolytic *S. agalactiae* collected by RIFAFE, to evaluate ECP as a material vaccine for prevention of *S. agalactiae*. The results indicated that tilapia vaccinated with ECP of the first isolat with protein 283 µg/kg body weight had a RPS value 60%. Tilapia vaccinated with ECP of the second isolat with protein 408 µg/kg had a RPS value 68%. Parallely, Taukhid *et al*. (2014) compared the efficacious of 4 types of vaccine preparations (broth, whole cell, crude extracellular products, and pure extracellular products) derived from *S. agalactiae* bacteria with isolate code N14G as a master seed in vaccine production. The results showed that the efficacious vaccine preparation was evaluated by survival rate were: whole cell vaccine (76.0%), followed by broth vaccine (65.0%), crude extra cellular product (49.0%), and pure extra cellular product vaccine (36.0%). Survival rate of positive control was 25.0%, and 34.0% for negative control. Two types of vaccine preparation showed more effective on relative percentage survival (RPS), namely whole cell vaccine (68.00%), and broth vaccine (53.37%).

The effectiveness of *S. agalactiae* vaccination based on gonad maturation stages on tilapia brood stocks in which the released antibodies was able to be transferred to the seed. The results showed that vaccination in tilapia brood stocks at stage 2 of gonad developmental stages gave highest protection by maternal immunity transfer to the seed against *S. agalactiae* (Nisaa *et al*., 2016). Further studies were carried out by Rahmi *et al*. (2021), conducting maternal vaccination in tilapia broodstock by distinguishing the effectiveness of administering the vaccine once and twice before spawning. The results showed that egg hatching in the treatment group of broodstock vaccinated twice was significantly different compared to the group of broodstock vaccinated once. The mortality rate of the two-time vaccinated was significantly lower than the other treatments. The RPS value of twice-vaccinated broodstock was not significantly different from that of once-vaccinated broodstock on the 10th day but significantly higher on the 20th day.

Furthermore, Firdausi *et al*. (2018) evaluate the effectiveness of vaccination on tilapia seedlings resulted from the vaccinated parent by hyper-osmotic infiltration method at different salinity. The results showed that the 10 ‰ (part per thousand) salinity gave the best results compared to the others and control. The final RPS (10 ‰) value was 84.72%, 66.49%, and 47.06%, on the 10th, 20th, and 30th days of vaccination, respectively. The value of RPS and specific

antibody level at 10 ‰ salinity was significantly different compared to the other treatments.

Analysis a specific and nonspecific immune response of tilapia fry that has been given with *S. agalactiae* vaccine made of N3M and N4M strains and examine the protective immunity against homologous and heterlogous *S. agalactiae* N3M, N4M, N17O, NK1, and N14G strains infection. Relative percent survival in N3M vaccinated fish after challenged with N3M and N4M was 87.50% dan 64.70%, respectively, otherwise in N4M vaccinated fish was 62.50% dan 76.47%, respectively. It was concluded that N3M and N4M vaccine strain have better protection as only if it tested with similar bacteria strain (Sukenda *et al*., 2018). Further studied of streptococcosis vaccine preparation has been done by Reynalta *et al*. (2019) by using different coated vaccine (chitosan, skim milk, and maltodextrin at concentration 1 % or 10 %). The result showed that chitosan coating at doses 1 % and 10 % were the best in solubility and protein concentration test. Summary, they concluded that freeze dried vaccine with chitosan coated 1 % is effective to improve immunity system of nile tilapia.

Taukhid *et al*. (2021) studied to assess the efficacy of bivalent and trivalent vaccines containing *S. agalactiae* bacteria on tilapia. The formula of the bivalent vaccine contains 75% of S01-196-16 and 25% of N14G isolates (v/v). Trivalent vaccine contains 30%, 35%, and 35% of N14G, NP1050, and SG01-16 isolates (v/v), respectively. A challenge test assessed the efficacy of the vaccines, and it was carried out at 30, 90, and 150 days post-vaccination by artificially infection at LD_{60}. The results revealed that the highest RPS of a bivalent vaccine against *S. agalactiae* (S01-196-16) was achieved at the first challenge (61.84%), and trivalent vaccine against *S. agalactiae* (N14G) and *S. agalactiae* (S01-196-16) was achieved at the first challenge (61.53% and 76.20%, respectively).

The RIFAFE is one of the institutions conducting intense research and development of vaccines for the prevention of streptococcosis in tilapia culture, both within the scope of internal units, joint research with universities, and with national private companies. On 03 May 2013, one of the downstream research in the form of anti-streptococcosis vaccine with the trademark "Streptovac" was issued a register number by the Competent Authority (Directorate General of Aquaculture, Ministry of Marine Affairs and Fisheries) with the registration number: KKP RI No. D 1305224 BKC. In 2021, the institute collaborated in the down-streaming research with the national pharmaceutical industry in the

context of mass production and commercialization of fish vaccines to prevent co-infection of *A. hydrophila* and *S. agalactiae* bacteria in tilapia. Finally, in the following year, the product fulfilled the regulations and was approved for marketing under the trademark "Caprivac hydrogalaksi".

The successful vaccination of fish is not only determined by the efficacy of the vaccine used but also largely determined by how and when the vaccine should be administered. Things that need to be known related to vaccine application in fish include: (1). Fish vaccines are generally only able to provide a level of protection for a relatively short period, which is about 2 - 3 months so that to get a long level of protection, re-vaccination (booster) is needed, and (2). Vaccination must consider the age/size of fish that are susceptible to disease, as well as the time/season when the disease arises; so that the administration of vaccines can provide the best results when the disease occurs.

Fish vaccination can be administered by three methods: injection (intra peritoneal/IP or intramuscular/IM), immersion into vaccine solution, and orally (mixed with feed). These three methods have their advantages and disadvantages which may affect the level of protection, duration of immunity, and cost of vaccination (Taukhid *et al.*, 2015a & 2015b). In summary, the prospects and potential of using vaccines for the prevention of streptococcosis in tilapia are indicated, although, at the operational level, there is a need to demonstrate their comparative and competitive benefits, so as to ensure concrete benefits for farmers.

Unlike vaccines, immunostimulants do not elicit responses from fish by synthesizing antibodies, but rather by increasing the activity and reactivity of cellular or humoral defense cells. *In vitro*, one of the indicators of increased cellular response is shown by phagocytic activity as measured by the nitro blue tetrazolium (NBT) (Anderson & Siwicki, 1993). The phenomenon of phagocytic activity manifests an increase in cellular response and subsequently an increase in the humoral response. Immunostimulants often used for immunostimulation include lipo-polysaccharide (LPS); 1,3 β-glucan extracted from *Saccharomyces cerevisiae*, and Levamisole. Several vitamins such as vitamin A, B, and vitamin C may also be used as immunostimulants (Sohne *et al.*, 2000). One of the immunostimulants which are relatively inexpensive and readily obtainable in the market is vitamin C (ascorbic acid). This element has several functions, including a). Improves

resistance (immunity) against infectious diseases such as parasites, fungi, bacteria, and viruses; b). Serves as a coenzyme for biochemical reactions; c). Avoid bone deformities; d). To reduce the adverse effects of environmental disorders or stress, and e). Accelerating the wound healing process (Sohne *et al.*, 2000).

To date, there is not much information concerning the use of immunostimulant materials specifically targeted for the control of streptococcosis in tilapia. One particular study on the use of vitamin C (ascorbic acid) has been conducted by Dwinanti & Fitrani (2016), by using vitamin C incorporated to vaccine in fish feed as an alternative way to prevent *S. agalactiae* infection on tilapia. Preparation of vaccine and vitamin C to the fish feed was conducted through incorporated feed (re-pelleting) technique. Dosage of vitamin C was 600mg/Kg feed. The result showed that vitamin C-vaccine enhanced tilapia protection from *S. agalactiae.* Relative Percent Survival (RPS) value for vitamin C-vaccine was to 66,7% while single vaccine was to 51,9%.

The use of antibiotics to control strepto-coccosis on nile tilapia aquaculture may give results if practiced in accordance with the rules of proper fish disease treatment. Based on experience, antibiotic application are only effective in treating outbreaks of streptococcosis if the treatment is implemented early. In most cases, oral antibiotic treatments are ineffective as the infected fish have a reduced appetite. Therefore, antibiotics are only able to partially control mortality rates during the period of application. Once the course of antibiotic is over, mortality usually increases again. This phenomenon leads to non-sustainable behaviour; as mortality increases again after a normal antibiotic course, farmers are tempted to extend the duration of antibiotic application to longer periods or use higher doses. This in turn increases selection pressure toward resistant bacteria. The negative consequences of using antibiotics, such as emergence of antibiotic resistant bacteria and antibiotic residues in meat, must be carefully evaluated. In Indonesia, farmers tend to use erythromycin and oxytetracycline to treat streptococcosis in tilapia as well as a prophylactic agent in healthy fish.

There are several advantages of rational use of drugs/antibiotics, including guaranteeing the effectiveness of drugs against targeted pathogens, avoiding the emergence of microbial resistance to antibiotics, and economic benefits. According to MMAF Regulation No. 1/2019 concerning Fish Drugs, it is required that the

treatment of fish diseases must be based on (a). diagnosis results from observations of clinical symptoms and/or laboratory analysis, (b). using fish medicines registered in the MMAF, in accordance with the provisions and instructions on the packaging/label, (c). recorded and documented, and (d). used treatment media, syringes, drug packaging, and/or expired drugs do not pollute the environment. However, in order to prevent development of antibiotic resistant bacteria and antibiotic residues in meat, farmers must practice prudent use of antibiotics, while referring to the local Fisheries Department Officer, veterinarians, fish health specialist and experienced aquaculturist is also recommended.

Recently, fish disease control techniques have begun to be developed in ways that are more environmentally friendly. It is then expected that in the future, fish disease control may no longer rely on the use of drugs and antibiotics, instead using environmentally friendly disease control materials such as herbal medicine (Caruso et al., 2013; Lusiastuti & Taukhid, 2013; Ramudu & Dush, 2013; Caruso et al., 2017) and the use of functional feeds.

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