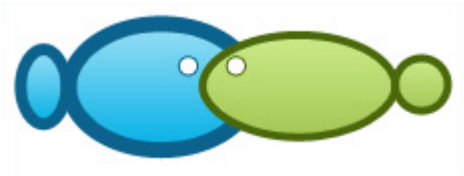

Streptococcus agalactiae whole cell bacteria toxin protein in Nile tilapia *Oreochromis niloticus*

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Abstract. Streptococcosis has become the main problem in Nile tilapia (*Oreochromis niloticus*) cultivation, and this disease has spread geographically to all the *O. niloticus* farms and hatcheries in the world. This study aimed to identify the toxic protein in pathogenic and non-pathogenic *Streptococcus agalactiae* based on molecular weight using the SDS-PAGE method. The treatments consisted of two groups of bacteria, the pathogenic and non-pathogenic bacteria. The tilapias were kept in (60x30x40 cm) aquariums with a density of 10 fish aquarium^{-1}. The fish were injected intraperitoneally at a dose of 0.1 mL fish^{-1} each with pathogenic and non-pathogenic bacteria. Then the fish were kept for 7 days and observations were made of the external anatomy, changes in swimming patterns, changes in feeding behavior, and fish mortality. The two groups of bacteria injected to the tilapia had their cellular protein tested by protein fractionation using SDS-PAGE. The study results revealed that in the all the proteins found in the non-pathogenic bacteria were also present in the pathogenic bacteria and there were new proteins not found in the non-pathogenic bacteria weighing 20.15, 28.75, 58.53, and 64.79 kDa. There were 4 new proteins found in the pathogenic bacteria not found in the non-pathogenic bacteria. These four proteins are believed to be toxic proteins that emerged and caused bacteria to become pathogenic. Therefore, there needs to be further investigation to study the toxicity of each of these proteins for vaccine development.

Key Words: Streptococcosis, *S. agalactiae*, SDS-PAGE, whole cell bacteria, pathogen, toxicity.

Introduction. Streptococcosis has been commonly reported as a highly pathogenic infectious disease for Nile tilapia (*Oreochromis niloticus*) caused by *Streptococcus agalactiae*. Geographically, this disease has been spreading to all tilapia growing out farms and hatcheries in the world. This species of bacteria has been isolated from several species of freshwater fish and marine fish (Cai et al 2016). This disease has become a major problem in the cultivation of tilapia (Pereira et al 2010; Kayansamruaj et al 2017; Shoemaker et al 2017). Cases of bacterial infectious diseases were recorded in fish farms in South Sumatra, Indonesia (Yuasa et al 2008), China (Li et al 2009; Chen et al 2012), and Thailand (Kayansamruaj et al 2015).

Streptococcosis in fish is often found to be caused by several types of *Streptococcus* sp. The symptoms are almost identical in each bacterial species and may result in central nervous system damage. It is indicated by clinical symptoms of exophthalmia (pop-eye) and meningoencephalitis. In warm water conditions with temperatures above 15ºC, it may lead to death. These symptoms can be caused by *Lactococcus garvieae, Streptococcus iniae, S. agalactiae and S. parauberis.*

Fish pathogenesis involves septicemia, clinical signs appearing immediately after infection, including depression or excitability, anorexia, C-shaped bodies, swimming erratically, irregular spinning, and death (Evans et al 2002), erratic swimming movements, whirling, bleeding from the eyes, cataracts, pop-eye, or bleeding around the anus and the base of the fins. The internal organs change, the part of the brain becomes soft and watery, and the liver is swollen and pale (Musa et al 2009).

The symptom levels may increase to chronic and acute infection (Evans et al 2006). The chronic level is indicated by the presence of wound-like bruises on the surface of the body, red spots on the fins, slow swimming and the fish stay more often at the bottom of the aquarium followed by appetite loss. Other symptoms often seen are prominent eyes

and whirling. If an acute attack occurs, sudden death may occur due to toxins, fluid loss from the digestive tract and malfunction of some organs. Transmission can occur through contact with infected fish. In some cases, infection causes different pathological damage to tilapia fish, depending on the cell type.

Virulence factors are highly affected by the characteristics of bacterial virulence related to its ability to evade the host's immune system. *S. agalactiae* virulence factors can be divided into structural and non-structural virulence factors. Structural virulence is formed by the components of cell constituents, both surface components and components of the bacteria's cell wall. These virulence factors consist of polysaccharide capsule antigens, protein antigens and lipoteichoic acid. Non-structural virulence factors are the bacteria's extracellular products including hyaluronidase, protease, nuclease hypuricase, C5a-ase, hemolysis and CAMP factors (Pritchard & Lin 1993). *S. agalactiae* is able to survive in professional phagocytes and non-pathogenic cells which cause septicaemia and meningoencephalitis in both freshwater and marine fish, including *O. niloticus* (Cai et al 2016).

This study aimed to identify toxin proteins in pathogenic and non-pathogenic *S. agalactiae* bacteria based on molecular weight using the SDS-PAGE method. This study is useful in studying the pathogenicity of bacteria that can stimulate immune responses in fish. Furthermore, the information can be used as a reference in the development of fish vaccines (Amrullah et al 2014a; Rauta et al 2016; Beck et al 2017; Jørgensen 2017) in the framework of disease-resistant fish production.

Material and Method. The experiment was conducted from May to August 2017 in the Fish Health Laboratory of the Department of Aquaculture, Pangkep State Polytechnic of Agriculture, Indonesia.

Bacteria and experimental animals. Bacterial isolates of *S. agalactiae* $N_{14}G$ were obtained from the Research and Development of Fish Disease Control, Depok, Indonesia. The species confirmation test was conducted using conventional morphological and biochemical characteristic testing and polymerase chain reaction (PCR). One loop of bacteria from agar medium was cultured into 5 mL of BHIB medium and incubated in a water bath at 29-30°C and 140 rpm for 24 hours. After 24 hours, 1 mL of the bacterial culture from the liquid culture medium was cultured in 9 mL of BHIB. Incubation was carried out in a water bath shaker at temperatures from 29 to 30°C and 140 rpm for 4 hours. After this, bacteria were ready for further tests.

O. niloticus were used as the experimental animal. Acclimatization was carried out for 14 days to prevent infection or contamination and isolations of bacteria from the eyes, kidneys and brain on BHIA media proved that *S. agalactiae* was not found in the animals.

Experimental design and rearing conditions. Treatment consisted of two groups of bacteria, namely pathogenic bacteria and non-pathogenic bacteria. The non-pathogenic bacteria were bacteria that had been grown and regenerated on BHIB media until the experiment was conducted, while the pathogenic bacteria were a group of bacteria that passaged in vitro, so that the bacteria became pathogenic to *O. niloticus*. Pathogenic bacteria were cultured in vitro. This was done by injecting the bacteria *S. agalactiae* to healthy tilapia at a density of 10^7 CFU.mL^{-1} intraperitonially (i.p.). This passage process was performed four times to produce the pathogenic *S. agalactiae* isolates.

O. niloticus were reared in fiberglass tanks (75 L capacity) at a density of 10 fish.tank^{-1}. The fish were injected 0.1 mL i.p. according to the treatment with pathogenic bacteria or non-pathogenic bacteria. Then the fish were reared for 7 days and the fish were observed for clinical symptoms, changes in swimming behavior, changes in eating behavior, and mortality. Fish mortality was calculated to obtain the percentage of mortality. Subsequently, both groups of bacteria injected to *O. niloticus* were tested for their cell proteins by protein fractionation using SDS-PAGE.

Protein fractionation. Fractionation of *S. agalactiae* bacterial proteins was performed to separate the proteins contained in bacterial toxins based on their molecular weight.

Fractionation of proteins was done using the SDS-PAGE method (Laemmli 1970). Before fractionation, sonication was first carried out to break down the bacterial cell. Then the protein fractionated was conducted using the SDS-PAGE method with 12% gel concentration.

Gel staining. The staining was started by fixing the gel with a fixative solution (25% methanol, 12% acetic acid) for 1 hour, then immersing in 50% ethanol for 20 minutes. Next it was immersed in 30% ethanol for 20 minutes twice, dipped in an enhancer (0.1 g $Na_2S_2O_3$ $5H_2O$) in 500 mL of distilled water for 1 minute, washed for 20 seconds and repeated 3 times. Then it was dipped in a silver nitrate solution (0.4 g of $AgNO_3$ mixed with 70 µL formaldehyde in 200 mL of distilled water (pure, microfiltrated, demineralized water) for 30 minutes. The gel was rinsed with distilled water twice for 20 seconds and dipped into a solution of 15 g Na_2CO_3 + 120 µL formaldehyde + 250 mL distilled water. When the black ribbon was visible, it was washed with a fixative solution until a bubble emerged and then it was washed with distilled water.

Parameters and statistical analysis. The parameters of the study consisted of the molecular weight of the bacterial proteins, external anatomical changes, changes in fish behavior and mortality. The parameters were analyzed descriptively.

Results

Identification of bacteria. The results of the identification of *S. agalactiae* type $N_{14}G$ as the test bacteria using the PCR method based on Mata et al (2004) showed that the bacteria had a fragment length of 192 bp (Figure 1).

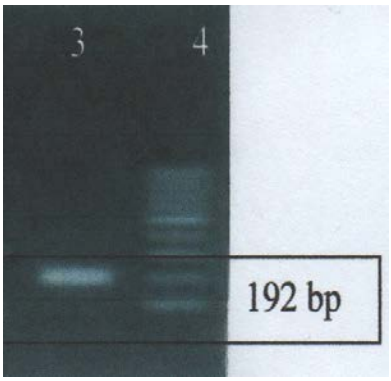

Figure 1. The identification results for the bacteria *Streptococcus agalactiae* type $N_{14}G$ using the PCR method. 4 (Marker) and 3 (*S. agalactiae* bacteria).

Post bacterial infection changes. Based on the infection results, the *O. niloticus* infected with the pathogenic bacteria *S. agalactiae* demonstrated changes in their external anatomy, swimming pattern, feeding behavior, and there even mortality occurred. All these changes demonstrated the characteristic clinical signs of streptococcosis caused by the bacteria *S. agalactiae*. On the other hand, the *O. niloticus* infected with the non-pathogenic bacteria did not demonstrate any abnormalities or fish mortality (Table 1).

Table 1

Changes in external anatomy, swimming patterns, feeding patterns, and *Oreochromis niloticus* mortality after being infected with *Streptococcus agalactiae* bacteria

Treatment	*External anatomical changes*	*Changes in swimming behavior*	*Changes in behavior*	*Fish mortality (%)*
Pathogenic *S. agalactiae* bacteria	Darkening in body color, a clear operculum and demonstrated shrinking of the eyes, exophthalmia and fish's body became curved like the letter C	Whirling and solitary at the bottom of the aquarium	Reduced appetite	High mortality (55.57%)
Non-pathogenic *S. agalactiae* bacteria	No anatomical changes	No changes in swimming patterns	No changes in feeding behavior	No changes in mortality

Whole cell bacterial protein fractionation. Fractination of *S. agalactiae* whole cell protein toxins using the SDS-PAGE method was aimed to separate proteins based on their molecular weight. The results of electrophoresis and silver staining are presented in Figure 2, where there were four protein bands in the non-pathogenic bacterial cells and 8 protein bands in the pathogenic bacterial cells.

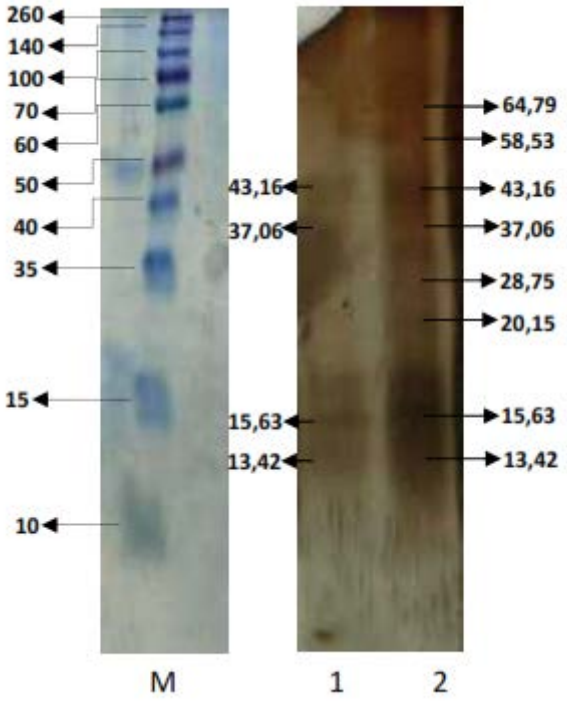

Figure 2. Results of protein fractionation of whole cell *Streptococcus agalactiae* using the SDS-PAGE method. M (marker), 1 (Non-pathogenic *S. agalactiae*) and 2 (Pathogenic *S. agalactiae*).

Based on the results of fractionation using SDS-PAGE, it can be seen that there was the difference in the structure of bacterial proteins. All the proteins present in non-pathogenic bacteria appeared in pathogenic bacteria. However, the pathogenic bacteria revealed a new protein with molecular weights of 20.15, 28.75, 58.53, and 64.79 kDa. These four proteins were not present in non-pathogenic bacteria.

Discussion. The results of this study revealed that there were differences in the proteins between pathogenic and non-pathogenic bacteria (Figure 2). The new proteins that emerged in the pathogenic bacteria are suspected to be toxin proteins which caused the bacteria to be pathogenic. The bacteria whole cell proteins obtained in this study were similar to the ECP S protein. Pathogenic *S. agalactiae* (Hardi 2011) consists of 62.3 and 55.8 kDa in BHI medium for 72 hours and 65.8, 60.1, and 55.8 kDa in BHIA medium for 24 hours. This study's results were also similar to the protein obtained by Chen et al (1997) with a protein range of 14-65 kDa in ECP *Mycobacterium* spp. Unlike Sugiyani (2012), the same isolates resulted in 55.80 and 17.10 kDa proteins on BHIB media for 72 hours.

The present study's results were in line with Pasnik et al (2007) who compared fresh immunogenic vaccine to vaccine that had been stored for one year. The fresh immunogenic vaccine, the result of SDS-PAGE silver staining and western blot immunostaining demonstrated a band with a molecular weight of 54 and 55 kDa, whereas the in the stored vaccine the 55 kDa band was not found and it was not immunogenic. This demonstrated that the 55 kDa protein was an immunogenic protein.

A study that demonstrated that the pathogenicity of bacteria is determined by a certain material in the bacteria has been performed by Kim et al (2015) using the model zebrafish *Danio rerio*. The bacteria were pathogenic and lethal to the fish model when they contained the GBS capsule, surface anchored lipoteichoic acid (LTA), and produced toxin, and became non-pathogenic due to the lack of these factors. *S. agalactiae* produces several virulence factors, including polysaccharide capsule antigens, protein antigens and lipoteichoic acid. Non-structural virulence factors are the bacteria's extracellular products including hyaluronidase, protease, nuclease hypuricase, C5a-ase, hemolysis and CAMP factors (Pritchard & Lin 1993).

Changes in cellular proteins that are believed to be toxic in pathogenic bacteria are rarely studies despite the relevance of this information with efforts to build specific immunity in order to develop vaccines. On the other hand, studies pertaining to the pathogenicity between different strains of bacteria are quite abundant such as by Wang et al (2017) who compared the genomics between virulent and non-virulent serotype V ST1 S and concluded that, generally, the serotype V ST1 GBS consisted of virulent and non-virulent strains in *O. niloticus* and that gene rearrangement could be the cause of different virulence levels between strains.

Another study was conducted by Kayansamruaj et al (2015) who compared the genomics of *S. agalactiae* serotype Ia*,* β-hemolytic GBS, sequence type 7 from complex clonal 7 which was the main cause of the streptococcosis outbreak in *O. niloticus* cultivation in Thailand. Wang et al (2017) evaluated the miRNA expression profile, potential function, and the relationship with genes involved in the inflammation pathway in the brains of *O. niloticus* infected by *S. agalactiae*, where MicroRNAs are a class of non-coding RNA molecules which have an important role in a number of biological processes. Cai et al (2016) had evaluated the phoB mutant in *S. agalactiae* as a live vaccine in pompano *Trachinotus ovatus*. The phoB gene was cloned from pathogenic *S. agalactiae* strain TOS01 and the mutant strain SAΔphoB constructed via allelic exchange mutagenesis.

Protein purification is a process to isolate proteins from complex mixtures. The various steps in the purification process release the protein from the matrix that binds it and separates the protein and the non-protein portion of the solution mixture to obtain the desired and pure protein (Coligan et al 1995). One technique used to separate proteins is SDS-PAGE which is based on the length of the polypeptide chain or the molecular weight in an electric field. The protein mixture to be dissolved was dissolved in the SDS, an anionic detergent type. This SDS denatures the secondary structure and

non-disulfide bond from the tertiary structure to the linear/primary structure. In addition, SDS also provides a negative charge on the linear structure of proteins by comparison depending on the weight of the protein.

Proteins with molecular weights of 20.15, 28.75, 58.53, and 64.79 were suspected as toxin proteins in the pathogenic bacteria that caused external anatomical changes, changes in swimming behavior and changes in diet and mortality in tilapia. In contrast to non-pathogenic bacteria, the proteins 20.15, 28.75, 58.53, and 64.79 kDa were absent; therefore, it was not toxic. The results of pathogenic testing caused anatomical abnormalities such as darkening in body color, shrinkage of the eyes, exophthalmia and C-shaped bodies. There were changes in swimming behavior which included whirling and changes in the fish's eating behavior. The exhibition of clinical symptoms, swimming and feeding behavior in this study were similar to the findings by Amrullah et al (2015) and supported the research of Baya et al (1990), Eldar et al (1995), Evans et al (2002), Salvador et al (2005) and Pasnik et al (2007), and ended in the death of the fish.

After being injected with pathogenic *S. agalactiae*, the mortality of the *O. niloticus* was 51.67%. Unlike the fish injected with non-pathogenic bacteria, no fish died. This indicated that the pathogenic bacteria can kill the tilapia. The pathogenic *S. agalactiae* injected into the fish would have the ability to quickly adjust to environmental changes during the infection, obtain nutrients, evade the fish's immune response, and spread to various tissues (Litwin & Calderwood 1993).

The results of this study indicated that *S. agalactiae* virulence is determined by the protein with molecular weights of 20.15, 28.75, 58.53, and 64.79 kDa. The identification of this toxin protein is an early stage and is the most important stage in vaccine development. Further study should be conducted on its potential as a fish vaccine, in the framework of disease-resistant fish production along with the application of probiotics (Amrullah et al 2014b; Park et al 2016; Speranza et al 2017) and immunostimulants (Wang et al 2016; Awad & Awaad 2017; Sahoo et al 2017).

Conclusions. The result of whole cell protein fractionation of pathogenic *S. agalactiae* showed that there were new proteins emerging that were not found in non-pathogenic bacteria i.e. 20.15, 28.75, 58.53, and 64.79 kDa proteins. The four proteins in these bacteria are likely to be the toxin proteins that determine the pathogenicity of *S. agalactiae*. Therefore, further evaluation is required to determine the toxicity of each toxin protein and then evaluate its immunogenic potential.

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