UNIVERSITI PUTRA MALAYSIA

HISTOPATHOLOGICAL COMPARISON BETWEEN EXPERIMENTAL *STREPTOCOCCUS AGALACTIAE* AND *STREPTOCOCCUS INIAE* INFECTIONS IN *OREOCHROMIS SP.*

DZULKIFLI BIN JAMALLUDIN

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FACULTY OF VETERINARY MEDICINE

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DZULKIFLI BIN JAMALLUDIN

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Histopathological comparison between experimental *Streptococcus agalactiae* and *Streptococcus iniae* infections in *Oreochromis* sp.", by Dzulkifli Bin Jamalludin and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement of the course VPD 4901 – Project.

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DEDICATION

Alhamdulillah. In the name of ALLAH, the most gracious and the most merciful.

I dedicate this thesis to my beloved parents & family,

Lecturers,

Friends,

Thank you for your endless love & support and everyone who reads and benefits from this thesis.

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, UPM in partial requirement to fulfil the course of VPD 4999- Final Year Project.

HISTOPATHOLOGICAL COMPARISON BETWEEN EXPERIMENTAL *STREPTOCOCCUS AGALACTIAE* AND *STREPTOCOCCUS INIAE* INFECTIONS IN *OREOCHROMIS SP.*

By

Dzulkifli Jamalludin

2018

Supervisor: Dr Annas Salleh

Streptococcus agalactiae and *streptococcus iniae* are two main pathogens contributing to streptococcosis in fish. Streptococcosis is an important disease, leading to economic losses in aquaculture industry. The aim of this study was to compare the histopathological evaluations between *S. agalactiae* and *S. iniae* infections in *Oreochromis* sp.. Two experiments were conducted. In the first experiment, 60 healthy fish with the size of less than 4" were divided equally into three (3) groups; one control (G1) (n=20) and two treatment groups (G2, G3) (n=20) each. Each group was further divided equally into two groups, consisting of 10 inoculated fish and 10 fish were not inoculated but served as commingling fish. The former were inoculated with sterile PBS, *S. agalactiae,* and *S. iniae*, intraperitoneally at the rate of 0.03 ml/g of $1x10^7$ cfu/ml of bacteria. In the second experiment, the same

experimental design was used, with the exception of the size of fish. Fish with more than 4" were used. G4 was inoculated with sterile PBS, G5 was inoculated with *S. agalactiae,* and G6 was inoculated with *S. iniae* at the aforementioned rate. All fish were observed every 6 hours for a period of 5 days. During the observation period, any dead fish was subjected to necropsy. After the observation period, any remaining fish were euthanized for necropsy. Samples of spleen, liver, and brain were collected and fixed in 10% neutral buffered formalin, and subjected to routine histopathology process. Gross lesions observed included cerebral oedema, ascites, integumentary haemorrhage, presence of pustules, and intestinal congestion. Tissue section of each organ was examined under microscope at 40x magnification and lesions were described and scored. In all infected groups, infiltration of inflammatory cells and haemorrhage were the most frequently observed lesion. All groups infected by *S. agalactiae* showed significantly ($p<0.05$) more severe lesions compared to the groups infected by *S. iniae*, regardless of the size. Significant difference ($p<0.05$) was also observed in comparison between the lesion score in fish of different sizes. Fish of more than 4" showed higher lesion severity score compared to fish of less than 4". In both streptococcosis, *S. agalactiae* is more pathogenic than *S. iniae* in *Oreochromis* sp., and fish more than 4" is more susceptible towards both streptococcosis.

Keywords: Streptococcosis, *Streptococcus agalactiae*, *Streptococcus iniae,* histopathology, *Oreochromis sp*, (tilapia)

ABSTRAK

Abstrak daripada kertas project yang dikemukakan kepada Fakulti Perubatan Veterinar UPM untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Tahun Akhir.

PERBANDINGAN HISTOPATOLOGIKAL DIANTARA JANGKITAN EXPERIMENTAL *STREPTOCOCCUS AGALACTIAE* DAN *STREPTOCOCCUS INIAE* DI DALAM *OREOCHROMIS* SP.

Oleh

Dzulkifli Jamalludin

2018

Supervisor: Dr Annas Salleh

Streptococcus agalactiae dan *Streptococcus iniae* adalah dua patogen utama yang menyebabkan streptococcosis di dalam ikan. Streptococcosis merupakan satu penyakit yang sangat penting di mana ia boleh membawa kepada kerugian ekonomi di dalam industry akuakultur. Tujuan penyelidikan ini adalah bagi membandingkan penilaian histopatologikal diantara jangkitan *S. agalactiae* dan *S. iniae* di dalam *Oreochromis* sp.. Dua eksperimen telah dijalankan. Dalam eksperimen yang pertama, 60 ekor ikan tilapia yang sihat berukuran kurang dari 4” telah dibahagikan sama rata kepada tiga (3) kumpulan; satu kawalan (G1) (n=20) dan 2 kumpulan rawatan (G2, G3) (n=20) setiap satu. Setiap kumpulan dibahagikan lagi secara sama rata kepada dua kumpulan yang terdiri daripada 10 ekor ikan diinokulasi dan 10 ekor ikan tidak diinokulasi tetapi bertindak sebagai ikan yang bergaul. Ikan yang

diinokulasi telah diinokulasi dengan steril PBS, *S. agalactiae,* dan *S. iniae,* secara intraperitoneal dengan kadar 0.03ml/g mengandungi $1x10^7$ cfu/ml bakteria. Di dalam eksperimen kedua, kaedah eksperimen yang sama juga digunakan, dengan pengecualian saiz ikan yang digunakan iaitu ikan yang berukuran lebih dari 4". G4 telah diinokulasi dengan steril PBS, G5 diinokulasi dengan *S. agalactiae,* dan G6 telah diinokulasi dengan *S. iniae* menggunakan kadar yang telah disebutkan. Keseluruhan ikan telah diperhatikan setiap 6 jam dalam tempoh masa 5 hari. Ketika dalam tempoh pemerhatian, sebarang ikan yang mati akan terus melalui nekropsi. Selepas tempoh pemerhatian, mana-mana ikan yang masih hidup akan ditakai bagi tujuan nekropsi. Sampel; limpa, hati dan otak dipungut dan disimpan di dalam 10% neutral buffer formalin, dan diteruskan dengan proses rutin histopatologi. Lesi kasar yang dapat dilihat termasuklah permukaan berair di bahagian otak, air di dalam rongga abdomen, pendarahan integumentary, penampakan nanah dan penyesakan usus. Seksyen tisu setiap organ telah diteliti di bawah mikroskop pada kadar magnifikasi 40x dan setiap lesi dijelaskan dan dinilai. Di dalam semua kumpulan jangkitan, kehadiran sel keradangan dan pendarahan adalah merupakan lesi yang paling banyak dilihat. Kesemua kumpulan jangkitan dengan *S. agalactiae* menunjukkan secara signifikan ($p<0.05$) lesi yang lebih teruk dibandingkan kepada kumpulan jangkitan dengan *S. iniae,* tidak kira samaada saiz berbeza. Perbezaan signifikan ($p<0.05$) juga dapat dilihat dalam perbandingan di antara skor lesi di dalam ikan yang berlainan saiz. Saiz ikan yang melebihi 4” menunjukkan kadar lesi yang lebih tinggi jika dibandingkan kepada saiz ikan yang kurang dari 4”. Di dalam kedua-dua jangkitan streptococcosis, *S. agalactiae* adalah lebih patogenik daripada *S. iniae* di dalam *Oreochromis* sp. dan ikan berukuran lebih dari 4” adalah lebih cenderung kepada jangkitan kedua-dua streptococcosis.

Kata kunci: Streptococcosis, *Streptococcus agalactiae*, *Streptococcus iniae,* histopatologi, *Oreochromis sp* (tilapia)

1.0 INTRODUCTION

Oreochromis sp., also known as Tilapia, is a prolific species of fish. This fish is reared in most parts of the world as it is assumed to be resistant to infection and environmental stressors. However, recently, it is found that *Oreochromis* sp. is prone to streptococcosis infection. Streptococcosis is an infection by Gram-positive bacteria of the genus Streptococcus. This bacteria is non-motile and non-spore forming. *Streptococcus sp* are facultatively anaerobic and they require nutritionally-rich media for growth. On blood agar supplemented with 5% blood, the organisms produces greenish discolouration (α-hemolysis) or complete clearing (β-hemolysis). Additionally, it is also fermentative in metabolism, releasing lactic acid, but without gas and catalase-negative. (Amal and Zamri-Saad, 2011). This disease has become a major problem in the aquaculture and fisheries industry throughout the world (Hernandez *et al*., 2009).

Currently, *S. agalactiae* and *S. iniae* have been identified as the main pathogens causing this disease leading to severe economic losses in the aquaculture and fisheries industry throughout the world (Evans *et al*., 2006; Amal and Zamri-Saad, 2011; Costa *et al*., 2013). Amongst the S*treptococcus* sp., *S. agalactiae* has a broad host range, infecting both terrestrial and aquatic animals. This bacterium can causes neonatal meningitis and mastitis in humans and cattle, respectively. However, other animals such as mice, cats, dogs, hamsters, camels and frogs can also be infected (Hernandez *et al*., 2009). *S. agalactiae* has been isolated from numerous fish species in natural outbreaks of disease and has been shown to be pathogenic to several fish species in experimental trials using different routes of infection such as cohabitation, immersion, intraperitoneal and intra-muscular injections (Evans *et al*. 2002). According to Pretto Giordano and Scarpassa in 2015, *S. iniae* can be consider as an important pathogen in aquaculture and it is an emerging zoonotic pathogen. The first isolation of *S. iniae* occurred in the 1970s from skin lesions on dolphins (*Inia geoffrensis*). Then, it was subsequently identified in fish in North America, Middle East, Asia-Pacific region and Europe (Pretto-Giordano and Scarpassa, 2015).

The main clinical signs presented on the fishes are unilateral or bilateral exophthalmos, ocular haemorrhages, increase in corneal opacity, distended abdomen, curvature of the spinal cord, stiffness, erratic swimming, bleeding tendency at the base of the fins and difficulty in breathing. Some fish may not show clinical signs prior to death (Yanong and Floyd, 2002; Pulido *et al*., 2004). Gross findings reveals haemorrhagic ascites, mucous content with reddish-brown colour in the intestine, pale and enlarged liver and a haemorrhagic brownish appearance of the retro-bulbar tissue and meninges (Pulido *et al*., 2004; Zamri-Saad *et al*., 2010).

At microscopic levels, most tilapias develop a primary inflammatory response of mononuclear cells with the subsequent formation of granulomatous nodules. Lesions include severe haemorrhagic or granulomatous meningoencephalitis accompanied by large areas of encephalomalacia (Noraini et al., 2013). Similar tissue lesions can be found in the choroid, sclera and the eyeball. Histopathological changes may be found in organs such as spleen, brain, gut and liver. Findings are necrosis of epithelial gut, bacterial clumps in the blood vessel of liver and congestion and inflammation in the spleen. Severe lesions are haemorrhage of meningitis and erosion and necrosis of mucosal gut. Although both bacteria shows similar gross and histopathological lesion findings but currently, there were no studies conducted specifically to compare the gross lesions and severity of histopathological lesions between *Oreochromis* sp. infected with *S. agalactiae* and *S. iniae* infection via intra peritoneal route. Therefore, this study is proposed with the aim to compare the histopathological lesion in experimentally infected *Oreochromis sp*. following exposure to *Streptococcus agalactiae* or *Streptococcus iniae v*ia intraperitoneal injection.

2.0 LITERATURE REVIEW

2.1 Streptococcus sp.

2.1.1 *Streptococcus iniae*

Streptococcus iniae is a Gram-positive bacteria which appeared punctiform and transparently white. Biochemical test such as catalase and oxidase will yield negative results. It also produces complete β-hemolysis on blood agar. The agent was first isolated from multifocal subcutaneous abscesses in captive Amazon freshwater dolphins which is the Iniae geoffrensis (Dewi *et al*., 2015). *S. iniae* are able to be carried asymptomatically. However, it is also associated with several sporadic disease outbreaks. 30% to 50% of mortality rates have been linked to infection by *S. iniae* in aquaculture farms (Rahmatullah et al., 2017).

S. iniae is also a major concern as not only it is one of the major causative agent of streptococcosis besides *S. agalactiae* in the aquaculture industry but also an important zoonotic bacterial disease which can cause morbidity and mortality in humans. Fishes infected by *S. iniae* are commonly presented with clinical signs such as loss of orientation, lethargy, ulcers, exophthalmia and in fatal cases-meningoencephalitis (Dewi et al., 2015)

2.1.2 *Streptococcus agalactiae*

Streptococcus agalactiae is a Gram-positive, coccus which are organized in pairs or short chains. It will yield the biochemical test catalase and oxidase negative. It may or may not have haemolytic reaction (Ortega *et al*., 2016). The lesions are characterised by loss of appetite, exophthalmia, ocular haemorrhage, increase in corneal opacity (unilateral or bilateral), distension of the abdomen, curvature of the spinal cord and erratic swimming (Abuseliana *et al*., 2011).

S. agalactiae is known to affect different species of fresh, marine and estuarine water fishes. Among the species of fish that are known to be infected are cultivated rainbow trout and tilapia. (Pretto-Giordano *et al*., 2010)

2.2 Pathogenicity of *Streptococcus sp.*

Buchanan *et al.* (2005) identified enzyme phosphoglucomutase as the virulence factor for *Streptococcus iniae*. This enzyme inter-converts glucose-6-phosphate and glucose-1-phosphate which play important role in the production of S. iniae polysaccharide capsules. Unlike *Streptococcus iniae*, the regulatory proteins and enzymes associated with cell surface metabolism have been revealed as the virulence factors for *Streptococcus agalactiae*. Therefore, the removal of the genes that are involved in these functions can reduce the virulence. Fuller et al. (2002) found that the virulence factor could also be caused by the gene that is associated with β-hemolysis. However, additional research should be carried out to identify and characterize the genes and the virulence factors that regulate their expression.

(Jantrakajorn, Maisak, & Wongtavatchai, 2014) stated that some of the bacteria are able to evade the immune system using its particular properties to cause systemic infection :

a) Surface antigens of Streptococcal bacteria can attach to the fish's cell surface, which prevent them to be eliminated by fish's lysozyme. Consequently, the bacteria replicate in lymph and blood (septicemia) and spread to target organs, such as liver, kidney, spleen and brain.
b) Toxin production, the important hemolytic toxin of Streptococcus is streptolysin, which is further classified as streptolysin S and streptolysin O. The toxins cause complete hemolysis on blood agar. Streptolysin S produces hemolysis on the surface of blood agar (surface hemolysis), whereas streptolysin O produces hemolysis under anaerobic condition, in a deeper layer of blood agar (deep hemolysis). The toxin rapidly damages cells and tissues, including white blood cells, liver and heart.
c) Enzyme production, most of the enzymes produced by Streptococcus are capable of digesting large molecules, such as fibrin clump and connective tissue. This enables the bacteria to easily penetrate the tissue, especially at skin lacerations and mucous membrane of several organs.

2.3 Streptococcosis disease

Streptococcosis is an infection by Gram-positive bacteria of the genus Streptococcus. They are non-motile, non-spore forming organism. *Streptococcus* sp. are facultatively anaerobic and require a nutritionally rich media for growth such as Brain Heart Infusion broth. On blood agar supplemented with 5% blood, the organisms produces greenish discolouration (α-hemolysis) or complete clearing (β-hemolysis) of the red blood cells. Additionally, it is also fermentative in metabolism, releasing lactic acid, but without gas and catalase-negative (Amal & Zamri-Saad, 2011). This disease has become a major problem in the aquaculture and fisheries industry throughout the world (Hernandez *et al*., 2009).

2.4 Diagnosis of streptococcosis

In fish, the disease diagnosis and investigation will follow the same principle as in vertebrae animals. During any disease outbreak, it is important to get the complete history combined with physical examination of the fish. Followed by obtaining samples from any affected animals with clear clinical signs of the disease and corresponding apparently normal fish from the same site/pond/cage (Wongsathein 2012).

In the case of streptococcosis, the diagnosis should be based on typical clinical signs, lesions, and demonstration of Gram-positive cocci bacteria, isolated from internal organs of affected fish, pathological findings and confirmation of the bacterial species with other laboratory methods. The pathogen is routinely isolated from the spleen, kidney, eyes and brain using media such as tryptone soya agar (TSA), brain heart infusion agar (BHIA), Todd-Hewitt broth agar (THBA), blood agar or selective agar containing thallium acetate-oxolinic acid (Buller, 2004; Austin and Austin, 2007). The incubation period is reported at between 24-48 hr at 25-35°C (Wongsathein 2012).

The bacterial isolates are then characterised by morphological evaluation, supported with the biochemical and molecular tests including analytical profile index (API) and PCR to confirm the presence of the suspected aetiological agent (Kitao *et al*., 1981; Plumb, 1999). Some of the selected organ from affected fish including kidney, spleen, eyes, brain, liver, intestine, gills, heart and muscle should be fixed in 10% buffered formalin for histopathology (Roberts, 2001).

However, the diagnosis of streptococcal infections in fish has been complicated because similar clinical signs are seen in the same fish species due to other pathogens. There are several other closely related gram-positive cocci that share similar features with *S. agalactiae* and in natural infections may present similar gross clinical signs of disease including *S. iniae*. Therefore, the identification of *S. agalactiae* in tilapia should include a combination of standard conventional methods, biochemical characteristics, Lancefield serogrouping and species-specific PCRs to ensure that the right aetiological agent is identified.

2.4.1 Gross lesions of Streptococcosis

Streptococcosis infection will produce a similar gross lesions towards fish. Multiple studies conducted had reported different gross lesions findings. Previously, it has been reported that gross lesions such as presence of blood-tinged fluid in the body cavity, enlarged and reddened spleen, pale and enlarged liver, hemorrhagic lesions on the skin and internal organs and meningoencephalomalacia (Zamri-Saad *et al*., 2014) as well as exopthalmia (Alsaid *et al.*, 2013).

2.4.2 Identification and isolation of *S. agalactiae* and *S. iniae*

The first indication that be used to identify presence of *Streptococcus sp* was by observing the clinical signs associated with the demonstration of Gram-positive cocci from the brains, kidneys, eye or other internal organs. The causative bacteria are best detected in the brains of diseased fish (Zamri-Saad *et al*., 2014). Further confirmatory diagnostic plan that can be done requires culture of internal organs, specifically the brain and kidney, followed by identification of the bacterium (Yanong and Floyd, 2002). Several types of media that have been recommended for culture of *S. agalactiae* and *S. iniae* include bovine blood tryptose agar, brain heart infusion agar (BHIA), Todd-Hewitt broth, nutrient agar supplemented with rabbit blood or horse blood agar and incubated at 22-37°C for up to 48 hours. The resultant colonies are "dull grey" of approximately 1-2 mm in diameter. A single colony from pure culture should be Gram-positive cocci, oxidase, and catalase negative and either non-hemolytic or β-hemolytic on agar plate.

The carbohydrate group antigen test should be also among the first presumptive test performed. The only group B streptococcal species is *S. agalactiae*. In contrast, *S. iniae* does not have a carbohydrate group antigen. If the streptococci hydrolyse starch, it is also presumptive test for *S. iniae* (Evans *et al*., 2004).

Rapid kits such as API 20E, API Rapid Strep 32, and API CH50 could not be used to identify *S. iniae* because this particular bacterium is not included in the database system. However, these rapid kits can be used for the identification of *S. agalactiae* and other Streptococcus spp. (Evans *et al*., 2006a).

Molecular diagnosis using the PCR technique is useful to identify streptococcus. Many of the PCR techniques make use of the 16S rRNA gene as the molecular marker for the identification of *S. iniae* (Zlotkin *et al*., 1998). Besides, a PCR technique using 16S-23S ribosomal DNA intergenic spacers was found to be useful for the identification of *S. agalactiae* from fish (Berridge *et al*., 2001). However, the results of the PCR assay should be supported by presumptive techniques to ensure the accuracy of the detection.

2.5 Treatment, Prevention, and Control

In cases of streptococcosis outbreak, there is a treatment option available to be carried out and it is done through the use of antibiotics. According to (Zamri-Saad *et al*., 2014) the use of antibiotics for treatment of streptococcosis is useful only in the early stages. This is due to the fact that oral antibiotics in the feed will not be ingested by the affected fishes as they are having inappetance. It is however, a common practice in Malaysia, to administer antibiotics in the feed as a prophylactic treatment. Among the common antibiotics used are oxytetracycline, amoxicillin, ampicillin & erythromycin (Wongsathein, 2012).

Since there is only 1 treatment option globally available, it is therefore, crucial to carry out prevention and control measures. Besides more economical aspect, it is also helpful in preventing any potential outbreak to adjacent areas. According to a study done by Wongsathein in 2012, actions such as improving water quality & environmental conditions, and reducing stocking densities are the most common actions carried out in intensively farmed tilapia. This also supported by a study done by Zamri-Saad, Amal, Siti-Zahrah, & Zulkafli in 2014 with additional control and prevention measures such as avoiding overfeeding, minimising stress of the fish (handling or transportation), regular removal of dead fish, implementing an “all in, all out” system, proper biosecurity measures in the farm and practicing vaccination.

3.0 MATERIALS AND METHOD

3.1 Experimental Fish

A batch of sixty six red hybrid tilapia (*Oreochromis* sp.) with less than 4 inch of length and a batch of sixty six red hybrid tilapia (*Oreochromis* sp.) with more than 4 inch of length were obtained from an SA Agromax, Puchong, Selangor. The fish were maintained in 6 plastic aquarium with size approximately 32 cm x 20 cm x 20 cm and were acclimatized for 10 days before the beginning of the experiments at the Biology Department, Faculty of Science, Universiti Putra Malaysia. During the acclimatization period, the fish were fed twice daily with commercial fish feed at a rate of 2% of their body weight until a days prior to experiment.

The water was constantly monitored and renewed with 50% fresh water daily. During acclimatization, the fish were treated with anti-fungal, anti-parasite and white spot disease. Several fish were randomly selected for necropsies and sample swabs were taken from brain, liver and spleen to confirm that the fish were disease free. All experimental procedures were approved by Institutional Animal Care and Use Committee, Universiti Putra Malaysia (approval number: UPM/IACUC/AUP- U035/2018).

3.2 Preparation of *S. agalactiae* and *S. iniae* inoculums

S. agalactiae and *S. iniae* was obtained from frozen aliquoted stock culture which was isolated from previous outbreak of Streptococcosis. The aliquoted stock culture was thawed and cultured on the blood agar containing 5% horse blood and incubated at 37º C for 24 hours. It was then inoculated in BHI broth (Brain heart infusion) and incubated at 37º C for 24 hours by using shaking incubator. In order to verify the presence of the bacteria in the inoculum, the aliquot was inoculated in a blood agar and incubated at 37º C for 24 hours.

3.3 Pilot study

Each bacterial inoculum were injected into 2 fish with a dose of 0.03ml/g. it is then observed for a period of 5 days. This step was done as a pilot study to see whether the stock inoculum had the ability to cause infection and mortality towards the fish without having to be re-virulent. Dead fishes were then subjected for necropsy and organ swab was taken in order to confirm the presence of the bacteria. Upon confirmation of the presence of the bacteria along with the clinical signs, gross and histopathological lesions observed, it can be said that the stock bacterial inoculum had the ability to cause infection and mortality without having to be re-virulent.

3.4 Bacterial concentration determination

In this experiment, an infective dose of $1x10^7$ was chosen for each of the treatment group. To obtain the desired concentration of infective dose, serial dilution method was carried out from the virulent broth and diluting it in a 10 fold dilution set up.

3.5 Study design

In the first experiment, 60 healthy fish with the size of less than 4 inch of body length were divided equally into three (3) groups; one control (G1) (n=20) and two treatment groups (G2, G3) (n=20) each. Each group was further divided equally into two groups, consisting of 10 inoculated fish and 10 fish were not inoculated but served as commingling fish. The former were inoculated with sterile PBS, *S. agalactiae,* and *S. iniae*, intraperitoneally at the rate of 0.03 ml/g of $1x10^7$ cfu/ml of bacteria. In the second experiment, the same experimental design was used, with the exception of the size of fish. In this experiment, fish with more than 4 inch of body length were used. G4 was inoculated with sterile PBS, G5 was inoculated with *S. agalactiae,* and G6 was inoculated with *S. iniae* at the aforementioned rate. All fish were observed every 6 hours for a period of 5 days. During the observation period, any dead fish was subjected to necropsy. After the observation period, any remaining fish were euthanised for necropsy. Samples of spleen, liver, and brain were collected and fixed in 10% neutral buffered formalin, and subjected to routine histopathology process.

The treated fish from all groups were tagged with the use of 5 different colour (red, black, and white, orange, green) of thread. Each colour was tag at 2 different location. Therefore, each colour will only be present at 2 fish in each tank. Each individual colour can be further differentiated by the location of the tag (upper or lower position of the tail).

3.6 Histological Examination and Evaluation

Organs such as brain, liver and spleen were used for histological examination. Histological slides were prepared by initially performing post mortem and removing the mentioned organs and were immersed in 10% buffered formalin for 48 hours. This was followed by routine histotechniques. Briefly, the samples were transferred into a cassette containing all three organs, underwent tissue processing process for 15 hours, sectioned at 4µm, and stained with H&E.

Each organ was examined in 4 different views at x400 magnification power for 7 histological changes. The 7 histological changes were infiltration of inflammatory cells, oedema, haemorrhage, congestion, thrombosis, vacuolation and necrosis. Each changes were recorded and scored with a severity score of 0-3. An average score was calculated from the 4 views of a single organ for each histological changes. The average score of each histological changes were then summed up and averaged to represent the severity of histological change for the specific organ in the individual fish. This step was repeated for each fish in the same group (Table 1). The average values of all the fishes were then summed up and averaged to represent the severity of histological changes for the specific organ for that particular group.

Treatment fish with Streptococcus iniae							
Spleen							
	Inflammatory cell	Oedema	Haemorrhage	Congestion	Thrombosis	Vacuolation	Necrosis
1. (infected-euthanized)							

Table 1: Example of scoring table

3.7 Statistical Analysis

All data regarding severity of histological changes following different types of bacteria and different size of fish were analysed using one-way and two-way anova. The statistical analysis was analysed by IBM ® SPSS® Statistic Version 22.

4.0 RESULT AND ANALYSIS

4.1 Gross Lesions

At necropsy, gross lesions observed included increase in corneal opacity, distended abdomen (Figure 1), presence of blood-tinged fluid in the body cavity (Figure 2), darkening of the skin on the body (Figure 3), meningoencephalomalacia, and hemorrhages at the base of fin.

Figure 1: Increase in corneal opacity & distended abdomen

Figure 2: Presence of fluid in the abdominal cavity suggestive of ascites

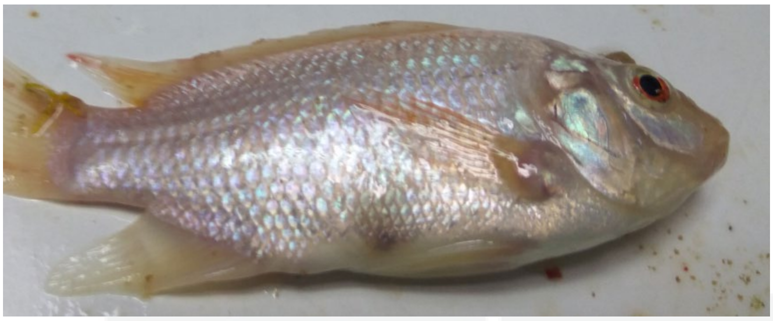

Figure 3: Darkening of the skin on the body

4.2 Histological lesions

All 7 lesions of infiltration of inflammatory cells, oedema, haemorrhage, congestion, thrombosis, vacuolation and necrosis were observed in each organ at varying degree of the severity between each individual fishes. It was also found that infected fish showed more severe lesion score compared to the commingled fish. Example of some of the lesions that were observed were congestion of the blood capillaries in the liver (Figure 4) and hemorrhages and presence of inflammatory cells in the brain (Figure 5).

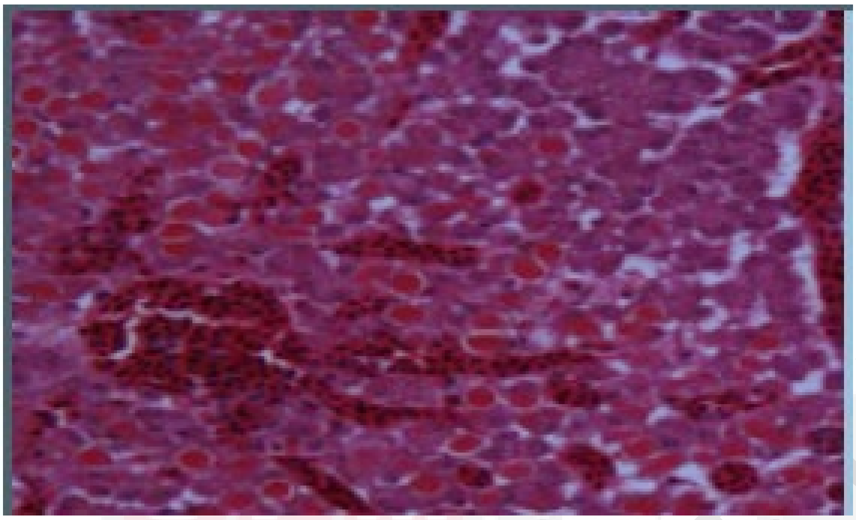

Figure 4: Liver of red hybrid tilapia infected with *S. agalactiae* showing severe congestion of liver blood capillaries (H&E, x400)

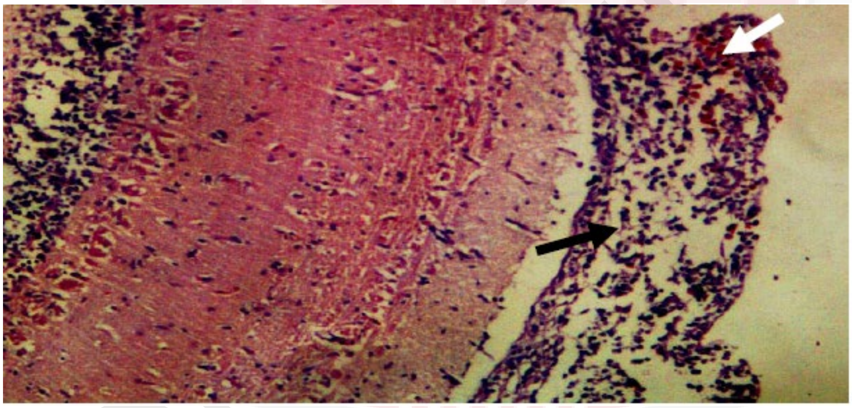

Figure 5: Brain showing haemorrhages (white arrow) and presence of inflammatory cells and increase intercellular space indicating oedema (black arrow) in the meninges (H&E, x400)

It was observed that the lesion with the most severe score in each organ regardless of the types of bacteria were presence of inflammatory cells and haemorrhage (Fig. 6). And, the brain and the liver were found to have the highest score for histopathological severity score.

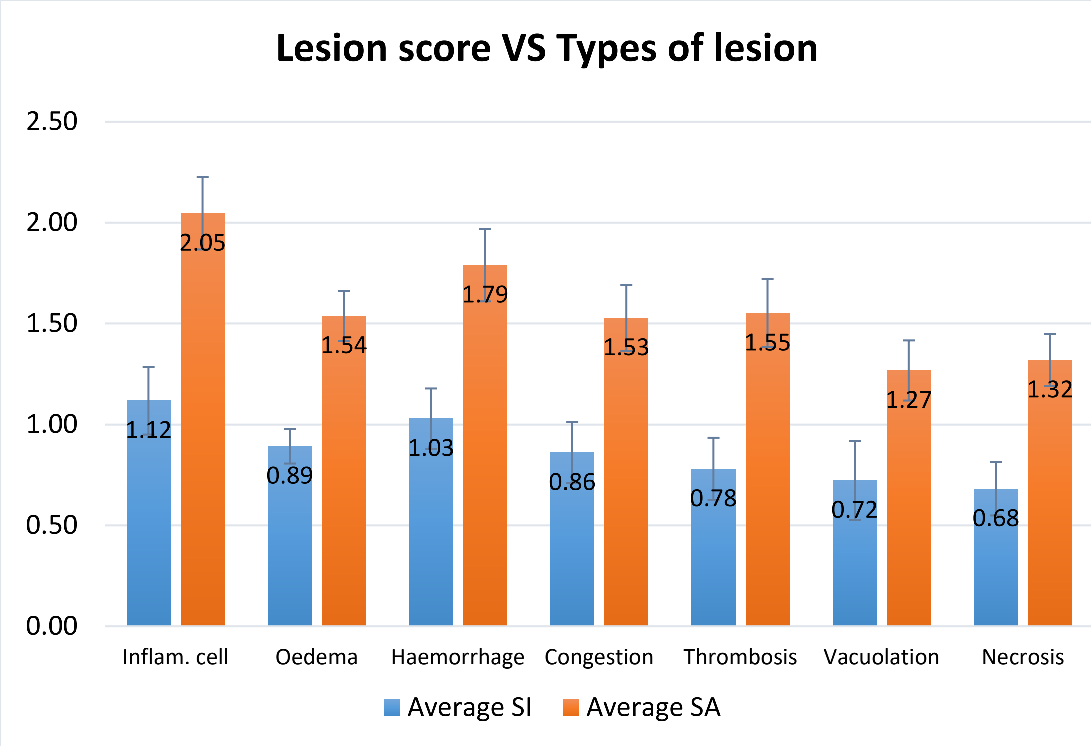

Figure 6: Graph of lesion score against types of lesions in both types of bacteria.

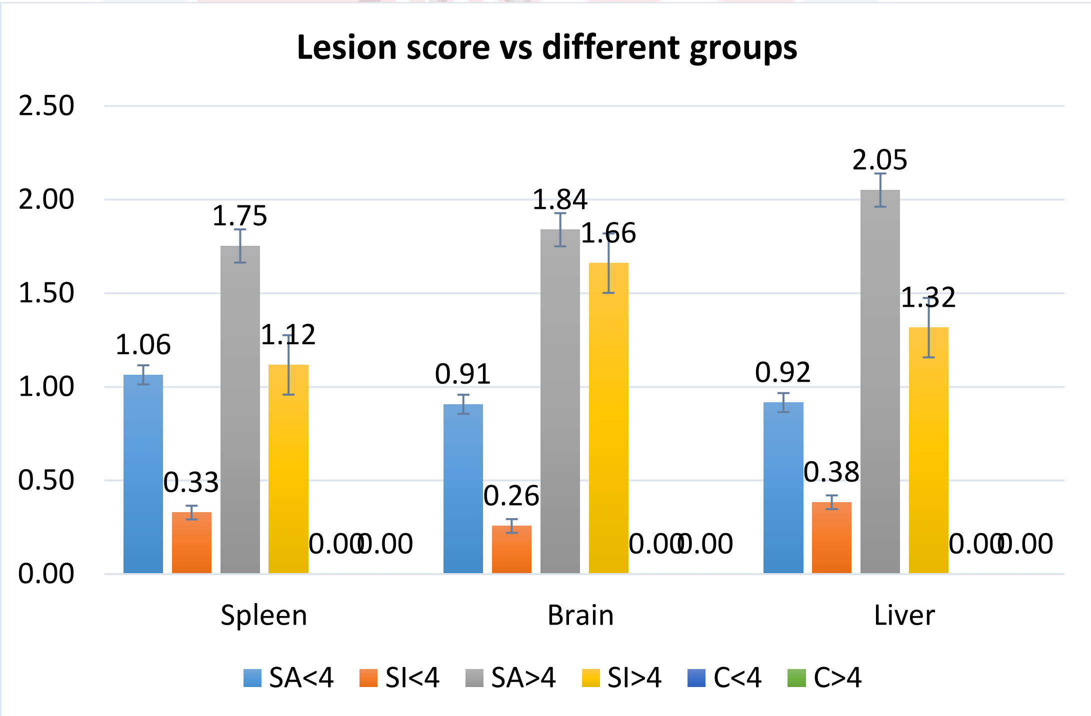

Figure 7: Graph showing lesion score against different groups in each organ

The severity of organs in *S. agalactiae* infected fishes were significantly (p<0.001) higher compared to the severity of organs in *S. iniae* infected fishes (Fig. 8). No lesion was observed in the control group. The severity of organs in larger sized fish (>4") were significantly (p<0.05) higher compared to severity of organs in smaller sized fish (<4") (Fig. 9).

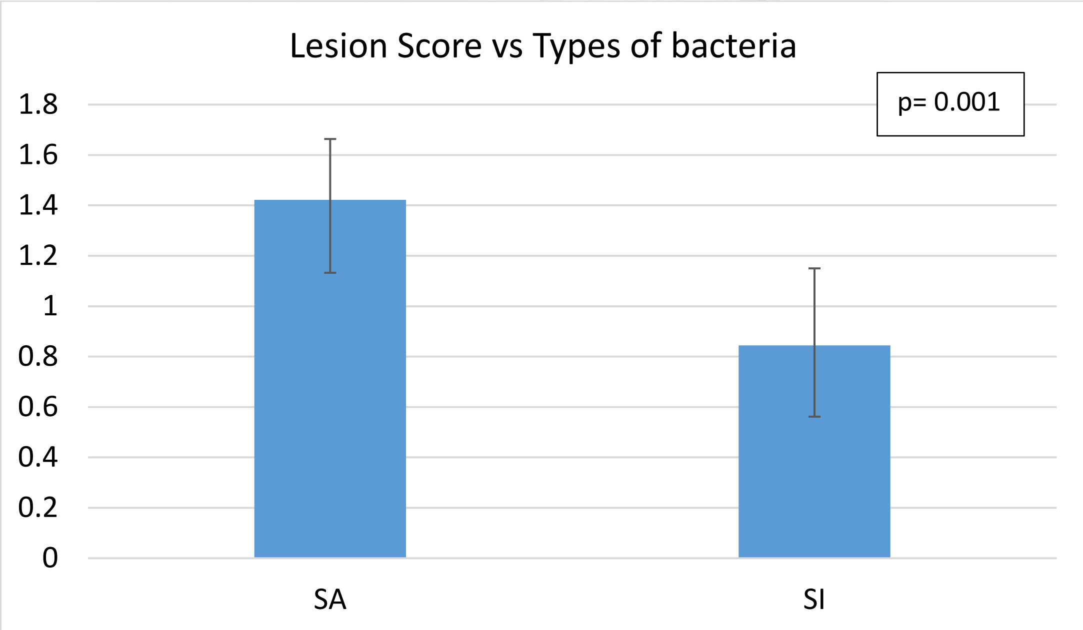

Figure 8: Graph showing lesion score against different types of bacteria regardless of the size of the fish.

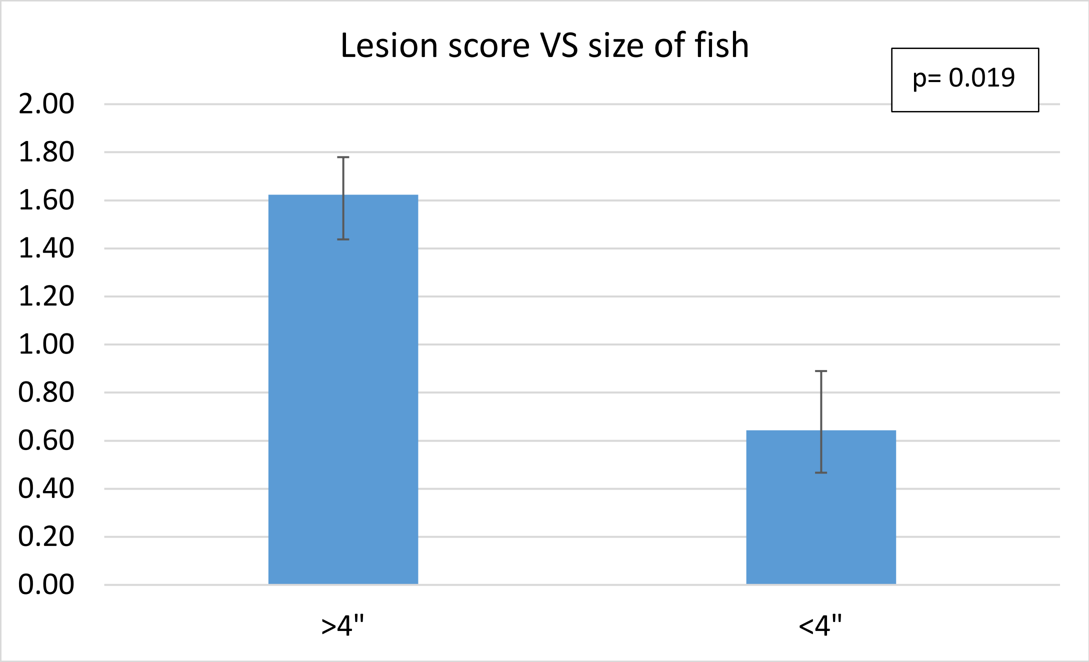

Figure 9: Graph showing lesion scores against size of the fishes regardless of the types of bacteria.

5.0 DISCUSSION

In this study, the gross lesions yield no different findings for both of the bacteria. The findings were classified as typical gross lesions findings for streptococcosis infection and were therefore, not suitable to be used as to differentiate one agent from another. Our findings in this study were similar with the study conducted by Chen *et al* in 2007 which also states that both bacteria will produce similar lesions.

The highest types of lesion that can be observed were presence of inflammatory cells and haemorrhages, regardless of the types of bacteria infecting the fish. The findings were consistent for *S. agalactiae* infection as reported by Alsaid *et al* in 2013. Similar findings were reported by Dewi *et al* in 2015 for *S. iniae.* However, since there are no specific studies which compares both the bacterial agent and its lesion, from our result, it was shown that *S. iniae* will exhibit a less severe lesion score compared to *S. agalactiae.*

From our results, it was found that the brain and the liver were the organs with the most severe lesion score. The severity of the lesion of the organ such as the brain would explain the clinical signs exhibited such as erratic swimming. More importantly, with the brain being one of the most affected organ of all, it was a presumptive diagnosis of streptococcosis infection in fish (Alsaid *et al*., 2013).

Statistically, there was a significant difference (p<0.05) observed when comparing the lesion score between both types of bacteria regardless of the size of the fish. *S. agalactiae* treatment groups showed a more severe lesion score compared to *S. iniae* treatment groups. Though no specific mechanism were stated to support this finding, however, a study carried out by Abuseliana *et al* in 2011 states that infections caused by *S. agalactiae* were the most commonly isolated agent in streptococcosis infection. There were also significant difference (p<0.05) in terms of the severity of the lesion caused in fishes of more than 4 inch of length compared to fishes of less than 4 inch of length regardless of the types of bacteria the group were treated. This showed that streptococcosis infection were more susceptible in fishes of more than 4" of length which was in accordance with a study conducted by Zulkafli *et al*. in 2009, Amal in 2011 and Amal *et al*. in 2013.

6.0 CONCLUSION

In conclusion, we have demonstrated, that *Oreochromis* sp. experimentally infected with *S. agalactiae* and *S. iniae* exhibited similar gross lesions and were not useful to be used as a criteria to differentiate between these two agents. This study has successfully shown that *S. agalactiae* will cause severe histopathological lesion compared *to S. iniae* at which *S. agalactiae* can be said to be of more pathogenic. Both *Streptococcus agalactiae* and *Streptococcus iniae* will produce similar histopathological lesion in spleen, brain and liver of *Oreochromis* sp when experimentally infected through intraperitoneal route. However, there were several studies which states that *S. iniae* tend to develop under a longer period of time therefore, it is suggestive that the observation period to be longer than the currently used method which is 5 days. Furthermore, the severity of the lesions can be noted occurring more prominent in the larger sized group of fishes. Therefore, streptococcosis infection were more susceptible and will produce more severe effect in larger than 4 inch length of fish. These findings will serve as guide for monitoring the streptococcal disease infection and will be useful as a baseline data for early diagnosis of the disease in *Oreochromis* sp.

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