UNIVERSITI PUTRA MALAYSIA

THE CLINICOPATHOLOGICAL EVALUATION OF 24 HOURS UPON CHALLENGE OF *Streptococcus iniae* IN RED HYBRID TILAPIA (Oreochromis sp.)

MUHAMMAD AQMAL HAKIM BIN MAZLAN

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CERTIFICATION

It is hereby certified that I have read this project paper entitled "THE CLINICOPATHOLOGICAL EVALUATION OF 24 HOURS UPON CHALLENGE OF *Streptococcus iniae* IN RED HYBRID TILAPIA (*Oreochromis* sp.)" by Muhammad Aqmal Hakim Bin Mazlan and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project.

_____

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DEDICATIONS

This project paper is dedicated to Allah SWT, who had made everything possible,

To my family,

Father

Mother

Brothers & Sisters

Nur Arina

To all the lecturers, staffs and my friends who were involved directly or indirectly in this project.

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It is with my deepest appreciation and gratitude that I thank God almighty and to all the people involved in making this project and this paper a reality.

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Last but not least, to the persons who always there for me and always encourage me throughout my studies. My family members, my best friends, and my significant other, Nur Arina Ahmad Jelani.

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LIST OF ABBREVIATIONS

%	Percent
Bp	Base pair
CFU	Colony forming unit
DAB	3,3'-diaminobenzidine
h	Hour
Hpc	Hours post challenge
IHC	Immunohistochemistry
min	Minute
ºC	Degree Celsius
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Rpm	Revolutions per minute
µl	Microliter
µM	Micromolar

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

PENILAIAN KLINIKOPATOLOGI 24 JAM SELEPAS DIUJI DENGAN *Streptococcus iniae* DALAM TILAPIA HIBRID MERAH (*Oreochromis* sp.)

Oleh

MUHAMMAD AQMAL HAKIM BIN MAZLAN

2016

Penyelia: Prof. Madya Dr. Md Sabri b. Mohd Yusoff

Kajian ini bertujuan untuk menggambarkan keterukan dan immunolokalisasi antigen di dalam otak, mata dan buah pinggang dalam setiap 6 jam untuk 24 jam dengan kehadiran atau ketiadaan faktor tekanan sebelum jangkitan. Lima belas tilapia hibrid merah di dalam setiap akuarium telah disuntik secara intraperitoneal dengan 10^9 CFU/mL dicairkan dalam PBS dan satu set lagi, telah disimpan untuk kawalan negatif. Tanda-tanda klinikal telah direkodkan dan diperhatikan, dan sampel dari insang, otak, mata dan buah pinggang telah dikumpulkan. Setiap sampel yang diperoleh daripada organ telah diuji dengan

kaedah pengasingan dan mengenalpasti jenis bakteria, dan histopatologi. Immunohistokimia (IHC) dan PCR konvensional juga telah dilakukan untuk mengesan kehadiran antigen. Walau bagaimanapun, tidak ada tanda-tanda jelas dan tiada penemuan makroskopik yang boleh diperhatikan sepanjang 24 jam selepas jangkitan penyakit ini. IHC telah dikesan seawal PCR dan pengasingan dengan nodaan yang teramat jelas dalam saluran darah, lumen dan dinding, makrofaj dalam koroid, pendarahan yang tertumpu dalam celahan buah pinggang dan meninges terutama dalam kumpulan yang diuji dengan tekanan haba diikuti oleh tiada tekanan. Immunolokalisasi oleh antigen dijelaskan dalam patogenesis streptokokosis dalam tilapia hibrid merah. Kesimpulannya, ikan yang tertekan lebih cenderung untuk membangunkan penyakit dan menunjukkan tanda-tanda yang lebih teruk penyakit berbanding ikan yang diuji dengan sebarang tekanan.

Kata kunci: Tilapia merah hibrid, *Streptococcus iniae*, intraperitoneal, immunolokalisasi, IHC, PCR

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project.

CLINICOPATHOLOGICAL EVALUATION OF 24 HOURS AFTER CHALLENGE OF *Streptococcus iniae* IN RED HYBRID TILAPIA (*Oreochromis* sp.)

By

MUHAMMAD AQMAL HAKIM BIN MAZLAN

2016

Supervisor: Assoc. Professor Dr. Md Sabri Mohd Yusoff

This study was aimed to describe the severity and immunolocalisation of the antigen of lesions in the brain, eyes and kidney in every 6 hours for 24 hours by presence or absence of stress factors before infection. Fifteen Red hybrid tilapia in duplicates were inoculated intraperitoneally with 10^9 CFU/mL diluted in PBS while another set, was kept for negative control. Clinical signs were recorded and observed, and samples from gills, brain, eyes and kidney were collected. Each of the samples was subjected to bacterial culture and isolation and histopathology. Immunohistochemistry (IHC) and polymerase chain reaction (PCR) were also done to detect the presence of the antigen. However, there were no obvious signs,

and no macroscopic finding can be observed throughout 24 hours post challenge (hpc) of the disease. IHC were detected as early as PCR and isolation with intense staining in a blood vessel, lumen and wall, macrophages in the choroid, focal haemorrhages in the renal interstitium and meninges especially in heat stressed followed by no stressors. The immunolocalisation of the antigen is explained in the pathogenesis of streptococcosis in red tilapia. In conclusion, fish that is stressed are more likely to develop diseases and showing more severe signs of disease compared to fish that was not subjected to stress.

Keywords: Red hybrid tilapia, *Streptococcus iniae*, intraperitoneal, immunolicalisation, IHC, PCR

1.0 INTRODUCTION

1.1 Study background

Tilapia is one of the most important aquaculture products that is expanding in Malaysia. It has a high demand and potential locally and also from international (Azeli, 2007). Tilapia was introduced to Malaysia in 1970's and a decade after that a cross between *Oreochromis niloticus* and *Oreochromis mossambicus* was introduced, which is Red hybrid tilapia. Initially, tilapia were regarded to be more resistant to bacterial, fungal, viral and parasitic diseases compared to other cultured fish species (Amal et al., 2011). However, recently tilapia has been found to be susceptible to these diseases. Among the fish species that are infected by *Streptococcus iniae* also include tilapia. *Streptococcus iniae* was first discovered on freshwater dolphin in Amazon, *Inia geoffrensis* with caused the dolphin to develop a "golf ball" lesion on the skin layer due to its appearance resembling a golf ball (Pier and Madin, 1976). Hence, the name *Streptococcus iniae* was derived from the "Inia", which refers to the genus of the river dolphin in South America. Phylogenetically, *S. iniae* is closely related to *S. parauberis*, both being clustered with *S. agalactiae* and *S. dysgalactiae*, and the whole group clustering with *S. pyogenes* according to sequence similarity in their 16S rRNA gene (Facklam, 2002). Streptococcosis is a general name for a variety of diseases caused by a group of bacteria called *Streptococcus* (strep-TOE-coccus). Some "strep" organisms normally live on the body of humans or animals and do not cause disease. Others may cause disease (sometimes severe) in both people and animals.

Justification to do the study on the disease was that from literature there are no reports or comparative study of clinicopathological evaluation of 24 hours upon the challenge of *Streptococcus iniae* in red hybrid tilapia. So from this study, we hope that the finding will help to better understanding of the clinicopathology of *Streptococcus iniae* in Red hybrid tilapia.

This study was done to fulfil the following objectives:

1. to determine the severity of lesions in the brain, eyes and kidney in every 6 hours to 24 hours by presence or absence of stress factors before infection.
2. to determine the immunolocalisation of the antigen in the brain, eyes and kidney by immunohistochemical detection (IHC).

For this study, the following hypotheses were proposed:

1. There is a different level of severity of lesions in the brain, eyes and kidney observed in every 6 hours to 24 hours by presence and absence of stress factors before infection.
2. Presence of the immunolocalisation of the antigen in the brain, eyes and kidney by immunohistochemical detection (IHC).

2.0 LITERATURE REVIEW

2.1 Aquaculture

The most rapid growing food-producing sector currently in the world is aquaculture. Up to 2013, the sector has an average annual growth rate of 8.9% since 1970 compared to 2.8% for terrestrial farmed meat production systems and 1.2% only for capture fisheries during the same period of time (Subasinghe et al., 2003). Aquaculture also continues to outpace the world's population growth with an increase in per capita supply from 0.7 kg in 1970 to 7.8 kg in 2006 from the sector itself, leading to an annual growth rate of 6.9% at that time (FAO, 2016). Aquaculture production is of great variety within each region. However, the sector is mainly dominated by the Asia-Pacific region, accounting for 89% of production in terms of quantity and 77% in terms of value with China being the biggest contributor (FAO, 2016).

Sustaining the supply of fish from capture fisheries will not be able to meet the growing global demand for aquatic food. Therefore, the Food and Agriculture Organization of the United Nations estimates that the global aquaculture production needs to reach 80 million tonnes by the year 2050 in order to maintain the current level of per capita consumption (FAO, 2016). As the human population increases by time, many developing countries are now focusing on aquaculture due to its economical and nutritional potential (Low et al., 2015). In Malaysia, the

aquaculture sector places a major focus on tilapia whereby it accounts for more than one-third of the total freshwater aquaculture production in the country (Low et al., 2015).

2.2 *Streptococcus iniae*

2.2.1. Taxonomy

According to the National Center of Biotechnology Information (NCBI), there are 9 taxonomy levels for *Streptococcus iniae*. They are cellular organisms under the domain of bacteria, phylum of Firmicutes, class of Bacilli, order of Lactobacillales, a family of Streptococcaceae, a genus of Streptococcus and with the species name of *Streptococcus iniae* (NCBI, 2016). *S. iniae* is a Gram-positive, oxidase-negative, catalase-negative, sphere-shaped bacterium (Rattanachaikunsopon & Phumkhachorn, 2010). The NCBI also lists strains of the bacteria which are *Streptococcus iniae* 9117, *Streptococcus iniae* IUSA1, *Streptococcus iniae* KCTC 11634BP, *Streptococcus iniae* SF1 and *Streptococcus iniae* SI25 (NCBI, 2016).

2.2.2 Streptococcosis

Streptococcosis is a serious disease in both humans and also other terrestrial animals (Suanyuk et al., 2010). Streptococcosis of fish is a generic term which is used to describe similarly but different diseases in

any one among at least six various species of cocci such as Streptococcus, Lactococcus and Vagococcus (Suanyuk et al., 2010). Among the other principal pathogenic species responsible for the streptococcal infections include *S. iniae* which is the main etiological agent of streptococcosis in a variety of saltwater and freshwater species of fish such as tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*), yellowtail (*Seriola quinqueradiata*) and many other species (Eldar et al., 1997; Sako, 1998; Abutbul et al., 2004).

The progression of the disease in fish is different and is shown to be dependent on the host species affected, the virulence of the isolate, its infection route, the age of the fish and also other water quality and environmental factors (Agnew & Barnes, 2007). Mortality due to *S. iniae* is often associated with meningoencephalitis which presents following the systemic dissemination of bacteria through major organs like liver and kidneys along with the bloodstream (Agnew & Barnes, 2007). The infection caused by *S. iniae* is a harmful disease, which leads to a huge number of deaths fish. Thus, it is regarded as one of the foremost economically critical pathogens in aquaculture (Rattanachaikunsopon & Phumkhachorn, 2010).

In aquaculture, *S. iniae* infection is usually controlled by antibiotics (Rattanachaikunsopon & Phumkhachorn, 2010). Some of the successful antibiotics that have been used to control the infection in fish include amoxicillin, enrofloxacin, erythromycin, oxytetracycline and

furazolidone (Stoffregen et al., 1996; Agnew & Barnes, 2007). One method of control that is most widely used is improving water quality and environmental conditions so that the challenge to culture fish stocks can be kept as low as possible (Agnew & Barnes, 2007). However, effective and long-term vaccination programs implementation is now the most likely aim for future control of the disease (Agnew & Barnes, 2007).

2.2.3 Epidemiology

The first report of *S. iniae* infection was in dolphins in San Francisco, California, USA in the year 1976 and later in 1978 at Niagara Falls, USA also in dolphins (Agnew & Barnes, 2007). Among other further cases that were reported are such as in Japan (1979), Singapore (1985), Israel (1986), Texas, USA (1991), Bahrain (195), North Queensland, Australia (1995), South-east Caribbean basin (1999) and Pennsylvania (2003) (Agnew & Barnes, 2007).

Based on data obtained from the countries sampled by MSD Animal Health, it is found that S. agalactiae is more prevalent than *S. iniae* (Merck Animal Health, 2015). The prevalence percentage of total streptococcal isolations from tilapia was 82% for *S. agalactiae* and only 18% for *S. iniae* (Merck Animal Health, 2015). There is notably no true epidemiological study of the disease outbreak since some sites have been visited on multiple occasions and thus, are overrepresented in the data set.

Figure 1: Distribution of *Streptococcus iniae* in the Asia-Pacific region

2.3 Tilapia

2.3.1 Taxonomy

Tilapias are freshwater fish under the class of Actinopterygii, the order of Perciformes, family of Cichlidae, a subfamily of Pseudocrenilabrinae and genus of Tilapia (Fish Base Organisation, 2015). Tilapia is actually the common name whereas *Oreochromis* spp. is the taxonomic name for these species of fish. There are over 70 species of these fish with at least eight species which are used for aquaculture (ISSG, 2006). Among them are Tilapia baloni, Tilapia cameronensis, Tilapia guineensis, Tilapia nigrans, Tilapia synderae and many more (Fish Base Organisation, 2015). The Red hybrid tilapia is a product of *Oreochromis mossambicus* crossed with *O. niloticus* (Nile tilapia) (Keshavanath et al., 2004).

2.3.2 Tilapias in Aquaculture

Tilapias have suitable characteristics for farming, and they are very domesticated earning them the title of “aquatic chicken” (Amal et al., 2011). Tilapias are fast-growing fish with firm white flesh. They can survive in poor water conditions, eat various food types and can be bred easily without special hatchery technology (Nandlal & Pickering, 2004). Tilapia is now a major aquaculture product, mainly because of its adaptability which allows the rapid production of commercial size fish that can fulfil the market demands (Zhang et al., 2011).

Contributing to more than a third of total freshwater aquaculture production in Malaysia, the Malaysian Annual Fisheries Statistics 2008 states that tilapia production was about 36, 000 tonnes per year (Red tilapia accounted for about 76%) and the estimated profit was more than 62 million USD in terms of wholesale value (Annual Fisheries Statistics, 2008). In addition, a more recent survey has shown that an increasing trend of fish cage culture activities has been operated in 6,000 fish cages growing majority Red hybrid tilapia *Oreochromis niloticus* (Najiah et al., 2012).

Initially, tilapia were regarded to be more resistant to bacterial, fungal, viral and parasitic diseases compared to other cultured fish species (Amal et al., 2011). However, recently tilapia have been found to be

susceptible to these diseases. Among the fish species that are infected by *S. iniae* also include tilapia.

2.4 Stresses in Fish

According to Medical Dictionary, stress is an "organism's total response to environmental demands or pressures" and the "reactions of the body to forces of a deleterious nature, infections, and various abnormal states that tend to disturb its normal physiologic equilibrium (homeostasis). In 1998, T. Ryan Gregory et al. suggested that fish which is treated with cortisol, which is the stress hormone, had lower growth rates compared to untreated group.

3.0 MATERIALS AND METHODS

3.1 Fish & Fish Culture

A total of 45 Red hybrid tilapia (*Oreochromis* sp.), weighing between 10 ± 3 g apparently healthy fish were obtained from an Aquaculture Extension Centre (AEC), Department of Fisheries Malaysia, Bukit Tinggi, Pahang. Prior to the experiment, tanks were disinfected and cleaned. The source of water was dechlorinated and continuously aerated using an aerator. Water quality was monitored and maintained throughout the experiment. The fish used were screened for bacteria (particularly for *S. iniae* and external parasites) before the experiment started to make sure that all the fish are free from any diseases. The fish were fed ad libitum with commercial feed before the experiment but throughout the experiment, the fish were off feed.

3.2 Bacteria & Bacteria Culture

S. iniae that was stocked in nutrient agar was subcultured onto the blood agar and incubated at 30 °C for 24 hours. The brain heart infusion broth (BHIB, Oxoid, UK) was used to subculture the colony and incubated in a shaker incubator at 30 °C for 24 hours. Four mL of the broth was then transferred into 4 spinning tube, each containing 1 mL broth. The tubes were spun at 3000 rpm for five minutes using a centrifuge. The solutions in all the tubes were decapped, and 1 mL of PBS was transferred into each

of the tubes. The process was repeated for two times and the final turbidity of the solution was compared to McFarland kit before the concentration will be expressed as colony forming unit per millilitre (CFU/mL). The last concentration of live *S. iniae* used for inoculation was adjusted to be 10^9 CFU/mL. About 50 µL of the solution was administered intraperitoneally to the fish.

The swabs from the organs were collected every six hours was immediately streaked onto the blood agar plates and incubated at 30 °C for 24 hours. Gram staining was performed to identify Gram-positive cocci in chain or paired and catalase test negative organisms. Finally, the colonies were further characterized using the commercialized test kit, API rapid ID 32 Strep® (bioMerieux SA, Marcy I'Etoile, France).

3.3 Experimental design

The experiment was conducted within 24 hours' time, by challenging the fish with 10^9 CFU/mL of live *S. iniae*. The fish (n=45) were divided into 3 groups. Group A (n=15), was subjected to heat stress at 31 °C before the challenge and throughout the experiment. The fish were gradually acclimatized to a higher temperature before being maintained at 31 °C for the bacterial challenge. Group B (n=15), was challenged without stressors and the water was maintained at room temperature. Group C (n=15) served as a negative control group without any challenge. Groups A and B were challenged with live pathogenic *S.*

iniae. The fish were kept for 24 hours off feed, and the clinical signs were observed continuously in the 24 hours duration of the experiment. The gills, brain, eyes and kidney were collected from three fish every six (6) hour within 24 hours from each group. The tissues samples were subjected to bacterial culture, PCR, histopathology and IHC.

3.4 PCR

Wizard Genomic DNA Purification Kit (Promega, USA) was used for confirmation of the desired species, *S. iniae* by extracting the total cellular DNA according to manufacturer's protocol. The extracted DNA then further evaluated by PCR for *S. iniae* primers LOX-1 (5'-AAG GGG AAA TCG CAA GTC CC-3') and LOX-2 (5'-ATA TCT GAT TGG GCC GTC TAA-3'), and cycling conditions described as follows; 1 cycle at 94 °C for 4 minutes, followed by 30 cycles at 94 °C for 1 minute, 52 °C for 1 minute, 72 °C for 1 minute and finally elongation at 72 °C for 10 minutes. Seven µL of the amplified products was electrophoresed using 1.0% (w/v) agarose gel in 1× TBE electrophoresis buffer (0.1 mM Tris/HCI, 0.1 mM boric acid, 0.002 mM EDTA, pH 8.3) (Sambrook et al., 1989). The gel was stained with 0.5 µg/mL RedGel stain.

3.5 Histopathology

Specimens were sacrificed with pitting technique, where the gills, eye, brain, and kidney tissue samples were collected for every 6 hours post challenge (hpc), then fixed it in 10% formaldehyde, and processed using a standard histological technique. After 24 hours fixation, the tissues were trimmed before dehydrated in an ethanol series, followed by embedding in paraffin, and finally serially sectioning at 5 µm. The sections were stained routinely with haematoxylin and eosin (H&E). Histopathological changes in each organ were semi-quantitatively ranked as none (0), mild (1), moderate (2), or severe (3).

3.6 IHC

Serial 5 µm thick sections were cut from the wax blocks onto silane-coated glass slides. The slides were dried for 15 minutes at 56 - 60 °C, dewaxed in xylene and rehydrated through a graded alcohol series. The slides then washed with phosphate-buffered saline-Tween 20 (PBST) for 10 minutes. Endogenous peroxidase activity was blocked with freshly prepared 3% hydrogen peroxide for 5 minutes at room temperature and rinsed and washed with PBST for 2 minutes. In enhancing the tissue to be immune-reactive, the heat-mediated antigen retrieval with citrate solution in a microwave oven was used. Sections were blocked with blocking buffer 1% normal serum (Bovine serum albumin) and PBST, and then sections were incubated with tilapia anti-*S. iniae* with the dilution of 1:50

for at least 1 hour at 37 °C in an incubator. After that, the sections were rinsed and washed with PBST for 5 minutes. Sections were incubated again at 37 °C for 30 minutes with secondary antibody (goat anti-tilapia) with the dilution of 1:100. The sections were rinsed and washed with PBST for 5 minutes before DAB was applied 1 mL diluents to a 1 drop DAB for colour change. Once the sections became brown, the slides immediately rinsed with distilled water and the slides will be stained using Mayer's haematoxylin solution for the background colour.

3.7 Statistical Analysis

Statistical analyses will be performed using MedCalc for Windows, version 12.2.1.0 (MedCalc Software, Mariakerke, Belgium) and tested at 5% level of significance. The differences in the data of lesion scoring will be analysed using a Kruskal-Wallis test with posthoc analysis tests if significant is applied.

4.0 RESULTS

4.1 Clinical Signs and Macroscopic Findings

The fishes were observed throughout 24 hours post challenge (hpc) with *S. iniae* for all the groups for any abnormalities or clinical signs. However, there was no abnormalities were observed, and all the fish appears to be completely healthy.

4.2 Bacteria Isolation and Identification

A small whitish umbonate colony approximately 1 mm in diameter with entire opaque border, an opaque centre and translucent ring of growth was observed on blood agar media. The bacteria were stained Gram-positive and encapsulated cocci in appearance when observed under a microscope.

Table 1: Bacteria culture from organs

Time (hours)	**Group A**			**Group B**			**Group C**		
	Brain	Eye	Kidney	Brain	Eye	Kidney	Brain	Eye	Kidney
6	X	X	√	√	√	√	X	X	X
12	√	√	√	X	√	√	X	X	X
18	√	√	√	√	√	√	X	X	X
24	√	√	√	√	√	√	X	X	X

√: Growth, X: No Growth

4.3 PCR

PCR amplification and sequencing showed a positive result for all the samples taken from the positive bacteria culture as shown in Figure 2.

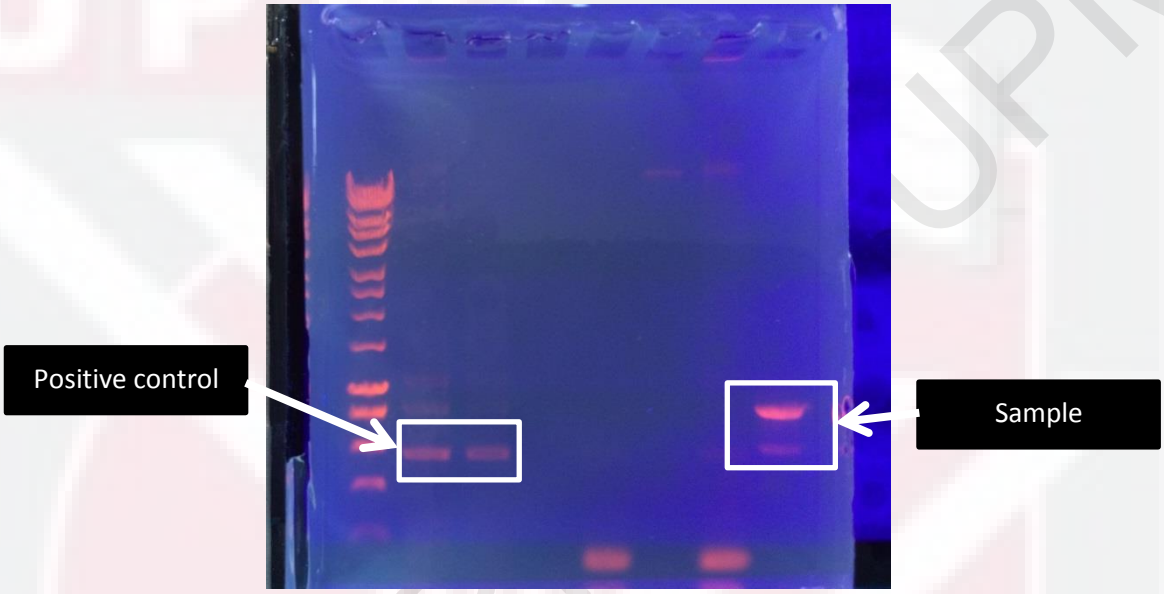


Figure 2: All samples *Streptococcus iniae* from sample yield 870 bp product, indicating the presence of 16s ribosomal ARN gene sequence.

4.4 IHC

Organs stained with immunohistochemistry showed a positive result when there is a presence of coccus shaped structures with brown staining with diffuse distribution can be observed in the slide. In this study, the immunolocalisation can be observed as early as 6 hours post challenge in the brain and kidney for both groups. On the other hand, for the eyes, the immunolocalisation occurs at 12 hpi for group A and 18 hpi for group B. For control, all the organs showed a negative result for IHC.

Table 2: Result of immunoperoxidase examination of different groups and organs of red hybrid tilapia for *S. iniae*

Group	Hour	Brain	Eye	Kidney
1	6	+	-	+
	12	+	+	++
	18	++	++	++
	24	++	++	+++
2	6	+	-	+
	12	+	-	+
	18	+	+	++
	24	++	+	++
3	6	-	-	-
	12	-	-	-
	18	-	-	-
	24	-	-	-

- Negative IHC; + Positive IHC

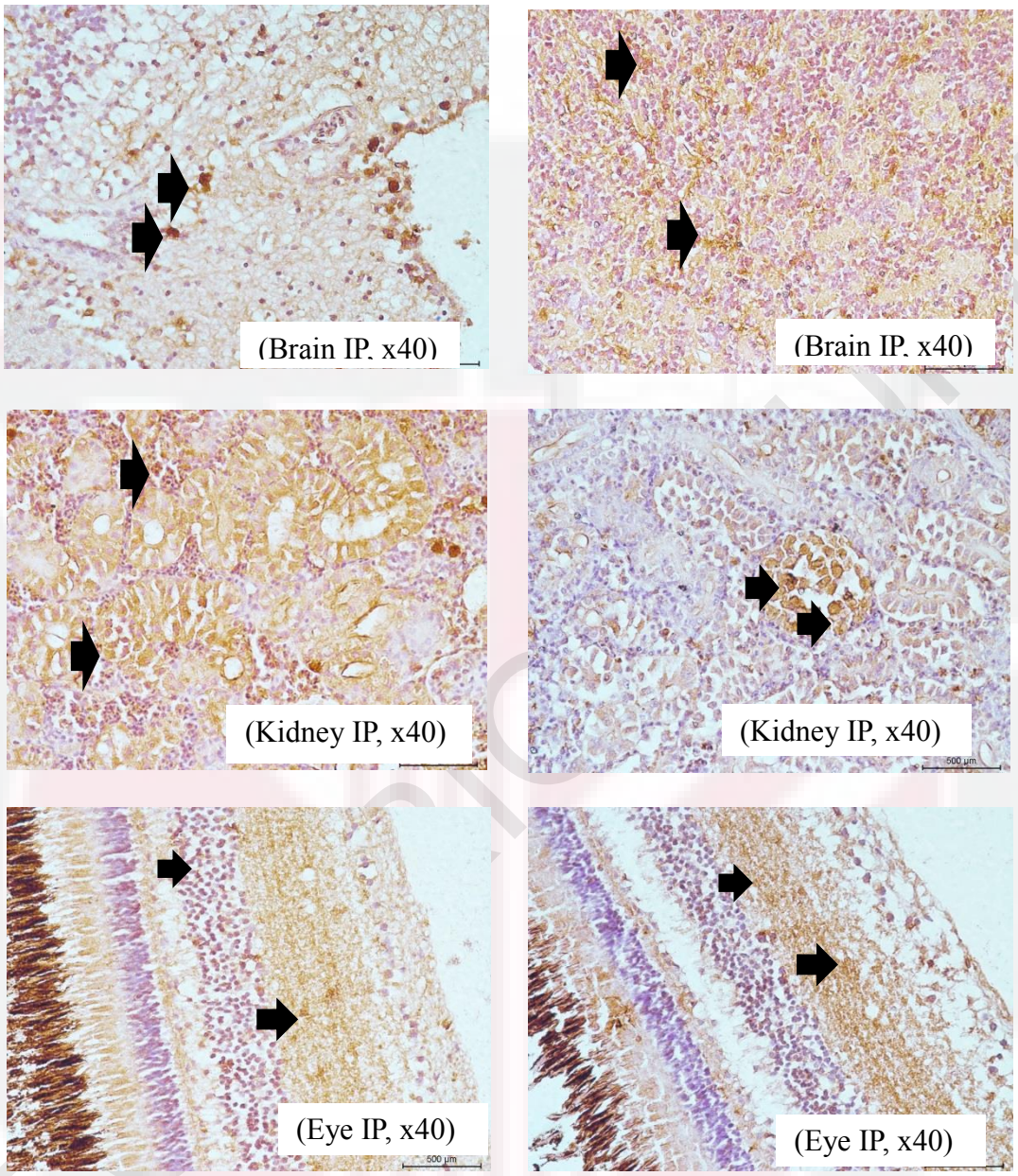


Figure 3: Examples of coccus shaped bacteria with brown colour staining with a diffuse distribution of *S. iniae* in brain, kidney and eye

4.5 Histology

Some of the common histological findings that can be observed in the eyes were a detachment of the blood vessel walls, infiltration of inflammatory cells including leucocytes, macrophages, fibrin and eosinophilic granular cells. On the other hands, in the brain, lesions such as congestion, infiltration by inflammatory cells and vacuolation can be observed throughout the experiment in the cerebellum and telencephalon. In the gills, lesions such as oedema, hyperplasia of secondary lamellae, fusion or clubbing of secondary lamellae, congestion and infiltration of inflammatory cells can be observed. Other than that, in the kidney, glomerulus atrophy, tubular cell swelling, hemosiderin, infiltration by inflammatory cells, congestion and degeneration of the structures can be observed. The change in normal structures can be observed as early as 6-hour post challenge for kidney, and it becomes more severe throughout 24 hours experiment.

The lesions become more severe throughout the experiment, and when compared between group A and group B, which are heat stressed and no heat stress, the lesions were more severe in the stressed group. The examples of the lesions that can be observed are shown in Figure 4-12.

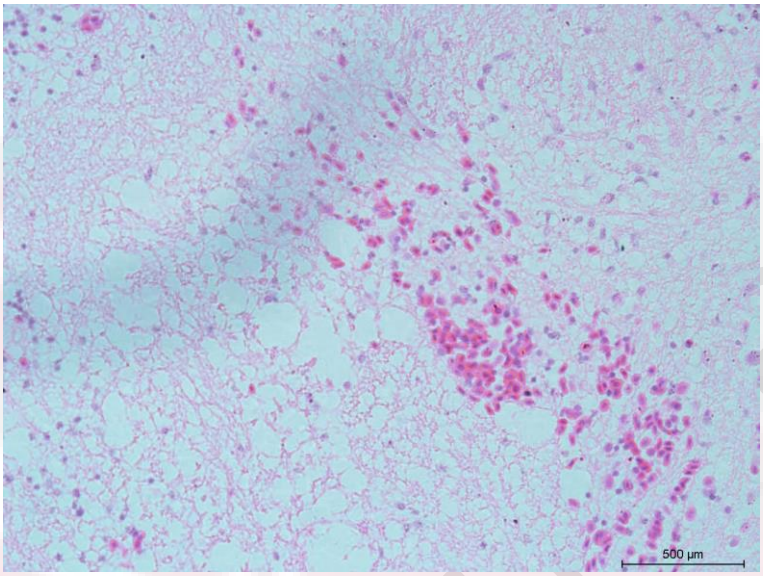


Figure 4: Brain. The meninges showed the degeneration of neuroglia cells and infiltration of inflammatory cells. Vacuolation also can be observed. (H&E, ×400)

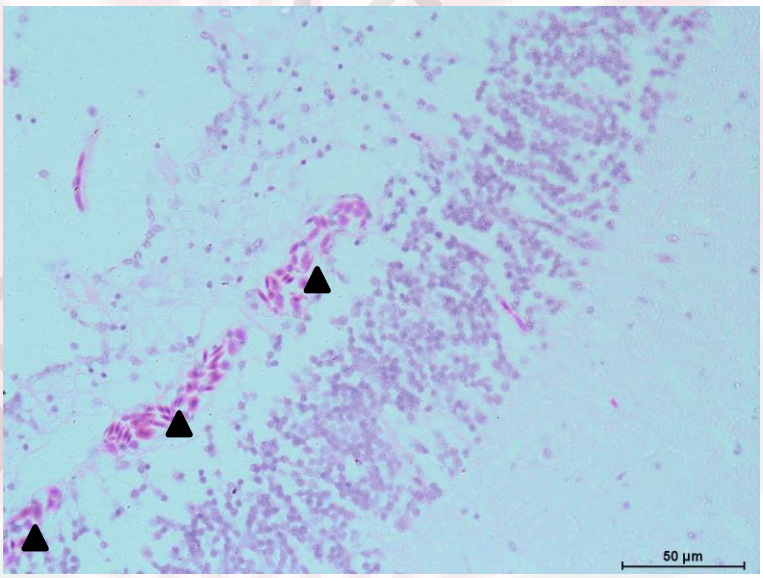


Figure 5: Brain. The optic tectum showed congestion (arrowhead) and detachment of the blood vessel wall. (H&E, ×400)

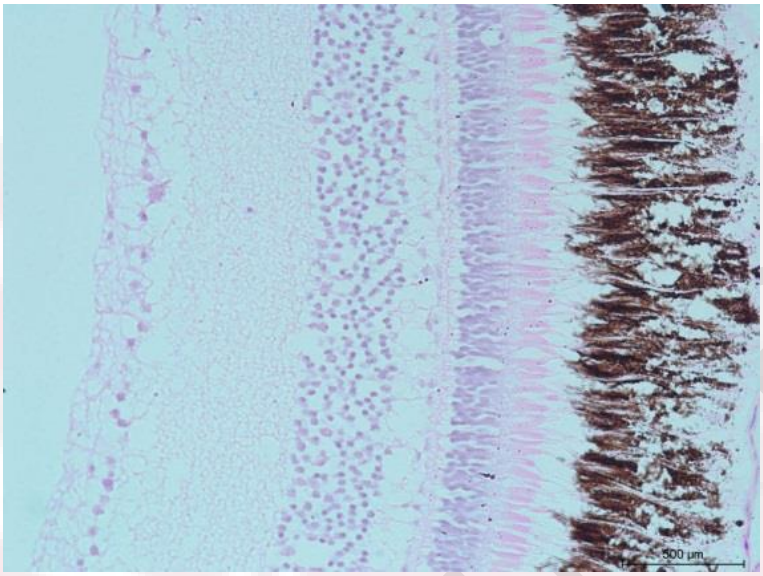


Figure 6: Eye. Thickening of the inner plexiform layer. (H&E, ×400)

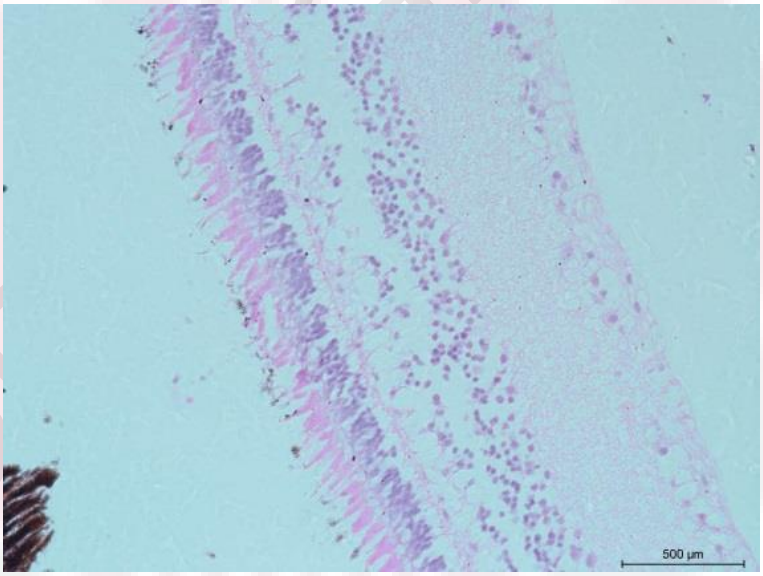


Figure 7: Eye. A detachment of pigment epithelium from photoreceptor layer. (H&E, ×400)

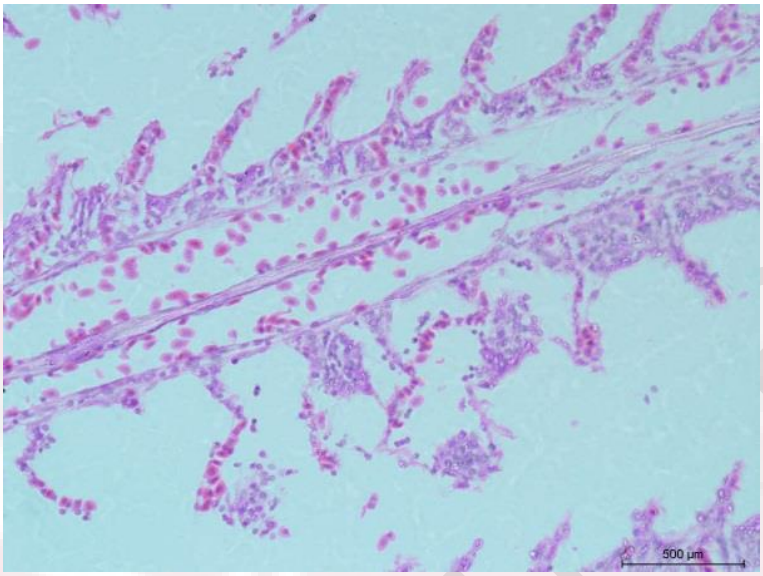


Figure 8: Gills. Gill epithelium degeneration, necrosis and numerous rodlet cells. (H&E, ×400)

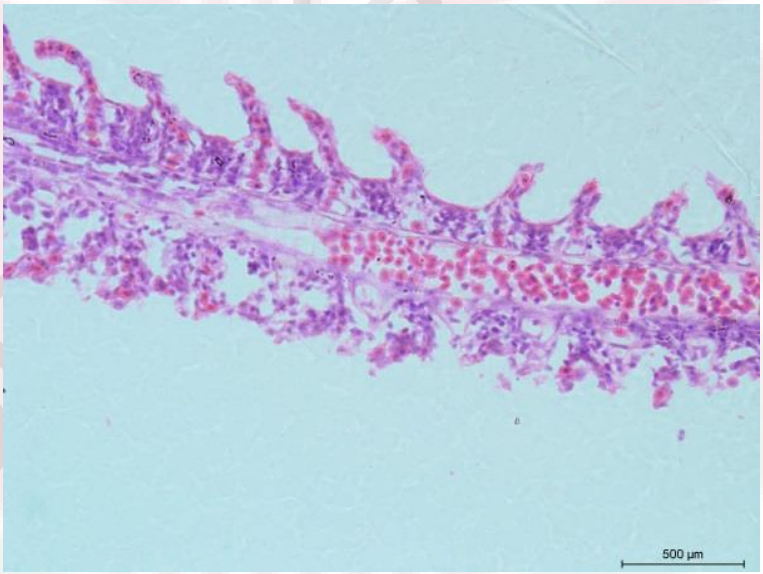


Figure 9: Gills. Congestion and fusion of secondary lamellae. (H&E, ×400)

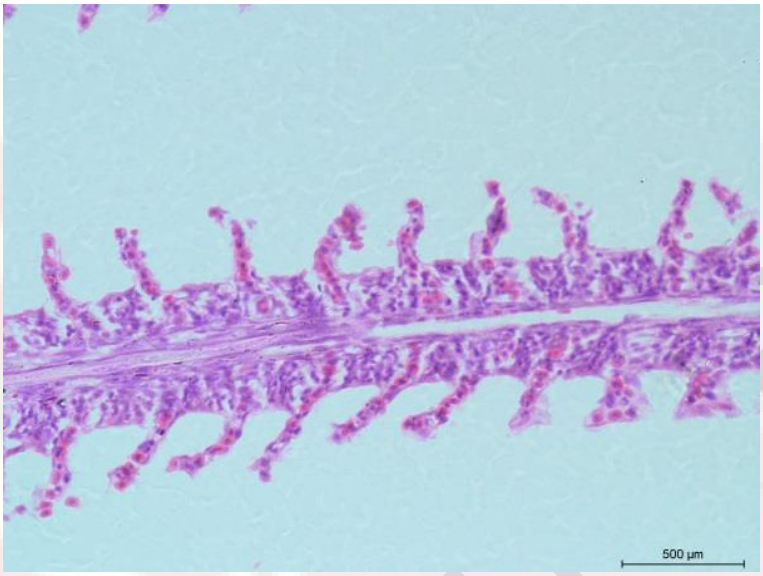


Figure 10: Gills. Hyperplasia. (H&E, ×400)

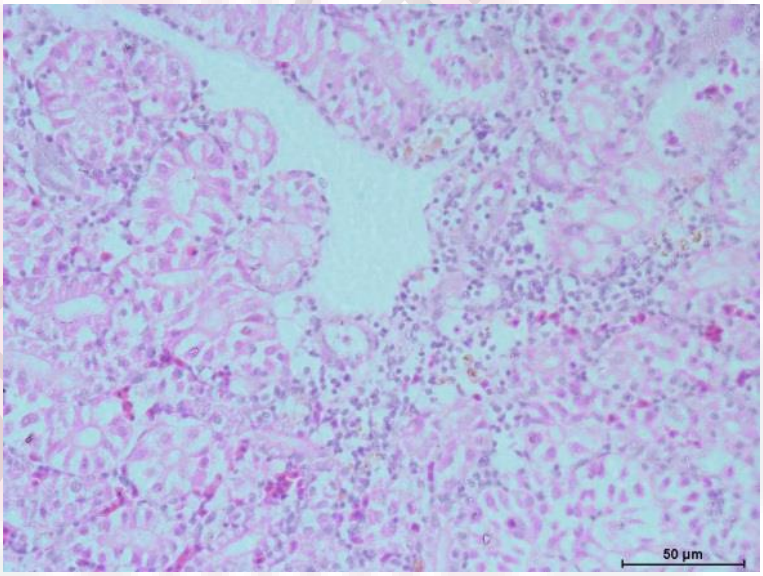


Figure 11: Kidney. Infiltration of numerous inflammatory cells. (H&E, ×400)

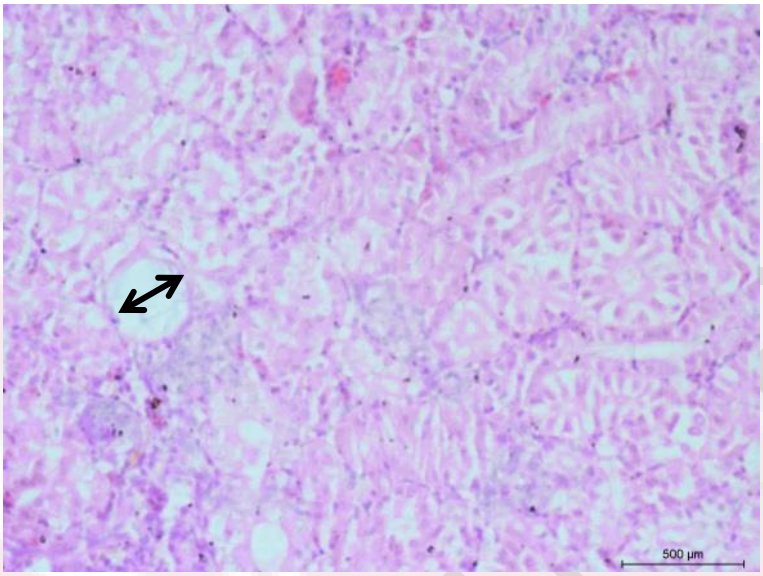


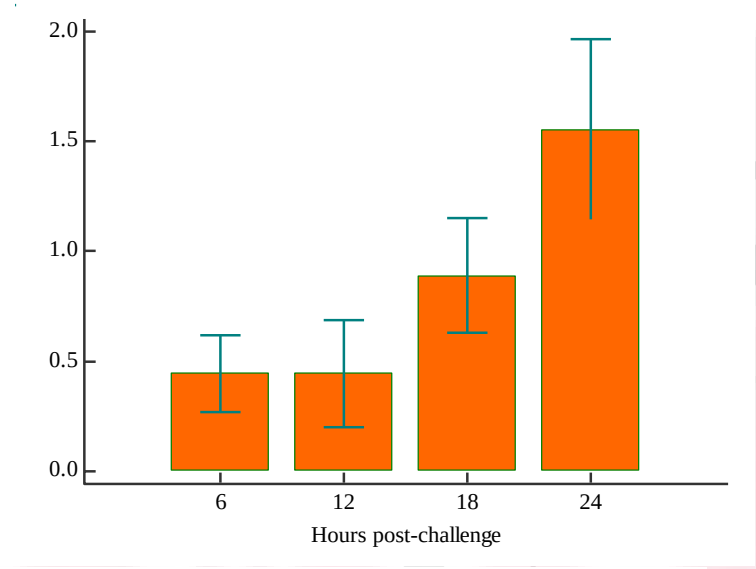
Figure 12: Kidney. Dilatation of collecting duct. (H&E, ×400)

4.6 Statistical Analysis

Table 3 shows the semiquantitative scoring done on the brain, eye, kidney and gills. The scoring on the lesions was ranked based on severity, which is 0 as none and 3 as the most severe lesion. MedCalc for Windows, version 12.2.1.0 (MedCalc Software, Mariakerke, Belgium) was used and tested at 5% level of significance. The differences in the data of lesion scoring were analysed using a Kruskal-Wallis test with posthoc analysis tests if significant is applied.

Table 3: Semiquantitative scoring of brain, eye, kidney and gills in Red hybrid tilapia (0-None, 1-Mild, 2-Moderate, 3-Severe)

Group		A				B				C			
	Lesion	**6**	**12**	**18**	**24**	**6**	**12**	**18**	**24**	**6**	**12**	**18**	**24**
Brain	Infiltration by	0	1	2	3	0	1	1	2	0	0	0	0
	inflammatory	1	0	1	3	1	0	1	2	0	0	0	0
	cells,	1	0	1	2	1	0	2	2	0	0	0	0
	congestion, Vacuolation												
Eye	Haemorrhage,	0	0	0	0	0	1	0	1	0	0	0	0
	infiltration,	2	1	0	0	0	1	1	0	0	0	0	0
	thickening of	0	0	2	2	1	0	1	2	0	0	0	0
	layers, detachment of layers												
Kidney	Glomerulus	0	1	1	1	1	1	1	1	0	0	0	0
	atrophy,	1	1	1	2	1	1	0	1	0	0	0	0
	tubular cell	1	2	2	2	1	1	2	2	0	0	0	0
	swelling, hemosiderin, infiltration, congestion, degeneration												
Gills	Oedema,	0	0	1	2	0	0	1	1	0	0	0	0
	hyperplasia,	1	0	1	3	1	1	2	2	0	0	0	0
	fusion or	1	1	2	3	1	1	1	2	0	0	0	0
	clubbing, congestion, infiltration												



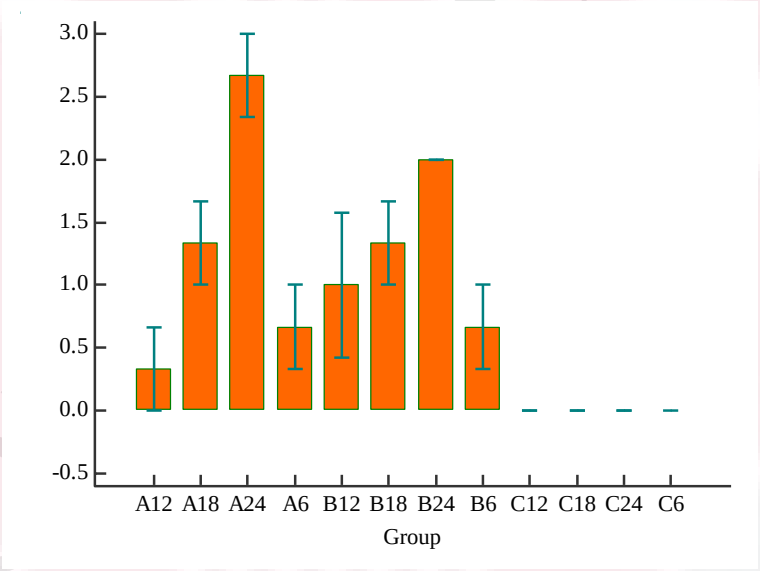


Figure 13: (Top) There is no significant difference between lesions when compared to hours. $P > 0.05$ despite the fact that the lesions become more severe with time. (Bottom) There is a significant difference between brain lesions versus the group with $P < 0.05$.

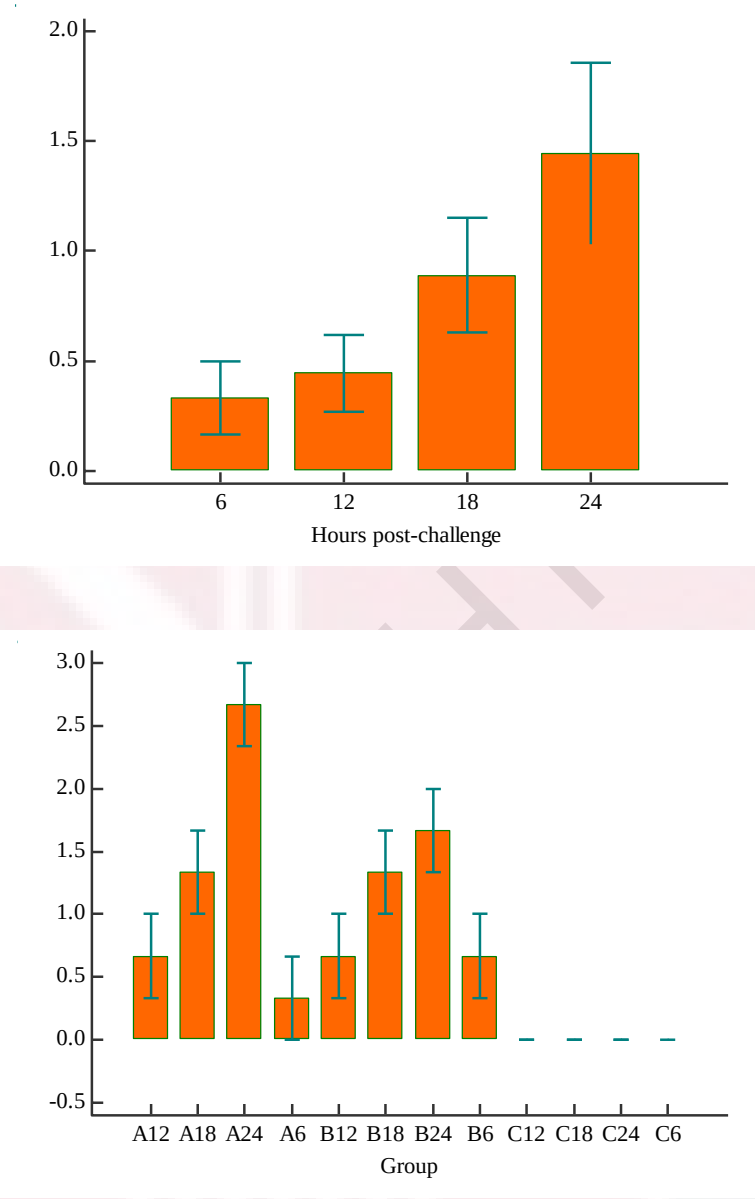


Figure 14: (Top) There is no significant difference between gill lesions when compared to hours with $P > 0.05$ despite the fact that the lesions become more severe with time. (Bottom) There is a significant difference between gills lesions versus the group with $P < 0.05$.

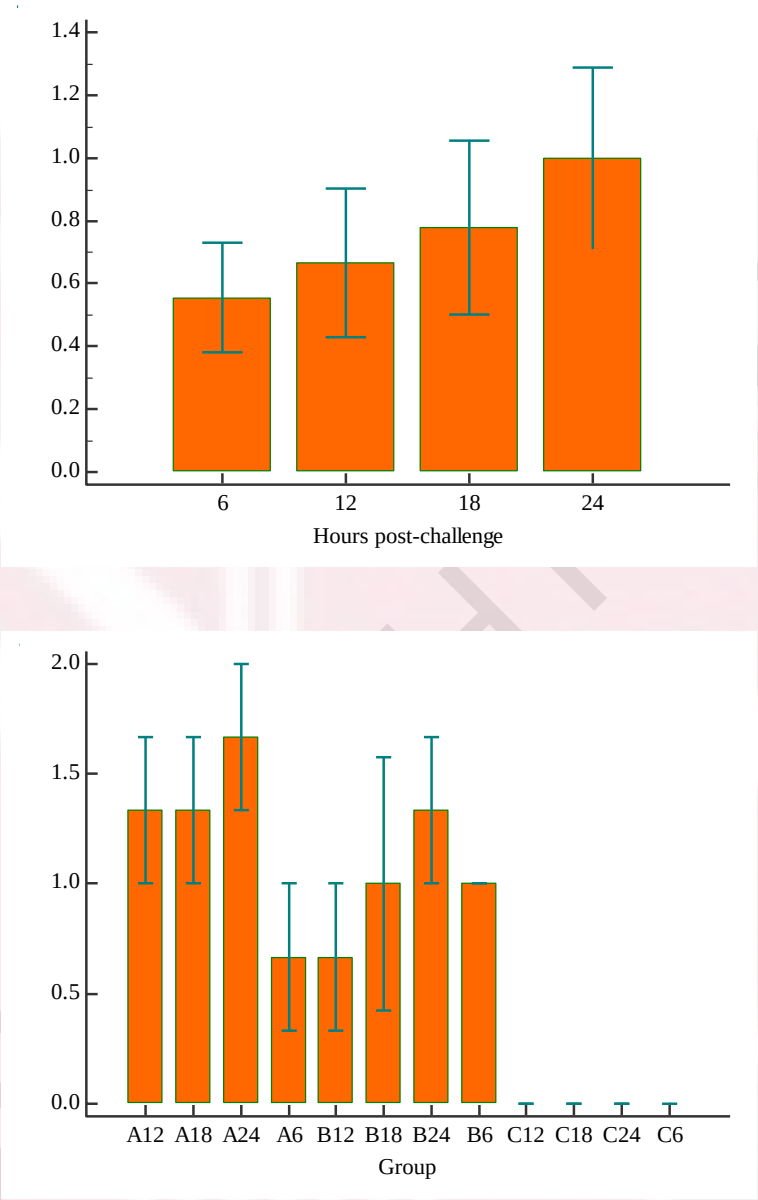


Figure 15: (Top) There is no significant difference between kidney lesions when compared to hours with $P > 0.05$ despite the fact that the lesions become more severe with time. (Bottom) There is a significant difference between kidney lesions versus group with $P < 0.05$.

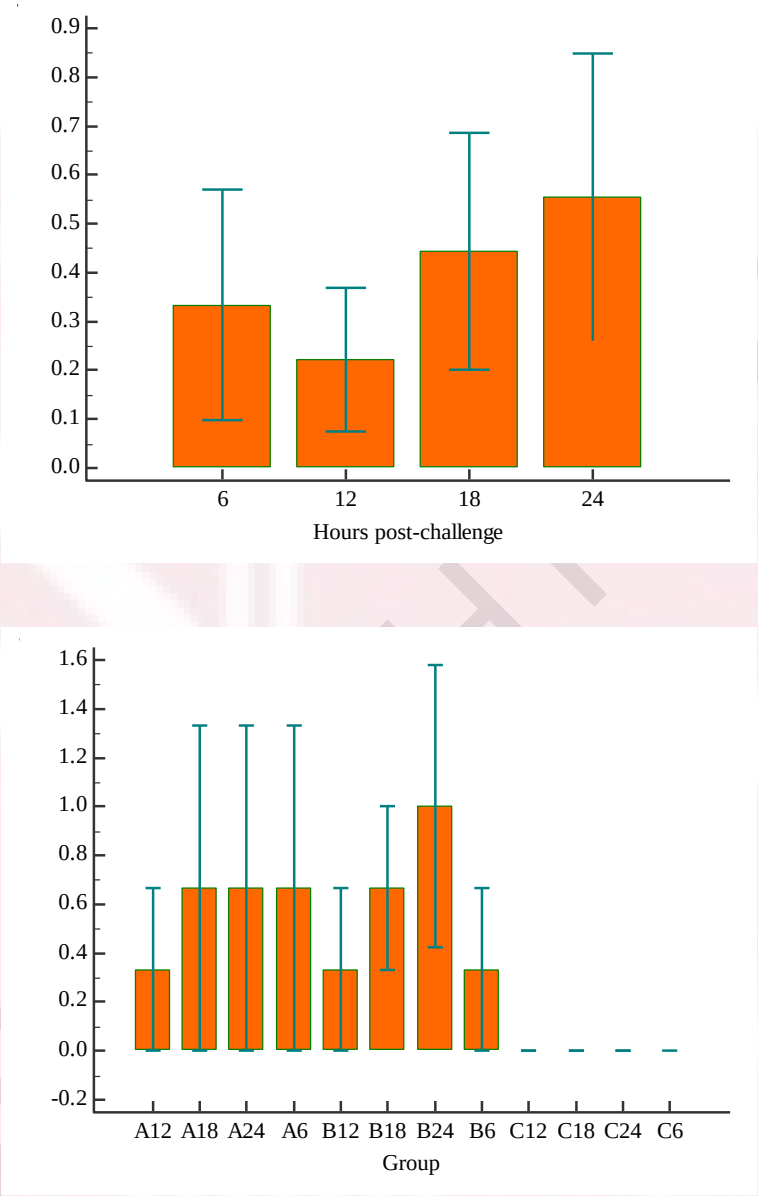


Figure 16: (Top) There is no significant difference between eye lesions when compared to hours with $P > 0.05$. (Bottom) There is also not a significant difference between eye lesions versus the group with $P > 0.05$. This could be due to technical error.

5.0 DISCUSSION

There was no mortality, and no macroscopic lesions that can be observed in this study because the experiment was conducted in 24 hours. These findings were supported by the statements made by Chen et al. in 2007 which states that tilapia may control natural *S. iniae* infections more effectively, resulting in a more chronic form of disease compared to that caused by *S. agalactiae*. Based on the statistical analysis done for histopathology, all the experimental fish which were subjected to the challenge whether subjected to the stress or not shows mild to severe pathological changes and lesions as the post time challenge increases throughout the experiment except on the eye. In this study, we speculated that the mortality and clinical signs can be observed after 24 hours post challenge. Another contributing factor to the pathological changes and lesions observed was the age and size of the experimental fish itself. Hernandez et al. (2009) state that larger fish found to be more susceptible to this disease than smaller fish despite the fact that generally juvenile animal are more susceptible to bacterial disease.

In this experiment, there is a significant difference in the brain, kidney and gills lesion when compared between group A and group B, which are stress induced and no stress induced. This finding is supported by a study done by Ali Farag et al., which states that stress will increase the virulence of bacteria. However, there is no significant difference between the lesions when it was compared to hours. This could simply mean that the disease is slowly progressive and less virulence than *S. agalactiae,* as Syuhaidah et al. (2012) state that the clinical signs and lesions can be observed as early as 2 hours post challenge. In

conclusion, although *S. iniae* is slowly progressive and less pathogenic, but when stress factor is induced, it becomes more pathogenic and shows a significant difference in the brain, kidney and gills. Histopathology is the most useful technique to study the pathogenicity of streptococcosis, where the gross lesion still cannot be observed within 24 hpi. The IHC results reveal the presence of bacteria in the cells tissue, therefore, it is highly specific, to detect the presence and localisation of antigen in the cells. In this study, the presence of the antigen can be detected as early as 6 hpi for group A and group B in the brain and kidney. For the eyes, the immunolocalisation of the antigen can be detected in 12 to 18 hpi for group A and group B. But nevertheless, the procedures need to be repeated for eyes due to a technical error.

6.0 CONCLUSION AND RECOMMENDATION

This study shows that that the severity of the lesion increases with time, and it also implies that the stressed fish show more severe lesion compared to fish with no stress-induced. This disease will not show any gross lesion and macroscopic findings, thus it is important to do screening for histopathology for the early stage of the disease as it can be detected via histology, IHC, and PCR.

As for the recommendations, firstly the experiment can be used and perform by subjecting another type of stressors such as stocking density, water quality and so on which are significant to the health of the fish. A different breed of fish at different age and size can also be suggested to carry out the experiment in the future. Other than that, a larger sample size in a larger tank can be used for a more accurate data collection and analyses.

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