UNIVERSITI PUTRA MALAYSIA

EVALUATION OF THE MUCOSAL IMMUNE RESPONSE OF RED HYBRID TILAPIA TO DIFFERENT DOSES OF SPRAY Streptococcus agalactiae VACCINE

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CERTIFICATION

It is hereby certified that I have read this project paper entitled "Evaluation of the mucosal immune response of Red hybrid tilapia to different doses of spray *Streptococcus agalactiae* vaccine", by Nur Izuin Binti Zainuddin and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirements for the course of VPD 4999 – Final Year Project

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ACKNOWLEDGEMENT

First and foremost, praises and thanks were dedicated to Allah SWT, the Almighty, for His showers of blessings upon me and giving me the strength to complete my final year project successfully.

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Not to forget my parents and family for their tremendous love, care and prayers for me. This project is dedicated to them. Last but not least, special thanks to my other half, Hariz for always being there for me and being my biggest supporter.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bp	Base pair
CFU/mL	Colony-forming unit per millilitre
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FKB	Formalin-killed bacteria
i.p.	Intraperitoneal
GALT	Gut-Associated Lymphoid tissue
GIALT	Gill-Associated Lymphoid Tissue
H&E	Haematoxylin & Eosin
NALT	Nose-Associated Lymphoid Tissue
PBS	Phosphate-buffered solution
PCR	Polymerase Chain Reaction
rpm	Rotation per minute
SALT	Skin-Associated Lymphoid Tissue
S. agalactiae	*Streptococcus agalactiae*
TSA	Tryptic soy agar
TSB	Tryptic soy broth

ABSTRAK

Abstrak daripada kertas projek yang dikemukan kepada Fakulti PerubatanVeterinar untuk memenuhi sebahagian daripada VPD 4999 – Projek ilmiah tahun akhir

PENILAIAN TINDAK BALAS IMUN MUKOSA TILAPIA HIBRID MERAH YANG DIVAKSINASI SECARA SEMBURAN TUNGGAL DAN TIGA KALI GANDA DENGAN *Streptococcus agalactiae* VAKSIN

Oleh

Nur Izuin binti Zainuddin

2020

Penyelia: Prof. Madya Dr Md Sabri Mohd Yusoff

Streptococcus agalactiae adalah salah satu daripada patogen yang penting yang boleh menyebabkan kematian dan morbiditi yang besar di antara pelbagai spesies ikan air tawar, muara dan laut. Kajian ini dijalankan bertujuan untuk menilai tindak balas imun mukosa pada ikan tilapia hibrid merah berikutan pemberian vaksin *S. agalactiae* tunggal dan tiga kali ganda secara semburan. 90 tilapia dipisahkan secara rawak kepada 3 kumpulan; Kumpulan 1, Kumpulan 2, dan Kumpulan Cx. Kumpulan 1 disembur dengan sel-sel bakteria *S. agalactiae* yang telah dimatikan oleh formalin sekali sehari (FKV). Bagi Kumpulan 2, ikan tersebut diberi vaksin sekali sehari selama 3 hari berturut-turut. Kumpulan 3 berfungsi sebagai kumpulan kawalan tanpa vaksinasi. Kumpulan 1 dan Kumpulan 2 diberi dos penggalak dua minggu setelah vaksinasi pertama. Ketiga-tiga kumpulan dicabar dengan jangkitan

S. agalactiae melalui suntikan intraperitoneum (i.p.). Sebarang petanda klinikal dan kematian dicatat setelah percubaan vaksinasi. Tiga ekor ikan daripada setiap kumpulan dikorbankan setiap minggu dan sampel kulit dikumpulkan untuk histologi. Perubahan dalam ketebalan epidermis kulit setiap minggu direkodkan. Analisis statistik perubahan ketebalan epidermis menunjukkan tidak ada perbezaan yang signifikan (p>0.05) dalam tindak balas imun kulit yang dihasilkan oleh kumpulan ikan tilapia yang terdedah kepada vaksin tunggal dan vaksin tiga kali ganda. Hal ini menunjukkan bahawa vaksinasi semburan satu dos sudah cukup untuk menghasilkan tindak balas imun pada ikan tilapia merah hibrid.

Kata kunci: Tilapia merah hibrid, *Streptococcus agalactiae*, semburan vaksin, ketebalan epidermis kulit.

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicines in partial for the course VPD 4999 – Final Year Project

EVALUATION OF THE MUCOSAL IMMUNE RESPONSE OF RED HYBRID TILAPIA TO DIFFERENT DOSES OF SPRAYED *Streptococcus agalactiae* VACCINE

By

Nur Izuin Binti Zainuddin

2020

Supervisor: Assoc. Prof. Dr Md Sabri Mohd Yusoff

Streptococcus agalactiae is one of the important pathogens that can cause substantial mortality and morbidity among various species of freshwater, estuarine and marine fishes. This study was aimed to evaluate the mucosal immune response in red hybrid tilapia following administration of a single- and triple-dose of spray killed vaccine of *S. agalactiae*. 90 tilapia were randomly separated into 3 groups; Group 1, Group 2, and Group Cx. Group 1 were sprayed with formalin-killed bacteria (FKB) of *S. agalactiae* once per day. For Group 2, the fish were vaccinated once per day for 3 consecutive days. Group 3 served as the control group without any vaccination. Both Group 1 and Group 2 were given a booster 2 weeks after the first vaccination. All groups were then challenged with pathogenic *S. agalactiae* through intraperitoneal (i.p.) injection. Any clinical signs and mortality were recorded following the vaccination challenge trial. 3 fish from each group were sacrificed each week and skin

samples were collected for histology. The changes in skin epidermal thickness every week was recorded. Statistical analysis of the epidermal skin thickness revealed that there is no significant difference in immune response produced by tilapia that is exposed to single-dose and triple dose spray-killed vaccine in tilapia. This showed that a single dose spray vaccination is sufficient to produce an immune response in Red hybrid tilapia.

Keywords: Red hybrid tilapia, *Streptococcus agalactiae*, spray vaccine, skin epidermal thickness.

1.0 INTRODUCTION

1.1 Study Background

Recently, the fisheries sector faces a major threat due to continuous outbreaks of streptococcal infections, particularly in the tilapia industry (Jiufeng et al., 2016). Tilapia is highly susceptible to this outbreak and resulting in deaths of up to 70% over about 7 days (Wongsathein, 2012). The main known reservoirs for *S. agalactiae* are humans and cattle. In humans, it is a causative agent of neonatal disease and mastitis in cattle (Delannoy et al., 2013). Abnormal behaviour related to meningoencephalitis and septicemia can be observed in streptococcal infected tilapia.

Vaccination is an important tool in the control of streptococcal disease as antibiotic therapy is not effective. A formalin killed *S. agalactiae* vaccine is proven to be effective in protecting tilapia when it is challenged with homologous strains (Evans at al., 2004). Mucosal immunization can be achieved in several ways including spray, oral vaccination or immersion. A study made by Noraini et al. (2013) proved that the spray vaccine was able to induce high protection in vaccinated fish after intraperitoneal and immersion challenge.

Therefore, in this study, boost vaccination will be done to enhance the efficacy of the killed spray vaccine. Antibody response of the fish will be evaluated by performing sprayed killed vaccine on the fishes through different degrees of exposure. A different set of tests such as immunohistochemistry, histopathology and

haematoxylin and eosin (H&E) staining will be performed to differentiate the efficacy of the single-dose vaccine and triple dose vaccine.

1.2 Justification

In teleost, there are a total of four mucosal-associated lymphoid tissues which are GALT, SALT, GIALT and NALT. SALT is one of the first lines of defence of innate immune response in fish. However, unlike GALT, SALT does not contain well-organized lymphoid aggregates. Hence, there are limited studies done focusing on the histology aspect of the SALT following spray vaccination on the fish. In response to any physical or chemical disturbances, SALT can influence direct changes in the skin and skin mucus cells (Bunnoy et al., 2019). One study has shown a significant increase in epidermal skin thickness and mucus cell density in bighead catfish administrated *Acinetobacter* KU011TH probiotic through feeding, as a water additive and their combination for 30 days (Bunnoy et al., 2019). We aimed to find out if the same result can be obtained in tilapia that is exposed to spray vaccination. Thus, this experiment is carried out to evaluate the SALT enhancement following spray vaccination by observing the changes in epidermal skin thickness

1.3 Objectives

1. To evaluate the mucosal antibody response of the Red hybrid tilapia by assessing the skin-associated lymphoid tissue development through the administration of a single- and triple-dose of spray *Streptococcus agalactiae* vaccine.

2. To determine the efficacy of the spray vaccine by evaluating the survival rate upon challenge with live pathogenic *Streptococcus agalactiae.*

1.4 Hypothesis

Ha: There is a significant difference in immune response produced by tilapia that is exposed to single-dose and triple-dose spray-killed vaccine in tilapia.

Ho: There is no significant difference in immune response produced by tilapia that is exposed to single-dose and triple-dose spray-killed vaccine in tilapia.

2.0 LITERATURE REVIEW

2.1 Red hybrid tilapia

Tilapia are freshwater fishes that are native to Africa but have been introduced into many tropical, subtropical and temperate regions of the world. They belong to the family of Cichlidae. The presence of an interrupted lateral line running superior along the anterior part of the fish and inferior along the posterior portion is one of the characteristics belong to this family. Presence of a single nostril can also be identified to distinguish with other families of bony fishes (Jobling, 2007). During the second half of the 20th century, the introduction of tilapia into many parts of the world are mainly for food fish, recreational fishing, aquatic weed control and research purposes (El-Sayed, 2006).

Tilapia is also considered a suitable fish for aquaculture purposes. This is mainly due to their exceptional traits such as fast growth, tolerance to a variety of environmental conditions, stressors and diseases, high reproductive capability and acceptance of artificial feeds immediately after yolk-sac absorption (El-Sayed, 2006). Tilapia is the second most important group of farmed fish after carps. According to Food and Agriculture Organization (FAO) statistics, by 2003, the largest country to be a producer of farmed Nile tilapia annually is China (806000 tonnes), followed by Egypt (200000 tonnes), Philippines (111000 tonnes), Thailand (97000 tonnes) and Indonesia (72000 tonnes).

Tilapia agriculture varies from rural sustenance or non-commercial purpose to a large-scale and market-driven level depending on the management intensity. Current trends might enhance the production of tilapia which includes the development of new faster-growing strains through selective breeding techniques, breeding procedures to produce genetically male tilapia without direct hormone use, pond polyculture systems and intensive cost-effective recirculation systems (FAO, 2020)

2.2 Streptococcus in Tilapia

Streptococcal infections in fish are known as red boil disease and it is caused by Gram-positive bacteria. The disease results in significant economic losses in the aquaculture industries. Different species of *Streptococcus* have been reported to cause the disease such as *S. iniae, S. agalactiae, S. dysgalactiae, S. faecium,* and *S. faecalis* (Woo & Bruno, 2006). These bacteria are opportunistic pathogens that are dependent on stress to establish pathogenicity in the fish (Bunch and Bejerano, 1997). Streptococcus is also a huge concern due to the bacteria capability to be transmitted from fish to humans. Several conditions such as low or high-water temperature, high salinity, high alkalinity, low oxygen content, high stocking density and high concentration of nitrite will make tilapia more prone to be infected with *Streptococcus* (Bunch and Bejerano, 1997).

Typical signs of tilapia infected with streptococcal infections are bilateral exophthalmia with cloudy corneal opacity, dark colouration of the skin, petechiae on opercula's inside wall, haemorrhage and fin erosion (Woo & Bruno, 2006). Other than

that, erratic swimming and lethargy can also be seen. Grossly, infected fish will show the presence of hepatomegaly, splenomegaly, ascites and congestion of brain, kidney, liver or spleen. Histopathological changes that can be seen are meningoencephalitis and panophthalmitis (Woo & Bruno, 2006). Amoxicillin is a broad-spectrum antimicrobial that has shown evidence to treat Streptococcal infections in tilapia (Plumb & Hanson, 2011).

2.3 Vaccination

In fish, the major lymphoid tissues consist of kidney, thymus, spleen and mucosa-associated lymphoid tissues. Unlike mammals, fish immune system lack of lymph nodes and bone marrow. In aquaculture, vaccination has been used widely as a prophylactic purpose which includes injection, immersion or oral feed vaccine (Adams, 2016). Immersion vaccination can be carried out by hyperosmotic immersion or directly by spraying the fish. Intraperitoneal is the most effective method. However, it produces more stress and is a time-consuming method. According to Gunnels et al. (1978), oral feed vaccination will not produce high levels of resistance although it is a desirable method compared to others.

Spray vaccination is also thought to produce a higher level of immunity when compared with oral vaccine administration. However, this method has a risk of producing low potency which may be due to inefficient uptake of antigens across the mucosal membrane. Small fish can be vaccinated using a hyperosmotic immersion or direct immersion. Several factors may affect vaccine efficacy which are the vaccine

dose, duration of immersion, antigen uptake during immersion, adjuvant performance, temperature, fish size and others (Bøgwald & Dalmo, 2019).

3.0 MATERIALS AND METHOD

3.1 Fish and experimental condition

A total of 90 fish were transported from Beranang Tilapia Farm, Selangor. The fish were divided into 3 groups which are Group 1, Group 2 and Group Cx (control group). Each group were duplicated into two aquariums. They were then let acclimatized for 3 days. The aquariums were cleaned and disinfected prior to the experiment. The water was filled half of the aquarium and anti-chlorine was added. The aerator was provided to each of the aquaria. The water quality was monitored throughout the experiment and replaced every 3 days. The fish were fed with commercial feed twice a day.

3.2 Bacterial and growth condition

Isolates of *S. agalactiae* were subcultured into tryptic soy agar (TSA) and left incubated at 37°C for 24 hours. A typical colony of *S. agalactiae* can be observed the following day which are small, white to grey raised colonies with smooth edges. Gram staining was done and we can observe Gram-positive bacteria which appears in pairs or short chains. *S. agalactiae* are non-motile, non-spore-forming and catalase-negative bacteria.

3.3 Formalin-killed bacteria (FKB) preparation

Colonies from TSA were subcultured into tryptic soy broth (TSB) in a falcon tube and incubated in a shaker incubator at 30°C for 24 hours. 0.5% formalin in phosphate-buffered saline (PBS) was added and kept at 4°C for 24 hours to kill the bacteria. The bacteria were then centrifuged at 6000 × g for 15 minutes. The

supernatant was then removed. 30 mL of PBS was added into the falcon tube to wash off remaining formalin. The solution was suspended using the vortex until it becomes homogenize and then centrifuged again. The process of washing the bacteria from the remaining formalin is repeated for 3 times. The concentrations were adjusted to 10^8 CFU/mL, by using McFarland method. The solution was cultured into TSA and incubated overnight to make sure no growth of bacteria can be seen.

3.4 Spray vaccine preparation

A commercial spray bottle was prewashed with 70% alcohol for 5 minutes followed by three 3-minutes washings with sterile PBS. The vaccine was then loaded into the sterilize spray bottle. The volume of the spray was measured by spraying into a 10 mL graduated cylinder. A single spray was equivalent to 1 mL.

3.5 Preparation of *Streptococcus agalactiae* inoculums for the challenge

The bacteria from TSA were subcultured into TSB and incubated in a shaker incubator for 24 hours at 37°C at 110 rpm. 200 µL of the bacterial culture was injected into naive Red hybrid tilapia intraperitoneally. This is to enhance the virulence of bacteria. The infected fish was euthanized 48 hours post-infection. Bacterial isolation of the brain, kidney and eye were done to re-isolate the *S. agalactiae.* A few colonies of *S. agalactiae* that have been isolated from the infected fish were then subcultured into TSB and incubated in a shaker incubator at 30°C at 110 rpm for 18 hours. *Streptococcus agalactiae* will then be used for vaccine challenge trial. The standard plate count method was done just before the challenge trial and the final concentration of live *S. agalactiae* is 2.8×10^7 CFU/mL.

3.6 Experimental design

Ninety tilapia were randomly divided into three groups; Group 1, Group 2 and Group 3. Group 1 were sprayed with formalin-killed bacteria (FKB) of *S. agalactiae* once per day. For Group 2, the fish were vaccinated once per day for 3 consecutive days. Group 3 served as the control group without any vaccination. Both Groups 1 and 2 were given a booster 2 weeks after the first vaccination. By the end of week 4, the fish were challenged intraperitoneally with live pathogenic *S. agalactiae.* Following the challenge, the fish were observed for any clinical signs and mortality. Throughout the experiment. Three fish from each group were euthanized weekly for five weeks. Their skin sample was taken and processed for histology.

3.7 Histology

3 fish each group were collected weekly from week 0 until week 4 for skin sampling. Fish were euthanized using a pithing method and the skin was cut approximately 5 mm thick at the dorsolateral area. The tissue was fixed in a 10% formalin for 24 hours. Next, the skin sample undergoes decalcification method for the complete removal of calcium salt and minerals from cartilage bones and scales. The decalcifying agent that is used in this study is nitric acid 10%. Following the decalcification process, the tissue will then be trimmed to smaller sizes. The trimmed tissue was then placed into a tissue cassette and labelled according to their group. Next, the tissue was processed to remove water in the tissues and replaced it with paraffin and making it possible for sectioning. Following tissue processing, the tissue was

placed in a mould (embedding) for sectioning. After sectioning, the tissue ribbons were placed into a water bath and then transferred onto a glass slide. Lastly, the slides were allowed to dry overnight and stained with H&E stain.

3.8 Bacterial isolation and Gram staining

Fish from each group were euthanized after the vaccine challenge. Samples of the eye, brain and kidney were taken for bacterial isolation and identification (PCR). The samples were cultured onto TSA agar and then incubated at 37°C for 24 hours. The colony that shows morphological characteristics that were similar to *S. agalactiae* was stained using Gram stain.

3.9 Polymerase Chain Reaction (PCR)

PCR technique was done to further confirm the presence of *S. agalactiae* in the challenge fish. Total cellular DNA was extracted with the FavorPrep Tissue Genomic DNA Extraction Mini Kit according to the manufacturer's protocol. Then, the extracted DNA was evaluated by PCR for *S. agalactiae* specific section of 16S-23S RNA intergenic spacer region with reverse primer 5'-AAG CCT TTA TTT GTT AAA TGA TAC GTG AAC-3' and forward primer 5'-GGA TCC ATG AAA ATG AAT AAA AAG GGA CT-3'. PCR cycle was performed using a thermal cycler machine. Thermal cycler machine had 3 major steps and repeated for 30 cycles. First is denaturation at 94°C for 1 minute, annealing at 57 °C for 1 minute, followed by extension at 72°C for 30 seconds. Agarose gel electrophoresis was conducted, and UV transilluminator was used to obtain the result.

3.10 Statistical analysis

Statistical analysis for this study was performed using IBM SPSS Statistics version 25.0 for Windows 10 and tested at 5% significance level. The skin epidermal thickness of all groups was measured from week 0 until week 4. The average value was recorded and analysed using One-way ANOVA.

4.0 RESULT

4.1 Thickness of epidermis

Immunization by spray vaccine of formalin-killed *S. agalactiae* on Red hybrid tilapia resulted in significantly (p<0.05) increased in the epidermal thickness for both vaccinated groups treated with single-dose vaccine and triple-dose vaccine which started from week 1 post-immunization. The epidermal thickness of both groups gradually increased until reaching week 4 upon challenge.

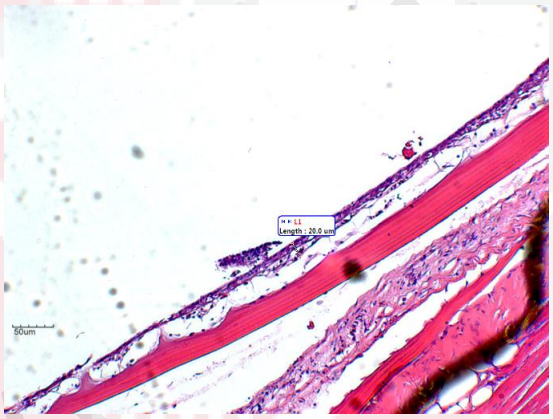


Figure 4.1.1: Thickness of skin epidermis in week 1 for Group 3 (scale bar: 50µm). The photos were captured with 20× magnification (scale bar: 50µm).

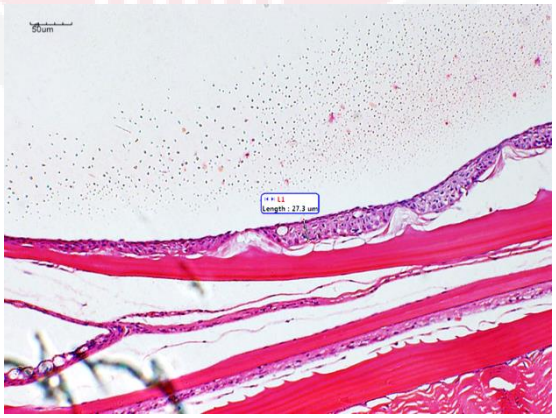


Figure 4.1.2: Thickness of skin epidermis in week 4 for Group 1 (scale bar: 50µm).

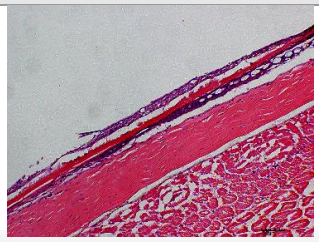
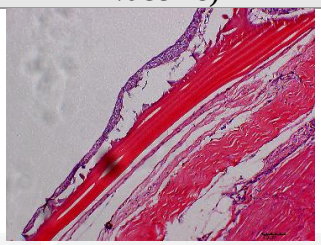
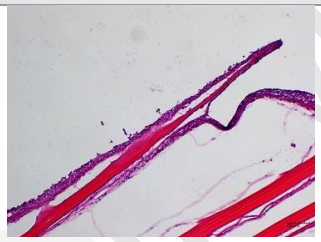
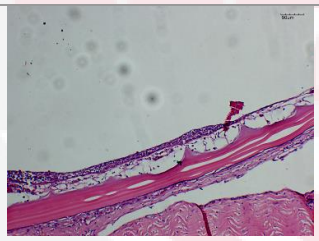
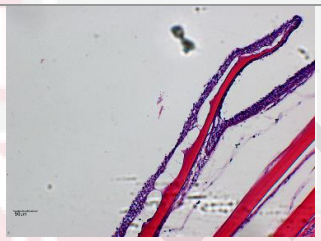
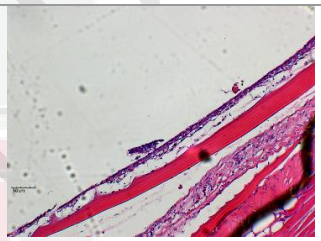
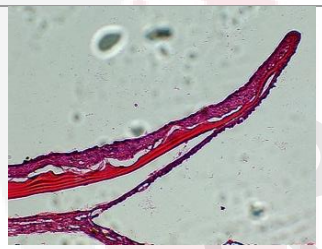
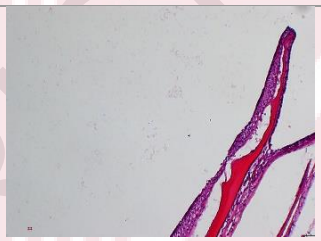
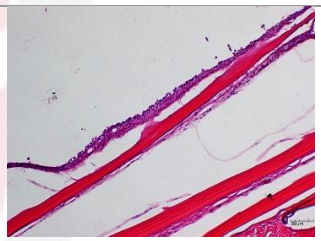
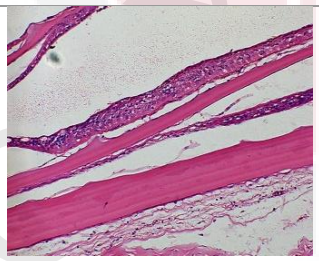
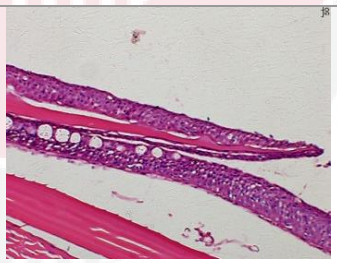
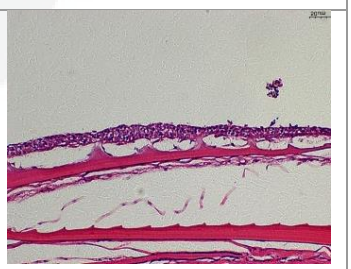
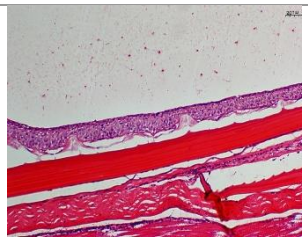
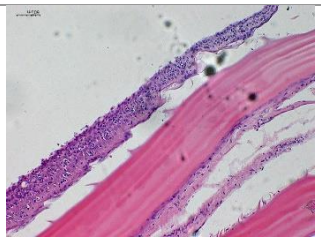
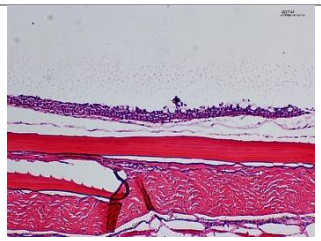
		TREATMENT GROUP		
		1 (Single dose vaccine)	**2** (Triple dose vaccine)	**3** (Control)
WEEK	0			
	1			
	2			
	3			
	4			

Figure 4.1.3: Overview of epidermal thickness in Group 1, 2 and 3 from week 0 until week 4.

However, on week 3 (after booster vaccination on week 2), we can observe the vaccinated group that was treated with triple-dose vaccine had a higher increase in epidermal thickness compared to the vaccinated group which received a single-dose vaccine. For the unvaccinated group (Cx), the epidermal thickness did not show significant (p>0.05) increasing or decreasing pattern from week 0 until the end of the experiment.

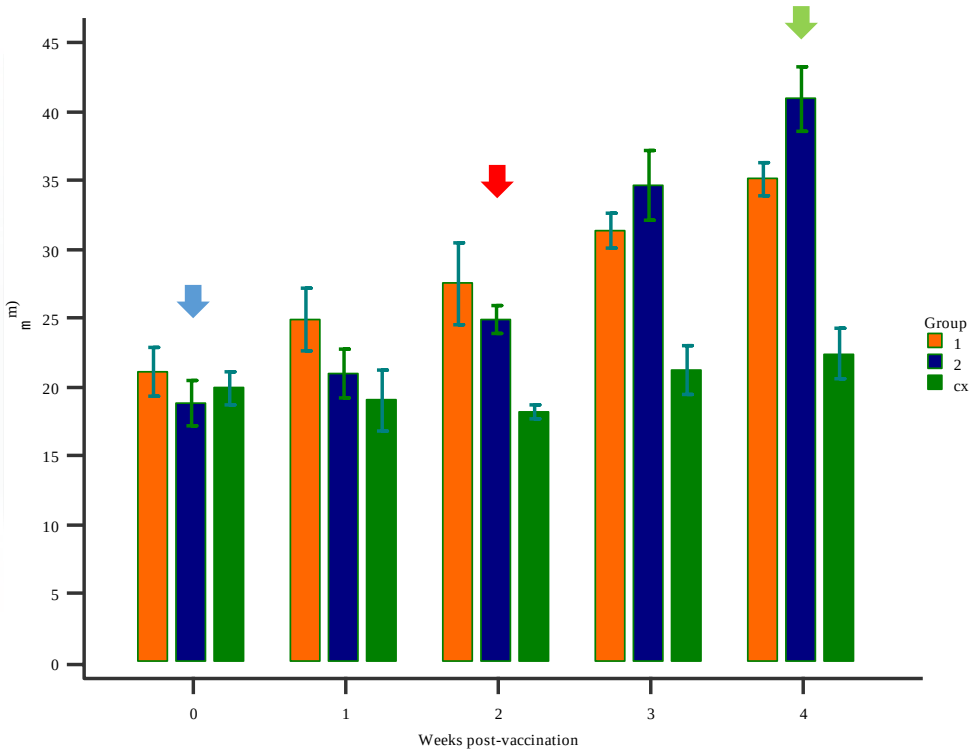


Figure 4.1.4: Thickness of skin epidermis in Group 1 (single-dose), Group 2 (triple-dose) and Group Cx (Control) were monitored weekly from week 0 until week 4. Vaccination was done on week 0 (blue arrow) and a booster dose was given on week 2 (red arrow) followed by a challenge on week 4 (green arrow) with 2.8×10^7 CFU/mL live *S. agalactiae* intraperitoneally.

Based on a statistical analysis of ANOVA, tests of between-subjects effects show that there is a significant interaction effect (p<0.05) between vaccine dose received and the sampling time (from week 0 until week 4). Upon the Tukey HSD test, there is a significant difference (p<0.05) between the control group and both vaccinated groups. However, there is no significant different (p>0.05) between the group that received a single-dose vaccine and group that received a triple-dose vaccine. When each week is analysed separately, it shows that there is a significant difference (p<0.05) of epidermal thickness in week 2, week 3 and week 4. However, there is no significant difference (p>0.05) showed in week 0 and week 1.

4.2 Clinical signs and mortality

Following 48 hours post intraperitoneal challenge with live *S. agalactiae*, the fish showed clinical signs of lethargic, unilateral exophthalmia and cloudy eyes, loss of appetite, erratic swimming and circling. The clinical signs are more prominent in the nonvaccinated control group. Within 14 days of post-challenge, 95% of mortality was recorded for the nonvaccinated control group, 65% and 50% of mortality were recorded for Group 1 and Group 2, respectively. The relative percentage survival (RPS) for each group revealed to be 32% and 47% for Group 1 (single-dose) and Group 2 (triple-dose), respectively.

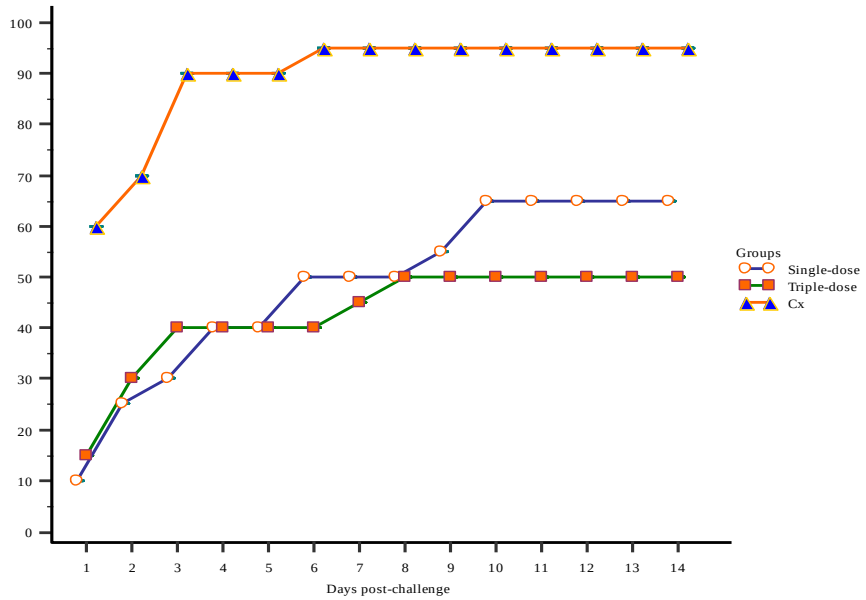


Figure 4.2: Cumulative mortality 14 days post-challenge with killed *S. agalactiae* spray vaccine.

4.3 Bacterial isolation

After 2 weeks of challenge trial, the fish were terminated and samples of eye, brain and kidney were collected. Bacterial isolation for each sample was done. From the bacterial culture, there is no growth in both vaccinated groups. However, bacteria growth can be observed from the nonvaccinated control group. The isolates revealed small, smooth, round whitish colonies. Upon Gram staining of the isolates, it shows Gram-positive, appear in pairs or small chains.

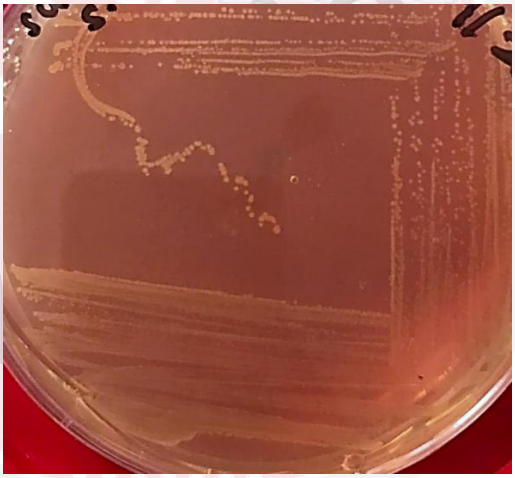


Figure 4.3.1: Small, pinpoint, whitish colonies which are presumptive characteristic of *S. agalactiae* infection on tryptic soy agar.

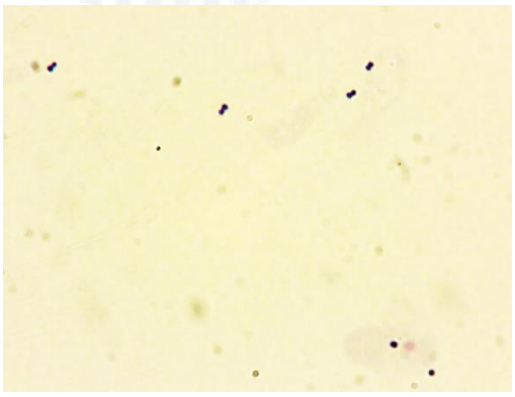


Figure 4.3.2: Gram-positive, cocci in pairs.

4.4 Polymerase chain reaction (PCR)

PCR amplification showed a positive result with a value of 1429 bp for the samples taken from the control group for *S. agalactiae* to be compared to the positive control.

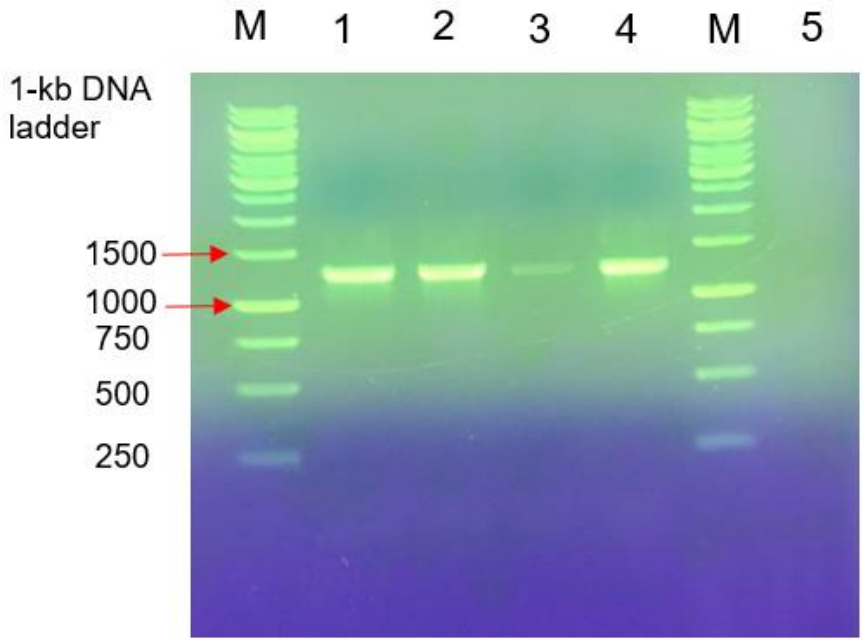


Figure 4.4: PCR showed bacteria culture from the control group were positive for *S. agalactiae* (~1429 bp).

5.0 DISCUSSION

This study investigated the immune response against streptococcosis in Red hybrid tilapia following spray vaccine of formalin-killed *S. agalactiae.* This route of vaccination is more convenient for smaller size fish as it will lessen the stress caused by handling, more rapid and economical. Other than that, this method can also provide a higher level of protection. In fish, the SALT is categorized as one of the first-line defence of innate immune response. The fish skin cells harbour a large number of nonspecific immune cells: mucus cells, club cells, alarm cells and sensory cells. Hence, the spray vaccine will allow direct exposure of antigen to the immune cells located in the fish skin and gills (Dadar et al., 2017). According to Bunnoy et al. (2019), in response to any physical or chemical disturbances, skin-associated lymphoid tissue (SALT) can influence direct changes in the skin and skin mucus cells. Thus, this experiment is carried out to investigate the immune response by evaluating the SALT enhancement following spray vaccination by observing the changes in the fish epidermal skin thickness.

This study is the first to report the immune response in tilapia from the histological aspect. Initial evaluation of immune response to vaccinated groups (single dose & triple dose) and nonvaccinated control group revealed a significant difference ($p<0.05$) in the changes of skin epidermal thickness. However, there is no significant difference ($p>0.05$) when we compare between single-dose group and triple dose group. This shows that a single spray with the following booster at 2 weeks interval is sufficient to elicit an immune response in the fish. Notably, histological analysis of

the skin strongly confirmed the histoarchitectural performance of SALT in all treatment groups compared to those in the control group.

Statistical analysis revealed that there is a significant difference ($p<0.05$) of epidermal thickness only seen in week 2, week 3 and week 4. Week 0 and week 1 is insignificant in term of the changes in epidermal skin thickness. This is probably due to the effect of the booster vaccine that was given on week 2. We can conclude that the booster effect will increase the level of immune response over time.

The relative percentage survival (RPS) revealed that for group 1 (single-dose), the RPS is 32% and for group 2 (triple-dose), the RPS is only slightly higher which is 47%. Hence, we can conclude that a single dose is sufficient to produce the same effect as the triple dose vaccine. However, it seems that the relative percentage survival (RPS) in our study was generally lower than the survival observed previously in other studies. This was contradictory to study done by Baltazar et al. (2020) where the relative per cent survival of fish in the vaccinated group is significantly higher than in a nonvaccinated group. Thus, the spray vaccine that is used in this study is not effective in protecting the fish against streptococcal infection.

This is probably due to several factors that can affect the efficacy of the vaccine. Stress caused by environmental or man-made factors such as photoperiod, seasonal changes, salinity, heavy metals, crowding, handling and transport, can induce immune suppression and be a limiting factor for vaccine efficacy (Pasnik et al., 2005). Other than that, the health status of the fish might also affect the efficacy of the

vaccine. And in our case, we found out there is a presence of parasite infestation in the gills in all of our groups. Low RPS can also be influenced by the route of vaccination. Several studies have revealed that immersion vaccination provided lower protection than those given by injection. Killed vaccines are generally administered by injection to achieve good efficacy. Evans et al. (2004) has demonstrated that bath immunization of tilapia resulted in RPS values that were two times lower than those achieved with intraperitoneal vaccination. Other than that, the incompatible vaccine challenge procedure can also lead to a low RPS value. According to Hoare et al. (2017), the intraperitoneal or subcutaneous injection can bypass the mucosal immunity stimulated by immersion vaccination.

Even though we can perceive a significant increase in the epidermal thickness in the vaccinated groups when compared to a nonvaccinated group, we can still see clinical signs from all groups post-challenge. Thus, we can assume that the spray vaccine used in this study may not be able to provide complete protection against systemic infection. According to a study made by Evans et al. (2004), she observed that the protection by the vaccine was comparable at 30- and 64-days post-vaccination and suggested that longer duration of protective immunity may be possible. Pasnik et al. (2005) in his study, also had findings that *S. agalactiae* vaccine provided significant protection against challenge between 47- and 180-days post-vaccination. Hence, we can conclude that fish in our study is not significantly protected by the spray vaccine due to the shorter period post-vaccination before the challenge.

6.0 CONCLUSION

The result of this study revealed that there is no significant difference (p>0.05) in the immune response produced by tilapia that is exposed to single-dose and triple-dose spray-killed vaccine. Based on this experiment, we can conclude that a single-dose of vaccine is sufficient to produce the same immune response as a triple-dose vaccine. Hence, it is very cost-effective for the farmer to only administer a single dose of the spray vaccine. This experiment also revealed that all groups showed clinical signs even though there is a significant increase in the epidermal thickness in the vaccinated groups when compared to a nonvaccinated group. From this result, we can conclude that the spray vaccine used in this study may not be able to provide systemic protection in the fish. However, other factors might also influence the efficacy of the vaccine.

7.0 RECOMMENDATIONS

In a further study, it is very important to screen the fish prior to the experiment to ensure that all fish are in a good condition to undergo the experiment. ELISA should also be done for the detection of *S. agalactiae* antibodies in the skin and we can relate the result to our RPS to make a better conclusion and further support our hypotheses. Other than that, further study may include a more suitable challenge method for efficacy testing. Intraperitoneal and intramuscular challenge method are often required for efficacy testing, but these do not mimic natural infections because the infective agents do not gain entry through the natural portals of entry. Future studies should seek to develop and use more relevant and suitable challenge models that mimic natural infections

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