UNIVERSITI PUTRA MALAYSIA

# EFFECTS OF DIFFERENT ROUTES OF VACCINATION AGAINST *Streptococcus agalactiae* IN RED HYBRID TILAPIA FINGERLINGS (*Oreochromis* sp.)

'AISYAH BT AMINUDDIN

**EFFECTS OF DIFFERENT ROUTES OF VACCINATION AGAINST *Streptococcus agalactiae* IN RED HYBRID TILAPIA FINGERLINGS (*Oreochromis* sp.)**

**'AISYAH BT AMINUDDIN**

**A project paper submitted to the**

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## CERTIFICATION

It is hereby certified that we have read this project paper entitled "Effects of different routes of vaccination against *Streptococcus agalactiae* in Red hybrid tilapia fingerlings (*Oreochromis* sp.)", by 'Aisyah Binti Aminuddin and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course of VPD 4999 – Project.

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**DEDICATIONS**

*To my dear and lovely parents, Mr Aminuddin Ahmad*

*And*

*Mrs. Ruhani Mohamed*

*For making me who I am today*

*To my dearest siblings,*

*Ahmad Syahiq Aminuddin*

*'Afifah Aminuddin*

*Ahmad Syamim Aminuddin*

*Ahmad Syazwan Aziz Aminuddin*

*For being there whenever I needed you*

*"You don't choose your family*

*They are God's gift to you, as you are to them."*

*-Desmond Tutu*

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| | |
|---|---|
| *S. agakactiae* | *Streptococcus agalactiae* |
| FKB | Formalin-Killed Bacteria |
| FCV | Formalin Crude Vaccine |
| PBS | Phosphate Buffered Saline |
| BHI | Brain Heart Infusion |
| ELISA | Enzyme-linked immunosorbent assay |
| PCR | Polymerase Chain Reaction |
| CFU/mL | Colony forming unit per mililiter |
| ˚C | Degree celcius |
| rpm | Revolutions per minute |
| PBST | Phosphate Buffered Saline + Tween-20 |
| BSA | Bovine serum albumin |
| IgM | Immunoglobulin M |
| DNA | Deoxyribonucleic acid |
| UV | Ultraviolet |
| ANOVA | Analysis of variance |
| mL | Mililiter |
| mm | Milimeter |
| µL | Microliter |
| < | Less than |
| Bp | Base pair |

## ABSTRAK

Abstak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada VPD 4999 - Projek ilmiah tahun akhir

**KESAN LALUAN VAKSINASI YANG BERBEZA TERHADAP *Streptococcus agalactiae* PADA ANAK TILAPIA HIBRID MERAH (*Oreochromis* sp.)**

**Oleh**

**'Aisyah Binti Aminuddin**

**2016**

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

Streptokokosis merupakan penyakit yang disebabkan oleh jangkitan *Streptococcus* sp. Penyakit ini merupakan masalah global bagi sektor pengeluaran ikan sedunia dan saling berkaitan dengan kerugian yang tinggi dalam sektor ekonomi. Kajian ini dilakukan bertujuan untuk mengenalpasti kesan terhadap penghasilan antibodi bagi laluan vaksinasi yang berbeza terhadap *Streptococcus agalactiae* dalam anak ikan tilapia merah. Sembilan puluh anak ikan tilapia merah dibahagikan secara rawak kepada tiga kumpulan iaitu kumpulan 1, 2 dan 3 yakni setiap kumpulan mempunyai tiga puluh ekor anak ikan. Dua formulasi bagi formalin vaksin tidak aktif telah dihasilkan iaitu vaksin berasaskan makanan dan semburan. Kumpulan 1 menggunakan vaksin secara semburan selama 3 hari berturut-turut pada minggu pertama dan pada minggu ketiga sebagai dos ransangan juga selama tiga hari berturut-turut. Bagi kumpulan 2, anak ikan divaksin secara semburan hanya sekali pada minggu pertama dan diberi vaksin berasaskan makanan pada minggu ketiga sebagai dos penggalak manakala kumpulan 3 kekal sebagai kelopok kawalan tanpa vaksinasi. Kesemua kumpulan dijangkitkan dengan *S. agalactiae,* $10^9$ CFU/mL pada kadar 100 µL melalui intraperitoneum. Setelah dijangkiti, anak ikan berada di bawah pemerhatian bagi sebarang petanda klinikal ataupun kadar kematian. Sampel mukus bagi lima ekor anak ikan daripada setiap kumpulan telah diambil dengan menggunakan steril calitan pada permukaan kulit ikan setiap minggu. Cecair lavaj usus juga telah diambil dan kedua-dua sampel diproses menggunakan asai imunoserap terangkai ensim (ELISA) tak langsung bagi mengenalpasti penghasilan antibodi IgM terhadap *S.* agalactiae. Keputusan ELISA menunjukkan penghasilan antibodi bagi sampel mukus dan cecair lavaj usus bagi anak ikan tilapia yang telah divaksin tidak mempunyai perbezaan bererti walaupun berlaian kaedah vaksinasi telah digunakan.

**Kata kunci:** *Streptococcus agalactiae*, Tilapia hibrid merah, vaksin semburan, vaksin berasaskan makanan, asai imunoserap terangkai  ensim (ELISA)



**ABSTRACT**

Abstract of the project paper presented to the Faculty of Veterinary Medicines in partial for the course VPD 4999 - Final year project.

**EFFECTS OF DIFFERENT ROUTES OF VACCINATION AGAINST *Streptococcus agalactiae* IN RED HYBRID TILAPIA FINGERLINGS (*Oreochromis* sp.)**

**By**

**'Aisyah Binti Aminuddin**

**2016**

**Supervisor: Assoc. Prof Dr. Sabri Mohd Yusoff**

Streptococcosis is a disease that develops following infection by *Streptococcus sp*. It is a major problem for fish production worldwide, and it is associated with high economic losses. This study was aimed at investigating the effects of different vaccination route against *Streptococcus agalatiae* in Red hybrid tilapia fingerlings. Ninety fingerlings were randomly divided into three groups, 1, 2 and 3 of 30 each. Two formalin-killed vaccine formulations were developed, feed based and spray. Group 1 was vaccinated using spray vaccine for 3 consecutive days in the 1$^{st}$ week and a booster dose for 3 consecutive days in the 3$^{rd}$ week. Group 2 was vaccinated once by using spray vaccine followed by a booster dose using the feed-based vaccine. While Group 3 served as a control group without any vaccination. All groups were challenged with 100 µL of *S. agalactiae* ($10^9$ CFU/mL) intraperitoneally. Following challenge, the fingerlings were observed for any clinical signs and mortality. Mucus samples of five fish from each group at sampling time were collected by using sterile swab at the surface of the skin. Gut lavage fluid was also collected, and both samples were subjected to indirect enzyme-linked immunosorbent assay (ELISA) to determine the IgM antibody levels against *S. agalactiae*. The results showed that the IgM antibody response in mucus and gut lavage fluids produced by the tilapia immunized with vaccination were not significantly different with each other even though different routes of vaccination were used.

**Keywords**: *Streptococcus agalactiae*, Red hybrid tilapia, spray vaccine, feed-based vaccine, enzyme-linked immunosorbent assay (ELISA)

# 1.0 INTRODUCTION

## 1.1 Study Background

Streptococcosis can be acute or chronic. Acute streptococcosis usually occurs in the seasons where the water temperature is high resulting in peaks of mortality that last 2 - 3 weeks (Intervet, 2006). The chronic take place when the water temperature is lower and does not cause peaks of mortality. Amal and Zamri (2011) reported that streptococcosis occur by the infection of *Streptococcus* sp. The shape is spherical or ovoid and 0.5 - 2.0 μm in diameter, occur in pairs or chains when grown in liquid media, non-motile, non-spore-forming and appears purple or blue when stained using Gram stain.

*Streptococcus* bacteria are part of the normal flora on animal bodies, but infection and disease also can occur when the bacteria enter through the cuts, abrasions, wounds or when the immune system becomes weakened especially involving the stress. *Streptococcus* spp., which can cause diseases in fish, includes *S. agalactiae* (Suanyuk *et al*., 2005) *Streptococcus iniae* (Shoemaker *et al*., 2000) and *Streptococcus difficile* (Berridge *et al*., 2001). Evans *et al. (*2002) said that Group B *S. agalactiae* is another emerging fish pathogen of freshwater and saltwater fish species throughout the world.

Environmental stress is playing a major role in influencing the fish immunity for examples such as water temperature, high nitrate level and low dissolved oxygen. When the line of immunity in fish is declined, the fish are highly susceptible to streptococcosis. Siti-Zahrah *et al*. (2004) and Amal *et al*. (2008) reported that high mortality of the fish infection is frequently recorded between April and July that depicts dry season in Malaysia. *When fish are infected with streptococcosis, the most common clinical signs* *shown include anorexia, exophthalmia, ascites and erratic swimming (Evans et al., 2002; Salvador et a., 2005) l*and meningoencephalitis in fish *(*Eldar *et al*., 1995). Mian *et al*. (2009) also said that streptococcosis caused by *S. agalactiae* is a major disease of many fish species, and it is characterized by septicaemia and meningoencephalitis.

Pathogenesis in infected fish involves septicaemia and colonization of several organs such as nares, brain, kidney and intestines (Pasnik *et al*., 2005). However, other than fish animals such as mice, cats, dogs, hamsters, camels and frogs can also be infected with streptococcosis (Evans *et al*., 2002).

The vaccine is an antigenic material that stimulates the immune system by developing the adaptive immunity to a pathogen. The main aim of vaccination against an infectious disease is to stimulate host adaptive immune responses to counteract the infection caused by a pathogen. Vaccination is the most effective method to combat disease (Karen and Scott, 2011) and it also an important disease prevention to maintain human and animal health worldwide. Craig *et al*. (2009) reported that vaccines developed for aquaculture have significantly reduced antibiot ic use in fish production. Lombart *et al.* (2007) also stated that vaccination is the most effective and cost-effective method of preventing infectious diseases.

## 1.2 Justification

The study in determining the antibody response between spray vaccine and a feed-based vaccine against *S. agalactiae* was never done before. Thus, this study will reveal the information regarding the antibody responses production between these two different methods and at the same time giving the choices of vaccination methods that can be more practical to be practiced in future. Besides, *S. agalactiae* is one of emerging fish pathogen reported in tilapia fish throughout the world including Malaysia.

### 1.3 Objectives

i. To determine the antibody response in the mucus and gut-lavage in Red hybrid tilapia fingerlings.

ii. To isolate *S.agalactiae* post challenge in Red hybrid tilapia fingerlings.

### 1.4 Hypothesis

**$H_O$:** Presence of antibody responses in mucus and gut-lavage in different route in Red hybrid tilapia fingerlings

**$H_A$:** Absence of antibody responses in mucus and gut-lavage in different route in Red hybrid tilapia fingerlings

## 2.0 LITERATURE REVIEW

**2.1 Tilapia**

Red hybrid tilapia, Oreochromis sp., had become the most important species on account of its fast growth rate, adaptability to a wide range of culture conditions and high consumer acceptability. For these reasons, it has been transferred throughout the world to over 100 countries to become the mainstay of tilapia farming in many different culture systems.

Tilapia is a generic term used to designate a group of commercially important food fish belonging to the family Cichlidae; the expression is derived from the African native Bechuana word “thiape,” meaning fish. According to a journal of Culture of Hybrid Tilapia: A Reference Profile, cichlids are classified in the large order Perciformes and inhabit the fresh and brackish waters of Africa, the Middle East, coastal India, Central and South America.

According to Fish Site, nowadays, it has emerged to become the second biggest aquatic species group after the carp group, with a worldwide harvest of over 2 million metric tons (MT), about 5% of global finfish aquaculture. Tilapia can tolerate water temperatures as low as 12 °C and can survive in water temperatures below 10 °C for prolonged periods of time. These species are also known to survive and grow in salt water. Because of the favourable culture characteristics mentioned above, tilapia is considered an ideal species for small scale fish farming as stated by Hepher and Pruginin (1990).

In intensive large scale aquacultures where tilapia are reared at high densities, tilapia is prone to various health problems since pathogens can be so easily transmitted between individuals. According to Laila Ali (2013), the risk is elevated if the keeper of the aquaculture fails to provide the tilapia with optimal conditions. For examples, when it comes to water quality, temperature and salinity.

Tilapia is a fairly low-value species; however, there is a thriving international trade at the top end of the business. Indeed, in the last 5 years or so, there has been an unprecedented development in the tilapia industry in terms of global production (double digit annual growth rate), international trade (26% increase per year in USA consumption), and industrialization of the farming industry (several integrated companies have emerged in recent years, each producing over 10,000 MT/year). So, tilapia has emerged from obscurity to become the number one commodity aquaculture species in the world. (Fish Site, 2010)

### *2.2 Streptococcus agalactiae*

Streptococcosis is a disease that develops following the infection by Streptococcus sp. They are spherical or ovoid in shape and 0.5 - 2.0 µm in diameter. They occur in pairs or chains when grown in liquid media, are non-motile, non-spore-forming and Gram-positive as stated by Zamri et al. (2011). A major identification feature of Streptococcus is that it is Gram-positive that appears purple/blue when stained using a procedure called a Gram stain. It is facultatively anaerobic, requiring nutritionally rich media for growth and commonly attacks red blood cell to produce greenish discolouration (α-haemolysis) or complete clearing (β-haemolysis) on blood agar.

In addition, it is also a type of bacteria that are fermentative in metabolism, producing mainly lactic acid, but without gas and catalase negative as according to Holt et al. (1994). Infection by Streptococcus agalactiae leads to various clinical signs, which include haemorrhages at the gill plate, loss of appetite, spine displacement, haemorrhages in the eye, corneal opacity, and haemorrhages at the base of the fins and in the opercula. The most prominent signs are uni- or bi-lateral exophthalmia, also known as “pop-eye”, and distended abdomen. The post-mortem examinations of the affected fish revealed the presence of blood-tinged fluid in the body cavity, enlarged and reddened spleen, pale but enlarged liver, as well as inflammations around the heart and kidney. Meanwhile, haemorrhagic lesions were observed on the skin according to a journal was written by Bullock (1981), Yanong & Floyd (2002) and Salvador et al. (2005).

Other clinical signs include darkening of the skin and erratic swimming, which is either spiralling or spinning just below the surface of the water. In some cases, however, the affected fish showed no obvious clinical signs before death and the mortality is mainly due to septicaemia and infection of the brain and nervous system. (Barham et al., 1979; Yanong & Floyd, 2002) Group B Streptococcus, also known as Streptococcus agalactiae, was once considered a pathogen of only domestic animals, causing mastitis in cows. S. agalactiae is now best known as a cause of postpartum infection and as the most common cause of neonatal sepsis (Zamri and Amal, 2011). According to a journal was written by Christian (2009), recently, numerous series have described S. agalactiae as a cause of infection in non-pregnant adults, providing descriptions of the clinical spectrum of disease, including clinical features, risk factors, therapy, and outcome of group B streptococcal infection in non-pregnant adults.

### 2.3 Vaccination in fish

Prophylactic treatments and good management practices can usually prevent or reduce the susceptibility to diseases. Although, antibiotics can overcome bacterial diseases, consumer health and food safety issues prevent their use in aquaculture. Moreover, established viral diseases cannot be treated. Therefore, vaccination is the best alternative to combat bacterial and viral diseases.

Vaccines are defined as various preparations of antigens derived from specific pathogenic organisms that are rendered non-pathogenic by K. Cendric et al. (2004). They stimulate the immune system and increase the resistance to disease from subsequent infection by the specific pathogen. It has been a key tool for the success of the livestock, pig, poultry and salmon industries in the fight against infectious diseases. Roy P. E. Yanong (2015) stated that the vaccine usage in the fish industry had dropped the industry's use of antibiotics. In fish farming, the use of vaccines is now so routine that all fish stocked in sea cages are vaccinated. It has been proven to be cost effective. However in all cases, only healthy fish should be vaccinated as it is a preventive measure.

There are three major modes of the application of vaccines as stated by RUMA, 2006 which are oral, immersion and injection. With oral vaccination, the vaccine is either mixed with the feed, coated on top of the feed (topdressed) or bio-encapsulated. When antigens are to be incorporated in feed, the heat sensitivity of the antigen has to be considered. When vaccines are used as top dressing in feed, a coating agent is usually applied, either to prevent leaching of the antigen from the pellets or to prevent the breakdown of the antigen in the acidic environment of the fish stomach. (Laila, 2013)

Skin epithelium and gills have mechanisms to protect fish in a broad as well as specific way. Immersion vaccination works on the ability of mucosal surfaces to recognize pathogens they had been in contact with. When fish are immersed in water containing the diluted vaccine, the suspended antigens from the vaccine may be absorbed by the skin and gills. (Laila, 2013). Then, specialised cells, such as antibody-secreting cells, present in the skin and gill epithelium will be activated and will protect the fish when fish are exposed to the live pathogen at a later stage. Other cells located in the epithelium of skin and gills, such as antigen presenting cells (macrophages), also absorb vaccine antigens and transport them to specialised tissues where the systemic immune response builds up.

In immersion vaccination, there are two application methods which are dip and bath. In dip vaccination, fish are immersed for a very short duration, usually for 30 seconds in a highly concentrated vaccine solution. With bath vaccination, fish are exposed for a longer period, usually one to several hours in a lower concentration of vaccine. (K.Cendric et al., 2004)

K. Cedric et al. (2004) reported that initially, fish farmers may not favour injection vaccination as they fear that the stress resulting from the handling and injection of the fish will cause high mortality. In contrast, many studies and farmers' experiences in the fish industry have shown that there is no mortality associated with the vaccination process per se, although some weak fish may die due to the handling process. Light anaesthesia of fish is needed for injection vaccination. This decreases the stress due to vaccination, prevents mechanical injuries and helps the fish to recover faster from the handling. When injection vaccination is performed properly, mortality immediately after vaccination should not exceed 0.25%. Higher mortalities indicate the incorrect use of anaesthetic, excessive handling/stress of the fish, incorrect needle insertion (e.g., rupturing the spleen), vaccination of diseased or weakened fish, lack of oxygen in the anaesthesia vessel, etc. Injection vaccines can be administered by intramuscular or intraperitoneal (in the abdominal cavity) injection, but the latter is by far the most common. As intraperitoneal injection vaccination involves depositing the vaccine in the abdominal cavity, it is important that the needle should penetrate the targeted abdominal wall of fish by 1 to 2 mm as stated by K. Cedric et.al, 2004.

## 3.0 MATERIALS AND METHODS

### 3.1 Fish and experimental conditions

Healthy cultured red hybrid tilapia fingerlings weighing about 10 to 15 g were transported from Aquaculture Research Station, UPM, Puchong and reared at the Moelcular Pathology Laboratory, University Putra Malaysia, Selangor. Prior to the experiment, all aquariums were cleaned and disinfected. The water was aerated continuously throughout the study. Ninety fingerlings were divided into three groups; group 1 and group 2 both as a positive control and group 3 as a negative control. Ten fingerlings were killed for bacterial and parasite screening. The fish approximately of the same size were assigned to three 80 L aquarium. The fish were maintained with 12 h light and 12 h dark per day. Water temperature was checked once a day, and they were fed with commercial feed twice a day. The temperature ranged 28.6 ± 0.1˚C, pH 7.4 ± 0.2, and dissolved 7.02 ± 0.4 mg/L.

### 3.2 Bacterial and growth condition

The isolates of S. agalactiae were obtained from outbreaks of streptococcus in Red hybrid tilapia kept in cage culture system at Kenyir Lake, Terengganu, Malaysia in 2013. The isolates were sub-cultured onto blood agar plate for 24 hours and incubated at 37˚C.

### 3.3 Formalin-Killed bacteria (FKB) preparation

Ten colonies of S. agalactiae from the blood agar plate were further sub-cultured into brain heart infusion (BHI) broth and incubated in a shaker incubator at 37˚C for 24 hours. Following incubation, the bacterial concentration was determined using Mc Farland methods. The bacteria were then killed by introducing buffered formalin to the end concentration of 0.5% formalin in phosphate-buffered saline (PBS) and kept overnight at 4˚C. After that, the bacteria were washed by centrifugation at 6000 g for 15 minutes before the supernatant was removed and PBS was added to the 50 mL falcon tube

(Greiner Bio-One). The remaining pellet was re-suspended till homogenize, and the solution was centrifuged again. The process was repeated for five times.

**3.4 Feed-based crude vaccine (FCV) preparation**

The pellet was prepared at the Nutrition Laboratory, FPV and Aquaculture Laboratory, FP. To incorporate the vaccine, the FCV will be re-suspended in PBS solution to a concentration of 109 CFU/mL at 1 L until a homogenous solution was obtained. Then, the FCV solution was sprayed onto the pellet mixture during the mixing process. Mixing will be done thoroughly with the use of a homogenizer to impregnate the FCV vaccine into the pellet. Finally, the pellet mixtures will be loaded into the pelleting machine to obtain the pellet size of 2 mm × 2 mm and dried up for 24 hours at 70˚C in the oven prior to the experiment.

**3.5 Experimental Designs**

Ninety fingerlings were equally divided into three groups. Group 1, will be vaccinated using spray vaccine in week 1 for 3 consecutive days and booster dose in week 3, also for 3 consecutive days. Group 2, will be vaccinated using spray vaccine in week 1, once and followed the booster dose using the feed-based vaccine in week 3, once. Group 3 will be served as a control group without any vaccination. All groups will be challenged with live pathogenic S. agalactiae through intraperitoneal route. Following challenge, all fish will be observed for clinical signs and mortality for the next 14 days.

**3.6 Preparation of *S. agalactiae* inoculum for challenge**

The bacteria were sub-cultured onto blood agar and incubated at 37˚C for 24 hours before five colonies were further sub-cultured into each six bottles of 20 mL of the brain heart infusion (BHI) broth and incubated in a shaker incubator at 37˚C for 24 hours by shaking at 110 rpm to obtain exponentially growing cells. The final concentration, as determined by Mc Farland was 109 CFU/mL. Following incubation, 1 mL of the broth was injected intra-peritoneal into the Red hybrid tilapia fingerlings to enhance bacterial virulence. The infected fish will be killed at 24 hours post-infection for re-isolation of S. agalactiae.

**3.7 Mucus and gut lavage collection**

Five fingerlings from each group; group 1, 2 and 3 were used to collect both mucus and gut lavage sample. The mucus samples were collected using sterile cotton to swab the mucus at the surface of the fish body. Swabbing was done ten times using the same cotton swab from head to tails on one side of the fish body. The swabs were then immersed into 1 mL of sterile PBS with 0.02% (w/v) sodium azide in bijou bottles and kept at 4˚C overnight. The solution will be then centrifuged at 4˚C, 3,000 rpm for three min before 500 µL of the supernatant will be collected and kept at -20˚C until used.

To collect the gut-lavage fluids, approximately 10 cm length of the hindgut were selected before the gut is infused with 1 mL of sterile PBS with 0.02% (w/v) sodium azide using sterile pipette and tips. Then the gut was gently massaged with fingers before the fluid were collected by directly poured into a bijou bottle. The collected fluid was kept at 4˚C for 4 hours before being centrifuged at 4˚C, 3,000 rpm for three minutes to remove the debris. Finally, 500 µL of the supernatant will be collected and kept at -20˚C until further use.

**3.8 Enzyme-linked immunosorbent assay (ELISA)**

Mucus and gut lavage fluid were subjected to indirect enzyme-linked immunosorbent assay (ELISA) according to the method of with minor modifications to determine the IgM antibody levels against *S. agalactiae*. The microtitre plates were coated with 100 µL of a suspension containing 105 CFU/mL and incubated overnight at 4˚C. On the next day, plates were washed with washing buffer, PBS + 0.05% Tween-20 (PBST), for 3 times before 200 µL of the blocking buffer (PBST + BSA) were added to each well and incubated for 1 hour at 37˚C. After incubation, the plates were washed again twice by using the washing buffer. This will be followed by the addition into each well of 100 µL the samples, which is either serum or gut lavage fluid that had been diluted at 1:1000. The solution was incubated at 37˚C for 1 hour. The plates were washed again for three times with the washing buffer before 100 µL of goat anti-tilapia hyperimmune serum, diluted at 1:5000, were added to each well and incubated at 37˚C for 1 hour. Again, the plates were washed using the PBST for 3 times before 100 µL of rabbit anti-goat IgMhorseradish peroxidase (Nordic, The Netherland), diluted at 1:5000, were added to each well and incubated at 37˚C for 1 hour. The plates were washed with the washing buffer for 3 times before 100 µL of a bound conjugate of TMB One Solution Substrate (Promega, USA) were added to each well and incubated at 37˚C for 30 minutes. The reaction was stopped by adding 50 µL of stopper solution (2.5 M sulphuric acid), and the optical density value was measured at 450 nm wavelengths (Anthos Zenyth 340st, Austria).

### 3.9 Bacterial isolation, Gram stain and PCR

Samples for bacterial isolation were taken from brain, eyes and kidney at second-week post challenge. Bacterial isolation was done by sub-cultured onto blood agar and incubated at 37˚C for 24 hours. Gram stain was used to identify Gram-positive bacterium. Samples of *S. agalactiae* were obtained from the pure culture colonies from positive bacterial isolations samples. For identification and confirmation of *S. agalactiae*, total cellular DNA was extracted with Wizard Genomic DNA Purification Kit (Promega, USA) according to manufacturer's protocol. This process was called DNA extraction. The extracted DNA was evaluated by PCR for *S. agalactiae*-specific section of 16S-23S RNA intergenic spacer region with primers STAUR 4 [ACG GAG TTA CAA AGG AC] and 6 [AGC TCA GCC TTA ACG AGT AC]. The PCR mixture was subjected to amplification in a thermal cycler machine which consists of three major steps and repeated for 30 cycles according to the following programme: denaturation at 94˚C for 1 minute, annealing at 57˚C for 1 minute, extension at 72˚C for 30 seconds followed by 72˚C for 2 minutes.

Agarose gel electrophoresis was conducted, and the result could be obtained by using the UV transilluminator.

### 3.10 Statistical Analysis

All the statistical analyses were performed using MedCalc for Windows, version 12.7.0.0 (MedCalc Software, Mariakerke, Belgium) and tested at 5% level of significance. The differences of each time point separately using a one-way analysis of variance (ANOVA) and posthoc test using the Student-Newman-Keuls pairwise comparison test.

## 4.0 RESULTS

### 4.1 Mean graph of antibody response from gut

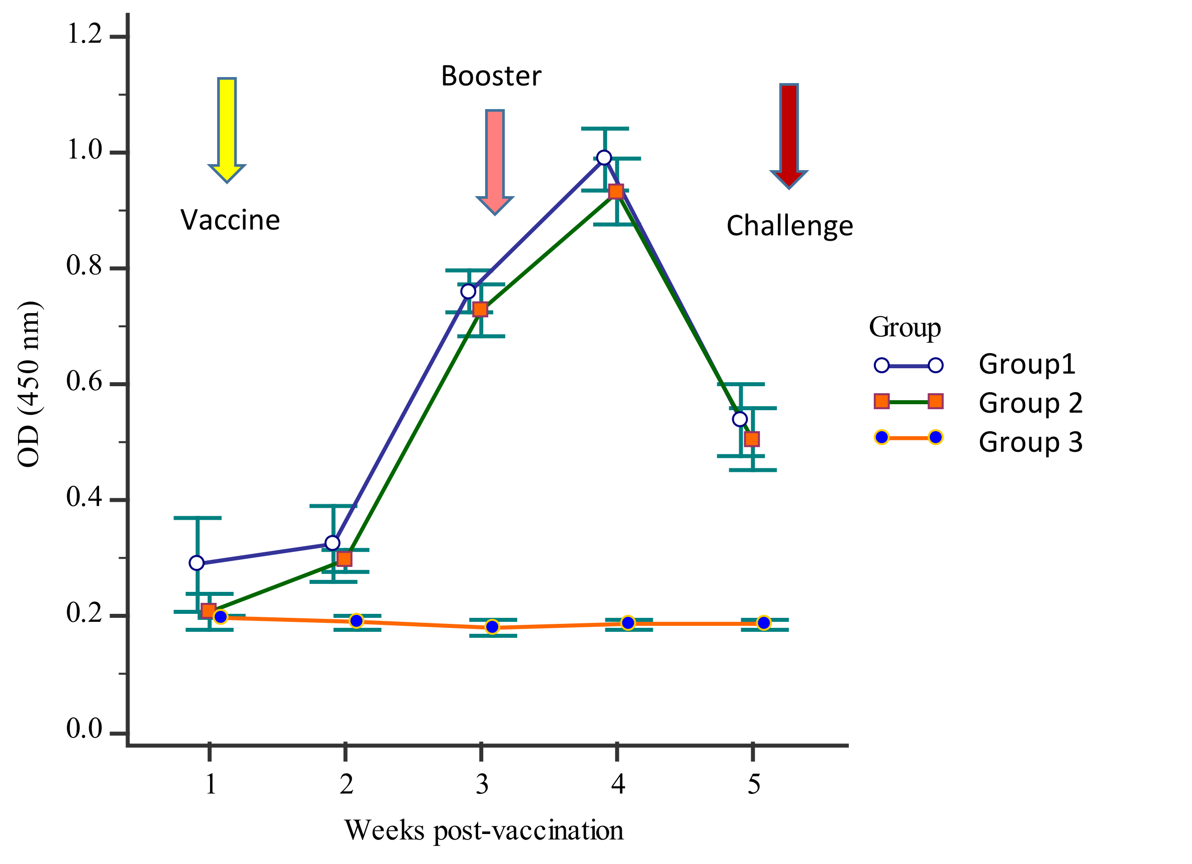



**Figure1**: Mean graph of Antibody response from gut

The mean value of antibody levels produce by Group 1 and Group 2 was much higher compared to the Control group which was Group 3. The antibody response was subjected to analysis of variance revealed significant (*P* < 0.05) difference between the vaccinated and non-vaccinated group. Based on the graph in Figure 1, the antibody response produced by the vaccinated group reaching a high value once the booster dose was given in week 3.

Based on all pairwise comparison analysis, the results revealed a significant ($P<0.05$) difference among the vaccinated and non-vaccinated group. The results shows that both two groups which is Group 1 and Group 2 are not significantly different from one another based on the table shown in Appendix 1which means that the antibody response produce from the gut were just about the same level despite giving spray vaccine once or thrice and despite giving spray vaccine or feed-based vaccine at the booster level. Control group which is Group 3 was significantly different from Group 1 and Group 2.

**4.2 Mean graph of antibody response from mucus**

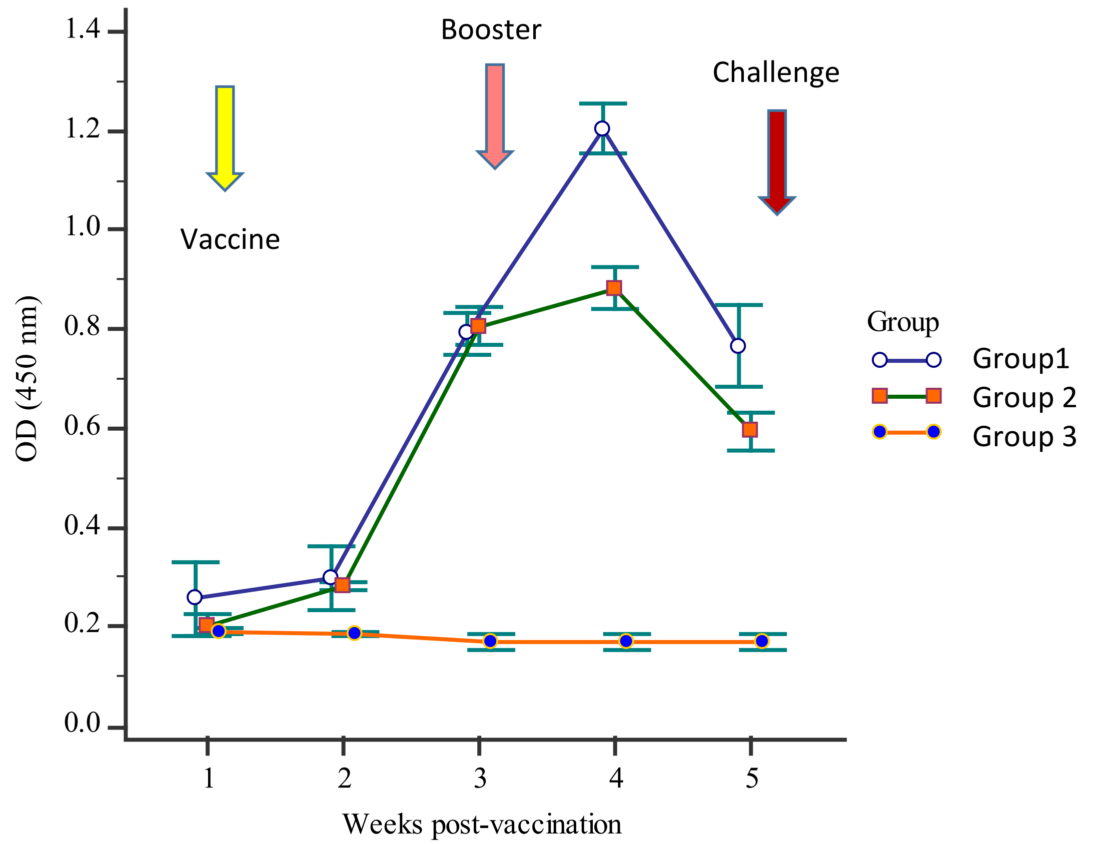


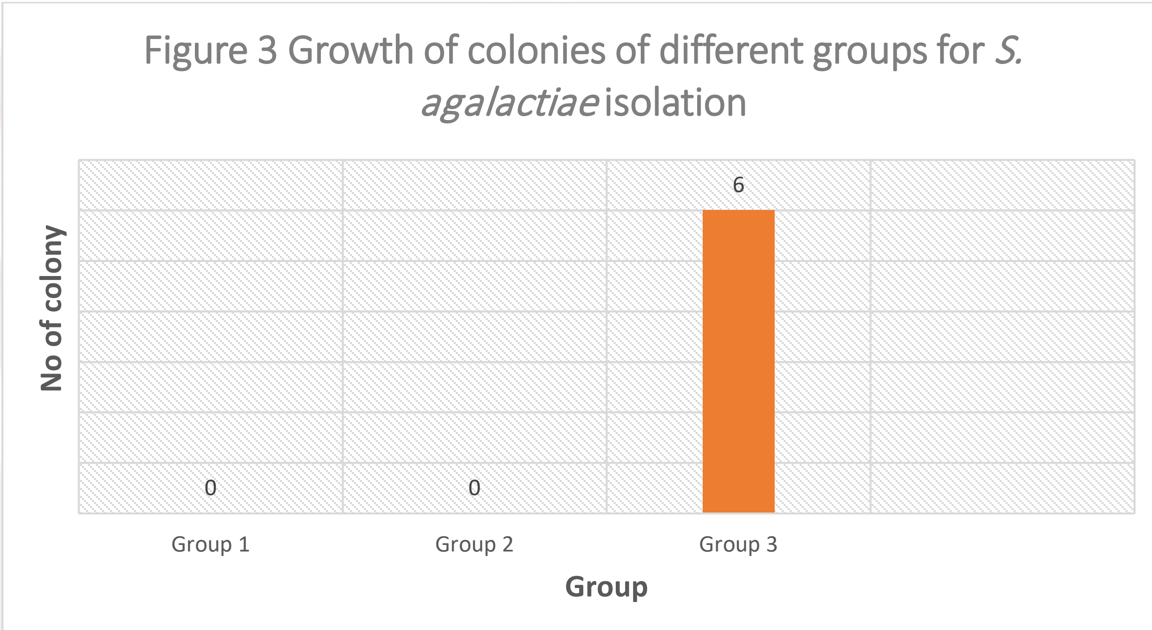

**Figure 2**: Mean graph of Antibody response from mucus

The mean value of antibody levels produce by Group 1 and Group 2 was much higher compared to the Control group which was Group 3. The antibody response was subjected to analysis of variance revealed significant (*P* < 0.05) difference between the vaccinated and non-vaccinated group. Based on the graph in Figure 2, the antibody response produced by the vaccinated group reaching a high value once the booster dose was given in week 3.

Same pattern can be observed in all pairwise comparison analysis shown in Appendix 2 for the mucus sample.

**4.3 Bacterial isolation**

The samples organ for bacteria isolation post challenged were the brain, eye and kidney. Eight samples were taken from each group, and the results reveal that there was no growth of colonies from both group 1 and group 2 which were the vaccinated group. However, there was growth observed from the control group. The primary isolates revealed small pin-point colonies on the blood agar. The colonies were spherical in shape. They were characteristically presumptive of Streptococcal spp., occur in pairs or chains and Gram-positive upon Gram's staining. However, the only sample from kidney exposed growth of the colonies and no growth in eye and brain sample. Biochemical profile of the isolates was confirmed by PCR. Figure 3 showed a graph of the growth of the colonies from different groups for *S. agalactiae*.




**4.4 Polymerase chain reaction**

PCR amplification showed a positive result with a value of 220bp for the samples taken from the control groups for *S. agalactiae* to be compared to the positive control as shown in Figure 4.

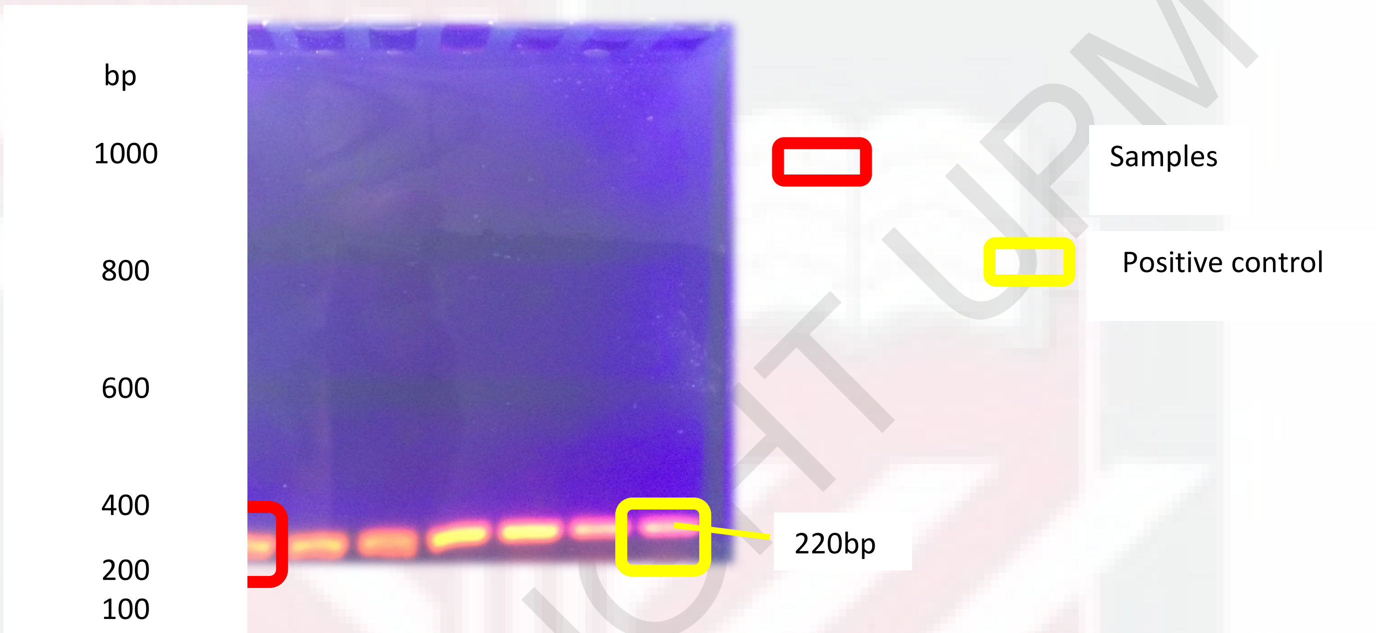



**Figure 4:** GelRed-stained 1% agarose gel of multiplex PCR products.

**5.0 DISCUSSION**

Based on the graph of the antibody, the vaccinated group did produce a much higher antibody level compared to unvaccinated one despite different route of vaccination. According to Garrison et al. (1980), spray administration is a modification of vaccination by immersion, and it has a high possibility of providing a high level of protection. A study done by Noraini et al. (2013) reveals that single spray exposure of formalin-killed cells of S. agalactiae vaccine was able to induce antibody. Besides, immersion vaccination is recommended for fry and fingerling tilapia of hatchery stage as stated by Le Breton (2009) and Merck (2013).

The all pairwise comparison analysis showed that there was no significant difference for antibody production both, from gut and mucus for different methods of vaccination. Therefore, different methods of vaccination can be applied in fish industry. A specific route of administration or even multiple applications using different methods may be necessary for adequate protection as said by Komar et al. (2004) and some method is impractical for larger size fish due to cost-effectiveness and the stress that could be induced by vaccination.

One important consideration for development and commercialization of vaccines includes the application methods and procedures that can be integrated into the normal production protocols of the target fish species that are relevant to the typical ecology and epidemiology of the disease. (Toranzo et al., 2009). Therefore, the most effective method will depend upon the pathogen itself and its natural route of infection also the life stage of the fish.

Vaccination is becoming an increasingly important part of aquaculture since it is considered a cost effective method of controlling different threatening diseases. In this study, oral vaccination which is in the form of feed-based vaccine manages to produce antibody just as the same level compared to spray vaccine. However, oral vaccines have a very short term stability once mixed with the feed. In most cases, only limited protection can be obtained, and the duration of protection can be rather short as stated by Evenson (2009).

Despite of having those problem, a journal written by Laila (2013) reveals that when vaccines are used as top dressing in feed, a coating agent is usually applied such as adjuvant, either to prevent leaching of the antigen from the pellets or to prevent breakdown of the antigen in the acidic environment of the fish stomach.

Streptococcus outbreak was first recorded in the late 1990s in Malaysia. The first outbreak of *S. agalactiae* in red hybrid tilapia (*Oreochromis sp*.) was observed in 1997 in Pahang River, Pahang. The disease affected tilapia weighing between 300 and 40 g causing 60% mortality. Subsequently in 2000, outbreaks of *S. agalactiae* infection were reported in Kenyir Lake, Terengganu and Pergau Lake, Kelantan, killing approximately 50% of the cultured tilapia population. (Zamri et al., 2014).

## 6.0 CONCLUSION AND RECOMMENDATIONS

The result of this experiment showed that there was no significant difference between giving the spray vaccine once or thrice. The level of the antibody produced by the fingerlings was still the same. Same goes with the results for giving the vaccine through different route; spray and feed based vaccine as it shows no significant difference between those two methods. However, there is a significant result to be compared between vaccinated and the control group. Hence, the vaccinated group did produce antibody against the related bacteria despite different methods of vaccination used.

Based on this experiment, it is proven that different route of vaccination can be practiced depending on the size of the fish in order to reduce the stress level to the animal upon vaccination. Besides, some of the methods of vaccination were more practical to the farmer rearing large scale of fish. Last but not least, vaccination is an important method of prevention of infectious diseases in farmed fish as it can induce antibody against those specific bacteria.

This project is recommended for future study since the mortality of the fish after challenge could not be observed due to time constraint. For this project, some suggestions are to increase in sample size of the animal and a duplication of each group of fish should be done in order to get accurate results.



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**APPENDIX 1**

**All pairwise comparison analysis of gut**

| Factor | n | Mean | SD | Different (P<0.05) from factor nr |
|---|---|---|---|---|
| (1) 1 | 20 | 0.5797 | 0.2931 | 3 |
| (2) 2 | 20 | 0.533 | 0.2864 | 3 |
| (3) 3 | 18 | 0.1866 | 0.01862 | (1)(2) |

**All pairwise comparison analysis of mucus**

| Factor | N | Mean | SD | Different (P<0.05) from factor nr |
|---|---|---|---|---|
| (1) 1 | 20 | 0.6634 | 0.3787 | 3 |
| (2) 2 | 20 | 0.5533 | 0.285 | 3 |
| (3) 3 | 18 | 0.1757 | 0.02545 | (1)(2) |