
UNIVERSITI PUTRA MALAYSIA

# EFFICACY OF IMMERSION AND ORAL VACCINATION AND THEIR COMBINATION AGAINST *Aeromonas hydrophila* IN JUVENILE CATFISH (*Clarias* sp.)

SURIANI BINTI KHALID

# EFFICACY OF IMMERSION AND ORAL VACCINATION AND THEIR COMBINATION AGAINST *Aeromonas hydrophila* IN JUVENILE CATFISH (*Clarias* sp.)

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A project paper submitted to the
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## CERTIFICATIONS

It is hereby certified that we have read this project paper entitled "Efficacy of Immersion and Oral Vaccination and their Combination against *Aeromonas hydrophila* in Juvenile Catfish (*Clarias* sp.)", by Suriani Binti Khalid and in our opinion is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the Final Year Project (VPD 4999) course.



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## LIST OF ABBREVIATIONS

| | |
|---|---|
| *A. hydrophila* | *Aeromonas hydrophila* |
| CFU/mL | Colony-forming unit per mililitre |
| FKB | Formalin-killed bacteria |
| i.p. | Intraperitoneal |
| GALT | Gut-Associated Lymphoid Tissue |
| MALT | Mucosal-Associated Lymphoid Tissue |
| TSA | Tryptic Soy Agar |
| TSB | Tryptic Soy Broth |
| rpm | revolutions per minute |
| sp. | species |
| *P* | p-value (significance) |
| MAS | Motile Aeromonas Septicaemia |
| CF1 | (Immersion × Oral) Group |
| CF2 | (Oral × Immersion) Group |
| CF3 | (Immersion × Immersion) Group |
| CF4 | (Oral × Oral) Group |
| CF5 | (Control) Group |
| °C | Degree Celsius |
| µL | Microliter |
| < | Less than |
| H&E | Hematoxylin and Eosin |

# ABSTRAK

Abstrak daripada kertas kerja projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keseluruhan kursus VPD 4999 - Projek Ilmiah Tahun Akhir.

**KEBERKESANAN VAKSINASI RENDAMAN DAN VAKSINASI BERASASKAN MAKANAN SERTA GABUNGANNYA TERHADAP *Aeromonas hydrophila* KEPADA KELI JUVANA (*Clarias* sp.)**

**Oleh**

**Suriani Binti Khalid**

**2022**

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**Penyelia bersama: Dr Mohd Fuad Bin Matori**

Penternakan ikan keli sering diancam penyakit akibat ketidakseimbangan interaksi antara ikan, keadaan persekitaran dan organisma penyebab penyakit. Jangkitan *Aeromonas hydrophila*, juga dikenali sebagai MAS (Motile Aeromonas Septicaemia) atau penyakit bintik merah, mengakibatkan kerugian besar kepada penternak akibat kematian, rawatan antibiotik dan kehilangan hari pemberian makanan. Pelbagai kaedah vaksinasi yang ideal seperti melalui rendaman atau makanan boleh mencegah penyakit ini. Walaubagaimanapun, keberkesanan dan kos buruh setiap kaedah masih menjadi persoalan. Matlamat kajian adalah untuk memahami keberkesanan vaksinasi secara rendaman dan pemberian makanan serta gabungannya terhadap *A. hydrophila*

menggunakan bakteria yang dibunuh formalin dalam keli juvana (*Clarias* sp.). Kajian ini dijalankan dalam masa 4 minggu. 50 ekor ikan keli juvana telah diagihkan sama rata kepada 5 kumpulan rawatan. Kumpulan 1 berfungsi sebagai gabungan vaksinasi rendaman dan pemberian makanan, Kumpulan 2 berfungsi sebagai gabungan vaksinasi pemberian makanan dan rendaman, Kumpulan 3 berfungsi sebagai vaksinasi rendaman sahaja, Kumpulan 4 berfungsi sebagai vaksinasi pemberian makanan sahaja, dan Kumpulan 5 berfungsi sebagai kumpulan kawalan. Semua kumpulan telah dicabar pada minggu ke-4 dengan 100 µL $1 \times 10^9$ CFU/mL *A. hydrophila* hidup melalui suntikan intraperitoneal (i.p). Berikutan cabaran, semua ikan diperhatikan untuk tanda klinikal, kematian dan pengasingan bakteria ikan mati. Tiga ikan daripada setiap kumpulan telah dikorbankan untuk pensampelan. Sampel kulit diambil untuk histopatologi. Analisis statistik taburan sel inflamasi merentasi tisu kulit menunjukkan terdapat perbezaan yang signifikan ($p<0.05$) sel radang dalam kulit apabila membandingkan kumpulan rawatan dengan kumpulan kawalan. Ringkasnya, kedua-dua jenis vaksinasi tidak menghasilkan perbezaan yang ketara ($p>0.05$) dalam perkembangan tisu limfoid, kerana tisu limfoid yang berkaitan dengan mukosa (MALT) tidak dapat dinilai dalam tempoh yang singkat selang vaksinasi 1 minggu.

**Kata kunci**: Ikan keli juvana; *Aeromonas hydrophila*; Motil Aeromonas Septicaemia; bakteria yang dibunuh formalin@formalin nyahbakteria; tisu limfoid berkaitan mukosa (MALTs)

# ABSTRACT

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

**EFFICACY OF IMMERSION AND ORAL VACCINATION AND THEIR COMBINATION AGAINST *Aeromonas hydrophila* IN JUVENILE CATFISH (*Clarias* sp.)**

**By**

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**2022**

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

**Co-supervisor: Dr Mohd Fuad Bin Matori**

Cultivation of catfish is often threatened by disease due to the imbalance in the interactions between fish, environmental conditions and disease-causing organisms. *Aeromonas hydrophila* infections, also known as MAS (Motile Aeromonas Septicaemia) or red spot disease, result in significant losses to farmers due to mortality, antibiotic treatments, and lost feeding days. Various routes of vaccination against the organism such as immersion or oral route can be ideal methods of preventing the disease. However, the effectiveness and labour costs of each route are still questionable. The aim of the study was to understand the efficacy of immersion and oral vaccination and their combination against *A. hydrophila* using formalin-killed

bacterin in juvenile catfish (*Clarias* sp.). This study was conducted in 4 weeks. 50 juvenile catfish were equally distributed into 5 treatment groups. Group 1 serves as a combination of immersion and oral vaccination, Group 2 serves as a combination of oral and immersion vaccination, Group 3 serves as immersion vaccination only, Group 4 serves as oral vaccination only, and Group 5 serves as control group. All groups were challenged at week 4 with 100 µL $1 \times 10^9$ CFU/mL of live *Aeromonas hydrophila* via intraperitoneal injection (i.p.). Following challenge, all fish were observed for clinical signs, mortality and bacterial isolation of dead fish. Three fish from each group were sacrificed for sampling. Skin samples were taken for histopathology. Statistical analysis of the distribution of inflammatory cells across the skin tissue showed that there is a significant difference ($p<0.05$) of inflammatory cells in the skin when comparing the treatment groups to the control group. In summary, both vaccination types did not result in a significant difference ($p>0.05$) in the development of the lymphoid tissue, since mucosa-associated lymphoid tissues (MALTs) cannot be assessed in a short period of 1 week vaccination interval.

**Keywords:** Juvenile catfish; *Aeromonas hydrophila*; Motile Aeromonas Septicaemia; formalin-killed bacterin; mucosa-associated lymphoid tissues (MALTs)

# 1.0 INTRODUCTION

## 1.1 Study Background

*Clarias* sp. has successfully adapted to various continents and is now can be found throughout Asia and Africa. *Clarias batrachus* is a medicinal fish found in various parts of India, particularly West Bengal and Tripura, and has traditionally been a staple food for pregnant and breastfeeding women, the elderly, and children (Debnath, 2011). *Clarias batrachus* has an exceptionally high level of tolerance across a wide range of environments, implying an adaptive evolutionary trait (Debnath, 2011). This species has successfully invaded various areas including eastern India, Pakistan, the Philippines, and southern Florida due to human encroachment. They are considered as an aggressively invasive species due to their mobility and tolerance and have thus been blacklisted in various countries.

*Clarias batrachus* is valued as a food fish in parts of its native range and is a focus of both subsistence farming and commercial agriculture. *Clarias batrachus* can be sold and traded alive due to its ability to survive extended periods of time without water, ensuring a relatively fresh food supply. Many institutions are conducting research on the production of various species of catfish, including *C. batrachus*, and the high-density culture has been reported to have the capacity to yield 100 tonnes/ha (Sahoo et al., 2010).

*Aeromonas hydrophila* has been described as the cause of outbreaks of haemorrhagic septicaemia. It is the cause of MAS (Motile Aeromonas Septicaemia) in both farmed and wild fish. Swollen abdomen, red mouth, and haemorrhages on

the exterior surface and around the anus are all symptoms of the disease (Monir et al., 2017). This bacterial attack is latent (prolonged), so although it has been detected on the body of fish, it does not exhibit signs of disease. This bacterial attack occurs only when the fish's bodily resistance is compromised as a result of stress induced by poor water quality, lack of food, or improper handling (Kartikaningsih, 2020).

Outbreaks of Aeromonas infections result in significant losses to farmers due to mortality, antibiotic treatments, lost feeding days and also the costly chemicals. This highly virulent pathotype of *Aeromonas hydrophila* (vAh) was first reported in the US catfish industry in 2009 (Shoemaker et al., 2018). Controlling vAh is difficult because farm mortality is generally acute, and the mortality is usually evident in larger, more valuable market-sized fish. There is little time before harvest to start antibiotic treatment, and the withdrawal period following antibiotic feeding consumes time and money. Therefore, at farm level, other management techniques such as vaccination are really needed (Shoemaker et al., 2018).

These studies allowed testing of alternative control strategies, such as the immunisation of the virulent pathotype of *A. hydrophila* (vAh), using reliable and realistic challenge methods (other than injection). Vaccination against various motile strains of *A. hydrophila* using formalin-fixed preparations has been used and shown to be effective, at least in laboratory experiments, to protect fish from the disease (Shoemaker et al., 2018).

### 1.2 Justification

The basis of this study is to determine whether the juvenile catfish (*Clarias* sp.) develop mucosa-associated lymphoid tissue (MALTs) such as skin-associated lymphoid tissue (SALT) and mucosal immunity following vaccination with formalin-killed *A. hydrophila* by immersion and oral administration. Effective vaccine is able to prevent the disease occurrence and thus helping farmers to increase their production with healthier fish and reducing/avoiding antibiotic use.

The study was done to determine the antibody response of fish when exposed to different vaccination routes which is between incorporated feed vaccine and immersion vaccination against *A. hydrophila*. Information on the mucosal immunity responses production can be revealed through this study and thus giving more choices of vaccination routes that can be practiced in the future.

### 1.3 Objectives

i. To compare the efficacy of immersion vaccination and oral vaccination of formalin-killed *A. hydrophila* in juvenile catfish (*Clarias* sp.).

ii. To evaluate the mucosal immunity in skin of juvenile catfish (*Clarias* sp.) following different routes of vaccination.

iii. To evaluate the mortality rate and clinical signs of juvenile catfish (*Clarias* sp.) following post-challenge with *A. hydrophila*.

## 1.4 Hypothesis

**Ho:** There is no significant difference in mucosal immunity between immersion vaccination and oral vaccination against *A. hydrophila* using formalin-killed bacterin in juvenile catfish (*Clarias* sp.).

**Ha:** There is a significant difference in mucosal immunity between immersion vaccination and oral vaccination against *A. hydrophila* using formalin-killed bacterin in juvenile catfish (*Clarias* sp.).

## 2.0 LITERATURE REVIEW

### 2.1 Catfish (*Clarias* sp.)

For years, the sector that produces the most food has been aquaculture, and its yield has surpassed that of wild-catch fisheries (FAO, 2014). Recently, Malaysia has developed aquaculture facilities for at least 49 freshwater and marine fish species. Asian seabass, snapper, and grouper are the marine fish species that are most frequently farmed. In contrast, catfish, tilapia, and riverine catfish are the most commonly cultivated freshwater fish species, with a combined production of 121,553.75 metric tonnes in 2020. (Ridzuan et al., 2022)

Around 10% of people worldwide make their living from aquaculture and fisheries, the majority of which are small-scale businesses. Fish consumption has been rising sharply around the world and has just surpassed one million tonnes (Sellegounder et al., 2018). However, fish become more susceptible to illnesses as a result of elevated stress levels caused by increasing stocking numbers. The prime challenges in breeding, maintenance and large-scale production of catfish are bacterial and viral infections (Muroga, 2001).

Thus, catfish in particular are regarded as an excellent model for studying aquaculture diseases because of their sensitivity to certain bacterial illnesses, particularly *A. hydrophila*, and their habitat in muddy water with low oxygen levels. In addition to having a supra-branchial organ that makes air breathing easier and enables them to withstand low oxygen environments as well as brief periods of desiccation, *Clarias gariepinus* exhibits extremely high levels of adaptation for life in stagnant environments (Weyl et al., 2016).

The unbalanced interaction of the cultivation pond's three major components, environmental factors, and disease-causing organisms leads to disease attack in the fishpond. The disease known as MAS (Motile Aeromonas Septicaemia), commonly known as red spot disease and caused by *Aeromonas hydrophila*, is frequently detected in catfish.

**2.2 *Aeromonas hydrophila***

*Aeromonas hydrophila* is a Gram-negative, facultative anaerobic, motile bacterium that is the causative agent of Motile Aeromonas Septicaemia (MAS) in fish. This bacterium is commonly found in aquaculture and can result in substantial losses when a predisposing stressor is present (Plumb and Hanson, 2010).

In research done by Abdelhamed et al. (2017), hemolysins, cytotoxins, enterotoxins, proteases, lipases, leucocidins, endotoxins, surface polysaccharides (capsule, lipopolysaccharide, and glucan), iron-binding systems, exotoxins, extracellular enzymes, secretion systems, fimbriae and other non-filamentous adhesins, motility, and flagella have all been linked to the pathogenic potential.

According to Kartikaningsih et al. (2020) the morphology of catfish infected with *A. hydrophila* exhibited clinical symptoms such stomach and intestinal enlargement, bruising, and haemorrhaging. Acute haemorrhagic septicaemia can cause oedema, bleeding, and diffuse necrosis in fish, while chronic ulcerative syndrome, can cause deep skin ulcers (Huizinga et al., 1979; Cipriano et al., 1984).

Catfish, bass, and other teleost fish are among the species of freshwater fish most commonly impacted by the disease. *A. hydrophila* is regarded as an emerging zoonotic and foodborne pathogen because the infection is frequently observed in

both marine and terrestrial species. Additionally, the high incidence of haemolytic and drug-resistant *A. hydrophila* in farm animals and seafood poses a major risk to human health (Igbinosa et al., 2012).

### 2.3 Vaccination in fish

Currently, vaccines are available for the majority of aquaculture fish species whereby the majority of these vaccines target bacterial diseases, while very few are developed to protect against viruses. Commercial vaccinations can be given orally (by combining with the feed), submerged (in a dip or bath), or injected via the intraperitoneal (i.p.) or intramuscular (i.m.) route, depending on the age and size of the fish. Injection vaccination typically provides the best protection. Nevertheless, it is also associated with extensive handling and stress on the fish (Embregts & Forlenza, 2016). Vaccinations administered orally or by immersion are typically used on fish that are too small to receive injections, however these methods typically have limited efficiency and short protection. Mass vaccination of fish exclusively through the mucosal pathways is not a typical practise due to the high costs of vaccine manufacture necessary for immersion immunisation or the low efficiency of the present oral formulations.

Currently, there is limited number of approved oral vaccines used in veterinary purpose. The ineffectiveness of oral vaccine is partially caused by antigen degradation in the harsh stomach environment, but it is also a result of the gut's strong tolerogenicity and poor vaccine design (Embregts & Forlenza, 2016). However, the mucosal route of vaccination, and particularly the oral route, would be the best way to give vaccines in terms of animal welfare and handling costs.

The great variety of fish species kept in captivity and their genetic diversity may add to the difficulties of oral vaccination development in fish. Therefore, even for the same disease, various fish species may require a distinct approach for the creation of oral vaccinations. Fish with and without stomachs, as well as carnivorous, herbivorous, and omnivorous fish species, exhibit significant changes in gut shape and intestinal environment (Embregts & Forlenza, 2016).

Fish without stomachs may require less antigen protection after oral immunisation since they are not subjected to the stomach's harsh environment and low pH. However, factors outside stomach pH are at play, and there are significant variances even within fish species that have stomachs. For instance, fish with a thin stomach wall, like rainbow trout, have high stomach acidity because their ability to knead and break down food depends more on pH than on muscle power. Contrarily, fish with a thick, muscle-filled stomach wall, like African catfish, are better able to knead their food and are consequently less dependent on stomach pH for food breakdown (Weber, 2014).

## 3.0 MATERIALS AND METHODS

### 3.1 Fish and experimental conditions

50 juvenile catfish were obtained from Beranang Farm with no history of Aeromonas outbreak and were used in this study. Prior to the experiments, five aquariums were cleaned and disinfected. Continuous aeration was provided throughout the experiment from the automated aerator. The aquariums used can accommodate up to 70 litres of water. The juvenile catfish weighing about 7 to 10 grams were equally divided into five groups; Group 1 serves as combination of immersion and oral vaccination, Group 2 serves as combination of oral and immersion vaccination, Group 3 serves as only immersion vaccination, Group 4 serves as only oral vaccination and Group 5 serves as control group. The fish were feed with commercial starter feed twice daily at the rate of 2% of body weight. All of the fish were acclimatized for more than one week before the experiment was started.

### 3.2 Bacterial isolate and growth condition

An isolate of *Aeromonas hydrophila* was obtained from the stock which previously was isolated from Kenyir Lake, Terengganu. The isolates were subcultured into Tryptic Soy Agar (TSA) plate and incubated for 24 hours at 37 °C.

### 3.3 Formalin-Killed Vaccine (FKV) preparation.

10 colonies of *Aeromonas hydrophila* from the TSA plate were sub-cultured into brain heart infusion (BHI) broth and incubated in shaker incubator for 24 hours at 37°C. The incubation was done when cloudy appearance was observed. Then,

bacterial concentration was determined using colony forming unit (CFU). The bacteria were then killed by pouring 0.5% buffered formalin in phosphate-buffered saline (PBS) and was kept overnight at 4°C. The killed bacteria then were placed into falcon tube and centrifuged at 10,000 rpm for 5 minutes and the supernatant was removed leaving only the pelleted cell. The remaining pellet was re-suspended until homogenize, and the solution was centrifuged again. After that, the pellets were washed with phosphate-buffered saline (PBS) for three times before it is ready to be used.

### 3.4 Preparation of Top-Dressed Feed Vaccine (Oral Vaccine)

500 ml of formalin-killed vaccine (FKV) was sprayed directly into 1 kg of commercial feed and mixed thoroughly. The feed was dried in an incubator at 30°C for 24 hours before being fed to the fish.

### 3.5 Preparation of *Aeromonas hydrophila* for challenge

The bacteria were subcultured into BHI and incubated for 24 hours in the shaking incubator. 100 µL of the bacterial culture was injected into naive juvenile catfish and observed for the development clinical signs. The dead juvenile catfish post-injection was taken for sampling. Bacterial isolation from skin and gills was performed to re-isolate *A. hydrophila* onto BHI agar. Skin samples were taken for histopathology.

### 3.6 Experimental design

The study was conducted within 4 weeks. The first vaccination was carried out at week 1. At week 1, Group 2 and Group 4 were fed with top-dressed vaccine at 2 % of body weight for 3 consecutive days. While Group 1 and Group 3 were vaccinated by immersion for 30 seconds and vice versa for booster on week 2. Group 5 served as a control group which was fed with commercial feed throughout the experiment. All groups were challenged at week 3 with 100 µL of $1 \times 10^9$ CFU/mL of live *Aeromonas hydrophila* via intraperitoneal injection. Following challenge, all fish were observed for clinical signs, mortality and bacterial isolation. Three fish from each group were sacrificed for sampling. Skin, gut and gill samples were taken for histopathology.

### 3.7 Observation of fish post-challenge

Skin and gills samples were used for bacterial isolation. Dead fish were subjected to the bacterial isolation. Bacterial isolation was performed by culturing the sample on a BHI agar plate and incubated at 37°C for 24 hours. The pure colony, which showed morphological characteristics of *A. hydrophila* was subjected to Gram stain to identify the Gram-negative rod-shaped bacterium. All clinical signs of *A. hydrophila* infection were observed and recorded. The mortality percentage was calculated to assess the protective ability of the fish in each group throughout the experiment.

### 3.8 Statistical Analysis

Statistical analysis for this study was performed using IBM SPSS Statistics 28.0 for Windows 10 and tested at a significance level of 5%. The distribution of inflammatory cells across the skin of juvenile catfish post-challenge with *A. hydrophila* for all 5 groups was measured at week 4. The mean value was recorded and analysed using the Kruskal-Wallis test.

## 4.0 RESULTS

### 4.1 Distribution of Inflammatory Cells Across Skin of Juvenile Catfish Post-Challenge with *A. hydrophila.*

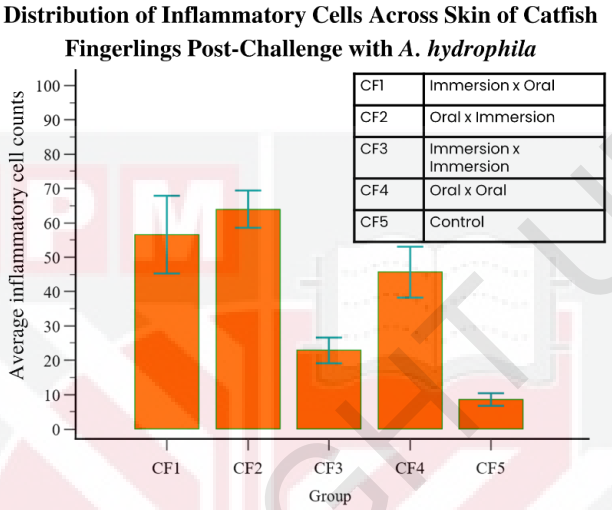


**Figure 4.1.1:** The graph shows the distribution of inflammatory cells across the skin of juvenile catfish post-challenge with *A. hydrophila* assessed on histology slides, taking the average number of 3 fish from each group. The test reveals a significant difference when *p*-value is less than 0.05 ($p<0.05$) of inflammatory cells in the skin when comparing the treatment groups to the control group.

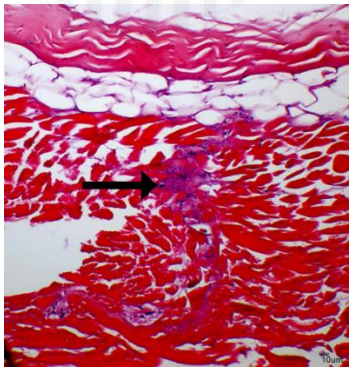

**Figure 4.1.2:** Aggregation of inflammatory cells (arrow), at 200× magnification, stained with H&E.

## 4.2 Clinical Signs of Juvenile Catfish Post-Challenge with *A. hydrophila*

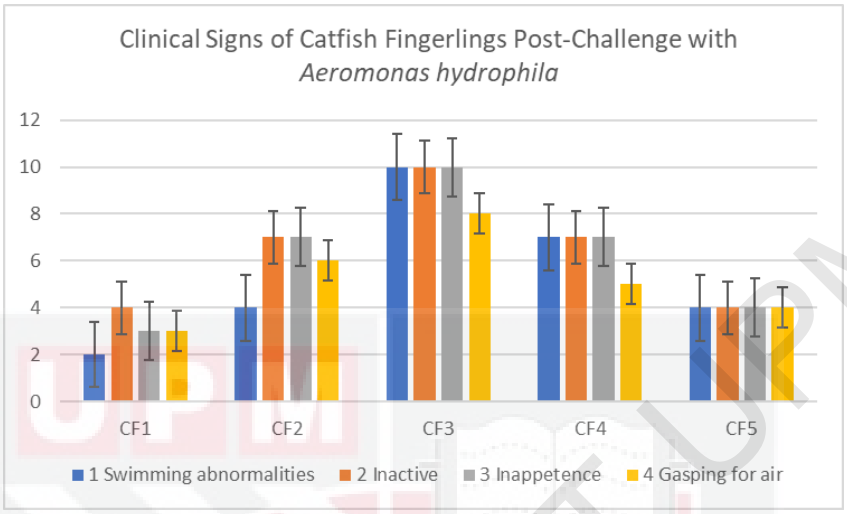


**Figure 4.2.1:** The graph shows acute clinical signs in all treatment groups and the control group shows similar signs of *A. hydrophila* infection. The graph shows that CF3 has the highest number of clinical signs compared to other groups.

## 4.3 External Lesions of Juvenile Catfish Post-Challenge with *A. hydrophila*

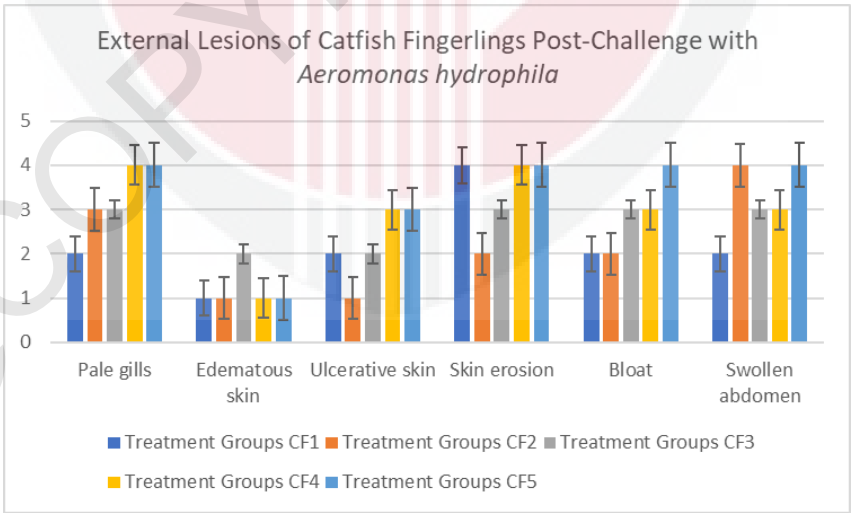


**Figure 4.3.1:** The graph shows signs of external lesions in all treatment groups and the control group relevant to the signs of *A. hydrophila* infection. There is not much difference between the groups. These signs are inconsistent, since individual fish elicit different signs depending on the bacterial load.

### 4.4 Internal Lesions of Juvenile Catfish Post-Challenge with A. hydrophila

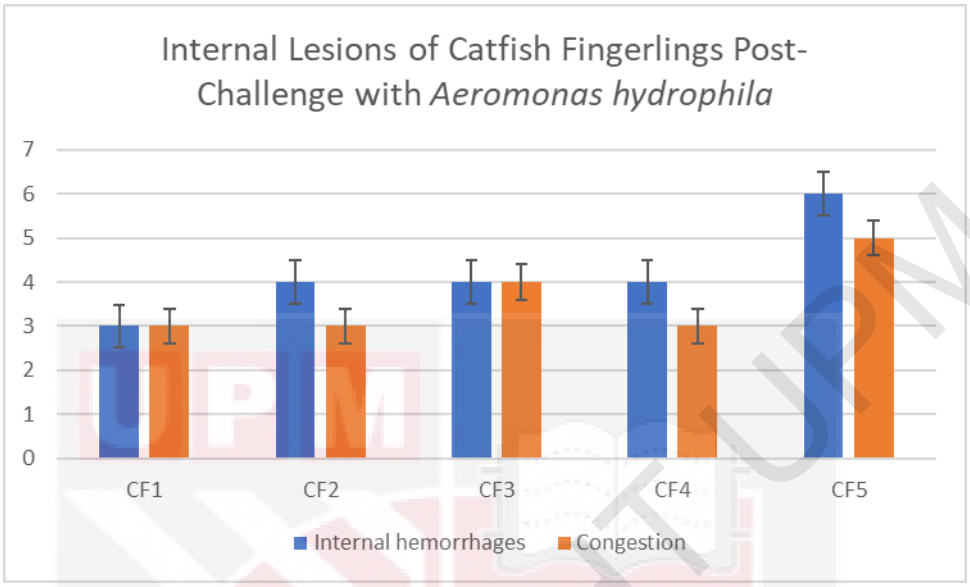


**Figure 4.4.1:** The graph shows the external lesions observed post-challenge with *A. hydrophila*. The control group shows more lesions compared to the treatment groups, and *A. hydrophila* infection usually causes severe intestinal lesions.

### 4.5 Mortality rate

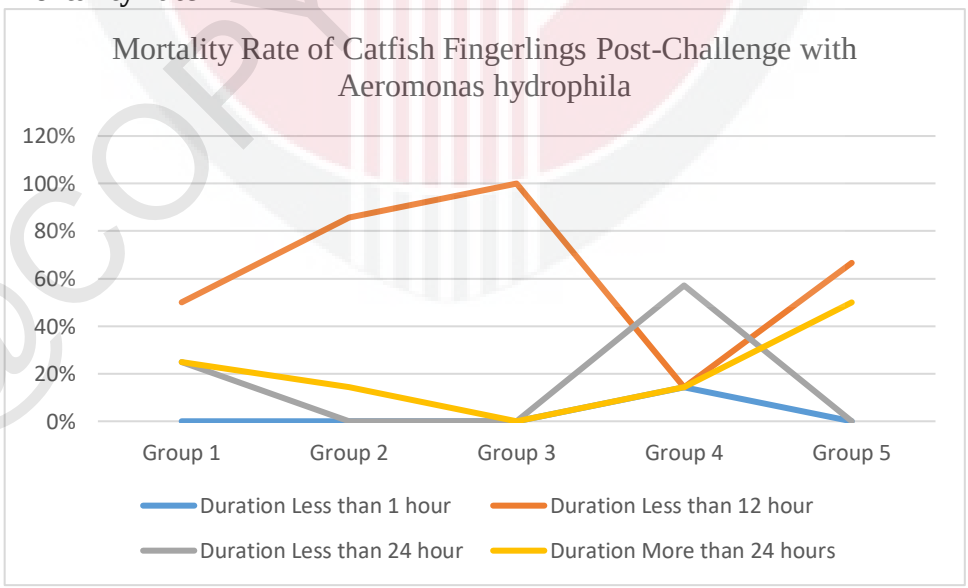


**Figure 4.5.1:** The graph shows 100% mortality was observed in all treatment and control groups, occurred within 5-48 hours post-challenge with *A. hydrophila*.

## 5.0 DISCUSSION

The aim of this study was to determine the presence of mucosal immunity, antibody response (MALT) and histopathological findings when the juvenile catfish were challenged with *A. hydrophila* by different vaccination routes. Intraperitoneal (i.p.) injection has been used in previous research to assess the virulence of *A. hydrophila* isolates and to study the impact of prophylactic therapies on MAS prevention (Pridgeon and Klesius, 2011; Zhang et al., 2014). Similarly, this study used an intraperitoneal injection method to challenge the juvenile catfish with the organism. The injection method is known to be more reproducible than the immersion or bath method.

Unfortunately, the presence of MALTs cannot be assessed in this study due to time constraint and the short vaccination interval. The juvenile catfish may not be able to produce antibodies in the short term. Thus, the distribution of inflammatory cells across skin tissues was observed to assess mucosal immunity post-challenge with *A. hydrophila*.

Immunization by different vaccination routes against *A. hydrophila* in juvenile catfish resulted in a distribution of inflammatory cells across the skin tissue showing that there is a significant difference ($p<0.05$) of inflammatory cells in the skin when comparing the treatment groups (C1, C2, C3, C4) to the control group (C5). Based on a study by (Abdelhamed et al., 2017) *A. hydrophila* possesses haemolysins, cytotoxins, enterotoxins, proteases, lipases, leucocidins, endotoxins, surface polysaccharides, iron-binding systems, exotoxins, extracellular enzymes, secretion systems, fimbriae and other non-filamentous adhesins, motility, and flagella have all been linked to the pathogenic potential. Therefore, inflammatory

reactions can occur and clinical signs such as impaired swimming, inactive, inappetence and gasping for air can also be observed. Increased inflammatory cells in treatment groups (C1, C2, C3, C4) due to fish initially may not be in good condition and disease free. Apart from that, it can also be due to that the immersion route has direct contact with the mucosal layer of the fish and gills. An immune response occurs and because these groups have undergone immersion vaccination twice, the fish are at a higher risk becoming infected and showing clinical signs compared to oral groups.

This study shows a 100% mortality rate in juvenile catfish post-challenge with *A. hydrophila*. Most of the mortalities occurred within 5–48 hours post-challenge. This scenario is supported by a study of Nadiro et al. (2020) according to which this bacterium usually causes a disease with a high mortality rate (80–100% mortality rate) in a short period of time in freshwater aquaculture, especially catfish (1 – 2 weeks).

There is no doubt that mucosal vaccination, and particularly oral administration would be the best form of vaccine delivery in terms of animal welfare and handling costs. However, mass vaccination of fish solely via the mucosal route is not a typical practice due to the high cost of vaccine production required for immersion immunization or the low efficiency of current oral formulations (Embregts & Forlenza, 2016).

## 6.0 CONCLUSION

The efficacy of immersion, oral vaccination and their combination cannot be determined in this study due to the factors discussed previously. The result of this study shows that the distribution of inflammatory cells across the skin tissue shows a significant difference ($p<0.05$) of inflammatory cells in the skin when the treatment groups are compared to the control group. This experiment also revealed that acute clinical signs of *A. hydrophila* can be observed in all treatment and control groups. The mortality rate post-exposure to *A. hydrophila* is 100%, with most of the mortalities occurring within 5-48 hours of exposure, supported by a previous study. Based on this experiment, we can conclude that both types of vaccination did not result in a significant difference ($p>0.05$) in the development of the lymphoid tissue, since mucosa-associated lymphoid tissues (MALTs) cannot be assessed in a short period of 1 week vaccination interval.

## 7.0 RECOMMENDATION

For further study to be conducted, screening of the fish should be performed prior to the experiment. This is to ensure that the fish to be used is disease free, healthy and fit for the experiment. Stress should be reduced during handling, especially environmental changes and transportation. Fish should be stable or acclimatized for at least 1 to 2 weeks beforehand. Water quality should be checked regularly to ensure the fish is in a healthy environment and is stress free. ELISA should be performed prior and post-challenge with the bacterin in the assay for antibody detection of *A. hydrophila* in skin. Apart from that, further study may include histopathological analysis of other organs such as spleen, liver, gills and brain to get better results and better understanding of fish immune response to vaccination. Extending the vaccination interval may be necessary to provide time for the fish to develop immunity. Real-time quantitative PCR (qPCR) was used to assess the level of targeted immune gene expression in catfish skin to confirm the host immune response to *A. hydrophila* challenge.

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