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DETERMINATION OF OPTIMAL RATIO OF FREUND'S INCOMPLETE AND PALM OIL ADJUVANTS INCORPORATED INTO FEED-BASED ORAL VACCINE FOR FISH

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Adjuvant plays an important role in the effectiveness of vaccines. It causes slow release of the vaccine thus, inducing long-term protection in animals and man. Optimal ratio of the adjuvant to be added must be determined to ensure optimal effect. In this study, two different adjuvants were compared in the newly developed feed-based vaccine against streptococcosis to determine the optimal ratio of adjuvant. They were the Freund's incomplete adjuvant (FIA) and the palm oil adjuvant (POA). The ratios were 0%, 3%, 5%, 7% and 10% adjuvants. 500 red hybrid tilapia of approximately 100g ± 10g bodyweight were divided into 5 groups for FIA and 600 red hybrid tilapia in 6 treatment groups for POA. Each replicate was vaccinated with double booster regime (3 vaccinations) using Feed Adjuvanted Vaccine (FAV) on weeks 0, 2 and 6. Other than that, normal commercial feeds were given. On week 10, the fish were challenged intraperitoneally with 2.6 x 10^{9} CFU/mL of live *Streptococcus agalactiae.* Serum samples collected at weekly intervals from all replicates for a period of sixteen weeks were subjected to ELISA to determine the systemic antibody responses. Immunization by FAV resulted in significant ($p<0.05$), p= 0.032 increase in the serum antibody levels (IgM) as early as week 2 in all vaccinated groups, while the level in the control group was insignificant ($P>0.05$), p=0.134. Groups with 5% to 10% adjuvants showed highest antibody levels. In fact, the antibody response of 7% and 10% showed no significance difference. In general, the 10% ratio of palm oil adjuvant stimulated better systemic immune responses resulting in good protection with 70% survival rate after challenge. Thus, the 10% palm oil ratio is the adjuvant of choice as the price is cheaper and easily available in Malaysia compared to Freund's incomplete adjuvant and at the same time gives good protection level to the fish.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENENTUAN NISBAH OPTIMUM ADJUVAN *FREUND'S* TIDAK LENGKAP DAN ADJUVAN MINYAK KELAPA SAWIT YANG PERLU DISEBATIKAN KE DALAM VAKSIN LISAN BERASASKAN MAKANAN UNTUK IKAN

Oleh

SA'AIDATUN ASYIKIN BINTI AMINUDIN

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Adjuvan memainkan peranan yang penting di dalam keberkesanan vaksin. Ia mendorong pelepasan vaksin secara perlahan-lahan seterusnya mencetuskan perlindungan jangka masa panjang pada haiwan dan manusia. Nisbah optimum adjuvan yang perlu ditambah ke dalam vaksin perlu ditentukan untuk memastikan kesan yang optimum. Dalam kajian ini, dua adjuvan yang berbeza dibandingkan dalam penghasilan vaksin baru yang diolah berasaskan makanan, keatas penyakit *streptococcosis* untuk mengetahui nisbah adjuvan yang optimum. Adjuvan yang digunakan adalah adjuvan *Freund's* tidak lengkap (FIA) dan adjuvan minyak kelapa sawit (POA) . Nisbah adjuvan yang dikaji adalah di antara 0%, 3%, 5%, 7% & 10%. 500 tilapia hibrid merah yang menghampiri berat 100g ± 10g telah dibahagi kepada 5 kumpulan untuk FIA dan 600 tilapia hibrid merah dalam 6 kumpulan rawatan untuk POA. Setiap kumpulan kajian telah divaksinasi mengikut rejim penggalak ganda dua (3 vaksinasi) menggunakan pelet makanan yang digaulkan bersama adjuvan dan vaksin (FAV) pada minggu 0, ke-2 dan ke-6. Selain dari itu, hanya makanan komersial biasa telah diberikan. Pada minggu ke-10, ikan telah dicabar dengan suntikan intraperitoneum 2.6×10^{9} CFU/mL bakteria *Streptococcus agalactiae* yang hidup. Sampel serum yang diambil pada setiap minggu dalam semua kumpulan kajian untuk tempoh masa enam belas minggu telah melalui prosedur ELISA untuk menentukan respon terhadap antibodi. Pengimunan menggunakan FAV mengakibatkan peningkatan bererti ($p < 0.05$), p= 0.032 paras serum antibodi (IgM) seawal minggu ke-2 dalam semua kumpulan vaksin, manakala paras antibodi kumpulan kawalan tidak menunjukkan perbezaan bererti ($p > 0.05$), p=0.134. Kumpulan dengan nisbah adjuvan 5% ke 10% menunjukkan tahap antibodi tertinggi. Malah, respon antibodi pada kumpulan adjuvan 7% dan 10% tidak menunjukkan perbezaan yang bererti dalam kedua-dua kumpulan. Secara keseluruhan, nisbah 10% adjuvan minyak kelapa sawit ke dalam vaksin merangsang gerak balas imun sistemik yang lebih baik dan memberi perlindungan yang bagus dengan 70% kadar kemandirian, walaupun selepas dicabar. Oleh itu, 10% adjuvan minyak kelapa sawit adalah nisbah adjuvan pilihan kerana harganya yang lebih murah dan mudah didapati di Malaysia berbanding adjuvan *Freund's* tidak lengkap, dan pada masa yang sama memberikan tahap perlindungan yang baik kepada ikan.

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I certify that a Thesis Examination Committee has met on 8 December 2017 to conduct the final examination of Sa'aidatun Asyikin binti Aminudin on her thesis entitled "Determination of Optimal Ratio of Freund's Incomplete and Palm Oil Adjuvants Incorporated into Feed-Based Oral Vaccine for Fish" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

BA	Blood Agar
BHIB	Brain Heart Infusion Broth
CFU	Colony Forming Unit
ELISA	Enzyme-Linked Immunosorbent Assay
FIA	Freund's incomplete adjuvant
FAO	Food Agriculture Organization
FAV	Adjuvanted Vaccine Incorporated Feed
FKB	Formalin Killed Bacteria
FNV	Vaccine Incorporated Feed
FRV	Recombinant Vaccine Incorporated Pellet
g	gram
GAT	Goat Anti Tilapia
Ig	Immunoglobulin
IPI	Intellectual Property Intermediary
M	Molar
mL	milliliter
MT	Metric Tonne
OD	Optical Density
PBS	Phosphate Buffer Saline
PBS/T	Phosphate Buffer Saline plus Tween 20
PCR	Polymerase Chain Reaction
POA	Palm oil adjuvant
ppt	parts per thousand

RAG	Rabbit Anti Goat
RM	Malaysian Ringgit
rpm	rotations per minute
SPSS	Statistical Package for the Social Science
USD	United States Dollar
µL	microliter
µg	microgram

CHAPTER 1

INTRODUCTION

1.1 Introduction

Tilapia takes the lead on become the second largest farmed fish produce worldwide and its production has quadrupled over the past decade following improvements in aquaculture system, marketability and stable market price (Wang & Lu, 2016). Subsequently, tilapia is one of the fastest growing fish in the world mostly due to the low production price. Furthermore, tilapia is increasingly demanded in worldwide markets ranging from the poorest segments in developing countries to highly developed western markets (Norman-López & Bjørndal, 2010). During the first half of 2016, approximately 170, 000 tonnes of tilapia (whole, fillets and breaded) entered the international market (Globe Fish, 2016).

Tilapia is known to be a hardy freshwater fish species. Although tilapia is more resistant to unfavorable water quality than other freshwater fish (Amal and Zamri-Saad, 2011), it has been reported to succumb to *Streptococcus* infection. Infection by *Streptococcus*, known as streptococcosis was first observed among the population of rainbow trout (*Oncorhynchus mykiss*) farmed in Shizouka Prefecture in Japan in April 1957. Since then, streptococcosis has been reported in many other fish species and contributes to profit loss of roughly USD150 million every year (Amal and Zamri-Saad, 2011). Streptococcosis is a septicemic infection that distress both freshwater and marine fish in farmed and feral populations (Ferguson *et al.*, 1994).

Streptococcosis is an infection by Gram-positive bacterium of genus *Streptococcus.* Several types of *Streptococcus* spp. are recognized as the etiological agents of streptococcosis in many aquaculture species of the world. However, the main pathogens that are reported frequently in tilapia farm fish are *Streptococcus agalactiae* and *Streptococcus iniae*. *Streptococcus agalactiae* nonetheless, involves in majority of the case at 80% (Yuasa *et al.,* 2008; Suanyuk *et al*., 2010; Channarong *et al.,* 2011; Jantrakajorn e*t al.,* 2014) leading to considerable morbidity and mortality around the world with huge estimated losses every year (Klesius *et.al*, 2000a; Klesius *et al*., 2008) There are three types of streptococci isolates found from the infected fish: the alpha hemolytic (Minami *et al.*, 1979), beta hemolytic (Robinson & Meyer, 1966; Boomker *et al.,* 1979) and non-hemolytic (Plumb *et al.,* 1974) streptococci. *Streptococcus agalactiae* is classified as a Lancefield group B streptococcus and the only streptococcus species that is classified within group B (Facklam, 2002).

In treating streptococcosis outbreaks, antibiotics are only effective if the treatment is applied early. Normally, oral antibiotic treatments are ineffective, as the infected fish have lost the appetite. Besides, antibiotic only control the mortality rate partially during application time, once the course of antibiotic is over, mortality rate usually surged again. As a result, farmers usually extend the duration of antibiotic application or increase the dose, which leads to several problems such as emergence of antibiotic resistance bacteria and antibiotic residue in meat (Zamri-Saad *et al.,* 2014). In Malaysia farmers frequently applied erythromycin and oxytetracycline to treat streptococcosis in tilapia as well as prophylactic agent in healthy fish (Najiah *et al*., 2009).

Streptococcus agalactiae isolated from tilapia in Malaysia were found to be resistance towards several antibodies, possibly due to the excessive and unappropriated use of the antibiotic previously to fight bacterial diseases in farm fish (Najiah *et al*., 2009). Thus, antibiotic treatment for streptococcosis in general is incompetent and production of suitable vaccines are required (Klesius *et al.*, 2000a). Lombard *et al*. (2007) reported that vaccination has been acknowledged as the key in prevention of fish from infectious disease. The main idea of providing vaccination is to offer a great immune response against the antigen applied, which capable to deliver a long-term protection against a pathogen (Klesius *et al.,* 2008).

Vaccines boost the immune system to assist in protection against the disease infection. Thus, the vaccination treatment in controlling the infection have become more crucial. Improving the role of adjuvants and its administration approach are necessary to meet the demands to ensure the safe supply of healthy fish products (Evensen, 2009). There are three most common vaccine delivery methods; the injection, bath immersion and oral administration. The route applied usually depends on the size of fish, the efficacy of the vaccines, the degree of stress and the cost effective. The most common aquatic vaccines delivery method is by injection, which undeniably the best method compared to immersion and oral routes. However, the weaknesses of injection delivery method include needs for high labor, pricey and not practicable for mass number of fish especially small fish under 20g (Plant & LaPatra, 2011).

Moreover, lesions at the injection site are noticeable and inside the fish body, adhesion were detected either between organs or between organs and peritoneal wall (Midtlyng *et al.,* 1996b; Mutoloki *et al.,* 2004). For bath immersion method, it has the advantage of stress free handling by lowering the water level, but this method has the drawback of demanding large amount of vaccine (Nakanishi & Ototake, 1997).

In Southeast Asian countries such as Malaysia, Vietnam, Indonesia and Thailand, most fish farmers operate on small-scale basis with little technical support. Thus, the costs of manpower and technical supports and facilities required to carry out vaccination using injection and immersion routes are unaffordable (Najiah *et al*., 2012; Ismail *et al*., 2016). Therefore, oral vaccination presents an ideal method for delivery of an efficacious vaccine to fish of any size, without the drawbacks related to injection immunization or handling required for most immersion delivery methods (Ghosh *et al*., 2015). In general, oral vaccination provides less pressure to the fish, feasible to operate on all production lines, practical to various fish sizes, less time spend on vaccine administration and labor saving making it the most preferable method for vaccination in aquaculture (Le Breton, 2009).

Freund incomplete adjuvant (FIA) is highly efficient in vaccination with a significant reduction in toxicity level (Tafalla *et al.*, 2013). A 10% addition of FIA in the newly developed feed-based vaccine against streptococcosis, resulted in 70% survival of fish (Ismail *et al.*, 2016), which was considered a good vaccine (Chettri *et al.*, 2015). Nevertheless, this commercial adjuvant is extremely expensive, especially for the commercial preparation of the vaccine. Thus, an alternative adjuvant, which provides good stimulation of immunity and subsequent protection as FIA at a cheaper rate, should be considered.

Studies have suggested the use of palm oil as adjuvant as palm oil is easily found in tropical country, cheap and safe (Wanasawaeng *et al.*, 2009). However, it has not been used extensively in animal vaccines, including the vaccines for fish. Thus, it is essential to demonstrate the efficacy of palm oil used as adjuvant in fish vaccine.

1.2 Problem statements

Previous study (Ismail *et al*., 2016) provide understanding on booster dose vaccine and gave 70% survival rate using 10% FIA. However, the cost of using 10% FIA in the vaccine is too expensive. Thus, we need to find a lower ratio of adjuvants to reduce the cost of vaccine production and give comparable protection to the fish. Due to the increasing awareness of animal welfare and environmental issues, a safer and natural ingredient were suggested to replace the commercial adjuvants currently use that give harmful side effect to the fish. Palm oil was selected as the best candidate to substitute the present adjuvant. Thus, this research provides a framework for a better understanding of the use of adjuvant in fish vaccine and demonstrated the efficacy of palm oil as an adjuvant in fish vaccine.

1.3 Objectives

1. To compare the optimal ratio of Freund's incomplete and pam oil adjuvant to be used as an adjuvant in the preparation of feed-based vaccine against streptococcosis.

2. To evaluate the efficacy of feed-based vaccine using Freund's incomplete and palm oil as adjuvant in protecting tilapia from streptococcosis.

1.4 Research hypotheses

1. The use of palm oil and lower ratio of Freund's incomplete adjuvants at the rate of 5-7% stimulates the immune response of fish.

2. The newly prepared palm oil adjuvant in feed-based vaccine against streptococcosis maintain good survival rate and produce vaccine efficacy of more than 60%.

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