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Effect of incorporating different concentrations of palm oil as adjuvant in fish vaccine

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Abstract

Adjuvants play important role in vaccine efficacy due to the slow release that leads to prolong immune response. This study determines the advantage of palm oil as adjuvant in the newly developed feed-based killed vaccine against streptococcosis. One thousand two hundred red tilapia of approximately 100g bodyweight were divided into 3 major groups. Group 1 consisted of 500 fish and was further divided into 5 sub-groups with replicate. Group 2 consisted of 600 fish and was further divided into 6 sub-groups while Group 3 with 100 fish in replicate. Fish of Group 1 were vaccinated with the feed-based killed vaccine containing 0%, 3%, 5% and 7% Freund's incomplete adjuvant (FIA) at weeks 0, 2 and 6. Group 2 was similarly vaccinated with the vaccine containing palm oil adjuvant (POA) at concentrations of 0%, 3%, 5%, 7% & 10%. Group 3 was control without vaccination. On week 10, all fish were challenged intraperitoneally with 2.6 x 10^9 cfu/ ml of live *Streptococcus agalactiae*. Serum samples were collected at weekly intervals from all replicates and were subjected to ELISA to determine the systemic antibody responses. Immunization by both POA and FIA resulted in significant ($p<0.05$) increase in the serum antibody levels (IgM) as early as week 1, while the level in the control group remained insignificant ($p>0.05$). The 10% palm oil adjuvant (POA) stimulated the best systemic immune responses resulting in 70% survival rate after challenge.

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Introduction

Streptococcosis is one of the important bacterial diseases of tilapia. The disease leads to considerable morbidity and mortality resulting in huge losses every year (Klesius *et al.*, 2000; Klesius *et al.*, 2008). To reduce losses, disease prevention by vaccination is the most appropriate method (Tafalla *et al.*, 2013). Furthermore, oral vaccination presents an ideal method for delivery of an efficacious vaccine to fish of any size, without drawbacks related to injection immunization or handling required for most immersion delivery methods (Ghosh *et al.*, 2015).

Inactivated vaccines are weakly immunogenic, thus require adjuvants or immunopotentiators to elicited the immune responses. Freund incomplete adjuvant (FIA) is highly efficient in vaccination with low 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 is 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 that could provide good stimulation of immunity and subsequent protection 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 fish vaccines. Thus, it is essential to determine the efficacy of the newly developed feed-based vaccine that uses palm oil as adjuvant. This paper describes and compares the optimal level of palm oil as adjuvant to be incorporated in a feed-based killed vaccine.

Material and methods

Fish preparation

A total of 1,200 red tilapia (*Oreochromis* spp.) with an average body weight of 100±10g were selected and kept in two 2-tonne tanks at the National Fish Health Research Division (NaFisH), Penang for a period of 1 month to condition the fish. Prior to the start of the experiment, 10 fish were killed for bacterial and parasite screening to ensure that the fish were free from any disease before the fish were randomly assigned into 24 tanks consisted of 50 fish per tank.

Bacterial and growth condition

An isolate of *Streptococcus agalactiae* were obtained from the National Fish Health Research Division (NaFisH), Batu Maung, Penang, Malaysia. The isolates were initially obtained from outbreaks of streptococcosis in red tilapia in cage-cultured system at Kuala Lipis, Pahang in 2007. The selected isolates were sub-cultured onto blood agar plate (Merck) for 24 hours and incubated at 30° C.

Feed-based vaccine preparation

The formalin killed bacteria were prepared according to the method described earlier (Firdaus-Nawi *et al.*, 2014). Feed pellet containing 6.7 x 10^7 cfu/ml of *S. agalactiae* and the palm oil adjuvant concentrations of 0%, 3%, 5,% 7% and 10% were added (Firdaus-Nawi *et al.*, 2014). The palm oil was a commercial cooking oil obtained from a local supermarket (Vesawit®, Malaysia). Similar feed-based vaccine was prepared containing same concentrations of Freund's incomplete adjuvant (Sigma-Aldrich (Merck), United States) for comparison.

Experimental design and vaccination regime

The selected red tilapias were divided into three major groups. Group 1 consisted of 500 fish that were kept in 10 tanks (50 fish per tank), Group 2 with 600 fish kept in 12 tanks and Group 3 with 100 fish and were kept in 2 tanks. Group 1 was further sub-divided into 5 groups with 2 replicates each and vaccinated with the feed-based killed vaccine containing 7%, 5%, 3% and 0% of the Freund's incomplete adjuvant. Group 2 was similarly sub-divided into 6 groups with 2 replicates each and was vaccinated with the feed-based killed vaccine containing 10%, 7%, 5%, 3% and 0% palm oil as adjuvant. For both groups, the vaccine was administered orally via feed at 3% bodyweight at

the start of the experiment, week 2 and week 6 of the experiment (double booster vaccination strategy). Group 3 with the 2 replicates was the control unvaccinated and was provided feed without the vaccine.

Serum samples were collected from 10 fish of each treatment at weekly interval for a period of sixteen weeks. The samples were subjected to ELISA to determine the systemic antibody (IgM) responses (Grabowski *et al.*, 2004; Firdaus-Nawi *et al.*, 2014). On week 4 after the last vaccination, all fish were challenged intraperitoneally with $2.6x10^9$ cfu/ ml of live *Streptococcus agalactiae* (Fig.1). Following challenge, all fish were observed for clinical signs before all surviving fish were killed at the end of the 16-week study period for post-mortem examinations. The experiment was approved by the Institutional for Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (Approval no. R043/2015).

The survival rate and vaccine efficacy were eventually calculated. The calculation of vaccine efficacy was according to Rodrigues and Smith (1999) using the following formula:

$$VE = 100 (IU – IV) / IU$$

VE = Vaccine Efficacy
IU = Disease incidence/mortality in the unvaccinated group (control group)
IV = Disease incidence/mortality in the vaccinated group (treatment group).

All data were subjected to statistical analysis. One-way ANOVA using SPSS 16.0 was used to compare group means between each treatment group and between the two adjuvant groups. Student's t-test was used to compare the rate of mortality between each treatment group and between the two adjuvant groups.

Significant difference was determined at $p<0.05$.

Results

Antibody titer

In general, both Freund's incomplete and palm oil adjuvants stimulated higher antibody response with increasing percentage of the adjuvant.

Table 1. Percentage of surviving fish (%) that were vaccinated with different concentrations of FIA and POA and challenged with live *Streptococcus agalactiae*. Generally, POA resulted in significantly higher rate of survival.

Treatment	Percentage of fish survive (%)	
	FIA	POA
10%	NA	70^a
7%	45^b	50^b
5%	30^c	60^a
3%	35^b	45^b
0%	20^c	25^c
Control	15^c	15_c

Immunization by FIA (Group 1) resulted in significantly ($p<0.05$) higher antibody level as early as week 1 and remained high throughout the 16-week of experimental period. Following administration of the first booster at week 2, the antibody level increased significantly ($p<0.05$) from week 2 until week 4. Highest titer was observed in the group vaccinated with 7% FIA (Fig. 2). By week 6, the antibody level of all treatment groups had declined to the lowest, and below the cut-off value especially for groups that were vaccinated with 0% and 3% FIA. Following the second booster at week 6, the titer rose again to reach peak at week 8 before started to decline from week 9 but remained above the cut-off value until week 11 for the group vaccinated with 7% FIA. At the time of challenge at week 10, only those vaccinated with 7% FIA showed the antibody level above the cut-off value (Fig. 2).

All groups of fish vaccinated with palm oil adjuvant (POA) showed similar antibody pattern as those vaccinated with FIA (Fig. 3). Prior to the second booster dose at week 6, only those vaccinated with 7% and 10% palm oil showed antibody level above the cut-off value. However, upon challenge at week 10, all treatment groups showed the antibody titer above the cut-off line (Fig. 3).

Survival rate of fish

Table 1 shows the survial rates of fish following vaccination with different concentrations of FIA and POA. In general, all treatments resulted in significantly ($p<0.05$) higher rate of survival than the control unvaccinated group. The rate of survival was 45%, 30%, 35% 20% and 15% for the 7%, 5%, 3%, 0% of FIA and the unvaccinated fish, respectively. Each FIA concentration resulted in significant ($p<0.05$) different in the rate of survival.

Table 2. The efficacy of the feed-based killed vaccine containing various concentrations of Freund's incomplete and palm oil adjuvants.

Treatment	Survival Rate (%)	Mortality rate (%)	Percentage of vaccine efficacy (%)
7% FIA	45	55	35
5% FIA	30	70	18
3% FIA	35	65	24
0% FIA	20	80	6
10% POA	70	30	60
7% POA	50	50	37
5% POA	60	40	47
3% POA	45	55	27
0% POA	25	75	1
Control	15	85	0

The rates for POA were 70%, 50%, 60%, 45% and 55%, respectively. The 70% POA resulted in highest rate of survival but insignificant ($p>0.05$) compared to the 7% and 5% groups (Table 1). Nevertheless, they showed significantly ($p<0.05$) higher rates of survival compared to the respective treatment with FIA.

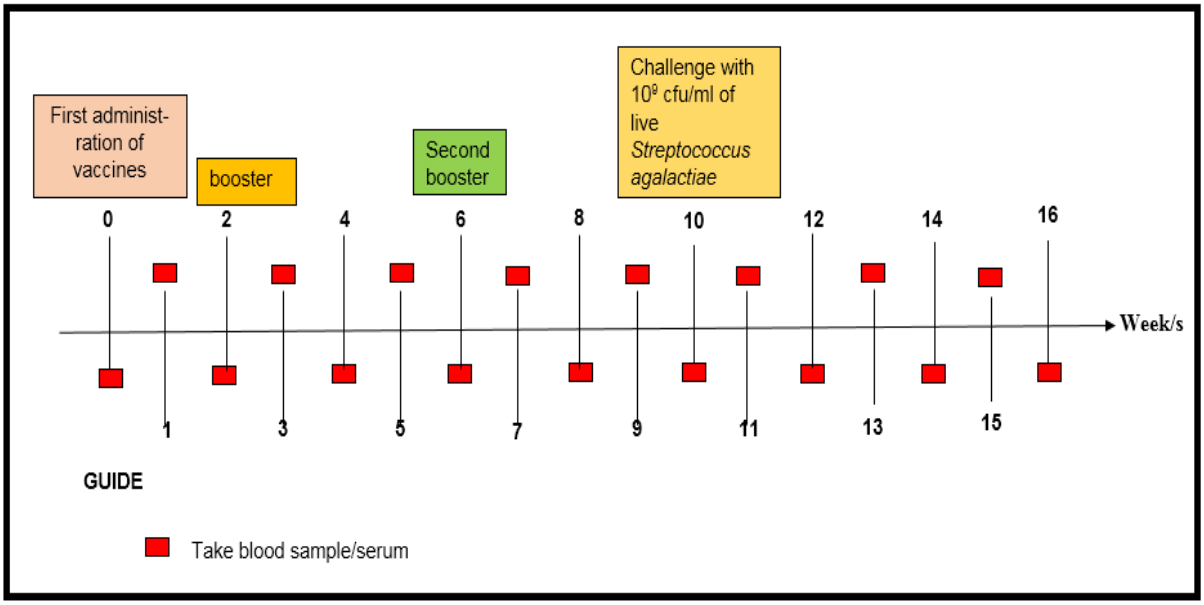

Fig. 1. Show the experiment timeline of 16-week study period. First booster is on week 2 while the second booster is on week 6. All fish were challenged on week 10.

Vaccine efficacy

Vaccine efficacy is the percentage reduction of disease in a vaccinated group of animals compared to an unvaccinated group. The vaccine efficacy for FIA was 35%, 18%, 24% and 6%, respectively (Table 2) while the efficacies of the POA were 60%, 33%, 47%, 27% and 40%, respectively (Table 2).

Clinical signs and Gross finding

The earliest clinical signs following challenge with *Streptococcus agalactiae* included self-isolation, loss of appetite and lethargy. These were followed by hemorrhagic fins cloudy eyes and unilateral or bilateral exophthalmia, erratic swimming and restlessness. The signs were detected as early as 24 hours post-challenge. Following post-mortem examination, the lesions observed included congested kidney, watery and hemorrhagic brain, pale liver and enlarged spleen.

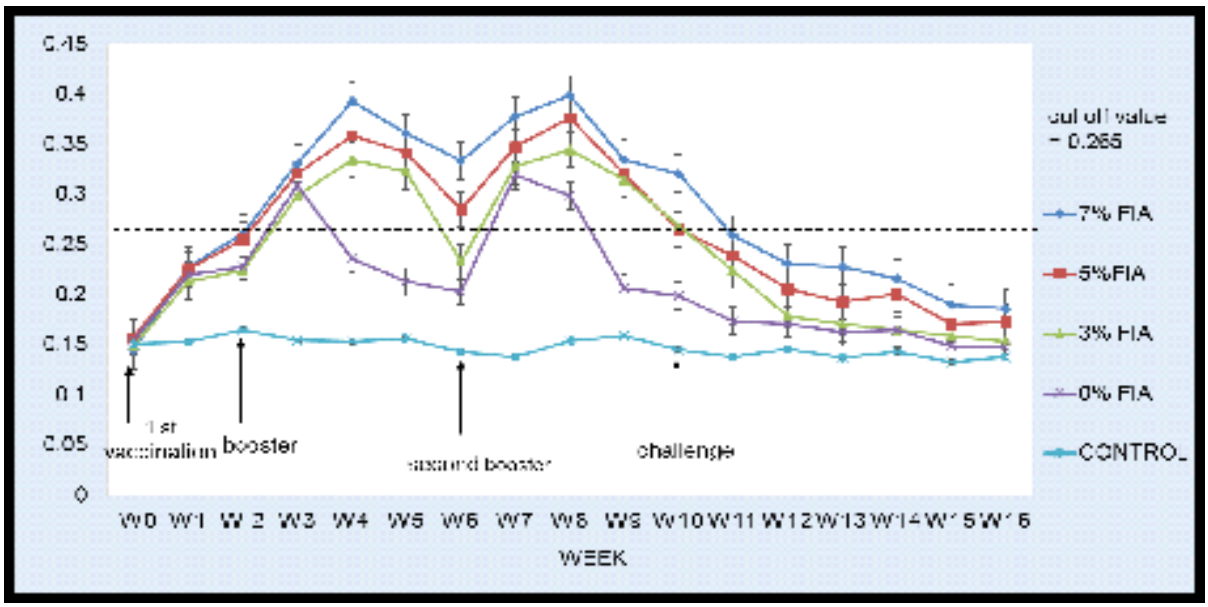

Fig. 2. The antibody patterns of tilapia following oral vaccination with the feed-based killed vaccine containing different concentrations of the Freund's Incomplete adjuvant (FIA). The groups vaccinated with 3%, 5% and 7% FIA showed the antibody levels above the cut-off value at the time of cha.

Discussion

Generally, the results of this study revealed that the antibody titer tends to be higher with increased concentration of both adjuvants. This is because the function of an adjuvant is to increase the immunogenicity of poor antigen, enhancing the vaccine efficacy and thus reducing the amount of antigen or number of immunization needed (McElrath, 1995). Nevertheless, vaccination regime requires double booster at weeks 2 and 6 to maintain the high antibody titer for the required period of 12 months.

At the time of challenge at week 10, the antibody levels of groups vaccinated with 7% Freund's incomplete adjuvant (FIA) and the 5%, 7% and 10% palm oil were higher than cut off value.

Similarly, fish vaccinated with feed-based vaccine containing adjuvant had higher antibody levels than those vaccinated with the vaccine without the adjuvant (0%). In fact, the POA stimulated much higher antibody responses at all concentrations than the FIA except at 10% concentration (Ismail *et al.,* 2016). Adjuvant act as an immune enhancer that is often used to boost the immune response toward a vaccine in the vaccination process that enhances the protective immunity against the targeted disease (Xu Dong *et al.*, 2010).

Consequently, vaccination with feed-based vaccine containing Freund's incomplete and palm oil adjuvants improved the rate of survival and vaccine efficacy, in agreement with the statement that adjuvant increase the vaccine efficacy (Tafalla *et al.*, 2013).

While both FIA and POA are as effective in preventing streptococcosis in this study, the POA seemed to stimulate much better immune response resulting in higher rates of survival. Eventually, the optimal concentration of palm oil to be used as adjuvant in the feed-based vaccine is 10%, similar to result reported for the 10% FIA (Ismail *et al.,* 2016).

The immune-stimulation effect of palm oil is due to the vitamin E content within the palm oil. It stimulates lymphocytes, macrophages and plasma cells at the sites of vaccination (Latshaw, 1991; Wanasawaeng *et al.*, 2009). Both humoral and cell-mediated immune responses appeared to be affected by Vitamin E supplementation (Meydani & Berhaka, 1998).

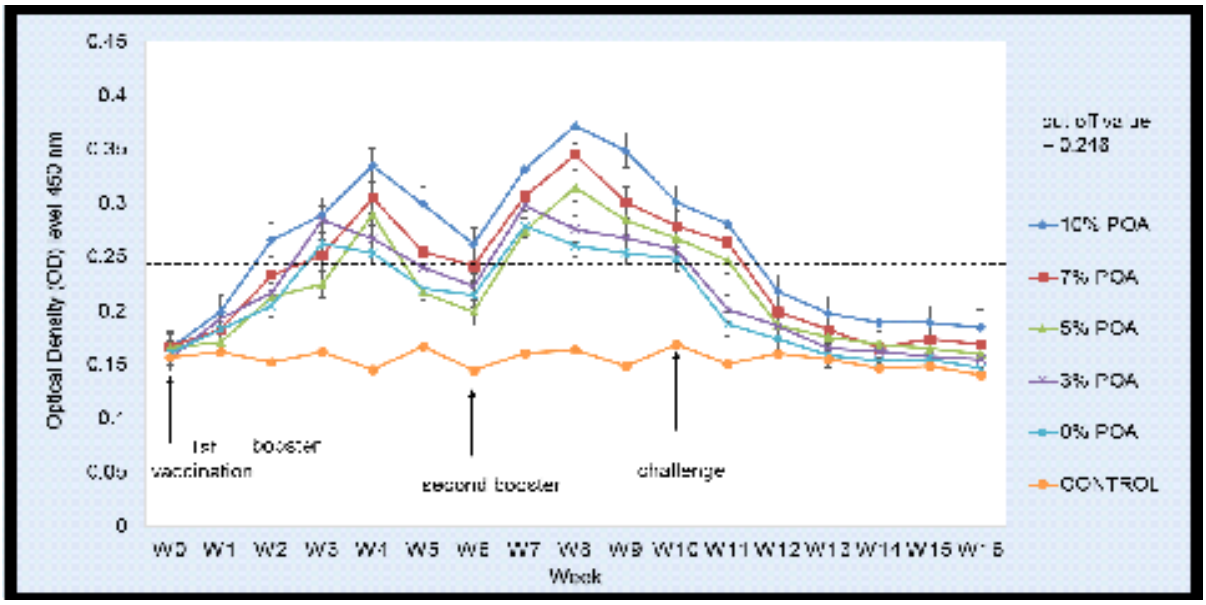

Fig. 3. Antibody level of tilapia following vaccination with the feed-based killed vaccine containing different concentrations of palm oil adjuvant (POA). At the time of challenge, all concentrations showed antibody levels above the cut-off value.

One of the main goals of using adjuvants in a vaccine is to decrease the dose of antigen in the vaccine and to reduce the cost of vaccination (Singh & O'Hagan, 2003). It is known that the prices of commercial adjuvants including the FIA are expensive leading to high cost of vaccine production, beyond the capability of local small-scale farmers. Therefore, this study tested the cheaper palm oil as adjuvant and was found to be comparable with the FIA. There are several benefits of palm oil adjuvant, which include convenience as it is a local source of material, safe from metal-pollutes because of its natural ingredient, can be combined with most types of vaccine, reduced side effect such as inflammation that comes with using mineral oil and the most important is it is far cheaper that can reduce the cost of vaccine production compared to mineral oil (Shamsuddin, 2012).

Conclusion

In conclusion, feed-based vaccine containing POA was as effective in providing protective against *S. agalactiae* infection as the Freund's incomplete adjuvant. At 10% palm oil with double booster strategy, the antibody levels remained higher than the cut-off value for a period of 12 weeks.

Acknowledgement

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