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The Effect of Adjuvants on Immunization of the Nile Tilapia, *Oreochromis niloticus* with *Aeromonas hydrophila* Bacterin

Hekmat M. Ali

Sciences and Mathematics Department, Riyadh Educational College
Girls College Presidency Agency, P.O. Box 2710, Riyadh 11417, Saudi Arabia

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Abstract. The effects of adjuvants on the immune response of the Nile tilapia, *Oreochromis niloticus* when injected intraperitoneally (I/P) 1, 4, 7 and 10 weeks post immunization with the bacterin prepared from *Aeromonas hydrophila* have been investigated. Fishes immunized with *A. hydrophila* bacterin, or with bacterin emulsified in either Freund's complete adjuvant (FCA) or in Freund's incomplete adjuvant (FIA) produced similar quantities of specific antibodies and were equally protected against challenge with *A. hydrophila*. However those fishes injected with saline, FCA or FIA were not protected against challenge and all died.

Introduction

The study of immune response in fishes was apparently attempted prior to 1903, when agglutinating antibodies against the bacterium, *Serratia piscitorum* were demonstrated in the blood of the carp [1]. Fishes are the most primitive vertebrates, yet they have an efficient immune system that protects them against various micro-organisms and parasites. Many studies were undertaken to stimulate the immune response of fishes [2,3, pp.221-229, 4]. Such response could be detected either by the presence of specific antibodies in the blood or by the protection of fish against various infections. The former could be revealed by the injection or immersion of antigen [5]. Trials were attempted to increase the production of antibodies and to prolong their prevalence in the blood by emulsifying the antigen in adjuvants [5-8].

The present study is intended to investigate the effect of various adjuvants on the

immune response of the Nile tilapia, *Oreochromis niloticus* when injected with bacterin prepared from *Aeromonas hydrophila*.

Materials and Methods

Fish

Three hundred and sixty Nile tilapia each weighing about 100 g were chosen from the fish center of the Suez Canal University in Ismailia. They were divided into 6 groups each comprising 60 fishes placed into 2 glass aquaria, each is 40 x 50 x 100 cms and is filled with dechlorinated tap water. The water temperature was maintained at 22°C throughout the experiment by an automatic heater. The fishes were allowed to acclimatize for 2 weeks before starting the experiment. They were fed a commercial fish diet at the rate of 5% body weight twice daily at 9.0 am and 3.00 pm 6 days / week.

Antigen

Formalin - killed *Aeromonas hydrophila* was prepared according to the method of Hudson and Hay [9, pp. 26-41] where the organism was inoculated in 3 liters of brain heat infusion (BHI) broth incubated at 35°C for 48 hrs. The bacterial culture was inactivated by the addition of formalin at a final concentration of 0.3% and was held night over at room temperature. The bacterial cells were harvested by centrifugation at 6240 xg for 15 min. and washed 4 times with sterile normal saline solution. For fish immunization, the inactivated bacterial cells were diluted with an equal volume of saline and were either used alone or were first emulsified with an equal volume of Freund's complete adjuvant (FCA) or with an equal volume of Freund's incomplete adjuvant (FIA). The six groups of fish were treated as follows: Group 1 injected with saline, group 2 with bacterin, group 3 with bacterin emulsified in FCA, group 4 with bacterin emulsified in FIA, group 5 with FCA and group 6 with FIA.

Each fish was injected I/P with 0.1 ml of the inoculum. For serological testing, the inactivated bacterial cells were diluted with sterile saline solution to a turbidity equaling to tube No.2 on the Mcfarland scale [7], and one drop of Loeffler's alkaline methylene blue, prepared as described by Cruickshank]10, pp. 112-125].

Antibody response

Weekly, 2 fishes were randomly collected from each group, properly wiped dry and the blood was collected from its caudal artery, according to the method of Lied *et al.* [11] into sterile screw capped bottles, that were kept overnight in the refrigerator and the serum was asecptically aspirated into a standard microtitre plate (U shaped wells). Serial two - fold dilutions of the serum were made with sterile saline solutions, using 0.025 ml pipette dropper. To the diluted serum, 0.025 ml of stained antigen was added, the suspensions

were mixed and incubated overnight at room temperature (22°C - 25°C). A positive serological reaction was indicated by bacterial agglutination and the last well in which agglutination occurred is considered as the end point and its dilution is taken as the titre of the serum.

Efficacy of the immune response

To determine the efficacy of the immune response of immunized fishes, 10 fishes from each group were exposed to artificial infection by the I/P injection of *A. hydrophila* at 1, 4, 7 and 10 weeks post-immunization, the bacterial solution was prepared by suspending 24 h. *A. hydrophila* culture from nutrient agar surfaces incubated at 28°C in sterile saline solution. The bacterial concentration was 2.0 mg/ml by wet weight and estimated to be between 0.7×10^3 - 0.3×10^3 cells/ml. The fishes were injected with 1.0 mg bacterial cells per each 100 g fish body weight. The challenged fishes were observed for 2 weeks and the dead ones were collected for re-isolation of *A. hydrophila.* The relative level of protection (RLP) in each fish group was determined using the method of Newman and Majnarich [12].

Results

Antibody response

The reciprocal antibody titres of fishes injected with saline, FCA and FIA were 2 at 7 days post injection and remained constant at that level throughout the experimental period. Those fishes injected with bacterin, bacterin emulsified in FCA and bacterin emulsified in FIA had also an antibody response, where the reciprocal antibody titres were 3.4 and 3.5 at 7 days post immunization and the maximum reciprocal titres were 10, 13.5 and 10.5, respectively 7 weeks post-immunization (see figure). Fishes injected with bacterin emulsified in FCA showed granulomta at the sites of injection besides visceral adhesions.

Efficacy of the immune response

The fishes injected with saline were not protected against the challenge with *A. hydrophila* since a 100% mortality rate was recorded. Yet, those immunized with bacterin, bacterin emulsified in FCA and bacterin emulsified in FIA were equally protected against the challenge, since 30, 20 and 30% mortality rates were respectively recorded after the first challenge and 0.0% mortality rate was recorded for the other 3 challenges. The fishes injected with FCA and FIA had some protection against the first challenge, since there were 60% and 80% respective mortality rates in either group, but a 100% mortality rate was observed with the other 3 challenges in both groups (see Table). All fishes dying after each challenge, have revealed the presence of *A. hydrophila* infection and the organism, was re-isolated from them.

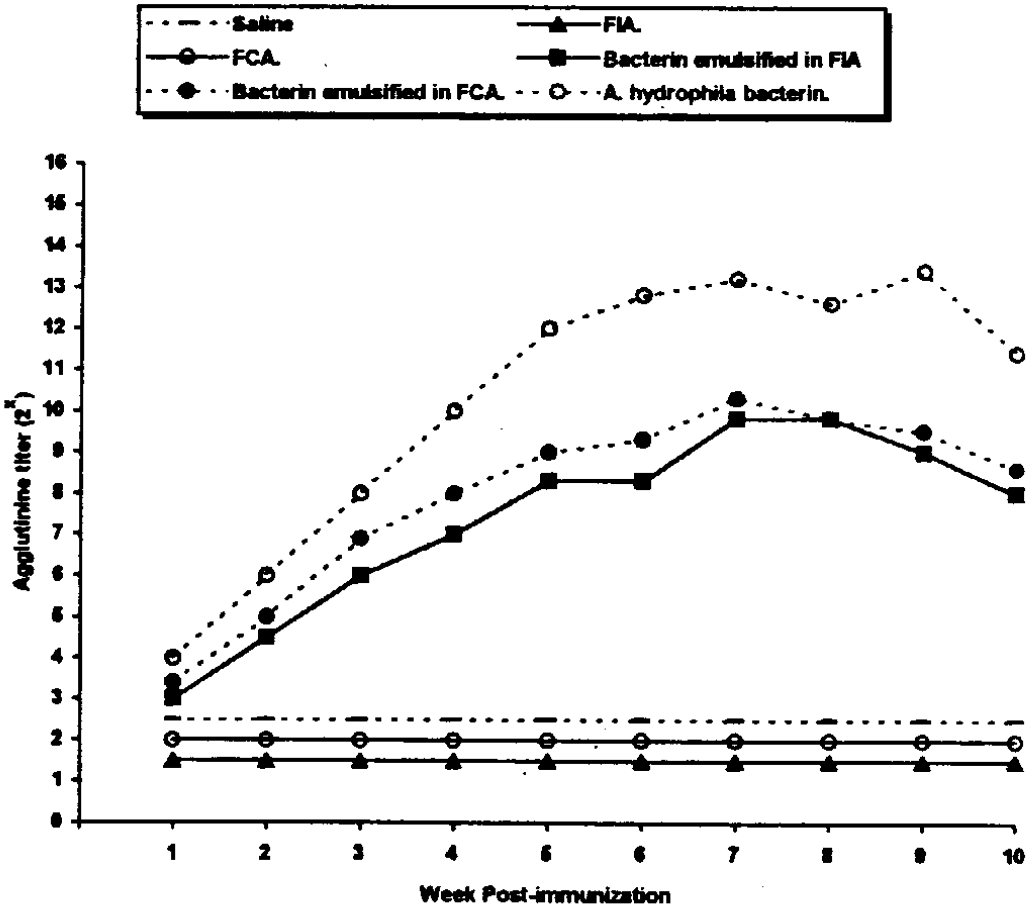

Fig. Results of agglutination test, on sera from fishes post-immunization with different sorts of antigen FCA = Fruend's complete adjuvant; FIA = Fruend's incomplete adjuvant.

Table. The results of challenge of fishes immunized with various types of antigens

Group	Immunization antigen dose injected intraperitoneally (IP)	Results of challenge expressed in mortality rate (M) and in relative level of protection (RLP %)							
		1st chall		2nd chall		3rd chall		4th chall	
		M	RLP	M	RLP	M	RLP	M	RLP
1	Controls (0.1 ml saline)	100	0.0	100	0.0	100	0.0	100	0.0
2	0.1 ml *A. hydrophila* saline	30	70	0.0	100	0.0	100	0.0	100
3	0.1 ml *A. hydrophila* bacterin emulsified in FCA	20	80	0.0	100	0.0	100	0.0	100
4	0.1 ml *A. hydrophila* bacterin emulsified in FIA	30	70	0.0	100	0.0	100	0.0	100
5	0.1 ml FCA	60	40	100	0.0	100	0.0	100	0.0
6	0.1 FIA	80	20	100	0.0	100	0.0	100	0.0

FCA = Fruend's complete adjuvant; FIA = Fruend's incomplete adjuvant.

Discussion

The present results have indicated that fishes immunized by *A. hydrophila* bacterin or by bacterin emulsified in either FCA or in FIA were equally protected against challenge by *A. hydrophila* sincspecific antibodies were produced against the injected antigens. However, thc : fishes injected with saline, FCA or FIA were not protected against challenge anc all died (100% mortality rate). Fishes immunized with *A. hydrophila* bacterin had a .iigh level of antibodies compared with those injected with saline. The titre of agglutinating antibodies was higher in the sera of immunized fishes compared to that of the controls. Similar to the observation of Dooly *et al.* [13 and 14], the formalin treated *A. hydrophila* bacterin in antigenic as it has stimulated the immune system of the fish to produce specific agglutinins. The addition of FCA to the bacterin has enhanced the immune reponse of the immunized fishes. Similar results were obtained by Post [15] and by Khalifa and Post [16] with other fish species. However, similar to the observations of Ellis [17, pp. 20-31], the use of FCA though enhancing to the immune response of fish, its use is however undesirable due to the abscesses, muscle necrosis, visceral adhesions and granuloma it brings about in fishes. On the other hand the addition of FIA to the bacterin did not enhance the immune response much more than bacterin alone. Similar observations were reported by Ward *et al.* [3] in the rainbow trout vaccinated with *Vibrio anguillarum* vaccines.

Fishes immunized with bacterin emulsified in either FCA or FIA were equally protected against challenge with slightly lower relative level of protection (RLP) against the first challenge (one week post immunization) compared to further challenges undertaken. Hence, similar to the observations of Plumb [2] in the Channel catfish, higher antibody titres do not necessarily mean a protective level of immunity. Similar observations were also made by Ruangpan *et al.* [4].

Although the immune response of fishes injected with either FCA or FIA did not differ from that of the controls, some protection was observed in the first challenge of those fishes which could be attributed to a stimulation of a nonspecific immune response especially in the presence of the muramyl dipeptide fraction (n-acetyle - muramyl - alanyl - Disoglutamine) of the *Mycobacterium* in FCA [18, pp. 126-161].

Hence, the addition of adjuvants to the bacterin prepared from *A. hydrophila* is not recommended for the immunization of fishes. This is because the same level of protection can be obtained by the vaccine, with or without the addition or adjuvants. Moreover, the adjuvants, especially FCA, have many undesirable side effects of the immunized fishes.

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تأثير المساعدات الأنتيجينية على الاستجابة المناعية لأسماك البلطي الـ يلي نوع *Oreochromis niloticus* بواسطة اللقاح المحضّر من بكتيريا *Aeromonas hydrophila*

حكمت محمد علي

كلية التربية ، وكالة الرئاسة العامة لكليات البنات ، ص. ب ٢٧١٠ ، الرياض ١١٤١٧ ، المملكة العربية السعودية

(استلم في ١٦ رجب ١٤١٥هـ؛ قبل للنشر في ٣٠ محرم ١٤١٧هـ)

ملخص البحث. تّم في هذه الدراسة تحديد دور المساعدات الأنتيجينية (Adjuvants) مساعد فروند التام ومساعد فروند الناقص على الاستجابة المناعية في أسماك البلطي النيلي نوع *Oreochromis niloticus* ، الناتجة عن التطعيم باللقاح المحضّر من بكتيريا *Aeromonas hydrophila* ، ولقد استخدمت ٦ مجموعات من أسماك البلطي النيلي ، بكل مجموعة ٦٠ سمكة وزن كل منها حوالي ١٠٠ جرام ، ولقد تم حقن كل مجموعة من تلك المجموعات في التجويف البريتوني بالآتي : المجموعة الأولى (مجموعة الضبط) بمحلول ملح فسيولوجي ، المجموعة الثانية باللقاح المحضّر من *A. hydrophila* ، المجموعة الثالثة باللقاح المستحلب في مساعد فروند التام ، المجموعة الرابعة باللقاح المستحلب في مساعد فروند الناقص ، المجموعة الخامسة بمساعد فروند التام والمجموعة السادسة بمساعد فروند الناقص . هذا وقد تم قياس معدلات الأجسام المناعية (Titres) أسبوعيًا في كل مجموعة ، كما تم قياس مقاومة تلك الأسماك للإصابة ببكتيريا *A.hydrophila* بحقنها في التجويف البريتوني بعد تطعيم الأسماك ب١ أو ٤ أو ٧ أو ١٠ أسابيع . وقد استخدم مؤشران لقياس تلك المقاومة هما نسبة نفوق الأسماك التي أصيبت بالبكتيريا والنسبة المئوية لمستوى الحماية ، وهي النسبة المئوية من الأسماك التي بقيت حية بعد الإصابة بالبكتيريا .

ولقد أوضحت النتائج أن المعدلات العكسية لمستوى الأجسام المناعية (Reciprocal antibody titres) في مجموعة الضبط من الأسماك، وكذلك في المجموعتين الخامسة والسادسة المطعمتين بمساعد فروند التام والناقص، كانت ٢ في اليوم السابع بعد التطعيم وظلّت على هذا المستوى طيلة مدة التجربة. أما الأسماك المحقونة باللقاح (المجموعة الثانية)، والمحقونة باللقاح المستحلب في مساعد فروند التام (المجموعة الثالثة)، والمحقونة باللقاح المستحلب في مساعد فروند الناقص (المجموعة الرابعة) فلقد كانت تلك المعدلات العكسية فيها ٣، ٤ و ٣,٥ على التوالي بعد أسبوع من الحقن، ولقد وصلت إلى ١٠، ١٣,٥ و ١٠,٥ على التوالي، كأعلى معدلات عكسية بعد ٧ أسابيع من الحقن.

أما بالنسبة لمقاومة العدوى فقد وجد أن أسماك الضبط (المجموعة الأولى) ليس لديها أي مقاومة حيث نفقت جميع أسماك هذه المجموعة التي حقنت بالبكتيريا. أما أسماك المجموعات الثانية، الثالثة والرابعة فلديها درجات متفاوتة من المقاومة، حيث بلغت نسبة الوفيات فيها على التوالي ٣٠٪، ٢٠٪ و ١٠٪ نتيجة لإصابتها بالبكتيريا بعد أسبوع واحد من التطعيم، ولم ينفق أي من تلك الأسماك (١٠٠٪ مقاومة للإصابة) نتيجة لإصابتها بالبكتيريا بعد ٤ أو ٧ أو ١٠ أسابيع من التطعيم، أما الأسماك التي طعّمت بمساعد فروند التام (المجموعة الخامسة) والتي طعّمت بمساعد فروند الناقص (المجموعة السادسة) فلقد قاومت الإصابة بالجرثومية إلى حد ما عند إصابتها بعد أسبوع واحد من حقنها بأي من المساعدين (نسبة النفوق في المجموعتين على التوالي ٦٠٪ و ٨٠٪)، ولكنها فقدت تلك المقاومة تمامًا عند إصابتها بالجرثومة بعد ٤ أو ٧ أو ١٠ أسابيع من حقنها بأي من المساعدين (نسبة النفوق ١٠٠٪).

الأسماك التي حقنت باللقاح المستحلب في مساعد فروند التام وجد بها أورامًا حبيبية (Granulomata) في مواقع الحقن وكذلك التصاقات عديدة في الأحشاء (Visceral adhesions)، أما كل الأسماك التي نفقت نتيجة لإصابتها بالجرثومة فلقد كان واضحًا عليها آثار تلك الإصابة، كما وتم عزل تلك البكتيريا في كل منها.