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Effect Of Different Prepared Antigens Of *Pseudomonas fluorescens* On Specific And Non-Specific Immune Response Of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The current work aimed to study four different prepared *Ps. fluorescens* antigens (formalin killed bacterin, extracellular product (ECP) suspension, sonicated cells (SC) suspension and mixture of ECP and SC suspension) to develop the best adequate strategy to control such infection in cultured Nile tilapia.

The nitroblue tetrazoilum (NBT), neutrophil adherence and lysozyme activity of vaccinated fish showed significant increases in all immunized groups in comparison with control at 2 and 4 weeks post vaccination. Serum bactericidal activity and antibody titer were significantly increased in all immunized groups at all periods of the experiment. The mixture of ECP and SC antigen showed the best serum bactericidal activity and antibody titer against *Ps. fluorescens.*

The relative percent of survival (RPS) after challenge with *Ps. fluorescens* at 4, 6 and 8 weeks post vaccination was significantly increased in all immunized groups in comparison with control. There were significant increases in RPS among a group immunized with a mixture of ECP and SC antigen than other three immunized groups at 4 weeks only. The higher values of the relative percent of survival were seen in the mixture of ECP and SC antigen followed by formalin killed antigen, sonicated cell antigen then the extracellular product antigen.

It could be concluded that all prepared vaccines are efficient against *Ps. fluorescens* infection, however a mixture of sonicated and extracellular product antigen seemed superior to other vaccines especially in bactericidal activity, antibody titer and RPS against *Ps. fluorescens*

INTRODUCTION

Bacterial diseases among fish caused by a variety of pathogens and represent a significant economic problem in the commercial aquaculture *(1)*. *Pseudomonas fluorescens,* is Gram-negative bacteria of the families *Pseudomonadaceae*, among the recognized bacterial pathogens that commonly associated with reared aquaculture species *(2)*.

Ps. fluorescens is a pathogen for a wide range of fish species including tilapia (*Oreochromis*) *(3-4)*. Infection of fish by *Ps. fluorescens* leads to the development of the so called Red Skin Disease, which is characterized by hemorrhage, scale falling off, and fins ulceration. Disease outbreaks often occur under stress conditionsThe use of vaccines in the aquaculture industry was important in reducing economic losses which occur as a result of disease *(5-6)* and in the reduction in use of antibiotics *(7)*. A number of different types of vaccines have been developed in fish against Gram negative, such as whole cell (WC) *(8-9)*, outer membrane protein (OMP) *(10)*, extracellular products (ECPs), lipopolysaccharide (LPS) preparations *(11)* and also biofilms *(12)*. Although the vaccinations are efficient, their mode of action remains unclear to determine its efficiency. Vaccination can be done by several ways where the injection route reliably delivers a small, known amount of antigen directly to the fish that most likely to be effective and provide protection of long duration *(13)*. Although vaccines have provided varying degrees of protection in fish, still until now no commercial vaccine available for *Ps. fluorescens (2)*.

In this work, four different *Ps. fluorescens* antigens were prepared and assayed to prevent *Ps. fluorescens* infections in cultured Nile tilapia.

MATERIAL AND METHODS

Fish: One thousand and fifty apparently healthy, Nile tilapia (*O. niloticus*) of both sexes (70±12 g) were collected from the WorldFish Center, Abbassa, Egypt and checked to be free from *Ps. fluorescens*. Fish were used for vaccination trial and treated of the ethic committee. Fish were divided into 5 equal groups (each 210 fish) and each group subdivided into three equal replicates, that kept in 15 fiberglasses (3 X0.6 X0.5m). Fish, throughout the experiment, fed on a balanced diet and provided with fresh water that partially replaced and air using electrical air pumping compressors with temperature adjusted at 26 ±1 °C.

Bacterial strains: The pathogenic strain, *Ps. Fluorescens,* was obtained as a reference local strain, from the Fish Health Laboratory at The WorldFish Center, Abbassa, Egypt.

Preparation of *Ps. fluorescens* antigens

a) Preparation of formalin killed bacterin: A formalin-killed vaccine was prepared as previously described *(14)*. *Ps. fluorescens* was grown in Trypticase Soy Broth (TSB;Difco) at 28° C for 24 h. Bacterial cells were killed by addition of formalin to achieve a final concentration of 0.7% and incubated for 3 h at 25 °C and then at 4°C overnight. Cells were collected by centrifugation at 6500xg for 30 min at 4°C and washed three times with phosphate buffered saline (PBS; pH 7.4), and then they were re-suspended in PBS at a final concentration of $1x10^9$ cells/ml.

b) Preparation of Extracellular product (ECP): The supernatants that obtained after centrifuging 24 h old cultures of *Ps. fluorescens* in brain heart infusion broth were filtered (0.22 µm). The toxic activities of ECP were neutralized with 0.5% formalin overnight at 4°C. Formalin was neutralized by the addition of a 15% solution of sodium metabisulphite ($10mL/L^{-1}$ of inactivated culture supernatants) with overnight incubation at room temperature *(15)*.

c) Preparation of sonicated cell (SC): *Ps. fluorescens* was grown on brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI, USA) at 28° C for 24 h. Bacterial cells were collected by centrifugation at 6500xg for 30 min at 4°C and washed three times with Sodium phosphate buffered saline (PBS; 10 mM Sodium phosphate buffer, 150 mM NaC1, pH 7.0) and re-suspended in PBS at 10^9 cells ml^{-1}. The suspension were kept on ice and sonically lysed with two 10 sec bursts using a probe sonicator with power level at 60 W. The sonicated cells were stored at -20°C *(16)*.

Immunization trial: The fish of groups 1, 2, 3, 4 and 5 were injected intraperitonial with 0.2 ml from each of sterilized saline (0.85 %), Formalin killed bacterin (corresponding to $4X10^8$ cfu/ml), ECP suspension (corresponding to $4X10^8$ cfu/ml), SC suspension (corresponding to $4X10^8$ cfu/ml) and 0.2 ml mixture of ECP & SC suspension (corresponding to $4X10^8$ cfu/ml); respectively.

Blood sampling and analysis: At 2, 4, 6, and 8 weeks post vaccination (pv), ten fish were randomly collected from the control (1) and treatment groups (2-5). The fish were anesthetized by immersion in water containing 0.1 ppm tricaine methane sulfonate (MS- 222). Whole blood (0.5 ml) was collected from the caudal vein of each fish using syringes (1-ml) and 27-gauge needles that were rinsed in heparin (15 unit ml^{-1}), to determine the NBT, and neutrophil adherence tests. A further 0.5 ml blood-sample was centrifuged at 1000 x g for 5 min in order to separate the plasma that stored at -20 °C to be used for lysozyme activity test. For separation of serum, blood samples (0.5 ml) were withdrawn from the fish caudal vein and transferred to Eppendorf tubes without anticoagulant. The blood samples were centrifuged at 3000 x g for 15 min and the supernatant serum was collected and stored at -20 °C until used for the serum bactericidal test.

Nitroblue tetrazolium activity (NBT): Blood (0.1 ml) was placed in microtiter plate wells, to which an equal amount of 0.2% NBT solution was added and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.05 ml) was added to a glass tube containing 1 ml N,N-dimethyl formamide and centrifuged for 5 min at 3000 rpm. The supernatant fluid was measured in a spectrophotometer at 620 nm in 1 ml cuvettes *(17)*.

Adherence/NBT assays: NBT-glass adherent assays were performed by placing single drops of blood (0.1 ml) on 2 glass cover slips and incubating them for 30 min at room temperature. The cover slips were then gently washed with phosphate buffered saline (PBS). Drops (0.1 ml) of 0.2% NBT were placed on microscopical slides and covered by a cover slip, then incubated at room temperature for 30 min with the NBT solution. The activated neutrophils were then counted under a microscope (x 400) *(18)*.

Lysozyme activity: The lysozyme activity was measured using the turbidity assay. Chicken egg lysozyme (Sigma) was used as a standard and 0.2 mg ml^{-1} lyophilised *Micrococcus lysodeikticus* in 0.04 M sodium phosphate buffer (pH 5.75) was used as substrate. Fifty ml of serum was added to 2 ml of the bacterial suspension and the reduction in the absorbance at 540 nm was determined after 0.5 and 4.5 min incubation at 22 °C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1} *(19)*.

Serum bactericidal activity (SBT): Bacterial cultures of *P. fluorescens* were centrifuged, and the pellet was washed and suspended in phosphate buffered saline (PBS). The optical density of the suspension was adjusted to 0.5 at 546 nm. This bacterial suspension was serially diluted (1:10) with PBS five times. The serum bactericidal activity was determined by incubating 2 ml of the diluted bacterial suspension with 20 ml of the serum in a micro-vial for 1 h at 37 °C. PBS replaced the serum in the bacterial control group. The number of viable bacteria was determined by counting the colonies after culturing on trypticase soya agar plates for 24 h at 37 °C *(20)*.

Serum antibody titer (Agglutination test): Serum antibody titer was measured using the agglutination protocol described by *Klesius et al (21)*. *Ps. fluorescens* were grown in TSB for 24 h at 28°C, harvested by centrifugation at 2000xg for 15 min, and washed with PBS. Bacteria were washed in phosphate buffered saline (PBS) twice more before adjusting the bacterial suspension concentration to each separated bacteria to $1.0x10^9$ CFU of bacterial cell/ml. The agglutination test was assayed in 96-well U-bottom microtiter plates. Tilapia sera (15 µl) were serially diluted two-fold in PBS. Each Pseudomonas sp. suspension (15 µl) was applied to each well prior to incubating plates overnight at room temperature (28 °C). Agglutination titers were reported as log10 of the reciprocal of the highest dilution of the sera showing agglutination of bacteria. Positive (*Pseudomonas* infected serum) and negative (normal serum) were included in each plate as controls.

Challenge test: Thirty fish from each treatment (10 from each replicate) were collected at 4, 6 and 8 weeks pv and subjected to experimental infection with *Ps. fluorescens.*

Ps. fluorescens was grown in TSB for 24 h at 28 °C with shaking on an orbital shaker (100 revolutions per minute) *(21)*. Fish were i.p. injected with 4×10^{8}CFU/fish. Following challenge, mortality was monitored and recorded daily for 15 days. Cumulative percent mortality and RPS were calculated *(22)*. Freshly dead fish were cultured for the presence of *Ps. fluorescens* to confirm the cause of mortality using standard bacteriological procedures.

RPS= 1-(percent of mortality in immunized group\ percent of mortality in control group) X 100.

Statistical analysis: Analysis of Variance (ANOVA) and Duncan's multiple Range Test *(23)* was used to determine the differences between treatments. The mean values were significant at the level of $P < 0.05$. Standard errors, of treatment-means, were estimated. All the statistics were carried out using Statistical Analysis Systems (SAS) program *(24)*.

RESULTS

The NBT, Neutrophil adherence and lysozyme activity of vaccinated fish through the injection route against Ps. fluorescens showed significant increases in all immunized groups in comparison with control group at 2 & 4 weeks post vaccination, while at 6 & 8 weeks pv a non- significant increase was seen in same groups (Fig 1).

Serum bactericidal activity of immunized Nile tilapia against *Ps. fluorescens* by different antigens showed significant increases in all immunized groups in comparison with control group at all periods of experiment. Antibody titer of experimented Nile tilapia was significantly increased in all immunized groups in comparison with control at all periods. Mixture of sonicated and extracellular product antigen showed the best values of serum bactericidal activity and antibody titer (Table 1).

The percentage of mortalities after challenge with *Ps. fluorescens* at 4, 6 and 8 weeks post vaccination were significantly decreased in all immunized groups in comparison with control. There was no significant difference betweens the immunized groups at all periods. Also, no significant differences in-betweens the different periods within the same treatment except in formalin killed antigen which showed a significant decrease values at 6 & 8 weeks than at 4 week post vaccination. The most decreased values were showed in mixture of sonicated and extracellular product antigen followed by formalin killed antigen and sonicated cell antigen then extracellular product antigen (Table 2).

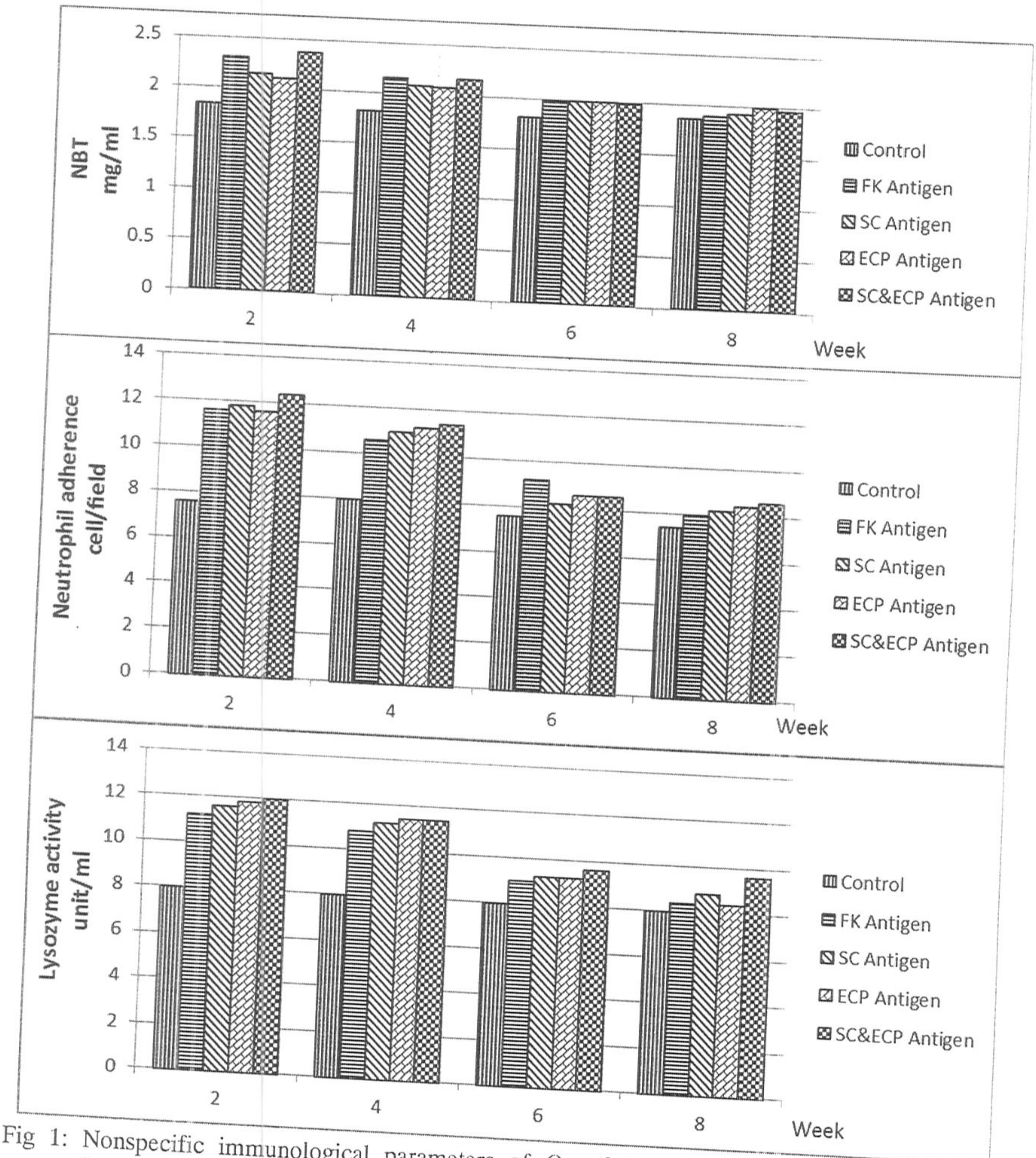

Fig 1: Nonspecific immunological parameters of *O. niloticus* immunized by different *Ps. fluorescens* antigens. FK, Formalin killed antigen, SC Sonicated cell antigen, ECP Extracellular product antigen, ECP &SC mix of Sonicated cell and Extracellular product antigens.

Table 1. Serum bactericidal activity and antibody titer against *Ps. fluorescens* of Nile tilapia immunized by injection using several *Ps. fluorescens* antigens

Period (week)	Test	Control	Formalin killed vaccine	Sonicated cell vaccine	Extracellular product vaccine	Sonicated &extracellular product
Two	Colony count	81.60 Aa ± 4.65	63.00 BCb ± 2.98	58.80 Bb ± 3.54	56.80BCb ± 2.92	57.40 Bb ± 3.04
	Antibody titer	2.20 Ac ± 0.20	5.20 Cb ± 0.37	5.20 Bb ± 0.38	5.40Bb ± 0.24	6.80 Aa ± 0.58
Four	Colony count	80.80 Aa ± 4.59	56.40 CDb ± 2.32	48.20 Cbc ± 2.78	45.80Dc 2.20	46.20 Cc ± 1.45
	Antibody titer	2.40 Ac ± 0.24	6.20 Bb ± 0.37	6.40 Ab ± 0.24	6.00ABb 0.45	7.80 Aa ± 0.37
Six	Colony count	81.20 Aa ± 5.76	54.00 Dbc ± 1.87	47.80 Cc ±1.39	48.40Cc ± 0.40	58.40 Bb 1.81
	Antibody titer	2.40 Ac ± 0.24	7.40 Aab ± 0.24	7.20 Aab ± 0.37	6.80Ab 0.20	8..00 Aa ± 0.32
Eight	Colony count	84.00 Aa ± 3.78	64.80Bbc ± 3.83	54.20 BCc ± 3.15	59. 20Bbc ± 3.72	66.00 Ab ± 2.91
	Antibody titer	2.20 Ac ± 0.20	7.00 ABab ± 0.32	6.60 Aab 0.24	6.20ABb 0.37	7.60 Aa ± 0.51

- *The column or raw with the same letters has no significant different.*
- *Capitals letter compare between the different periods within the same treatment.*
- *Small letters compare between the different treatments within the same period.*

The relative percent of survival (RPS) after challenge with *Ps. fluorescens* at 4, 6 and 8 weeks post vaccination showed significant increases in all immunized groups in comparison with control. There were significant increases in group immunized with a mixture of sonicated and extracellular product antigen than the other three immunized groups at 4 weeks only. The higher values of the relative percent of survival was seen in the mixture of sonicated and extracellular product antigen followed by formalin killed antigen, sonicated cell antigen then extracellular product antigen (Table 2).

Table 2. Mortalities percentage and relative percent of survival among Nile tilapia immunized by injection with different *Ps. fluorescens* antigens

Period (week)		Group				
		Control	Formalin killed vaccine	Sonicated cell vaccine	Extracellular product vaccine	Sonicated &extracellular product
Four	Mortality %	73.33 Aa ± 1.67	45.00 Ab ± 2.89	38.33 Ab ± 3.33	40.00 Ab ± 2.89	30.00 Ab ± 0.00
	RPS %	0.00 c	35.82 Bb ± 2.91	45.08 Ab ± 5.55	42.81 Ab ± 4.43	57.11 Aa ± 1.00
Six	Mortality %	68.33 Aa ± 1.67	31.67 Bb ± 1.67	33.33 Ab ± 3.33	35.00 Ab ± 2.89	28.33 Ab 1.67
	RPS %	0.00 b	53.67 Aa ± 0.45	51.23 Aa ± 4.88	48.79 Aa ± 4.22	58.55 Aa ± 2.44
Eight	Mortality %	68.33 Aa ± 1.67	33.33 Bb ± 1.67	36.67 Ab ± 1.67	36.67 Ab ± 0.32	31.67 Ab ± 1.67
	RPS %	0.00 b	51.23 Aa ± 2.44	46.35 Aa ± 2.43	46.35 Aa ± 2.44	53.67 Aa ± 2.44

- *The column or raw with the same letters has no significant different.*
- *Capitals letter compare between the different periods within the same treatment.*
- *Small letters compare between the different treatments within the same period*
- *RPS= Relative percent of survival.*

DISCUSSION

The way of the antigen preparation and the route of administration to fish are principal factors in obtaining effective vaccination *(25)*. During past 1–2 decades, much attention has been given to develop suitable vaccine based on antigenic nature of used strains and route of administration for controlling diseases in aquaculture *(26,27)*.

Although several vaccine delivery methods are available, injection is the most effective and the most reliable *(28)*. Non-specific immune parameters, lysozyme activities, NBT activity and neutrophils adherence in fish are important defense mechanism to protect it from bacterial infections but there is a paucity of information on the influence of vaccine on the activity of non-specific immune mechanisms *(29)*.

In the present study, there was a significant difference in the lysozyme activity, NBT activity and neutrophils adherence between the control and immunized groups in the 1st, 2nd and 4th weeks of immunization. The same results were reported by Dash et al *(30)*.

In fish, bactericidal activity is considered one of the major defence mechanisms in the early stages of microbial infections *(31)*. In the present study, serum bactericidal activity of immunized Nile tilapia against *Ps. fluorescens* was significantly increased in all immunized groups in comparison with control group at all periods of experiment except at 1st week post vaccination. On the other hand, in a parallel study, serum bactericidal activity analyses of vaccinated fish (32%) against *Edwardsiella tarda* was 2.3-fold lower than unvaccinated fish (72%) *(32)*. Also similar vaccine-induced serum killing effect against *V. anguillarum* has been observed previously in Atlantic cod *(33)*.

In the current study, antibody titer of Nile tilapia immunized by different *Ps. fluorescens* antigens was significantly increased in all immunized groups in comparison with control group at all periods. Antibody has been shown to correlate with protection for *Streptococcus iniae (34)* and *A. hydrophila (35)* in cultured fish species.

The mixture of sonicated and extracellular product antigen showed the best values of bactericidal activity and antibody titer against *Ps. fluorescens* followed by formalin killed antigen and sonicated cell antigen then extracellular product antigen. Bjõrnsdóttir et al. *(36)* showed that, a significant antibody response against sonicated bacterial cells was detected after vaccination but a significant response was not observed against the ECP antigens before or after challenge with *Aeromonas salmonicida*. ECP antigens were reported to has a stronger immune response to *Vibrio harveyi* and *Flavobacterum psychrophilum (37)*. The extracellular products (ECP) of bacteria is a factor responsible for a number of biological effects including immunostimulatory activities in different animals including fish *(38)*.

The percentage of the relative percent level of survival after challenge with *Ps. fluorescens* at 4, 6 and 8 weeks post vaccination were significantly increased in RPS in all immunized groups in comparison with control. Some results indicated that immunity is produced by systemic injection (IP or IM) of the ECP and FKC1ECP vaccines *(39)*. The RPS values obtained for *Y. ruckeri* using the ECP-vaccine ranged between 74.0 and 81.4%. These values are similar to or lower than RPS values reported previously for the vaccine against yersiniosis on the rainbow trout *(40)*. The higher values of the relative percent of survival was seen in the mixture of sonicated and ECP antigen followed by formalin killed antigen, sonicated cell antigen then extracellular product antigen. Unpurified ECP has been shown to be protective when 'toxoided' by formalin and chloroform and then established with lysine *(41)*. *S. salar* immunized with *Aeromonas salmonicida* sp. achromogenes ECP were found to elicit better protection than formalin killed whole-cells *(42)*. The mixture of killed-cells and concentrated ECPs from Jeju-45 led to significant protection against the homologous isolate of *S. iniae* in olive flounder, followed by killed cells then the ECPs.

It could be concluded that, although all prepared vaccines are efficient against *Ps. fluorescens* infection through injection route, a mixture of sonicated and extracellular product antigen seemed superior to other vaccines especially in bactericidal activity, antibody titer and RPS against *Ps. fluorescens*. However, as injection vaccination is difficult to apply in a large scale, further trials on Nile tilapia using same vaccines through immersion is advised to test applicability and the cost-effectiveness for the commercial use.

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الملخص العربي

تأثير اللقاحات المختلفة المحضرة من ميكروب السيدومونس فلورسنس علي الاستجابة المناعية النوعية والغير نوعية في أسماك البلطي النيلي

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تم دراسة اربعة لقاحات مختلفة محضرة من ميكروب السيدوموناس فلورسنس (لقاح الفورمالين الميت، معلق المنتج خارج الخلية، معلق الخلية المعاملة بالالترسونك وخليط المعلقين الاخيرين) وذلك بغرض التعرف علي افضل الطرق للسيطرة علي العدوي بهذا الميكروب في اسماك البلطي النيلي.

وقد تبين زيادة معنوية في مستوي الاختبارات المناعية المقاسة (نيتروبلوتترازوليم ، نيتروفيل ادهرينس ونشاط الليزوزيم) في كل المجموعات الملقحة بالمقارنة بالمجموعة الضابطة خلال الاسبوع الثاني والرابع بعد التحصين. كما لوحظ زيادة معنوية في اختبار النشاط القاتل للبكتيريا ومعدل الاجسام المضادة ضد ميكروب السيدوموناس فلورسنس في كل المجموعات المحصنة خلال التجربة. وقد ابدي خليط اللقاحين المعلقين افضل النشاط القاتل للبكتيريا ومعدل الاجسام المضادة ضد ميكروب السيدومونس فلورسنس.

وقد أوضحت نسبة الاعاشة النوعية بعد اختبار التحدي بإستخدام ميكروب السيدوموناس فلورسنس بزيادة نوعية بعد التحصين في جميع المجموعات عند الاسبوع الرابع والسادس والثامن بعد التحصين بالمقارنة بالمجموعة الضابطة.

وقد كانت أكبر نسبة اعاشة نوعية في المجموعة المحصنة بخليط اللقاحين المعلقين تتبعها لقاح الفورمالين الميت ثم لقاح الخلايا المعاملة بالالترسونك ثم لقاح منتجات خارج الخلايا.

وقد خلصت الدراسة الي ان كل اللقاحات المحضرة كانت مؤثرة ضد الإصابة بميكروب السيدوموناس فلورسنس بالرغم من ان خليط المعلقين الخلايا المعاملة بالالترسونك و منتجات خارج الخلايا يعطي أكثر فاعلية عن اللقاحات الاخري وخاصة في النشاط القاتل للبكتيريا ومعدل الاجسام المضادة ونسبة الاعاشة النوعية الخلايا المعاملة بالالترسونك ثم لقاح منتجات خارج الخلايا.