Vaccination Of *Dicentrarchus labrax* (European Sea bass) Against *Vibriosis*

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ABSTRACT

Vibriosis is one of the most important diseases of *Dicentrarchus labrax* (European Sea bass. Two protective vaccines were prepared from formalized cultures of *Vibrio alginolyticus* and *Vibrio damsela*. Relative percent survival index (PBS), lysozyme activity and nitric oxide production were detected in vaccinated fish by oral, immersion and injection routes. RBS ranged from 82.86 to 91.99 % in fish vaccinated by the two vaccines. Detectable rise of lysozyme and nitric oxide by both vaccines compared with that of control group. It could be concluded that *Vibrio* formalized vaccine protect against *Vibriosis* and enhance immune parameter.

Key words: *Vibriosis*, Sea bass, Formalized Killed Vaccine FKV, RPS, lysozyme activity and respiratory burst.

INTRODUCTION

Mariculture is one of the main sources of income in many countries worldwide *(1)*. European Sea bass *(Dicentrarchus labrax L.; Moronidae; Perciformes)* is a marine fish of great economic importance particularly in Mediterranean aquaculture *(2)*. *Vibrio* species remain among infectious disease agents posing a potentially serious threat to aquaculture *(3)*.Chemotherapy, vaccination and other prophylactic measures are generally adopted to control microbial infection *(4)* but development of drug resistant microbes *(5)* has prompted alternatives treatment of infection. Vaccination is becoming an increasingly important part of aquaculture, since it is considered a cost effective method of controlling different threatening diseases *(6)*.Vaccine is an effective protective measurement for controlling of *Vibriosis (7)*. Most bacterial vaccines used in aquaculture to date have been inactivated vaccines obtained from a broth culture of a specific strain subjected to subsequent formalin inactivation.

Injection and immersion methods are suitable only for intensive aquaculture and both require the fish to be handled or at least confined in a small space during the procedures. Oral vaccination is the only method economically suited to extensive aquaculture but can be administered in an artificial diet *(8)*. It was necessary to define the vaccination strategy term to include diseases vaccinate against, the vaccine type, vaccination method, the timing of vaccination and the revaccination *(6)*.

Thus this work was conducted for controlling of *Vibriosis* formalized killed bacterial vaccine.

MATERIALS AND METHODS

Experimental fish Two hundred and fourty apparently healthy Sea bass with an average body weight (60±10 g) were obtained from National Institute of Oceanography and Fisheries (NIOF) fish farms and acclimatized to experimental condition for two weeks in circular fiberglass aquaria each of 1000 L capacity. Fish were fed dry pelleted commercial

ration containing 40% crude protein twice daily at 3% of their body weight (ALLER AQUA EGYPT). About 25% of water volume was changed daily, water temperature was (25 ±3^{O}C), Dissolved Oxygen D.O 5±3mg/l, pH 7.4-8.8, salinity at 12±3‰ salinity and supplied with air systems. Fish were divided into two groups each of 120 fish; G1 vaccinated with *Vibrio alginolyticus* FKV and G2 vaccinated with *Vibrio damsela* FKV, each group was divided into four subgroups in three replicates each of 10 fish replicate, first subgroup served as control, second, third and fourth subgroups served as injection, immersion and oral subgroups respectively.

Preparation of *Vibrio alginolyticus* and *Vibrio damsela* antigen

The antigens were prepared using *V. alginolyticus* and *V. damsela* strains. They were identified morphologically and biochemically ***(9)***. Both strains were grown on tryptone soy broth with 2.5% NaCl and incubated for 24 hr. at 28^{O}C ***(10)***. The cells were harvested by centrifugation at 4000 rpm for 10 minutes, then washed twice with sterile Phosphate Buffered Saline (PBS) and then re-suspended in (PBS) to the desired concentration 1.5 × 10^{8} CFU ml^{-1} under sterile condition. Bacterial cells with 1.5 × 10^{8} CFU ml^{-1} were inactivated by overnight soaking in 0.3% formalin ***(11)***. Prepared formalized killed vaccines were kept at room temperature for 24 h and then stored at 4 ^{O}C. For test sterility, 0.1 ml of the suspensions was streaked on TSA with 2.5 % NaCl plates and incubated for48 h at 28^{o}C***(12)***.

Adjuvant: Adjuvant was prepared by mixing sodium alginate with antigen and formulated with ration ***(11)***.

Route and dose of administration:The vaccines were kept at room temperature before vaccination process and all fish were anaesthetized in MS222 (100 mg l^{-1}) ***(13,14)***.

1. Injection route: -Prepared vaccine was injected intraperitoneally I/P with 100 µl of formalized killed vaccine ***(10)***.
2. Immersion route: Fish were immersed in diluted vaccine solution at 1:10 in sea water and the exposure time around 30 second and returned to the aquaria ***(15)***.
3. Oral route:-Preparation of fish pellets incorporated with formalized killed *V. alginolyticus* and *V. damsela* vaccine Under aseptic condition the vaccines were manually incorporated into commercial dry pellets at rate of 1.5×10^{8} CFU g^{-1} for treated fish. Prepared fish pellets were kept in 4^{O}C until use. Immunization was repeated two times at two weeks intervals ***(12)***.

Vaccination process evaluation

After performing challenge test at the thirty days of experiment, the immunological parameters were assessed. The fish were starved for 24 h and anaesthetized before sampling. Blood samples were taken from the caudal blood vessels and centrifuged at 4000 rpm for ten minutes. Serum samples were stored at -20 ^{O}C until further analysis.

Challenge test

Preparation of bacterial inoculum: Selected isolates were grown onto tryptone soy agar at 27^{O}C for 24 hours. The pure culture of *V. alginolyticus* and *V. damsela* strains was suspended into a sterile saline. A suspension of the organisms was adjusted to (1.5x 10^{8}CFU/ml), using McFarland standard tubes ***(16)***.

Experimental design

Bacterial isolates were diluted in 0.9% sterile saline solution. The first *D. labrax* fingerlings group (G1) was injected intraperitoneally with 0.1 ml of *V. alginolyticus* bacterial suspension at approximately 1.5 × 10^{8} CFU/ml. The second group (G2) was injected intraperitoneally with 0.1 ml of *V. damsela* bacterial suspension at approximately 1.5 × 10^{8} CFU/ml. The control group was injected with 0.1 ml of sterile saline solution. After bacterial inoculation vigorous aeration was given to reduce the stress. All infected fish were collected and observed for pathological signs and mortality. Mortality was considered positive when a pure culture of the same strain was recovered from dead fish. The mortality and survival (%) was calculated by Relative

Percentage Survival index (RPS). RPS value is the survival rate of fishes after vaccination when compared with control fishes.

$$RPS = \left[1 - \frac{\text{Mortality rate in vaccinated}}{\text{Mortality rate in non vaccinated}}\right] \times 100$$

Lysozyme activity

Lysozyme activity was tested by using the lyso-plate assay, which is based on the lysis of the bacterium *Micrococcus lysodeikticus*. Briefly, 2 mm wells punched on agarose plates containing *M. lysodeikticus* (50 Ag ml^{-1}) were filled with 5 µl of phosphate buffer (14.04 g/l KH2PO4, 5.2 g/l Na2HPO4, pH 6.2) or sample of serum. Plates were incubated at 25 ^{O}C for 20 h, and the lysis halo was measured. Chicken egg white lysozyme (CEWL, Sigma) at different concentrations was used as standard. Assays were performed in triplicate and results are shown as the mean value *(17)*.

Detection of Nitric Oxide (NO)

Briefly, 75 µl of supernatants were removed from individual wells and placed in a separate 96-well microtitre plate. One hundred microlitres of 1% sulphanilamide in 2.5% phosphoric acid was added to each sample followed by 100 µl of 0.1% N-naphthyl-ethylenediamine in 2.5% phosphoric acid. The optical density of each well was determined using an automated plate reader (Biotek) at 540 nm. The approximate concentration of nitrite in the samples was determined from a standard curve generated using known concentrations of sodium nitrite *(18)*.

RESULTS AND DISCUSSION

A vaccine against *Vibriosis*, which is an important disease of farmed marine fish, has recently undergone successful field trials and will be commercially available in the very near future.

The formulated vaccine was Formalized Killed Vaccine (FKV) and designed into injection, immersion and oral routes. The main aim was to assess the efficacy of this three vaccination routes under field conditions. As shown in table (1) and figure (1), the protection level represented in Relative Percentage Survival index (RPS), Lysozyme activity and Nitric Oxide afforded against both *Vibrio alginolyticus* and *Vibrio damsela (Photobacterium damselae subsp. damselae)* infection. The cumulative mortality of *Vibrio alginolyticus* challenged control group was (50%), whereas vaccinated injection and oral were higher (8.57 %) than immersion vaccinated group (5%). Concerning *Vibrio damsela* challenged control group showed cumulative mortality of (83.33%), whereas the immersion vaccinated group was the highest (8.57%) followed by injection group (7.5) and (6.67 %) for the oral route.

A statistically significant difference ($P < 0.05$) was found for the Relative Percentage Survival index (RPS), lysozyme and Nitric oxide production were found among vaccinated and non-vaccinated groups. The Relative Percentage Survival index RPS in vaccinated group by *Vibrio alginolyticus* FKV was (82.86%) for injection and oral vaccinated groups followed by (90%) for immersed vaccinated group. On the other hand, the Relative Percentage Survival index in vaccinated groups by *Vibrio damsela* FKV was (89.72, 90.99 and 91.99%) for immersed, injection and oral vaccinated groups respectively.

At the same time, vaccination elicited significant ($P<0.05$) stimulation of immune response represented in significant increase in lysozyme production in vaccinated groups than non-vaccinated groups. Vaccinated group by *Vibrio alginolyticus* FKV showed the highest lysozyme activity 288.3±2.55 U $mL^{-1}$for immersed followed by injection and oral vaccinated groups (244.17±5.31, 200.5±6.55) respectivly and the lowest (194.3 ±5.61 UmL^{-1}) was unvaccinated groups. The highest Relative Percentage Survival index (RPS), might be related to advantageous mode of the fast

protection indicated by higher lysozyme production *(8)*. Also, a degree of protection can be achieved by simply injecting the killed bacterial suspension *(15)*.

Concerning oral vaccination, the lowest lysozyme production and subsequent the highest (RPS) might be related to some factors related either to extrinsic manufacturing intrinsic digestive circumstances. The vaccine antigens have to be exposed very high temperatures and pressures associated with the feed manufacturing and extrusion process. Also, it can be difficult to ensure that all of the target fish receive sufficient vaccine. Moreover, the foregut of fish is highly acidic, and this can cause a problem in the presentation of the antigens in oral vaccination *(15)*.

On the other hand, vaccinated group by *Vibrio damsela* formalized killed vaccine showed the highest lysozyme activity in injected groups (266.23±1.65) followed by oral and immersed vaccinated groups (222.1±4.23 & 200.5±2.65 U mL^{-1}) respectively. Lower lysozyme production and subsequent some extent higher Relative Percentage Survival index (RPS) might be related to some levels of short term immunity achieved by immersion vaccination. This technique limited by some factors including size, individual value and reaction to the general stress of handling *(8, 15)*.

Another immunological judgment of vaccine efficacy is Respiratory burst assays. Respiratory burst (sometimes called oxidative burst) is the rapid release of reactive oxygen species (superoxide radical and hydrogen peroxide) from different types of cells. Reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) work together to damage cells and have been implicated in the pathogenesis of several disease states. RNI are a family of molecules derived from nitric oxide (NO) and superoxide anion (O_2^-), produced via nitric oxide synthase (NOS) and NADPH oxidase, respectively *(19)*.

The Nitric Oxide was significantly stimulated ($P < 0.05$) in vaccinated groups than non-vaccinated control groups. Vaccinated group by *Vibrio alginolyticus* formalized killed vaccine showed the highest Nitric Oxide production (396.6±2.61 µM) for immersed followed by injection and oral vaccinated groups (318±3.31 & 311±3.16) respectively and the lowest (295.7±2.6µl µM) was unvaccinated group. On the other hand, vaccinated group by *Vibrio damsela* formalized killed vaccine showed the highest Nitric Oxide production in injected groups (365.4±6.21) followed by oral and immersed (326.9±1.35 & 322.8±3.65 µM) vaccinated groups respectively. It was observed a slight increase of the respiratory burst of leukocytes from fish injected with formalin-killed bacteria post injection *(20)*. Also, infection of gilthead Sea bream, with *V. anguillarum* resulted in the priming of the respiratory burst (Oxidative burst) of leukocytes *(21)*.

In sharp contrast, Sea bass were injected intraperitoneally with formalin-killed *V. anguillarum* resulted in a partial inhibition of the respiratory burst at 4 h post-infection and a complete inhibition at 24 h post-infection. The compared behavior of these immunological parameters in the three vaccine routes might be related to the difference strength of each vaccine and the condition of each individual *(20, 22)*.

Conclusion

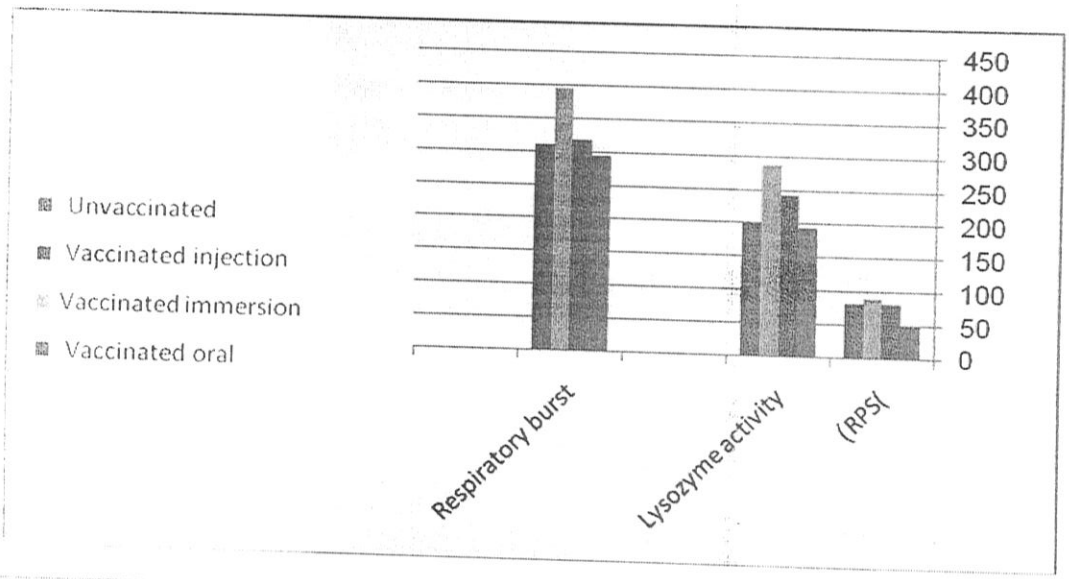
Each vaccination method have advantages and limitations depending upon different farm circumstances, the type of disease, nature of the vaccine and the target stock Therefore, it is the time to propose a complete immunization schedule for development of more effective vaccines for the diseases in aquaculture.

Table 1. Showing parameter assays of *Vibrio* vaccines in tested groups

Groups N=30	*Vibrio alginolyticus*				*Vibrio damsela*	
	Relative Percentage Survival index (RPS)	Lysozyme activity U mL-1	(Nitric oxide) µM	RPS	Lysozyme activity U mL-1	(Nitric oxide) µM
Control group		194.3 ±5.61	295.7±2.6		194.3 ±4.32	295.7±5.42
Injection group	82.86	244.17±5.31*	318±3.31*	90.99	266.23±1.65*	365.4±6.21*
Immersion group	90	288.3±2.55*	396.6±2.61*	89.72	200.5±2.65*	322.8±3.65*
Oral group	82.86	200.5±6.55*	311±3.16*	91.99	222.1±4.23*	326.9±1.35*

*Asterisk indicates that the value is significantly different from the control group ($p < 0.05$).

(A)



(B)

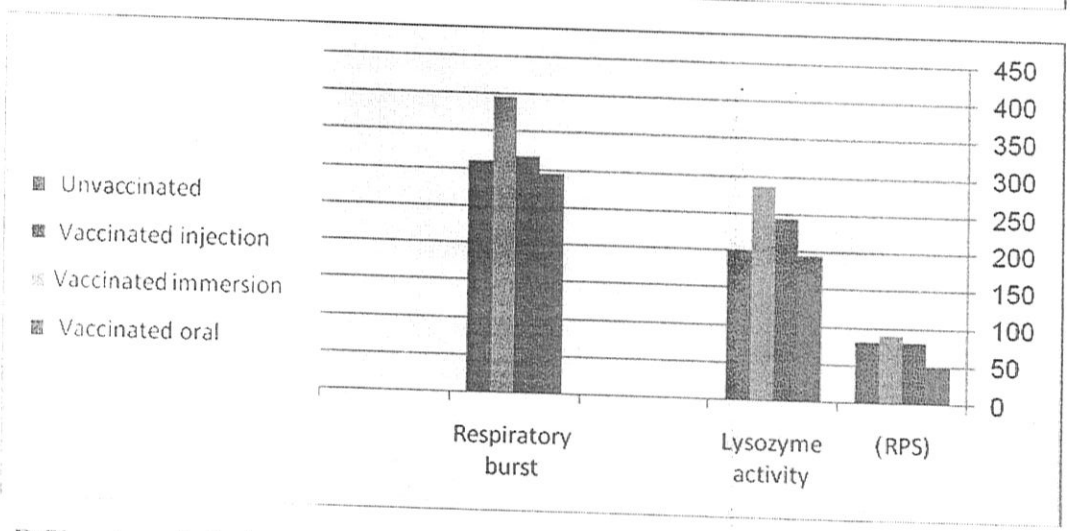


Figure A and B.Showing Relative Percentage Survival index RPS, lysozyme activity and respiratory burst (Nitric oxide) production for vaccinated and non-vaccinated groups by *Vibrio alginolyticus* (A) and *Vibrio damsela* (B) formalized killed vaccine.

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الملخص العربي

تحصين اسماك القاروص الاوروبى ضد مرض الفبريو

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يعتبر مرض الفبريو من أهم المشكلات البكتيرية المرضية التي تواجه الاستزراع البحري وبخاصة اسماك القاروص الأوروبي. ومن هذا المنطلق تم تحضير لقاح بكتيري ميت من عترات الفبريو المعزولة وهى فبريو الجينوليتيكس وفبريو دامسيلا وتم إجراؤها في ثلاث طرق بالحقن، التغطيس والفم من خلال تجربة استمرت ثلاثين يوما وبعدها تم تقييم عملية التحصين من خلال تحديد نسبة الإعاشة بعد إجراء العدوى التجريبية ، حيث كانت في المجموعات المحصنة بلقاح الفبريو الجينوليتيكس أعلاها في المجموعات المحصنة بالتغطيس ٩٠% يليها المحصنة بالحقن والفم ٨٢,٨٦%. على الجانب الأخر، ففي المجموعات المحصنة بلقاح الفبريو دامسيلا فكانت نسبة الإعاشة أعلاها في المجموعات المحصنة بالفم ٩١,٩٩ بعدها الحقن ٩٠,٩٩ ثم التغطيس ٨٩,٧٢%.نسبة الليسوزيم في المجموعات المحصنة بلقاح الفبريو الجينوليتيكس كانت أعلاها في طريقة التحصين بالتغطيس٢,٥٥± ٢٨٨,٣ $U\ mL^{-1}$ بعدها طريقة الحقن ٥,٣١± ٢٤٤,١٧ والفم ٦,٥٥± ٢٠٠,٥ واقلها المجموعات الغير محصنة فكانت ٥,٦١± ١٩٤,٣ $U\ mL^{-1}$. أما بالنسبة للمجموعات المحصنة بلقاح الفبريو دامسيلا فكانت أعلى نسبة لليسوزيم في طريقة التحصين بالحقن ١,٦٥ ± ٢٦٦,٢٣ بعدها طريقة الفم ٤,٢٣± ٢٢٢,١ثم التغطيس حيث كانت ٢,٦ ± ٢٠٠,٥ U mL^{-1}. قدرة خلايا الدم البيضاء على الأكسدة باستخدام النيتريك اكسيد.فكانت قيمة أكسيد النيتريك في المجموعات المحصنة بلقاح الفبريو الجينوليتيكس أعلاها فيطريقة التحصين بالتغطيس ٢,٦١± ٣٩٦,٦ µM يليها طريقة الحقن ٣,٣١± ٣١٨ ثم الفم ٣,١٦ ± ٣١١ وكانت اقل قيمة في المجموعات الغير محصنة٢,٦ ± ٢٩٥,٧ µM . أما بالنسبة للمجموعات المحصنة بلقاح الفبريو دامسيلا الميت فكانت اعلي قيمة لأكسيد النيتريك في طريقة التحصين الحقن ٦,٢١ ± ٣٦٥,٤ يليها الفم ثم التغطيس١,٣٥ ± ٣٢٦,٩ و ٣,٦٥± ٣٢٢,٨ µM ترتيبا. يتضح من خلال تلك المعايير إن هذين النوعين من اللقاح البكتيري كانا ذا كفاءة في الثلاث طرق بدليل ارتفاع معدل الإعاشة والليسوزيم وأكسيد النيتريك معنويا في المجموعات المحصنة عن المجموعات الغير محصنة. ومن ثم فان التحصين كأسلوب من الأساليب الوقائية من الأمراض وأدى إلى تحسين معدل الإعاشة والأداء المناعي لأسماك القاروص الاوروبى المصابة بمرض الفبريو.