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IMMUNOLOGICAL AND BIOCHEMICAL RESPONSES IN ASIAN SEABASS FINGERLINGS (LATES CALCARIFER) VACCINATED WITH VIBRIO ANGUILLARUM BACTERIN

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SUMMARY

Groups of apparently healthy Asian seabass fingerlings (*Lates calcarifer*) weighing from 20 to 30 g were immunized against vibriosis by a vaccine in the form of formalin killed bacteria of Vibrio anguillarum that originated from diseased fish. This immunization was administered by three different routes which were bath immersion, orally (by feeding) or intraperitoneal injection (IP). Immunization process was followed with booster dose by the same routes 2 weeks after the 1st dose. The efficacy of this vaccination was evaluated based on immune responses (specific and non specific) and protection against bacterial challenge performed by IP inoculation with 0.1 ml of bacterial suspension of live virulent *V.anguillarum* at concentration of 1.2 x 10^8 c /ml. Antibody titres (as specific immunity), phagocytic activity phagocytic index and serum bactericidal activity (as non-specific immunity) , mortality % and relative percentage survival (RPS) as challenge indicators against *V.anguillarum* were determined. Also, serum: total protein, albumin and globulin contents and albumin / globulin ratio (A / G) were estimated. In the present study serum antibody titres, phagocytic activity and phagocytic index were significantly increased in different vaccinated groups after one and two weeks from the 1st dose of vaccine and after one week of booster dose of vaccine. Except, only one week after the 1st dose of vaccine, bath immersion vaccinated group showed no changes in serum antibody titres. Detection of serum bactericidal activity showed that there were significantly higher levels against V. anguillarum as marked reduced survive index (SI) in all vaccinated fish compared with controls at different sampling times. Serum bactericidal activity in

oral and IP injection of vaccinated fish groups showed higher levels by significantly indicated reduced survive index (SI) more than in bath immersion vaccinated group at different sampling times. The vaccination conferred protection without any mortalities against *V. anguillarum* challenge as relative percentage survival (RPS) = 100 % for each group of oral and IP injection vaccination. RPS in the bath immersion vaccinated group was 60 % with 40% mortality, while, in control group there was no protection, where, RPS was zero and mortality was 100 % Serum total protein and globulin contents were significantly increased in all vaccinated groups one and two weeks after 1st dose of vaccination and also one week after the booster dose. Albumin contents of serum were significantly decreased in bath immersion and oral vaccinated groups after one week from the 1st and booster doses of vaccination. A / G ratios were significantly decreased in bath immersion and oral vaccinated groups after one week from the 1st and booster doses of vaccination Also, A / G ratios in IP injection vaccinated groups were significantly decreased after two weeks and one week from the 1st and booster doses of vaccination respectively Thus Asian seabass fingerlings gave good protective immune responses to Vibrio anguillarum vaccine when vaccinated by direct bath immersion, orally or IP injection but in the two last routes it was better and marked protective immunity were recorded

INTRODUCTION

Special care must be taken to control opportunistic fish pathogens in the intensive suboptimal culture conditions. Although prophylactic treatments using antibiotics have been used with some success in controlling such diseases, these chemotherapeutic remedies were widely criticized for their negative impacts (Anderson, 1992). Therefore, vaccination strategies are generally targeted to increase antibody levels in fish to provide protection against pathogens. The Vibrio group comprises microorganisms with a dynamic role in the marine and brackish environments They are isolated from seawater, sediment, plankton, invertebrates and fish. They are abundant members of the micro flora of aquatic organisms (Colwell, 1984). Vibriosis as a fatal per acute or acute disease caused by *V.anguillarum* bacteria and is characterized by a generalized haemorrhagic septicaemia with necrosis and inflammation of internal organs in case of acute form, while, in chronic form the lesion showing a swelling or haemorrhagic ulcer located on the caudal peduncle of the fish so called as "saltwater furunculosis" (Noga, 2001). This disease affects several marine fish species world wide especially intensively cultured fish (Toranzo et al., 2005; Zhou et al., 1997, Mo et al., 2002 and Austin and Austin, 1987), often with high mortalities in the stages of fry and fingerlings (Noga, 2001). For controlling this disease, antibiotics were used especially in aquaculture. There are increasing risks for the use of

chemotherapeutic agents due to their adverse effects on the environment, their residues in the food and the potential hazards of antimicrobial resistance (Ellis, 1988a & b and Jian and Wu, 2003). Vaccination as specific protective immunity with bacterial cell preparations was required against *V.anguillarum* in various fish species (Li et al., 2005; Espelid et al., 1991; Mikkelsen et al., 2004 and Mikkelsen et al., 2007). However, *V . anguillarum* is a particulate antigen, appears to be acting as a thymusñindependent antigen and survived better in seawater if pathogenic bacteria were present (Olafsen et al., 1981). Challenge experiments confirmed that antibody titres were correlated with protection from V. anguillarum challenge. Vaccination of fish against bacterial diseases using commercially prepared bacterin, namely for *V.anguillarum* and Yersina ruckeri has been employed in salmonid farming for many years (Busch, 1983; Tebbitt and Goodrich, 1983). The efficacy of Vibrio vaccine varied depending on V. anguillarum serotype isolate used for challenge (Mikkelsen et al., 2007). This was recorded in cod, Gadus morhua L., for classical vibriosis which caused by V. anguillarum as the major bacterial disease from the hatchery and through the whole life cycle of the cod (Schroder et al., 2006). As reported by Chen and Chen (2001) an ideal vaccine should be relatively inexpensive to produce, easy to administer and safe. Commercial vaccine against Vibriosis is formalin-killed (0.3% v/v) whole cell preparations (Austin, 1984). The present study is the first report on a successful vaccination of Asian seabass fingerlings (*Lates calcarifer*) under cultural and hypersaline conditions and environment of the Red Sea coast in the Middle East. The objectives of the present study was to evaluate the efficacy and ability of a laboratory formalinñkilled *V.anguillarum* bacterin which prepared from a strain originally isolated from diseased Asian seabass (lates calcarifer) and administrated in initial and booster doses, through investigation of the protective specific immunity as antibody titres and non-specific immune parameters as phagocytic activity, phagocytic index, serum bactericidal activity and challenge with live virulent *V.anguillarum.* Also, effects of vaccination on some serum biochemical parameters as serum: total protein, albumin and globulin contents and albumin / globulin ratio (A / G) were estimated in Asian seabass fingerlings (*lates calcarifer*).

MATERIALS AND METHODS

*** Fish and experimental design**: This study was carried out during November and December, 2006 at The Fish Farming Center (FFC), Jeddah, Saudi Arabia. Four groups of apparently healthy Asian seabass fingerlings (*lates calcarifer*) as 60 fish for each group in weight range of 20- 30 g / fish in triplicate were maintained in hemisphere fiberglass tanks of 500 l capacity for each tank where, one group was the control non vaccinated and the other groups were for experimental vaccination. Acclimatization of fish in ambient labora-

tory conditions for 10 days was applied using high pressure sand filtered seawater all over the period of study. About 25 % of water was replaced daily along with the removal of waste feed and faecal materials. The acceptable limits of water quality throughout the experiment were measured systematically every two days to maintain its optimal level as: temperature was from 25 to 28°C, dissolved oxygen concentration was >5.0 mg / l, pH = 7.44+/-0.76, salinity was 42- 43 %0, total nitrogen ammonia concentration not exceeded 0.06 mg / l and nitrite nitrogen did not exceed 0.03 mg / l during the whole period of the study. Fish were fed with formulated diet of commercial pellets (as 48 % of protein) at the rate of 3% of their body weight divided twice daily as it is recorded for the rate and frequency of the fish feeding program in The FFC. All the fish were anaesthetized with sedative dose of clove oil in concentration of 0.1 ml of clove oil for 10 l of seawater before any route of vaccination and before blood collection from the caudal blood vessels to minimize the possible stress induced during handling. Fish were starved 24 h before the time of vaccination and before sampling time

* **Bacterial strain and vaccine preparation**: V. anguillarum was isolated from mortalities among cultured Asian seabass fingerlings (lates calcarifer) in FFC, Jeddah, Saudi Arabia. These isolates were cultured on Thiosulphate Citrate Bilesalt Sucrose (TCBS) agar (Oxoid) plates at 28oc for 48 h These bacteria were locally identified by using MicrobactTM [GNB 24E] (Oxoid) gram negative identification system as small motile rods in the laboratory using biochemical reactions (Farmer and Hickman, 1992) as well as based on the characteristics described in Bergey's manual of systemic bacteriology (Holt et al., 1994). The cultures were stocked in TCBS slants at 4oc. These cultures were used to prepare the vaccine and to infect the fish as challenge experiment in this study.

(a) Propagation of *V. anguillarum:* According to the method of Lin et al. (2006), 1 L of Brain Heart Infusion Broth (Oxoid) contained in 2 L Fernback flask the colonies on the cultures were scraped (harvested) from the plates to be inoculated and mixed in this broth then incubated at 28oc with moderate shaking for 24 h. By centrifugation at 3000 rpm / 10 min the bacterial cells were collected as pellets and resuspended in sterile PBS at pH= 7.2 to a concentration of approximately 15 x 10^8 colony forming units (CFU / ml) as measured by a number 5 MacFarland nephelometry standard according to Magarinos et al. (1994a).

(b) Bacterin preparation: The already isolated and identified *V.anguillarum* strain was inactivated by 3 % formalin and incubating overnight. Then, the bacterial cells were collected by centrifugation at 3000 rpm for 20 min and washed three times with PBS and centrifugation at 3000 rpm. The cells were finally resuspended in PBS at a concentration of 15 x 108 / ml (McFarland Standard No. 5) according to Magarinos et al. (1994a)

and stored at 4°Ç until use. The inactivation of bacteria was confirmed by plating 0.1 ml of bacterial suspension on BHI agar (Oxoid) with 2%NaCl and incubating at 25°C for 24 h, where, absence of bacteria from plating indicating inactivation.

* **Vaccination:** Following the recommendations of Amend (1981) and Cardella and Eimers (1990), immunization for groups of the fish except not vaccinated control one was conducted after the acclimatization period by bath immersion, oral (mixed with feed) or IP injection with prepared bacterial suspension containing killed V. anguillarum whole cells bacterin (WCB). First group was the control; the 2nd one was exposed to vaccine preparation for 1 h after dilution with sterile seawater to obtain a concentration of approximately 10^8 cells / ml. The 3rd group of fish was vaccinated orally by feeding on a ration mixed with prepared vaccine in concentrations of 15 x 10^8 cells / g once at the time of vaccination, while, the fish of the 4th group were inoculated IP with 0.2 ml of the vaccine (WCB) in concentration of 15 x 10^8 cells / ml. After two weeks from the first dose of vaccination booster doses were administered to the experimental groups by the same doses, routes, suspensions and concentrations as in initial vaccination. In case of bath immersion vaccination (initial & booster doses) the water inlet was closed and the level of water was adjusted to 60 L, then vaccine was added to the tank, where, fish were exposed to the vaccine for 1 h with vigorous oxygenation before the water flow was restored to normal. In case of oral immunization (initial & booster doses) the bacterin suspension was mixed with quantity of feed of oral experimental group 24 h before time of administration of vaccination and this mixture was dried at room temperature and stored at 4°C till used.

* **Challenge experiment:** 60 fish as well in each experimental group as in control group were challenged by IP injection of 0.1 ml with virulent strain of V. anguillarum at concentration of 1.2 x 10^8 cells / ml at the same time after one week from the booster dose of vaccination. The injected fish were observed for a period of 3 weeks for pathological lesions, recording number of mortality and removing of dead fish from tanks. The dead fish were examined for lesions and investigation of the aetiological agents, Where, V. anguillarum was re-isolated from each dead autopsied fish. The challenge was terminated when the mortality had ceased in all groups and there were no any lesions. From the number of survival in control and experimental groups, percentage of mortality was calculated. Potency of vaccine by different routes was measured by calculating the RPS (Amend, 1981) using the formula:

RPS = 1 - {vaccinated mortality % / non-vaccinated (control) mortality %} x 100.

* **Collection of blood samples:** At 7 days intervals through the whole period of the present

study a six number of randomly chosen fish from each experimental and control groups were taken after 24 h of final feeding for blood collection. Blood was collected from the caudal blood vessels with 24 gauge needle and 2 ml plastic syringe. Blood samples were divided into two aliquots, one was transferred immediately into 2 ml micro centrifuge tubes containing 0.2 ml anticoagulant (12.5% sodium citrate) and stored at 4oc for leukocrit study (Blaxhall and Daisley, 1973). The remaining blood was kept in room temperature for one hour without anticoagulant to collect serum by centrifugation at 3000 rpm for 10 min and stored at ñ20 oc for immunological assessment and biochemical analysis. All the samples collected were analyzed in triplicate.

* **Determination of phagocytic activity and phagocytic index**: The ability of fish phagocytes to ingest foreign particles is usually demonstrated with an in vitro phagocytosis assay. In the present study, a laboratory in vitro phagocytosis assay. Rate of phagocytosis of blood samples was estimated according to Siwicki et al. (1994). Fifty Ug *Candida albicans* culture (previously adjusted to 1g Candida albicans / 100 ml saline) was added and mixed to 1 ml of freshly citrated collected blood from fish of both control and experimental groups and shaken in water bath at 23- 25°Ç for 3- 5 h. Then, smears of the blood were fixed with methanol for 5 minutes and stained with Giemsa stain (MERCK / Germany). Smears washed three times with distilled water and dried at 40°Ç. The slides were oil immersion microscopically examined at 100 xs for phagocytes engulfing Candida albicans. Phagocytosis was estimated by determining the proportion of macrophages which contained intracellar yeast cells in a random count of 100 macrophages and exposed as percentage of phagocytic activity (PA). The number of phagocytized organisms was counted in the phagocytic cells and called as phagocytic index.

$$\text{Phagocytic activity (PA)} = \frac{\text{Number of phagocytic cells containing yeast cells x 100}}{\text{Number of all phagocytic cells in the field}}$$

$$\text{Phagocytic index (PI)} = \frac{\text{Number of yeast cells phagocytized}}{\text{Number of phagocytic cells}}$$

* **Serum antibody levels:** The agglutinating antibody titer was the reciprocal of the highest serum dilution at which agglutination occurred against formalin- killed antigen of V. anguillarum strains. These titers were monitored using a standard micro-agglutination procedure (Roberson, 1990).

* **Serum bactericidal activity:** Serum bactericidal activity to V. anguillarum was determined according to Rainger and Rowley (1993). Briefly, in a sterile eppendorf tube 300 ul of fresh serum of each sample mixed well with 300 ul of V. anguillarum suspension (15 x 10^3 cells / ml) and in another sterile eppendorf tube as blank same quantity of *V.anguillarum* suspension of the same concentration was mixed well with 300 ul of sterile PBS (pH= 7.2). All tubes of samples

and blank were incubated at 28oc. 50 ul from these incubated tubes were removed at 0, 1, 2, 3 and 4 h. and different serial dilutions were plated on 2216E and incubated at 28oc for 24 h and colony-forming units (CFUs) were counted. The bactericidal activity of test serum was expressed as percentage colony forming units in the test group to that control group (% CFU / control), where, from the number of *V. anguillarum* colonies in each plate, total Colony Forming Unites (CFU) in each sample was estimated and the results were recorded as survival index (SI) values (Wardlaw and Unlles, 1978) calculated as follows:

SI = CFU at end / CFU at start X 100

* **Determination of some serum biochemical parameters:** By using of assay kits (from Spinreact, S.A.U. Ctra. Santa Colona, Spain) and spectrophotometer (APEL, UV- VIS, PD-303UV) collected sera samples were analyzed for total protein and albumin contents according to methods of Koller (1984) and Burtis et al. (1999) respectively. Serum globulin content (subtracting albumin from total protein) and the albumin- globulin ratio (A / G) were calculated.

* **Statistical analysis:** Obtained data from experiments were analyzed using one-way- unstacked ANOVA. Results were considered significant if $p<0.05$. Mortality in each group challenged was compared statistically with the control group using Chi-square test (Zar, 1974).

RESULTS

* **Antibody titres:**

Antibody levels were detected in different fish groups (non-vaccinated control, bath immersion, oral and IP injection) at different sampling times (zero time, one & two weeks after the 1st dose of vaccination (initial dose) and after one week from booster dose of vaccination). There were significantly increase in specific antibody levels against *V. anguillarum* in comparing with non-vaccinated controls. As shown in Table (1) these increases were significant at $p<0.01$ in bath immersion vaccinated groups after two weeks from initial dose of vaccination and after one week of the 2nd (booster) dose of vaccination respectively, while, the increases were highly significant at $p<0.001$ in oral and IP injection vaccinated groups after one and two weeks from the initial dose of vaccination and after one week from booster vaccination. On the other hand, specific antibody levels in bath immersion vaccinated fish were not affected after one week from the initial dose of vaccination.

* **Phagocytic activity and phagocytic index :**

Phagocytic activity and Phagocytic index were increased significantly in all vaccinated groups without any expatiation. These increases were significant at $p<0.01$ in bath immersion group after one and two weeks from the initial dose of vaccination for both parameters and for phago-

cytic index after one week from booster dose of vaccination. Increases in phagocytic activity in the same group were highly significant at $p<0.001$ (Table 2) after one week from booster dose of vaccination. Both parameters showed highly significant increases at $p<0.001$ (Tables 2&3) in oral and IP injection vaccinated groups at different sampling times except that the significant increases of phagocytic activity after one week from the initial dose of vaccination were at $p<0.01$ (Tables 2 & 3).

*** Serum bactericidal activity:**

There were significantly increased levels of bactericidal activities against *V. anguillarum* in vaccinated fish compared with not vaccinated controls as shown in Table (4). Bactericidal activity showed higher levels at $p<0.001$ in oral and IP injection vaccinated fish groups at different sampling times indicated by marked reductions in (SI). These higher bactericidal levels in oral and IP injection vaccinated groups were more than in bath immersion vaccinated group which were at $p<0.05$ and $p<0.01$ after one week from 1st dose and after two weeks from 1st dose and one week from booster dose of vaccination respectively. Blank showed highly significant ($p<0.001$) increase in (SI) in comparing with control at each sampling time indicating very low bactericidal activity in these tests .

*** Challenge tests and mortality:**

7 days post immunization program end (booster dose) all groups of Asian seabass fingerlings were subjected to IP injection challenges by using live virulent *V. anguillarum*. The cumulative percentage mortalities were recorded in all groups. Fish showed better protection levels in oral and IP injection vaccinated groups in comparison with control and bath immersion groups. Oral and IP injection vaccinated groups had no any mortality percentages and showed RPS 100 % in each group at the end of the study. After the end of 18 days from time of challenging with lethal dose of virulent live *V. anguillarum* cell suspension by IP injection non vaccinated control and bath immersion vaccinated groups showed 100 % and 40 % mortalities at significance of $p<0.01$ and $p<0.001$ respectively, while, RPS in both groups were zero and 60 % respectively. Lesions of challenging infection began to be appeared after 7 days and 11 days from IP injection of live bacteria in control and bath immersion groups respectively. The lesions were present as haemorrhages at the base of the fins, around the anus, in the lower parts of the opercular bone and in the skin. Dissection of infected fish showed large amounts of yellow ascites in the peritoneal cavity.

*** Changes in some serum biochemical parameters:**

Table (6) presented significant increased contents of serum: total protein and globulins at $p<0.05$ and $p<0.001$ in bath immersion, oral and IP injection vaccinated groups after one & two weeks

Table (1): The effect of vaccination with three different routs (bath immersion, oral and IP injection) against *V. anguillarum* on Asian seabass fingerlings (*Lates calcarifer*) antibody titres.

Time of sampling / Groups	Before the 1st dose of vaccination (zero time)	One week after the 1st dose of vaccination	Two weeks after the 1st dose of vaccination	One week after the booster vaccination
Control group#	2.333+/-0.516	2.667+/-0.516	3.000+/-0.000	2.667+/-0.516
Bath immersion group	3.000+/0.000	3.000+/-0.000	3.667+/-0.516**	4.000+/-0.894**
Oral group	2.667+/-0.516	4.000+/-0.000***	5.667+/-0.516***	6.333+/-0.516***
IP injection group	2.667+/-0.516	.333+/-0.516***	6.000+/-0.894***	7.667+/-0.516***

means non-vaccinated group
The observed values were expressed as mean value+/- standard deviation (S.D.).
Mean values with different superscripts are significance levels at (* = $p<0.05$), (** = $p<0.01$) and (*** = $p<0.001$) different from control.
Standard deviations (+/-S.D.) were based on a pooled estimate of variance from the ANOVA (one way- unstacked).

Table (2): The effect of vaccination with three different routs (bath immersion, oral and IP injection) against *V. anguillarum* on Asian seabass fingerlings (*Lates calcarifer*) phagocytic activity (PA).

Time of sampling / Groups	Before the 1st dose of vaccination (zero time)	One week after the 1st dose of vaccination	Two weeks after the 1st dose of vaccination	One week after the booster vaccination
Control group #	16.167+/-2.483	13.667+/-1.633	15.500+/-1.049	14.833+/-1.012
Bath immersion group	17.333+/-2.733	17.833+/-2.563**	18.500+/-1.517**	19.333+/-1.211***
Oral group	17.000+/-1.095	18.167+/-2.639**	20.333+/-1.862***	22.333+/-0.516***
IP injection group	15.667+/-1.966	18.500+/-1.049***	22.667+/-1.211***	26.000+/-0.632***

means non-vaccinated group
The observed values were expressed as mean value+/- standard deviation (S.D.).
Mean values with different superscripts are significance levels at (* = $p<0.05$), (** = $p<0.01$) and (*** = $p<0.001$) different from control.
Standard deviations (+/-S.D.) were based on a pooled estimate of variance from the ANOVA (one way- unstacked).

Table (3): The effect of vaccination with three different routs (bath immersion, oral and IP injection) against *V. anguillarum* on Asian seabass fingerlings (*Lates calcarifer*) phagocytic index (PI).

Time of sampling / Groups	Before the 1st dose of vaccination (zero time)	One week after the 1st dose of vaccination	Two weeks after the 1st dose of vaccination	One week after the booster vaccination
Control group #	18.833+/-2.137	15.66[illegible]+/-2.582	17.000+/-0.894	18.167+/-0.753
Bath Immersion group	20.667+/-1.751	22.000+/-3.347**	20.000+/-2.000**	21.000+/-2.000**
Oral group	17.667+/-1.033	23.667+/-2.805***	25.667+/-3.011***	29.000+/-0.894***
IP injection group	17.667+/-2.160	23.667+/-2.16[illegible]***	30.333+/-2.422***	36.000+/-0.632***

means non-vaccinated group
The observed values were expressed as mean value+/- standard deviation (S.D.).
Mean values with different superscripts are significance levels at (• = p<0.05), (•• = p<0.01) and (••• = p<0.001) different from control.
Standard deviations (+/-S.D.) were based on a pooled estimate of variance from the ANOVA (one way- unstacked).

Table (4): The effect of vaccination with three different routs (bath immersion, oral and IP injection) against *V. anguillarum* on the bactericidal activity in the serum of Asian seabass fingerlings (*Lates calcarifer*) as represented by survive index (SI).

Time of sampling / Groups	Before the 1st dose of vaccination (zero time)	One week after the 1st dose of vaccination	Two weeks after the 1st dose of vaccination	One week after the booster vaccination
Blank	85.667+/-4.546***	87.500+/-4.889***	86.167+/-4.875***	88.667+/-4.320***
Control group #	60.500+/-5.753	58.333+/-6.121	59.333+/-5.610	59.167+/-6.369
Bath immersion group	42.833+/-11.583**	42.333+/-11.396*	38.500+/-11.912**	39.333+/-11.776**
Oral group	47.167+/-10.907*	25.167+/-7.859***	21.500+/-5.992***	20.667+/-8.189***
IP injection group	44.500+/-9.094**	21.167+/-6.338***	18.000+/-6.033***	14.333+/-3.777***

means non-vaccinated group
The observed values were expressed as mean value+/- standard deviation (S.D.).
Mean values with different superscripts are significance levels at (• = p<0.05), (•• = p<0.01) and (••• = p<0.001) different from control.
Standard deviations (+/-S.D.) were based on a pooled estimate of variance from the ANOVA (one way- unstacked).

Table (5): Mortality percentage and relative percentage survival (RPS) in Asian seabass fingerlings (*Lates calcarifer*) after challenge with lethal dose as 0.1 ml at concentration of 1.2×10^8 cells / ml of live virulent *V. anguillarum* by three different routs (bath immersion, oral and IP injection).

Parameters / Groups	No. of dead fish	No. of survival fish	Mortality %	RPS
Control group #	60	0	100^{***}	0
Bath immersion group	24	36	40^{**}	60
Oral group	0	60	0	100
IP injection group	0	60	0	100

means non-vaccinated group
Values with different superscripts are significance levels at (•• = $p<0.01$) and (••• = $p<0.001$).
RPS values over 50 indicate positive effect of the vaccine (Amend, 1981).
Mortality in each group challenged was compared statistically with the control group using Chi-square test (Zar, 1974).

and after one week from 1st and from booster doses of vaccination respectively. Serum albumin levels and values of A / G ratios as well were significantly ($p<0.05$) decreased in bath immersion and oral vaccinated groups after one week from each 1st and booster doses of vaccination in comparing with controls as significantly ($p<0.05$) decreased values of A / G ratios in IP injection vaccinated groups after two weeks and one week from 1st and booster doses of vaccination respectively as shown in Table (6). Also, it was observed that the fish of the control groups in the present study showed increases in serum total protein, albumin and globulin contents and decrease of A / G ratio at sampling time of one week after booster dose of vaccination in comparing with controls at other sampling times.

DISCUSSION

Vaccination has proven to be a very efficient prophylactic method to prevent outbreaks of bacterial diseases of intensively cultured fish in aquaculture (Toranzo et al., 2005 and Larsen and Pedersen, 1997). It is as the alternative to chemotherapy and application of multifunctional phytochemicals in aquaculture is new venture and few work carried out in fish culture. Therefore, the only way of accurately measuring the potency of a vaccine against a pathogen is to determine under controlled laboratory conditions, whether or not the vaccinated fish are protected against the infectious agent from whom the vaccine is made (Ellis, 1988a & b). Protective immunity is usually assessed by the effect and the success of vaccination which relies on the recognition of specific bacterial antigens by the immune system (specific and non- specific) of the host (Mikkelsen et al., 2007) and by challenging with determining percentage of mortalities and RPS. Also, estimation of some serum biochemical parameters aid in evaluation of protective immunity. Antibody response is known to be an important component of the fish immune system and it was found that fish generated significant levels of serum antibody responses against *V. anguillarum* in vaccinated fish in comparing with non vaccinated ones. In the present study immunized Asian seabass fingerlings (Lates calcarifer) could produce specific antibody within one week of vaccination and this humoral immune response against *V. anguillarum* increased during the following weeks after vaccination as in agreement with Berg et al. (2007) in their vaccination study on Atlantic salmon (Salmo salar L.) . The first peak of antibody titre in the present study was observed at 7th day in oral and IP injection vaccinated groups in comparing with non vaccinated controls. In bath immersion there was no changes in antibody titres at the same time. This can be attributed to low vaccine concentration (antigen concentration) in the bath immersion or / and not enough duration of exposure to vaccine as reported by Schroder et al. (2006) when they vaccinated Atlantic cod (Gadus morhua L.), where, an insufficiently dip exposure in a diluted solution might result in a "low

dose" vaccination and poor production of immunity. Thus, for immersion vaccination, the protection seems to be dependent on the indicating that a prolonged exposure time might compensate for low antigen concentration. In other words, in case of bath immersion vaccination in this study, the antigen-specific antibody titres were enhanced in serum of fingerlings (Lates calcarifer) in the 2nd week after 1st dose of vaccination (initial dose) and more marked after the booster dose. Therefore, the value of a vaccine cannot only be measured by the antibody production of those animals that are vaccinated as investigated in channel catfish, Ictalurus punctatus, (Plumb and Vinitnatharat, 1993) because the presence or absence of humoral antibody does not necessarily reflect the degree of protection. However, there is no correlation observed between the level of agglutinating antibodies and protection as reported by Plumb and Vinitnatharat (1993) as well as antibody levels were not different as a result of size variations as recorded by Berg et al. (2007). Although oral and IP vaccination consistently produces higher titre antisera, but, the results of this study support the idea that oral and bath immersion vaccinations is more suitable and practicable routes for field aquaculture than IP vaccination. Indeed, there was some suggestion that fish experiencing two contacts with the antigen (first dose of vaccination and booster dose) mounted a secondary immune response. These findings can be explained by the failure of the microagglutinating assay to detect protective serum antibodies or by the fact that the cellular response is relatively more important than humoral immunity in protecting fish against Vibriosis. Similar results have been reported previously against Vibriosis in Ayu, Plecoglossus altivelis, (Kawano et al., 1984); in rainbow trout, Salmo gairdneri (Tatner and Horne, 1986) and in turbot, Scophthalmus maximus and rainbow trout, Onchorhynchus mykiss (Santos et al., 1991b). Also, similar results were recorded for other fish pathogens such as Aeromonas salmonicida in rainbow trout, Salmo gairdneri (Ellis et al., 1988) and Pasteurella piscicida in gilthead Sea bream, Sparus aurara (Magarinos et al., 1994a & b). Although the serum agglutination antibody titre increased in groups immunized, the migration and filtration of leucocytes in vaccinated fish was more characteristic and indicative. Immune response and non specific defense mechanisms may be important to withstand infection, especially in the early phases of the infection. As well granulocytes and macrophages play a central role in the cellular part of the non- specific defense of fish as phagocytic activity and phagocytic index were determined in this study. Non-specific defense in this study was marked and characteristic high levels of phagocytic activity, phagocytic index and bactericidal activity were in agreement with study of Li, et al. (2005) on efficacy of *V.anguillarum* antigen in Japanese flounder (Paralichthys olivaceus). Our results clearly indicated that serum bactericidal activities of three vaccinated groups were higher values in comparing with those of non vaccinated

control group from 7th day after vaccination and become more marked and internstic especially after booster dose. Vaccination in the present work conferred the best protection against V. anguillarum in oral and IP injection vaccinated groups represented as RPS = 100 % for each group, but in bath immersion vaccinated group the protection was moderate and it's RPS = 60 %. Mortality among non- vaccinated controls was 100%, while, it was 40% in bath immersion vaccinated fish and there was no mortality in fish immunized by oral or IP routes. All above present study results were in agreement with those of Espelid et al. (1991) on the humoral immune responses in cod, Gadus morhua when vaccinated with four strains of V. anguillarum; Mikkelsen et al. (2004) on the efficacy of vaccine in cod, Gadus morhua when vaccinated against Vibriosis; Schroder et al. (1992) on virulence, immunology and vaccination of V. salmonicida in cod, Gadus morhua; Schroder et al. (2006) on early vaccination against classical Vibriosis in Atlantic cod, Gadus morhua; Grontvedt et al. (2004) on bath vaccination and challenge against furunculosis in spotted wolfish, Anarhichas minor, and Johnson et al. (1982) on adult salmonids in response to vaccination against infection with *V. anguillarum* and Yersinia ruckeri. In the present study there were significant increase in contents of serum total protein and globulins in bath immersion, oral and IP injection vaccinated groups after one & two weeks and after one week from 1st and from booster doses of vaccination respectively. Serum albumin levels and values of A / G ratios were significantly decreased in bath immersion and oral vaccinated groups after one week from each 1st and booster doses of vaccination in comparing with controls. Also, values of A / G ratios were significantly decreased in IP injection vaccinated groups after two weeks and one week from 1st and booster doses of vaccination respectively. The increase in serum contents of total protein, albumin and globulin and decrease of A / G ratio in the fish of the control groups at sampling time of one week after booster dose of vaccination in comparing with non-vaccinated controls at other sampling times may be attributed to some stress conditions such as miss handling or oxygen depilation. This condition may be a reflex action of fishes as suggested by Ellis (1981) and Anderson et al.(1982) in their studies on stresses in fishes. Although the injection route, not reflecting the natural route of infection, it was more reproducible and provided a reliable indication for subsequent farm trials as recorded by Nordmo (1997), and therefore, vaccinated fish showed no mortality compared with the respective controls as well as oral rout of vaccination. Results of this study indicated that the prepared vaccine can induce cell- mediated immune responses and confer protection and high resistance against *V. anguillarum*. This is meaning that all surviving fish showed protection against the challenge with the high virulence strain of *V. anguillarum*. Calculated savings from an economically justified vaccination program should combine the value of vac-

cine that provides only moderately higher levels of protection (as vaccine efficacy) should combine the value of the disease risk level, increased survival, reduced antibiotic use, fewer compensatory fish and reduced carcass removal (disposal) costs as reported by Thorarinsson and Powell (2006). Therefore, these factors have a profound influence on the financial impact of prophylactic measures such as vaccines. In general, immersion and oral vaccination is an effective and practical method for mass vaccination of fish, while, IP injection vaccination unpractical and not used in intensively cultured fishes. Oral vaccines against bacteria may be disappointed presumably because protective antigenic determinants are destroyed in the gastrointestinal tract (Austin and Austin, 1987; Rodgers and Austin, 1985). Therefore, bath immersion vaccination may be the most suitable, practicable and available in mass cultured fishes for protection against Vibriosis.

It is concluded that Asian seabass fingerlings (Lates calcarifer) gave good protective immune responses to *V. anguillarum* bacterine when vaccinated by direct immersion, orally and IP injection and specific and non-specific immunity play an important role in conferring significant protection.

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الأستجابة المناعية و البيوكيميائية في أصبعيات القاروص الآسيوي (لاتيس كالكاريفر) الذى تم تحصينه بلقاح ميكروب الفيبرو أنجيلارم

صفوت إبراهيم يوسف شلبى (قسم الكيمياء الحيوية و التسمم الغذائي- معهد بحوث صحة الحيوان- مركز البحوث الزراعية- مصر)
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تم تحصين مجموعات من أصبعيات أسماك القاروص الآسيوي تزن الإصبعية الواحدة من ٢٠ إلى ٣٠ جرام ضد مرض الفيبريوزيز باستخدام لقاح في شكل بكتريا فيبرو أنجيلارم مأخوذة من أسماك مريضة و تم إماتة هذه البكتريا بالفورمالين. و تم إجراء هذا التحصين بثلاث طرق مختلفة و هي عن طريق حمام الغمر و عن طريق الفم (مع الغذاء) و عن طريق الحقن في داخل الغشاء البريتونى. و بعد أسبوعين تم أعطاء جرعة ثانية منشطة من نفس اللقاح بنفس الطرق السابقة. وقد تم تقييم كفاءة هذا التحصين استنادا إلى الاستجابة المناعية (النوعية و الغير نوعية) و الوقاية ضد تجربة التحدي البكتيري التي تمت بواسطة الحقن في الغشاء البريتونى بمعدل ٠,١ مل من معلق بكتريا الفيبرو أنجيلارم الحية و شديدة الفاعلية ذات تركيز ١,٢ x ١٠^٨ خلية / مل. و قد تم تعيين المناعة النوعية متمثلة بقيم الأجسام المضادة و كذلك تعيين المناعة الغير نوعية متمثلة في نشاط و في مؤشر الخلايا الأكولة و النشاط المميت للبكتريا في السيرم. أما النسبة المئوية للوفيات و معدل النسبة المئوية للبقاء (RPS) فقد تم اعتبارها مؤشرات تحدى ضد بكتريا الفيبرو أنجيلارم. وقد تم أيضا قياس محتويات السيرم من البروتين الكلى و الألبومين و الجلوبيلين و حساب نسبة الألبومين / الجلوبيلين.

و في الدراسة الحالية فإن قيم الأجسام المناعية و نشاط و مؤشر الخلايا الأكولة زادت جميعها معنويا في جميع المجموعات المحصنة بعد أسبوع و أسبوعين من الجرعة الأولى للتحصين و كذلك بعد أسبوع من الجرعة المنشطة (الثانية) من التحصين. باستثناء المجموعة المحصنة بالغمر بالحمام المائي فإنها بعد أسبوع واحد من الجرعة الأولى لم تظهر أي تغيرات في قيم الأجسام المضادة. و أظهر قياس نشاط إبادة البكتريا في السيرم مستويات عالية معنويا ضد بكتريا الفيبرو أنجيلارم ممثلة بانخفاض مميز في مؤشر البقاء في كل المجموعات المحصنة بالمقارنة مع المجموعة الضابطة عند كل أوقات أخذ العينات المختلفة. و قد كانت هذه النشاطات في كل من المجموعتين المحصنة عن طريق الفم و عن طريق الحقن في الغشاء البريتونى ذات مستويات عالية عنها في مجموعة التحصين عن طريق حمام الغمر و ذلك في كل أوقات أخذ العينات. و قد أعطى التحصين مناعة وقائية بدون أى وفيات عند إجراء تجربة التحدي للفيبرو أنجيلارم ممثلة بمعدل نسبة منوية للبقاء وصلت إلى ١٠٠% في مجموعتي التحصين عن طريق الفم و عن طريق الحقن في الغشاء البريتونى. أما في مجموعة التحصين عن طريق حمام الغمر فقد كان معدل النسبة المئوية للبقاء = ٦٠% مع نسبة وفيات = ٤٠%، بينما في المجموعة الضابطة كان معدل النسبة المئوية للبقاء = صفر و النسبة المئوية للوفيات = ١٠٠%.

محتويات السيرم من البروتين الكلى و الجلوبيلين زادت زيادة معنوية بعد التحصين بالجرعة الأولى وبعد الجرعة المنشطة الثانية في كل أسماك المجموعات المحصنة. أما محتوى السيرم من الألبومين فقد انخفض معنوياً في كل من مجموعتي التحصين بحمام الغمر و التحصين بالفم بعد أسبوع من كل من الجرعة الأولى و من الجرعة الثانية من التحصين. أما نسب الألبومين إلى الجلوبيلين فقد قلت معنوياً في مجموعتي التحصين بحمام الغمر و التحصين بالفم بعد أسبوع من الجرعة الثانية (المنشطة) من التحصين، و في مجموعة التحصين بالحقن في الغشاء البريتونى فقد قلت هذه النسبة معنوياً بعد أسبوعين من الجرعة الأولى من التحصين و بعد أسبوع من الجرعة الثانية (المنشطة) من التحصين على الترتيب.

و يستنتج من هذه الدراسة أن أصبعيات القاروص الآسيوي قد أعطت مناعة وقائية جيدة كاستجابة للتحصين بإحدى الطرق سواء بحمام الغمر أو بالفم أو بالحقن في الغشاء البريتونى ضد ميكروب الفيبرو أنجيلارم.