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Use of vaccines in controlling bacteria fish diseases caused by *Vibrio anuiliticus*

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ABSTRACT

The present study was carried out to evaluate the effect of vaccination strategies to control vibriosis in farmed marine fish in Damietta governorate-Egypt. Two types of vaccines were papered including formalin-killed bacteria as well as heat killed whole cell bacterins as administrated by intraperitoneal injection sea bream fish. Fish immunized with formalin killed vaccine gave rise disease resistance better than heat killed vaccine in challenge test as denoted by relative level of protection (RLP). Determination of single immune diffusion, micro agglutination techniques and total protein content showed that formalin vaccine produce a good disease protection than the heat killed vaccine. Complete blood count examination for detecting lymphocytic count, hemoglobin %, RBCs count and WBCs count showd that formalin vaccine was the best. The booster dose after three months declared elevation of immune status of the fish. Highly significant effect was observed in hemoglobin levels, circulating lymphocyte count and total protein content in fish immunized with formalin vaccine than heat killed vaccine

Key words: *Sparus aurata*, *Vibrio alginoticus*, Vibriosis, Vaccine, Vaccination, administration route.

INTRODUCTION

The aquacultures have grown rapidly over the last decades, and roof of this is the number of aquatic species, totaling to 600, that are being farmed worldwide (FAO, 2016). The rapid expansion and intensification of aquaculture production has led to the outbreaks of new pathogens and infectious diseases caused by viruses, bacteria and parasites, inflicting major problems in the fish farming industry (Geng *et al*., 2012). The majority of bacterial diseases in aquaculture production systems are caused by some causative agents include bacteria from short, Gram-negative rods belonging to the families Enterobacteriaceae, Pseudomonadaceae (*Pseudomonas*) or Vibrionaceae (*Vibrios*). Typically, they cause septicaemic and ulcerative disease conditions. The long, Gram-negative, myxobacteria of the family Cytophagaceae, which are not recognized as pathogens of warm-blooded animals, may also cause heavy mortality in fish stocks (Barbosa *et al*., 2011). Bacteria are the most common among the pathogens in cultured fish that cause mass mortality in aquaculture both marine and fresh water (Mancuso, 2014). Vibrio species are gram -ve bacteria of the family Vibrionaceae. Vibriosis is a deadly haemorrhagic septicaemic disease affecting various marine and fresh/brackish water fish, bivalves and crustaceans causing severe economic losses worldwide (Frans *et al*., 2011).

Treatment of diseases has focused on chemicals and antibiotics. Treatment of affected fish with antibiotics is effective, but gives rise to problems such as accumulated resistance in the bacteria, which renders the antibiotic useless (Choi and Oh, 2007). Accumulation of antibiotic residues in fish tissue and environment cause human and animal health risk. Vaccination is an effective prophylactic treatment for infectious diseases in fish

culture, but it may be very expensive and stressful to the fishes. A single vaccine is effective against only one specific type of pathogen, but limits the effectiveness for wide range of pathogens due to the complex antigenic structure **(**Ardo *et al*., 2008). The importance of vaccine development is associated with the prevention of diseases taking into account the triad of infection, were the interaction of the etiological agent, the host and the environmental conditions lead to the disease outbreak. Compared to terrestrial animals, controlling each one of these factors in aquaculture is almost impossible, water is the perfect vehicle for bacterial disease outbreak; therefore the vaccine preparation is specifically prepared for the affected site (Gudding, 2014).

Therefore, the main objective of the present study was to develop and compare the efficacies of various vaccine preparations against Vibriosis in silver sea bream. Also, to determine the potential protective mechanisms induced by each vaccination protocol. Moreover, to carry out field trails in vaccination and controlling of fish and detecting the efficacy of the prepared vaccines by Challenge test and estimating the Relative level of protection (RLP).

MATERIALS AND METHODS

Isolation and identification of vibrio species:

Vibrio species was isolated from naturally diseases sea bass, sea bream, mullet and eel. The diseased fish suffered from petechial hemorrhage, detached scales, exophthalmia and abdominal dropsy. The pathological changes varied according to the stage of the disease where severe congestion of all internal organs with serous to sero-hemorrhagic fluid in the abdominal cavity was characteristic in some cases.

Sampling and processing:

One hundred (100) marine fishes of four different species were freshly captured from two localities in Egypt, (Manzala Lake, Shatta Village) through the different seasons of the year. On each season, twenty-five fish of each species were collected and freshly examined. For each fish clinical signs, average body weights and P.M examination were carried out using the methods described by (Buller, 2004). For bacterial isolation of vibrio species samples from gills, liver, spleen, kidney and external lesions from fishes were cultured on general and selective media.

Culture media

Media used for the isolation of the vibrio were:

Liquid media

Tryptic Soya broth (TSB) (Difco, Detroit, MI, USA) supplemented with 3% NaCl which was used for the growth of some suspected isolates prior to plating.

Semi-Solid media

0.5 % Nutrient agar medium **(**Oxoid, 1982) which is supplemented with 3% NaCl which was used for the preservation of all isolated strains as well as for the detection of bacterial motility. Thiosulphate citrate bile salts sucrose agar (TCBS): (TCBS, Biolife, Milan, Italy) supplemented with 3% NaCl which was used as selective medium for the isolation of Vibrio species (Whitman, 2004).

Bacteriological examination:

Postmortem (PM) examination: was carried according to (Conroy and Herman, 1981).

Identification of bacterial isolates: was carried out according to the methods described by Austin and Austin (1999).

Serotyping of two of the isolated vibrio anguilirium bacteria using TCBS media was carried out.

PCR was performed for detecting 2 virulent strains of *Vibrio anguiliticus*.

Experimental design: (vaccine preparation):

From the isolated vibrio anguiliticus serotyped and isolated on TCBS media the pure culture were taken in 500 ml of 85% (Nacl)saline and washed three timed by centrifugation at 10 ppm, lastly the sediment of bacteria were taking for preparation of vaccine and store in refrigerator until the vaccine were papered. Formalin vaccine was prepared by addition 0.5% formalin overnight or heating at 65 °C for three hours, respectively. The prepared vaccines were stored in the refrigerator at 4 °C until the experimental technique was carried out. After washing the suspended bacteria, 500 ml of suspended bacteria in formalin as well as 500 ml of saline solution were used preparing the formalin-killed bacterins (FKC) and heat –killed bacterins (HKC), respectively.

Two kinds of whole cell bacterins, which were formalin-killed bacterins (FKC) and heat –killed bacterins (HKC), were prepared by treating the washed prepared bacterial suspension.

Sterility test:

The prepared vaccines were tested for sterility (completely inactivation of bacteria) by culturing on TCBS agar media and after incubation, no growth of colony was observed.

Safety test:

The prepared vaccine injected intraperitoneally in sea bream fish in two aquaria for formalin and heat killed vaccine and observed two weeks. The results were no changes in fish behavior or fish health.

Experimental fish vaccination:-

One hundred and seventy silver sea bream fishes, weighing approximately (50–100 g), were obtained from local fish farms in Damietta Governorate. Fish were transported to the laboratory and acclimated in Mediterranean seawater aquaria (20 L capacity) equipped with biological filtrates for three weeks prior to experiments. Fish, without any apparent infectious symptoms, were then randomly separated into experimental groups and maintained in aquaria (20 L capacity) equipped with seawater recirculation. Fish were fed with a pelleted diet purchased from commercials farms (25% protein) in ratio of 5% (fish body weight/day) (Woo and Kelly, 1995). Throughout the experimental period, seawater temperature was kept at 20–22 °C and salinity was maintained constant at 33 ppm.

A total of 160 fished (sea bream) were divided in to four duplicated aquaria (20 fish each). The first duplicated groups of fish were injected intraperitoneal with formalin killed vaccine (FKV), while the second duplicated aquaria were injected intraperitoneal with heat killed vaccine (HKV). The third duplicated groups injected with formalin broth and saline only in the control groups, the forth groups fish only as control negative, respectively. All groups were injected i.p. with 0.2ml/ fish for two months as initial dose and injected with booster dose after three months from the beginning of the vaccination process, respectively.

Hematological examination:

Fish under experimentation were anesthetized using lignocaine. Heparinized blood samples were collected from the caudal vein of vaccinated and control groups using sterile syringe. Serum samples were collected and stored at -20 °C until laboratory examination performed.

The challenge infection:-

A toxoid fresh 24 h bacterial culture colonies of a virulent (*V. alginolyticus*) used for preparing a toxiod experimental challenge as described by (li *et al*., 2003). All fish were injected intraperitoneally with 0.2 mL/fish of *V.*

alginolyticus at the dose of 5×10^5 CFU/fish. Fish mortality was monitored daily for two weeks. The survival rates and the protection (expressed as relative level of protection (RLP) of silver sea bream immunized with various vaccine preparations. Relative level of protection was evaluated according to the following formula described by (Amend, 1981). The efficacy of vaccines were performed by the following equation:

RLP = (1 − mortalities of vaccinated fish/mortalities of control fish) × 100

Serological experiments:

The micro-agglutination test:

Formalized whole culture vaccine was used as antigen in the serological tests according to Hay *et al*., (2002).

Single immune diffusion test:

From all vaccinated fish, heparinizedblood samples were collected. The formalized whole culture vaccine was used as antigen and the collected heparinized serum used as antibodies and the test subjected to the single immunodiffusion test, according to Ouchterloney (1962).

Determination of Total plasma protein:

Instrumentally, total protein concentration was determined in each sample according to Weicheselbaun (1946). Collected heparinized blood samples were used for determination of hemoglobin (Hb %), total erythrocytic count (RBCs), total and differential leukocytic count (WBCs)

The statistical analyses:

The data collected were statistically analyzed using one-way ANOVA adapted by SAS (2000). Means were statistically compared for the significance ($P \leq 0.05$) using Duncan (1955) multiple range test.

RESULTS AND DISCUSSION

Regarding to the immunity status of vaccinated fish, determination of antibody titer was conducted by micro agglutination test and single immune diffusion was recorded in Tables (1 & 2). The results detected by micro agglutination techniques (Table 1), two months later from the beginning of vaccination process indicated that the levels of the antibody titer were (51.25 µl ±3.77) and (45.83 µl ± 3.59) in the vaccinated fish groups by formalin and heat killed vaccine, respectively. On the other hand, after three months the antibody titer were (54.17 µl ±2.89) & (49.58µl ±3.34) in formalin and heat killed vaccine, respectively. Higher values of antibody titer was shown in formalin vaccinated fish than in fish vaccinated by heat killed vaccine whereas no changes in antibody titer in the fish of the control groups.

Concerning the results detected by single immune diffusion techniques, the formalized vaccine used in the vaccinated groups gave higher values (1.13 cm ±0.08) & (1.16 cm ±0.07), respectively at two and three months duration after the vaccination process than that in the groups of fish vaccinated by heat killed vaccine which were (1.05 cm ±0.11) and ((1.10 cm ±0.07), respectively (Table 2).

The current results showed significant higher values of immunological burden of fish groups vaccinated by formalized vaccine, than groups vaccinated by heat killed vaccine as illustrated in Tab1es (1 & 2). These were in agreement with those recorded by Jun *et al*. (2016) who reported elevations of serum agglutinating antibody titer in silver sea bream (*Sparus sarba*) vaccinated by formalized *Vibrio. alginolyticus* vaccine. Whereas results recorded by Colquhoun and Lillehaug (2014) showed high antibody titer by Formalin- or heat-killed whole cell vaccine.

The total levels of immunoglobulin after intraperitoneal injection showed significantly higher values ($P \leq 0.05$) in formalized and heat killed vaccine groups which were (3.83 g/dl ±0.08) and (3.63 g/dl ±0.14), respectively at two months

after vaccination as compared with the control groups which were (2.70 g/dl ±0.17 and 2.77 g/dl ±0.08) (Table 3 & Fig. 3). This confirms the effect of vaccination as a factor influencing the immune response in the silver sea bream which may be attributing that the bacterins can stimulate the antibodies production in fish.

The levels of the total protein after three months from the beginning of vaccination process were (4.15 g/dl ±0.14) and (3.78 g/dl ±0.11), respectively in vaccinated fish groups by formalized and heat killed vaccine.

Regarding total protein, significant higher values of immunological status of fish groups vaccinated by formalized vaccine were detected than in groups vaccinated by heat killed vaccine as illustrated in (Table 3). The results of the present study were in agreement with results by Hu *et al*. (2012) where they reported that total protein increased in the vaccinated fish than in control fish during their research about the development and efficacy of an attenuated *vibrio harveyi* vaccine candidate with cross protective against *vibrio algoliticus.*

RBCs count at two and three months duration after the beginning of vaccination process in the experimental groups (formalin and heat killed vaccine) were significantly increased compared with control groups (Table 4 & Fig.4). The results of the present study were in close contact with Bruno et al (2009) who recorded higher hematocrit values in vaccinated fish groups as well as number of erythrocytes and leukocytes than the non-vaccinated group. Intraperitoneal vaccination presented higher total number of leukocytes, lymphocytes and serum agglutination titer. In contrary our results were disagreed with that detected by Jun *et al*. (2016) who reported that no significant effect on the serum hematocrit and hemoglobin.

The present study showed that vaccination stimulates the haemopoesis and also induces the nonspecific immunity in fish. Similar results was given by Bailone *et al*. (2010) who showed that 10 days after immunization with a polyvalent vaccine at a concentration 1×10^8 CFU/mL, there was an increase on erythrocytes, leukocytes, thrombocytes and circulating lymphocytes production.

With regard for vaccine, the hemoglobin concentration means values recorded in (Table 5 & Fig. 5), were significantly ($P \leq 0.05$) increased compared with control groups at two and three months duration after the beginning of vaccination process in the experimental groups (formalin and heat killed vaccine). The results also revealed that hemoglobin concentration values were positively correlated with the RBCs count. Also, the present results were similar to Sajjad *et al*. (2012) who recorded that hemoglobin was elevated significantly, especially in the fish immunized by the formalin- and phenol-killed bacterins through various administration routes. On the other hand, the current results were disagreed with that detected by Jun *et al*. (2016) who reported that there was no significant effect on the serum hematocrit and hemoglobin.

Concerning the results recorded in (tab.6 and fig .6) clarify that there were significant increases ($P \leq 0.05$) in total and differential leukocytic counts at two and three months after vaccination in the experimental groups (formalin and heat killed vaccine) compared with control groups.

Significant higher total leukocytic counts values of immunological status of fish groups vaccinated by formalized vaccine, than that vaccinated by heat killed vaccine as illustrated in Table (7). These results are in agreement with the results of Salah *et al*. (2015) who recorded that the lymphocytes were significantly increased in vaccinated fish in comparison with unvaccinated group at all periods. Similar studies showed that 10 days after immunization with a polyvalent vaccine at a concentration 1×10^8 CFU/mL, there was an increase leukocytes, thrombocytes and

circulating lymphocytes production (Bailone *et al.,* 2010). On the other hand, Aly e*t al* .(2000) reported a marked increase in the number of lymphocytes around the activated melanomacrophage centers in the kidney together with a maximal splenic response in the form of activated melanomacrophage centers with marked increase in macrophages and lymphocytes together with proliferation of hematopoietic elements around the splenic sinuses. Diaz *et al*. (2006) reported that high lymphocytic ability in gilthead sea bream (*S. aurata* L.) specimens given a mixture of two inactivated bacteria.

It was clear from results in Table (8) that relative level of protection (R.L.P) at the end of the experiment of the vaccinated sea bream fish groups by formalin vaccine was 91.66 , while it was 83.33 in vaccinated fish groups produced by heat killed vaccine. Higher values of RLP showed in formalin vaccinated fish than in fish vaccinated by heat killed vaccine whereas the levels of RLP in the fish of the control groups were 16.66 and zero in control –ve and control +ve , respectively. The results of the RLP confirm the role of the humeral antibodies in protecting fish against *Vibrio. Alginolyticus* infection.

These results agree with that obtained by Jun *et al*. (2016) who found that the i.p. delivery route, fish immunized with formalin killed acquired the best protection, whereas the other vaccine preparations gave variable protective effects upon pathogenic *V. alginolytics* challenges.

Conclusion:

The present results indicated that formalin- or heat killed whole cell bacteria are currently the most popular vaccines employed in farmed fish, and good protection against vibriosis, however, formalin gives the best results..

Table (1): Antibody titer of fish after two and three months of vaccination.

Vaccination	Two months	Three months	T-test	
	Mean ± SD	Mean ± SD	T-test	P-value
Control-ve (N=12)	0.00 ± 0.00	0.00 ± 0.00	-	-
Control+ve (N=12)	0.00 ± 0.00	0.00 ± 0.00	-	-
Heat killed (N=12)	45.83 ± 3.59	49.58 ± 3.34	2.46	0.032*
Formalized (N=12)	51.25 ± 3.77	54.17 ± 2.89	3.02	0.012*

*****extremely significant.
Data in each Colum represented the mean ± standard deviation.
-ve control: fish only +ve control: fish injected with formalin.

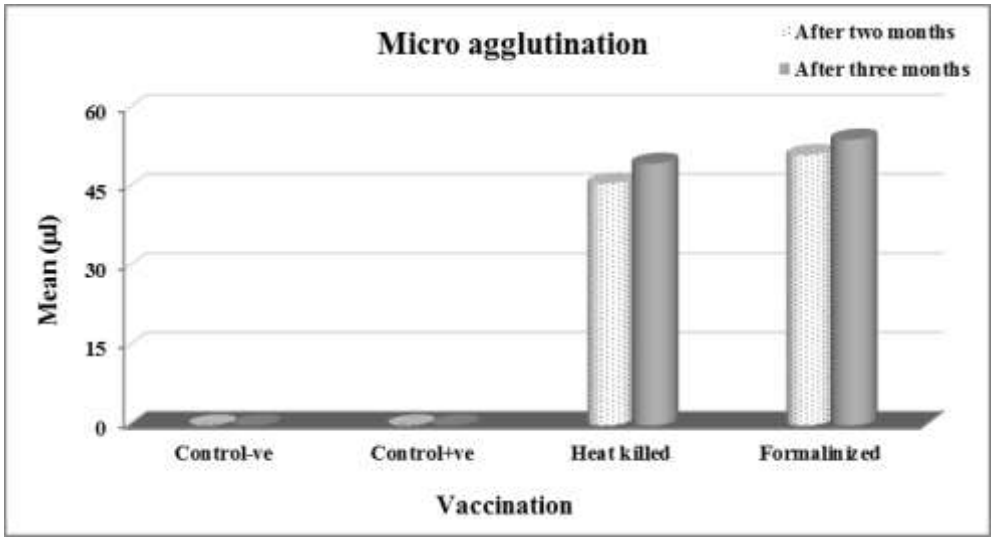

Fig. (1): Antibody titer of fish after two and three months of vaccination.

Table (2): Antibody titer after two and three months of vaccination.

Vaccination	Two months	Three months	T-test	
	Mean ± SD	Mean ± SD	T-test	P-value
Control-ve (N=12)	0.00 ± 0.00	0.00 ± 0.00	-	-
Control+ve (N=12)	0.00 ± 0.00	0.00 ± 0.00	-	-
Heat killed (N=12)	1.05 ± 0.11	1.10 ± 0.07	1.20	0.256
Formalized (N=12)	1.13 ± 0.08	1.16 ± 0.07	1.48	0.166

Data in each Colum represented the mean ± standard deviation.
-ve control: fish only +ve control: fish injected with formalin.

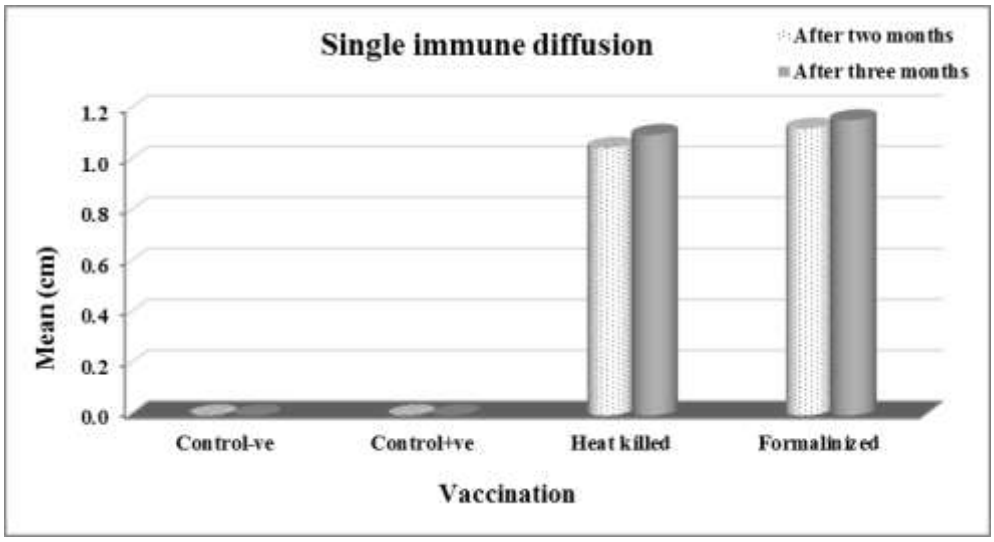

Fig. (2): Antibody titer after two and three months of vaccination.

Table (3): Total protein in vaccinated fish after two and three months of vaccination.

Vaccination	Two months	Three months	T-test	
	Mean ± SD	Mean ± SD	T-test	P-value
Control-ve (N=12)	2.70 ± 0.17	2.72 ± 0.14	0.26	0.799
Control+ve (N=12)	2.77 ± 0.08	2.77 ± 0.08	-	-
Heat killed (N=12)	3.63 ± 0.14	3.78 ± 0.11	4.78	0.001***
Formalized (N=12)	3.83 ± 0.08	4.15 ± 0.14	6.92	0.001***

*****extremely significant.
Data in each Colum represented the mean ± standard deviation.
-ve control: fish only +ve control: fish injected with formalin

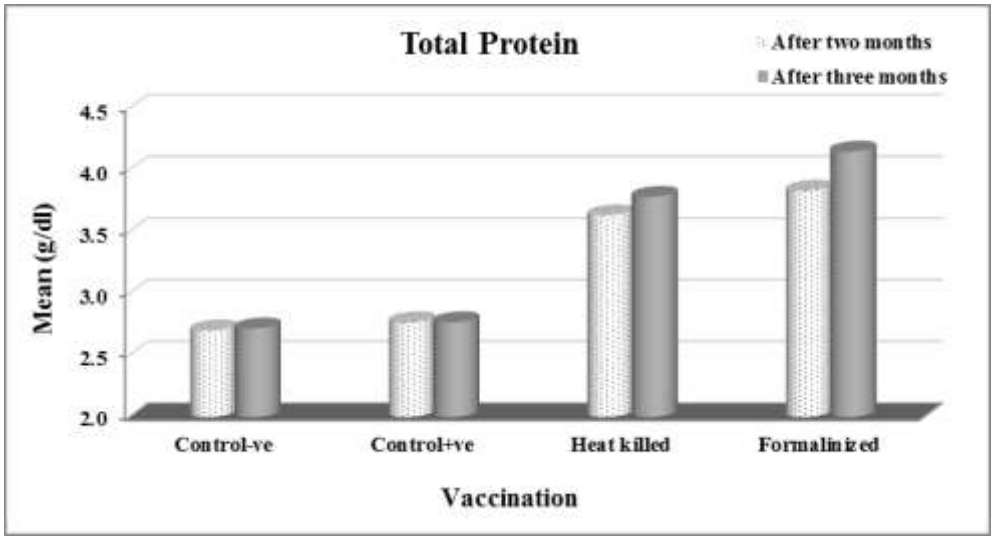

Fig. (3): Total protein in vaccinated fish after two and three months of vaccination.

Table (4): RBCs (10^6/dl) **in vaccinated fish after two and three months of vaccination.**

Vaccination	Two months	Three months	T-test	
	Mean ± SD	Mean ± SD	T-test	P-value
Control-ve (N=12)	1.38 ± 0.07	1.41 ± 0.04	1.15	0.273
Control+ve (N=12)	1.39 ± 0.06	1.40 ± 0.07	0.56	0.585
Heat killed (N=12)	2.13 ± 0.05	2.35 ± 0.02	13.72	0.001***
Formalized (N=12)	2.32 ± 0.01	2.41 ± 0.03	10.06	0.001***

*****extremely significant.
Data in each Colum represented the mean ± standard deviation.
-ve control: fish only +ve control: fish injected with formalin.

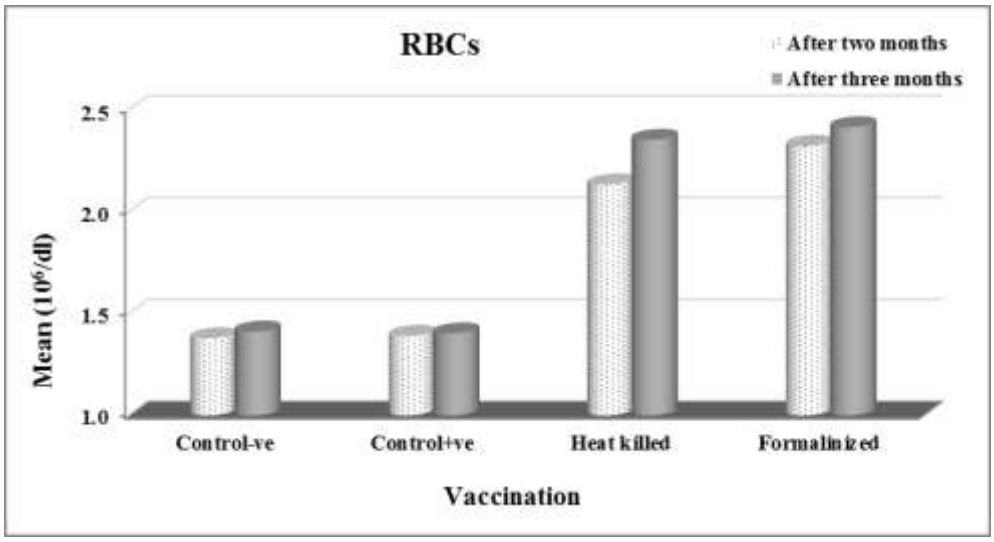

Fig. (4): RBCs in vaccinated fish after two and three months of vaccination.

Table (5): Haemoglobin in vaccinated fish after two and three months of vaccination.

Vaccination	Two months	Three months	T-test	
	Mean ± SD	Mean ± SD	T-test	P-value
Control-ve (N=12)	7.03 ± 0.39	6.99 ± 0.43	0.19	0.855
Control+ve (N=12)	7.00 ± 0.49	7.08 ± 0.55	0.44	0.665
Heat killed (N=12)	8.51 ± 0.27	9.48 ± 0.24	7.69	0.001***
Formalized (N=12)	9.39 ± 0.41	10.33 ± 0.36	8.87	0.001***

*****extremely significant.
Data in each Colum represented the mean ± standard deviation.
-ve control: fish only +ve control: fish injected with formalin.

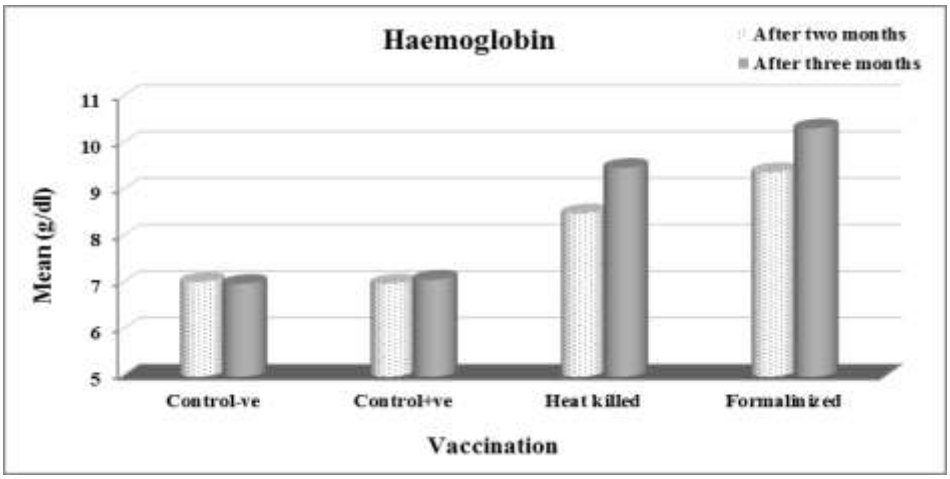

Fig. (5): Hemoglobin in vaccinated fish after two and three months of vaccination.

Table (6): WBCs (10^3/dl) **in vaccinated fish after two and three months of vaccination.**

Vaccination	Two months	Three months	T-test	
	Mean ± SD	Mean ± SD	T-test	P-value
Control-ve (N=12)	51.52 ± 1.94	51.95 ± 2.16	0.54	0.597
Control+ve (N=12)	49.88 ± 1.83	51.61 ± 2.53	1.61	0.137
Heat killed (N=12)	85.38 ± 1.59	88.31 ± 1.36	5.78	0.001***
Formalized (N=12)	90.29 ± 1.34	95.34 ± 2.19	9.55	0.001***

*****extremely significant.
Data in each Colum represented the mean ± standard deviation.
-ve control: fish only +ve control: fish injected with formalin.

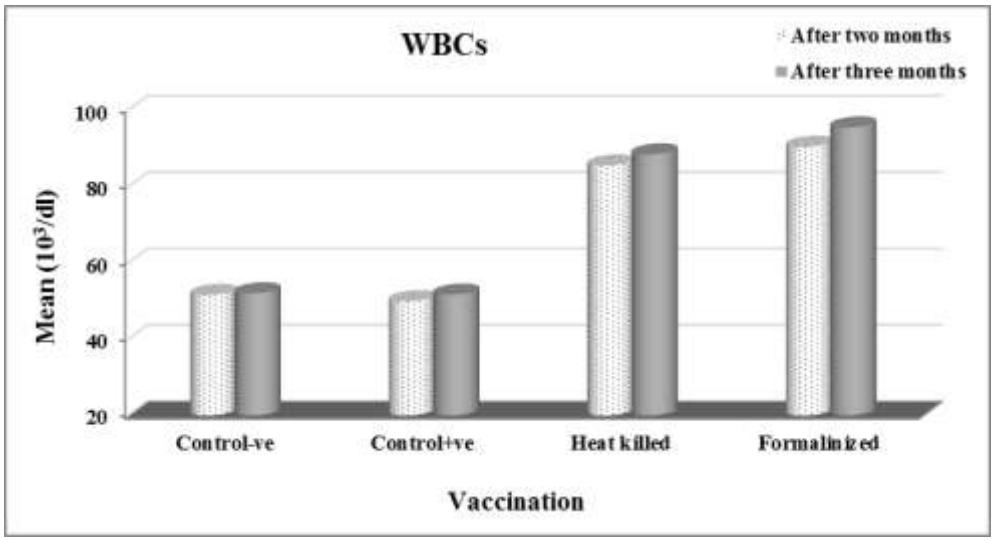

Fig. (6): WBCs in vaccinated fish after two and three months of vaccination.

Table (7): Lymphocytes in vaccinated fish after two and three months of vaccination.

Vaccination	Two months	Three months	T-test	
	Mean ± SD	Mean ± SD	T-test	P-value
Control-ve N=12	52.33 ± 2.90	52.17 ± 2.98	0.35	0.732
Control+ve N=12	52.33 ± 3.08	53.00 ± 2.80	0.60	0.560
Heat killed N=12	69.08 ± 0.67	71.67 ± 1.15	7.22	0.001***
Formalized N=12	71.00 ± 0.85	75.42 ± 0.90	11.10	0.001***

*****extremely significant.
Data in each Colum represented the mean ± standard deviation.
-ve control: fish only +ve control: fish injected with formalin.

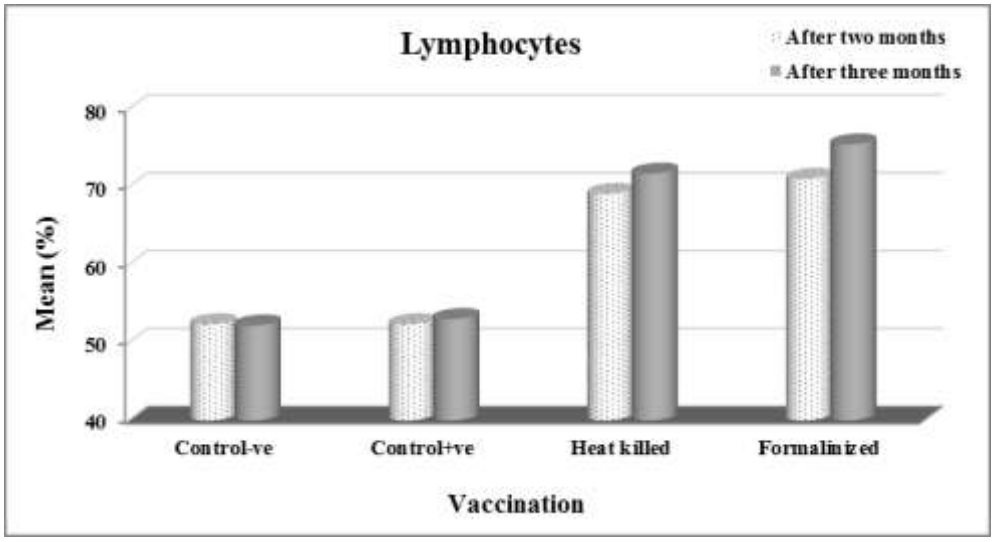

Fig. (7): Lymphocytes in vaccinated fish after two and three months of vaccination.

Table (8): Results of challenge test (Relative Level of Protection, RLP)) after two weeks.

Group	Total no. of fish	Mortality	Mortality (%)	R.L.P
Control -ve	20	10	50	16.66
Control +ve	20	12	60	0.00
Heat killed	20	2	10	83.33
Formalized	20	1	5	91.66

RLP = (1 − mortalities of vaccinated fish/mortalities of control fish) × 100

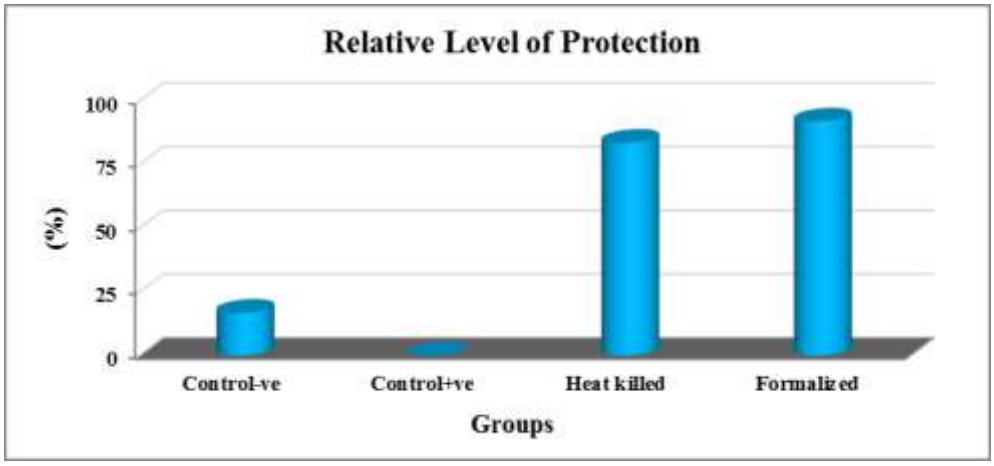

Fig. (8): Results of challenge test (Relative Level of Protection) after two weeks

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استخدام اللقاحات فى مكافحة الأمراض البكتيرية للأسماك التى تسببها فيبريو انجيوليتكس

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المستخلص

تم إجراء هذه الدراسة لتقييم تأثير استراتيجيات اللقاح فى السيطرة على مرض الفيبريو فى مزارع الأسماك البحرية بمحافظة دمياط . تم إعداد نوعين من اللقاحات وهى بكتريا الفيبريو الميتة بمادة الفورمالين والآخر البكتريا التى قتلت بالحرارة وتم تحصين مجموعات أسماك الدنيس باللقاحات المحضرة عن طريق الحقن البيريتونى . وجد أن الأسماك التى تم تحصينها باللقاح الميت بالفورمالين يعطى المقاومة للأمراض بشكل أفضل من اللقاح الميت بالحرارة فى اختبار التحدى ضد الميكروب وكانت أعلى معدل حماية فى اللقاح الميت بواسطة الفورمالين . تم عمل اختبارات تحديد انتشار المناعة وتقنيات التلصيق الجزئى ومحتوى البروتين الكلى الذى يظهر أن اللقاح الفورمالين ينتج وقاية جيدة ضد الأمراض عن اللقاح الميت بالحرارة

تم فحص تعداد الدم الكامل للكشف عن الخلايا الليمفاوية ونسبة الهيموجلوبين وعدد كرات الدم الحمراء وعدد كرات الدم البيضاء وأتضح من النتائج أن لقاح الفورمالين هو الأفضل . كما تبين انه بعد أعطاء الاسماك الجرعة الزائدة قد أدت الى ارتفاع الحالة المناعية للأسماك وقد لوحظ وجود تأثير كبير فى مستويات الهيموجلوبين وعدد الخلايا الليمفاوية ومحتوى البروتين الكلى فى الأسماك المحصنة بلقاح الفورمالين أعلى من تلك المحصنة بلقاح البكتريا الميتة بالحرارة.