Oral Vaccination of Nile Tilapia (*Orechromis niloticus*) Against Motile Aeromonas Septicaemia

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Abstract: The present study was planned for preparation of formalin inactivated wet-packed whole cells Aeromonas *hydrophila* bacterin for oral vaccination. The humeral antibody response of vaccinated Nile tilapia *(Orechromis niloticus (O. niloticus)* was determined by micro-agglutination test. Moreover efficacy of the prepared bacterin against infection with *Aeromonas hydrophila* was detection and calculated as a relative level of protection. Nile tilapia *(O. niloticus)* immunized orally with formalin-inactivated *Aeromonas hydrophila* .wet-packed while cells had low level of antibody titer reached 2 and 3 by log2 at first and fourth week post-immunization respectively while Nile tilapia *(O. niloticus)* fed on minced meat without vaccine had antibody titer reached 1 by log 2 throughout the experimental period . The relative level of protection among Nile tilapia *(O. niloticus)* immunized orally were 86.8. [Report and Opinion. 2010;2(1):46-51]. (ISSN: 1553-9873).

Keywords: *Aeromonas hydrophila* - bacterin -vaccination- humeral antibody- Nile tilapia.

1. Introduction

Recently many countries practice fish culture very successfully not only as food industry but also as major source of income. Bacterial diseases among cultured fish either primarily or secondarily are considered to be a major cause of fish mortalities Grisez,,L. and Ollevier,F.(1995). *Aeromonas hydrophila* is known to be one of the most important bacteria associated with diseases in marine and freshwater fishes. The diseases caused by *Aeromonas hydrophila* ranged from acute rapidly fatal septicemia to latent infections and has been referred as hemorrhagic septicaemia or *Aeromonas septicemia.* At present, most of the cultured fish diseases are treated with drugs such as antibiotics, sulfonamides, nitrofurans and others. The chemotherapeutic measures are effective, particularly when used as early as possible, and have wide spectrum of pathogen control. However, several difficulties are often encountered by chemotherapy as, the cost of drugs is expensive, the resistant strains of pathogens are easily induced in water and the drug residues may deposit in fish body may introduce potential hazard to public health and to the environment by the emergence of drug resistant microorganisms and antibiotic residues and retain in water system as toxicants or pollutants Sugita et al (1991). In order to avoid the side effects of chemotherapy, the control measure by immunization of fish with vaccines gains the effort and rapid development. Many experimental and practical approaches to stimulate the immune response of fish were reported (Badran 1984, Abdel-Kader 1994 and Aly *et. al*., 2000**)**. Such immune response could be detected either by the presence of specific antibodies in the blood or by protection against infection. In the past, the presence of antibodies in the blood is well revealed when the immune system is stimulated by the injection of the antigen, but not when given orally or by immersion. After which, the trials were attempted to increase the production of antibodies and prolonging their presence in the blood by emulsifying the antigen in adjuvant Krantz, *et. al* (1963) who used mineral oil emulsion, Collins, *et. al* (1976) Fruend's incomplete adjuvant (FIA) and Badran (1990**)** FIA and Fruend's complete adjuvant (FCA). Moreover, several techniques have been successfully used for fish vaccination. Such techniques included injection immersion and oral routes.

The present study was planned for: Preparation of formalin inactivated wet-packed whole cells for oral vaccination. Determination of the humeral antibodies by microagglutination test in parent fish and fingerlings. Examination of the efficacy of the prepared bacterines by infection of the tested fish with *Aeromonas hydrophila* and calculation the relative level of protection (R.L.P).

2-Material and methods

2-1-Fish: - A total of (210) live apparently healthy. Nile tilapia (*(O. niloticus*) divided as follow(Forty adult fish for biological test, Forty adult fish for innocuty test, Eighty adult fish for oral vaccination, Twenty-five male of body weight from 120:130 gm, Twenty five female of body weight from 100:120 gm.

2-2-Ponds:-Twenty-three cement ponds in a private hatchery fish in Kafr El Sheikh Governorate with dimensions of 3x 8x 2 meter and Twelve glasses aquaria with dimension of 70 x 53 x 53 cm. in Labe of Hydrobiology Dept. NRC, were used for the biological and innocuty test Cement ponds and glasses aquaria were supplied with dechlorinated water with a temperature(25:28 C^{o}**).**

2-3-Diet: diet with 35% and 25% protein for feeding of fingerlings and adult Nile tilapia respectively. Food in ratio of 3% of fish body weight per day was considered to be the optimal maintenance amount required for adult fish and 5% for fingerlings according Noor El Deen (2OO7) .

2-4-Bacterial strain used:-Aeromonas hydrophila were isolated from liver or ascetic fluid of diseased fishes on Brain heart infusion broth followed agar with 0.5, 1, 2 and 4% NaCl Chen and Levin (1975).

2-5-Biological test (Virulence test): To detect the level of virulence of the obtained *Aeromonas hydrophila* strain, laboratory test were conducted using Nile tilapia (*O. niloticus*) as the fish of choice. According to Wakabayashi, et. al (1981) the Bacterial solution for fish inoculation was prepared by suspending 20 hs culture from brain heart infusion agar of the obtained strain in sterile physiological saline solution to give a concentration of 5 mg bacterial cells by wet weight/ml which was estimated to be between 1.8 / 10^6 and 1.6 / 10^9 C.F.U/ml .

2-6-Vaccine preparation for oral vaccination: Wet-packed, whole cell bacterin was prepared according to Rohovec, et. al (1981) as: Ten ml of brain heart infusion broth were inoculated with *Aeromonas hydrophila* . After 12 hs. Incubation at 25°C, 2 ml of this broth culture were used to inoculate one liter broth culture which was in turn incubated for 12 hs at 25°C. The prepared one liter was used as an inoculum for 15 liters of the broth medium and subjected to an incubation period of 10 : 12 hs at 25°C. Finally, 250 ml of 20% dextrose solution was added and the culture was incubated for an additional 12 hs. The bacterial cells were killed by addition of formalin to give a final concentration of 0.3 % over night. The cells were harvested by centrifugation and stored at 20 °C.

2-7- Innocuity test: This test was performed according to Anderson , et. al (1970) by inoculation of susceptible fish (*O. niloticus*) intraperitonealy with the prepared bacterin to insure that there is no infection or disease will be occur from living bacteria .

2-8-Sterility test: This test was done as described by Aly (1981) by cultivation of the prepared bacterins on brain heart infusion agar to insure that there's no growth of *Aeromonas hydrophila* or other organisms may be occurred.

2-9-Preparation of stained antigen used in antibody titration. Preparation of *Aeromonas hydrophila* antigen for antibody titration was established. The formalin inactivated bacterial cells by wet-weight was diluted with equal volume of sterile physiological saline solution. One drop of Loffler's alkaline methylene blue, prepared as described by Cruickshank (1985) was added to each 10 ml of the diluted antigen

2-10-Biological test "virulence test":-Forty Nile tilapia with (110 ±10) g body weight were divided into two groups each contains Twenty fish. Fish of the first group were injected intramuscularly through the back with 0.2 ml of the bacterial suspension (5 mg bacterial cell by wet weight /ml) /100g body weight Badran (1987). The fish of the second group (control group) injected with 0.2ml of sterile physiological saline solution. The tested fish were placed under observation for 2 weeks. The strain of *Aeromonas hydrophila* can be classified into 3 categories of virulence:-High virulence: - All tested fish were dead in a week. Moderate virulence: - Not all tested fish were dead in a week Virulence: - No fish were dead without show any clinical signs.

2-11- Safety test (Innocuity test):-The safty test was performed according to Anderson et. al (1970) by inoculation of the susceptible Nile tilapia (*O. niloticus*) intraperitonealy (I.P) with washed bacterin cells from the prepared vaccine. Two groups of Nile tilapia corresponding to the vaccinated and control, each contained 20 fish with (100 ±10 g) body weight were used. Nile tilapia (*O. niloticus*) of the first group were injected intraperitonealy with 0.1 mg bacterin cells) / fish. The fish of the second group (Control) were injected intraperitonealy with sterile physiological saline solution . The fish of both groups were placed under investigation during 15 days after injection. After that, fish were tested for re-isolation of injected organism on brain heart infusion broth that incubated at $25C^{0}$ for 24 hs.

2-12-Sterility test : - An inoculum from the bacterin was cultivated on brain heart infusion agar and incubated at 25°C for 24 hs. The cultures were examined for positive bacterial growth.

2-13- Vaccination method:- Eighty fish of *O.niloticus* (Forty male and Forty female)were used with separation of male from female and placed under observation in 2 cement ponds for 2 weeks for acclimatization and insuring the freedom of fish from diseases. Forty male and Forty female) were placed in 10 groups each contain 4 male and 4 female with attention that 9 groups of vaccinated were fed on diet contain wet-packed whole cell bacterin at level of 5 mg/g of diet Fryer, et. al (1976), while the other Eight fish (Four male and Four female) fed diet without bacterin in one group as a control. Food containing vaccine was given at ratio of 3 % of the fish body weight per day for 8 days. The blood collected and sera separation was performed from Thirty-six fish (Eighteen

males and Eighteen females from vaccinated fish) and two male and two female from control. Antibody titration of the collected serum was evaluated by microagglutination (MA) test. The other Thirty-six fishes were placed in 6 groups each contain 3 male+3 female with attention that 5 groups of vaccinated and one group nonvaccinated fish to give chance for normal breeding. Fingerlings from each group collected and 50 of each group squeezed and body fluid collected for microa-gglutination and biochemical analysis while other 50 fingerling from each group used for challenge test.

2-14- Challenge test:-The 6 groups used for breeding gave fingerlings after different periods post vaccination: (1- first group gave fingerlings after 12 day p.v, Second group gave fingerlings after 18 day p.v ,third group gave fingerlings after 20 day p.v , Forth group gave fingerlings after 25 day p.v ,fifth group didn't gave fingerlings , 6th group (control group) gave fingerlings after 14 days. Six groups of Nile tilapia (*O. niloticus*) breeder fish and six groups of fingerlings each contain 50 fingerlings corresponding to each group of breeder fish were used .one group of 50 fingerling from nonvaccinated fish not exposed to challenge used as negative control. Breeder fish subjected to challenge one month post vaccination, while fingerling subjected to challenge at age of one month. The organism for challenge was cultured in brain heart infusion broth at 25 ± 1°C for 24hs. The cultured broth was diluted with sterile saline solution to give a final concentration of 1.0 g bacterial cell by wet weight/L Badran (1993).Before immersing the experimental fish in the diluted broth culture for 10 minutes. The fish were pre-immersed in 1.5% Nacl solution for 5 minutes. Then immersed in the prepared broth culture, the challenged fish were placed under observation for 2 weeks and the dead fishes were used for *Aeromonas hydrophila* resonation. The relative level of protection (RLP) in each challenge was determined according to Newman and Majinarish (1982). using the equation

$$(\text{RLP}) = 1 - \frac{\text{Present immunized mortality}}{\text{Present control mortality}} \times 100$$

3-Result:-

3-1-The results of biological test of *Aeromonas hydrophila* among Nile tilapia were documented in Table (1). The results explained that, sixteen fish died in the second day post-infection. Then Eight fish were died in the third day post-infection, then three fish died in the fourth day post-infection and finally, four fish was died in the fifth day post-infection. No fish of the control group were died during the experiment.

Table (1) : The result of biological test of *Aeromonas hydrophila* among Nile tilapia (Orechromis niloticus)

* Group 1			**Group 2			Days
No. of fish	%	% of Total mortality	No. of fish	%	% of Total mortality	
0	0	0	0	0	0	1st day
16	40	40	0	0	0	2nd day
8	20	60	0	0	0	3rd day
12	30	90	0	0	0	4th day
4	10	100	0	0	0	5th day
-	-	100	0	0	0	6th day
-	-	100	0	0	0	7th day

* The fish of group 1 were injected I/M with 0.2 ml of the bacterial suspension/ 100 g fish body weight.

** The fish of group 2 were injected I/M with 0.2 ml of sterile physiological saline solution/ 100 g fish body weight.

3- 2-Tests performed to insure safty and sterility of the bacterin:-The injected fish showed no signs of *Aeromonas hydrophila* infection and there were no postmortem changes. The cultures of resolution showed

no microbial growth of *Aeromonas hydrophila*.

4-3-The cultivated plates showed neither *Aeromonas hydrophila* nor other bacterial growth after 24 hs. of incubation at 25 °C . This result indicated that the prepared vaccine was sterile and safe to be used in the vaccination process.

3-4- The results revealed that, food supplied to fish in ratio of 2 and 2.5% of their body weight per day were not sufficient for maintenance while the food supplied in ratio of 3.5% of fish body weight was more than the fish requirement.

4-5-The results of immune response of Nile tilapia (*O. niloticus*) vaccinated orally with *Aeromonas hydrophila* wet-packed whole cells bacterin in comparison with those fed on untreated food were slight increase in the immune response of vaccinated fish where the antibody titers were 2 at 1st and 2nd week post-vaccination and 3 at 3rd and 4th week post vaccination. On the other side the antibody titer in the control group was 1 by log2 throughout the experiment. While, microa-gglutination and biochemical analysis increase in vaccinated fish than nonvaccinated (Table,2).

Table (2) :Results of the micro-agglutination titer, double immunodiffusion test, and total protein, g/dl, in Fingerlings samples at 4th week.

Fingerlings from control fish	**Fingerlings from oral vaccinated fish**	**Sample Test**
12	150	Micro -agglutination titer
-	+	Double immunodiffusion test
2	3	Total protein (g/dl)

3-6- The results of challenge were documented in Table (3). The results explained that, the fish vaccinated by oral methods were protected against challenge with *Aeromonas hydrophila* where the RLP were 86.8 .

Table (3): Comparison between the relative levels of protection (RLP) afforded by the different route of Aeromonas hydrophila vaccines of O. niloticus.

Route of vaccine	Results*	Percent of survivals	RLP
injection	4/50	92	91.2
immersion	5/50	70	86.8
oral	5/50	90	86.8
control	0/50	100	100

4-Discussion

Throughout this work the biological properties of *Aeromonas hydrophila* strain were tested for determining its virulence to Nile tilapia *(O. niloticus)* After which preparation of *Aeromonas hydrophila* bacterin (formalin inactivated bacterial cells) was performed.

The bacterin was tested for safety and sterility before immunization of Nile tilapia *(O. niloticus)* was done by the oral method of vaccination. Beside, the determination of humoral antibody titers and RIP of vaccinated and non-vaccinated fish. Several trials for vaccine preparation were performed on organisms other than *Aeromonas hydrophila* . The results of these trials were successful in the production of several vaccines for immunization against fish pathogens, such as *Aeromonas hydrophila* (Azad, et. al., 1999) and *Aeromonas salmonicida* (Cipriano, 1983) and some of them had been commercialized. The present trial also explained the ability of Nile tilapia *(O. niloticus)*, to produce high level of specific antibody titer after Indicated that the bacterin prepared from *Aeromonas hydrophila* after treatment with formalin was antigenic in nature being able to against the inoculated antigen.

The results obtained from this investigation recorded that Nile tilapia *(O. niloticus)*, collected for the present study had a light natural antibodies specific to *Aeromonas hydrophila* which detected by microagglutination test and calculated by log2. natural antibody titers were 1 throughout the experiment. These results agree with those reported by(Badran, 1994) who recorded low level of natural antibodies (1 by log2) against *Aeromonas hydrophila* in *O. niloticus*. The author explained that, the natural antibody against *Aeromonas hydrophila* produced as a result of contact of the normal fish with *Aeromonas hydrophila* present in the fish environment. The level of natural antibody against *Aeromonas hydrophila* was about the half of natural antibody against *Aeromonas hydrophila* Nile tilapia (*O. niloticus*) Badran, 1990 (1991 B). The high level of natural antibody against A. hydrophila was produced as a result of continuous contact of Nile tilapia (O. niloticus) with *Aeromonas hydrophila* was normally present in the fresh water and normally inhabit the intestinal tract of fish . Concerning to oral vaccination, the result revealed that, Nile tilapia *(O. niloticus)* vaccinated by wet –packed whole cells bacterin by oral route at ratio of 5mg bacterial cells/gm minced meat for 15 days produced low level of humeral antibody (2 at first and second week and 3 at 4th and 4th week post vaccination) not greatly different from those of control fish . Unfortunately, there are no available literature dealt with oral vaccination with *Aeromonas hydrophila* bacterin. On the other hand, the result of the present study nearly agree with those recorded by many authors on organisms other than P. *fluorescens* (Rohovec, et.al., 1975; Fryer,et. al., 1976; Rodegers and

Austin, 1981; Badran, 1991 B, and Azad et. al (1999). Regarding to the relative level of protection (R.L.P) Amend (1981) Suggested that the RLP of over 60% provided acceptable protection. Our result revealed that the RLP of Nile telapia *(O. niloticus)* vaccinated by injection, immersion, and oral rout were 91.2, 86.8 and 86.8 % respectively. These results explained that, there is no great difference between the R.L.P of fish vaccinated by different methods inspite of the humeral antibody titers resulted from immersion and oral vaccination were low when compared with those of injection vaccination. The protection against infection of fish vaccinated by immersion and oral routes was related to agglutinins secreted in the muscus of body surface, gills; and intestinal mucosa (Kawai et. al., 1981; Badran, 1991 B, 1995 A, 1995 B and Sabry. N.M.(2008). The secreted agglutinins inhibit the organism to move freely and grow on the surface of the body and the mucus with trapped organism are removed leaving the skin clean and intact Badran (1991 B).

Indeed, the protocol of oral vaccination is very attractive since it's suitable for mass administration to fish of all size, imposes on stress on the fish because handling is not required and therefore dose not interfere with routine husbanding practices. Moreover, oral vaccination is the only method studies concerning the success of laboratory and field application of oral vaccines, according to the R.L.P against vibriosis (Rohovec et. al., 1975 Kawai and Kusuda, 1985 Fryer et. al., 1987 and Kusuda et. al., 1987), furunculosis (Austin and Rodgers , 1981., Rodgers and Austin, 1985 and Fryer, 1987) and motile Aeromonas hydrophila (Badran, 1991 A and 1991 B and Aly et. al., 2000)

Conclusion : The bacterin prepared from *Aeromonas hydrophila* had antigenic in nature where it's able to stimulate the immune system of immunized Nile tilapia *(O. niloticus).* Nile tilapia *(O. niloticus)* vaccinated by oral routes had low level of humeral antibody titers not greatly different from those of control . The protection of vaccinated fish against infection not dependent only on the humeral antibody responds where immersed and orally vaccinated fish, which had low level of humeral antibody titer, were protected against infection at the same level of fish vaccinated by injection route.

5-References

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