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The use of coated and uncoated oral inactivated *Aeromonas hydrophila* vaccine on the immune response of Nile Tilapia (*Oreochromis niloticus*)

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Abstract

Coated and uncoated formalized killed vaccine of *Aeromonas hydrophila* (bacterin) were used in the immunization of Nile tilapia against Motile Aeromonad septicemia. Vaccination was conducted via oral route. Fish were fed on oral initial dose followed by 2 booster doses every 2 weeks at 2nd and 4thweek . Blood samples were taken at zero days to get the pre-immune serum and every two weeks 2, 4 and 6 weeks post immunization from the initial dose and at 8th week post challenge for antibody titration in the fish sera. The humoral antibody response of vaccinated Nile tilapia *(Oreochromis niloticus)* was determined by direct agglutination titer test. Moreover, lysozyme activity was assayed for non-specific immunity. Experimental challenge infection with the virulent strain of *Aeromonas hydrophila was* done and daily mortality was recorded for 14 days to estimate the cumulative mortality and relative percent survival (RPS) of the fish in both control and vaccinated groups. The results of the group that fed on coated formalized killed vaccine gave the highest Antibody titers, lysozyme values, and RPS percent.

Key words .*Aeromonas hydrophila*- bacterin -vaccination- - gelatin- Nile tilapia

Introduction

Fish diseases caused by *Aeromonads* and are considered the major bacterial problems facing the aquaculture development and causing mass mortalities that reduced production and low quality of aquatic organisms (**Ghittino, 1976; Abdel-Hadi, 2004**).Also, motile Aeromonas Septicemia

(MAS) is one of the most economic diseases affecting fish farms in Egypt (**Atallah *et al*., 1999**). *Aeromonas hydrophila* is considered among the most pathogenic organisms in both homothermic and poikilothermic hosts including tilapia species (**Amin *et al*., 1985; Zaki, 1991**). It affects not only *O. niloticus* but also, causes severe outbreaks in *Cyprinus carpio* and *Mugilcephalus* (**Marzouk and Nawal, 1991;Eissa *et al*., 1993**). So, the use of vaccines that combined with good health management techniques may prevent the disease and the production becoming more predictable. Moreover, vaccines are a preventative measure to avoid disease outbreak and it has been successful in reducing the use of antibiotics (**Badran and Eissa,1991**). Unlike antibiotics, which kill or stop disease-causing bacteria. Using vaccines stimulate the fish's immune system to produce antibodies that help and protect the fish from diseases. In assessing the most important bacteria, for Egyptian aquaculture, more consideration has been given to the organisms that cause the most commercial damage and the organisms that are the most difficult to treat or are the most persistent **(Ahmed and Shoreit, 2001**). Many experimental and practical approaches to stimulate the immune response of fish were reported by (**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.

Thus, this study was conducted to tackle the following objectives:

- Developing suitable and effective vaccine against *Aeromonas hydrophila* using coated inactivated vaccine with gelatin and uncoated vaccine without gelatin.
- Evaluating the immunity levels obtained by oral vaccination and lysozyme activity
- Estimating the Relative Percent Survival (RPS) percent after the challenge test.

Materials and Methods

Fish and experimental design

For this experiment, 135 Nile tilapia,*O. niloticus* with an average body weight (35±2g) were obtained from Central Laboratory for Aquaculture Research , Abbassa, Abo-Hammad, Sharqia, Egypt. Fish were transported to a laboratory then the health status of the experimental fish was inspected.. The fish were randomly distributed (15 fish per each glass aquarium). in 9 experimental glass aquaria (120 liters), fish have been used in 3 fully prepared treatments including 3 replicates per each treatment .

Three treatments groups (T1 = 0, T2 = coated vaccine and T3= uncoated vaccine) to evaluate the effect of the treatments on immune response of tilapia. The examined fish were kept for 2 weeks for acclimation in the laboratory conditions. During the experimental period, the water quality parameters were monitored every day and maintained at optimal levels by regular water exchange (temperature; 24.5°C, dissolved oxygen; 7.2mg L^{-} 1, salinity; 0.32ppt, pH; 6.44 units and ammonia- nitrogen <0.21 mg L^{-}1).

Bacterial strain

Aeromonas hydrophila was isolated from the liver of diseased (*Oreochromis niloticus*). Fish were obtained from El Abbassa fish farm on tryptic soy broth, nutrient agar, R.S. media, **Shotts and Rimler (1973)** and Tryptic Soy Agar with 5% sheep erythrocytes, **Chen and Levin (1975)**. Then,the physical and biochemical characters were carried out according to **Bergey's manual of systematic bacteriology, (2005).**

Preparation of formalized killed *A.hydrohila* vaccine

For the preparation of bacterin, *A. hydrophila* was inoculated into Tryptic soy broth (TSB) and incubated for 24 hrs at 25°C. Formalin (37 % w/v) was added to the broth culture at a final concentration of (0.5 % v/v) and left 48 hrs at room temperature. The inactivated cells were harvested by centrifugation at 4000 rpm for 10 minutes, then washed twice in PBS and resuspended to the density of 1.5 x 10^{10} cells/ml . After that, the bacterin was tested for its sterility (free from living cells) by streaking onto Tryptic soya agar according to **(Nayak *et al*., 2004**).

Sterility test

This test was done as described by **Aly (1981)** by the cultivation of the prepared bacterins on tryptic soy agar to ensure that there's no growth of *Aeromonas hydrophila* or other organisms may occur.

Safety test

The safety test of the vaccine was performed according to **Anderson et. al (1970).**

Preparation of stained antigen used in antibody titration

Aeromonas hydrophila antigen that used for antibody titration was prepard by dilution.of formalin-inactivated bacterial cells by wet-weight, an equal volume of sterile physiological saline solution. One drop of Loffler's alkaline methylene blue prepared and was added to each every 10 ml of the diluted antigen as described by **Cruickshank (1985)**

Diet preparation

Commercial diet powder containing 25% protein was mixed with formalized killed *Aeromonas hydrophila* vaccine (bacterin) solution contains the whole cell at the level of 10^{10}CFU g^{-1}concentration by spray then, pelleted. The diet was coated bacterin by soaking in melted gelatin flakes (International Co. for gelatin Manufacture, Egypt) according to **Midtlyng *et al.* (1996)** as the first treatment**.** The second treatment was uncoated bacterin (without gelatin).The commercial diet powder was mixed with saline free from the vaccine as a control group. All diets were pelleted and left to dry in air.

Oral vaccination

A total of 135 tilapia (15 fish/aquarium) were used in 3 treatments in three triplicates. First treatment (Coated V.) fish were fed on coated formalized killed *A. hydrophila* vaccine with gelatin,Second treatment (uncoated V.) fish were fed on uncoated formalized killed *A. hydrophila* vaccine without gelatin and third treatment as control (without the addition of any vaccine to the feed)**.**.The fish were fed on a feeding rate of 2 % of body weight per day for 10 days then the fish were fed on basal diet and two booster doses were given at 2nd and 4^{th}week.

Sampling

Blood samples were collected from 3 fish from each treatment from the caudal blood vessels **(Lied *et al*., 1975)**. Samples were taken at zero-day and every two weeks from the initial dose and post-challenge test for antibody titration by using the direct agglutination method and lysozyme activity. After that, the samples were centrifuged at 3000 rpm for 15 min. Finally, serum samples were kept at −80°C till use.

Activation of the bacterial isolates for the challenge test

The *A. hydrophila* which was used for the challenge test was activated (**Azad *et al*., 1997**) 10 days before the challenge by serial I/P passage through live tilapia fish for 3 times.

Challenge test

An experimental infection was induced in the remaining fish (n=10/group/replicate) with pathogenic bacteria *Aeromonas hydrophila*. The bacteria were grown overnight in trypticase soya broth (TSB, Difco, USA) and the concentration was adjusted to (1.5×10^8 CFU/ml) in PBS.

Fish groups were injected with 0.2 ml of the bacterial suspension intra-peritoneally (I.P.). The fish were observed daily up to 14 days after the challenge and Relative percent survival (RPS) was calculated according to the following formula, **(Amend, 1981)**.

$$RPS = 1 - \frac{(\%\ \text{mortality in challenged fish})}{(\%\ \text{mortality in control fish})} \times 100$$

Antibody titer assay

Antibody titers against *A. hydrophila* were determined by the direct agglutination method according to **Yildirim *et al.* (2003).** 96 well microtiter plate with round bottom wells were used. The assay was initiated with a dilution of 1:1 (50 µl of phosphate buffer: 50 µl of serum) and consequently two-fold serial serum dilutions were made by adding 50 µl of diluted serum till the 12th well. As a result the serum dilutions were 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048. Thereafter, 50 µl of inactive *A. hydrophila* (1×10^{10} CFUs) suspension was added to each well and then microplates were covered with plastic film and incubated at room temperature for 16-18 hr. The agglutination endpoint was established as the last serum dilution where agglutination was visible. Agglutination antibody titers were expressed as log2 (x+1) of the reciprocal of the highest serum dilution showing visible agglutination as compared to the positive control. The last well was used as a negative control, where there was only 50 µl PBS buffer.

Lysozyme assay

Lysozyme activity of blood serum was determined as described by **Anderson and Siwicki (1995)** with some modifications. Blood serum was prepared by centrifuging the blood at 3000 rpm for 5 min. Serum (0.1 mL) was placed in test tubes and 0.9 mL of a 0.75 mg/mL *Micrococcus lysodeikticus* (Sigma, MO, USA) suspension in phosphate buffered saline, pH 6.2 was added and mixed well. The absorbance was measured at 450 nm by a spectrophotometer at 1 min intervals for 10 min after mixing with bacteria and the rate of change of absorbance calculated. Lysozyme activities were calculated using hen egg white lysozyme (Sigma, MO, USA) as a standard.

Statistical analysis

All experimental data were subjected to one -way ANOVA to evaluate the effect of coated, uncoated vaccine and control groups per weeks. Differences between means were tested at the 5% probability level using Duncan test. All the statistical analyses were done using SPSS program version 15 (SPSS, Richmond, VA, USA) as described by **Dytham (1999).**

Results and Discussion

Vaccines for aquaculture have been successful in reducing the use of antibiotics. Unlike antibiotics, which kill or stop disease-causing bacteria, vaccines stimulate the fish's immune system to produce antibodies that help and protect the fish from diseases. There are various methods of vaccination, the oral route is one of them and it is simple, cheap and ideal for mass administration to fish of all sizes and for large-scale aquaculture in addition to the elimination of the stress caused by the parental administration and the possibility of quick vaccination a large number of fish with reduced costs **Tatner, et al. (1984).**

Antibody titer assay

The results revealed that antibody titers by using the direct agglutination method were higher in fish vaccinated with coated formalized killed vaccine against *A.hydrophila* with gelatin than those were obtained in fish vaccinated with uncoated vaccine which agreed with **Romalde *et al*. (2004**) who mentioned that oral vaccine that prepared by the addition of bacterial cells to feed without a carrier or protective coating might expose to the antigen and might be damaged during digestion process. So, they concluded that improvement or modification of bacterin was induced by incorporation in fish feed by the coating material. That is why; gelatin was used for coating the vaccine in this study. However, the best results of Ab titers were obtained from vaccinated fish with coated and uncoated vaccine groups and were significantly higher at 6th week (8.66±.4.05 and 6.00±.0.20 respectively) at $P < 0.05$ than those that obtained from non vaccinated or control group (2.66±1.33) and these results agreed with **Akhlaghi, (2000)** who recorded that the oral route of administration of antigens induced the high immune response in fish. Moreover, the results of coated inactivated vaccine groups were higher than those were obtained from uncoated group and control group which agreed with **Johnson and Amend (1983**) who found that one of the important factors for the poor response to oral vaccination is the digestive degradation of antigens in the foregut before the vaccine reaches the immune responsive areas in the hind gut and other lymphoid organs .Besides ,the antibody titers kept with the

best level post challenge at 8th weeks which agreed with **Rodrigues et. al, (2003)** who found that the oral route of administration of antigens induced higher immune response than the other routes of administration as in (Table 1).

Lysozyme activity

Lysozyme activity is an important index of the innate immune response of fish. It is well documented that fish lysozyme possesses lytic activity against bacteria. Also, it is known to be opsonic in nature and activates the complement system and phagocytes. Besides, the most important thing is that Lysozyme is a vital defense molecule of the non-specific immunity and plays a significant role in mediating protection against bacterial invasion (**Saurabh and Sahoo 2008**). Also, there was a significant increase in serum lysozyme activity, especially by vaccination. Therefore, in this study, the results of the coated vaccine group at 6th week of lysozyme value was (1.525±.397) and at 8th week (after the challenge) was (1.587±.418). Also, lysozyme values were higher in coated and uncoated vaccine groups than control group especially at 8th week post challenge (1.587±.418, 0.750±.094 and 0.657±.0638) respectively. These results agreed with **Stefaan *et al.* (2004)** who recorded that the skin mucus antibody level was higher after oral vaccination compared to the i/p route and the oral vaccination of fish could be successful when the antigens reach the second out of the intestine in sufficient quantities **Stefaan, *et al*, (2005) as in table (2).**

Challenge test

The results of relative percent of survival in the present investigation showed that it ranges from 86.67 to 93.3 % in tested vaccinated groups which agreed with **Esteve *et al*, (2004)** who demonstrated that the oral route of vaccine administration induced protection level higher than 80% and induced significant systemic and mucosal immune response. In the present study, fish were vaccinated orally with the coated vaccine of *A. hydrophila* (bacterin) in the form of coated pellets vaccine of gelatin flakes. Also, It was clear that the oral vaccination by this methodology of pellet preparation and coating with gelatin was capable of inducing 93.3% relatively high level of protection (RPS) in tested fish. Also, this method can be easily used in the field when compared with the injection route as in **(table 3) and figure (1).**

Table (1) Antibody titers (Mean ± SE) of Nile tilapia sera of different groups

Period ► Treatments	Zero w	2nd w	4th w	6th w	8th w
Control	3.33± 0.66bc	2..66±2.66bc	2.66±1.33^{bc}	2.66±1.33^{bc}	2.00±1.33^{c}
Coated vaccine	3.33±0.66 b	6.66±1.33abc	6.00±.0.20 abc	8.66±.4.05ab	10.66±2.66a
Uncoated vaccine	3.33±1.33^{ab}	5.33±1.33abc	6.66±1.33abc	6.00±.0.20 abc	6. 00±0.0abc.
P. value:			***P* < 0.05**		

High Ab = 10.66±2.66 low Ab =.2.00±1.33

Means having the same letters in the same column are not significantly different at $P < 0.05$.

Table (2) Serum lysozyme activity (Mean ±SE) of Nile tilapia sera of different groups.

Period Treatments	Zero w	2nd w	4th w	6th w	8th w
Control	0.496±.0831^{c}	0.772±.110^{abc}	0.787±.0269^{abc}	0.827±.0564^{abc}	0.657±.0638^{c}
Coated vaccine	1.046±.248^{abc}	1.032±.617^{abc}	0.712±.177^{a}	1.525±.397^{ab}	1.587±.418^{c}
Uncoated vaccine	0.614±.116^{c}	0.831±.240^{abc}	0.892±.022^{abc}	0.666±.238^{ab}	0.750±.094^{abc}
P. value:			***P* < 0.05**		
Treatments:			**0.176**		

Low lysozyme value µg/ml. =0.472±0.058. High lysozyme value = 1.587±.418.

Means having the same letters in the same column are not significantly different at $P < 0.05$.

Table (3) RPS% of examined fish groups challenged with *A. hydrophila*.

Treatments	No of fish	Dead	Survival	Mortality %	Relative percentage of survival (RPS) %
Control	30	27	3	90	10
Uncoated V.	30	4	26	13.3	86.7
Coated V.	30	2	28	6.7	93.3

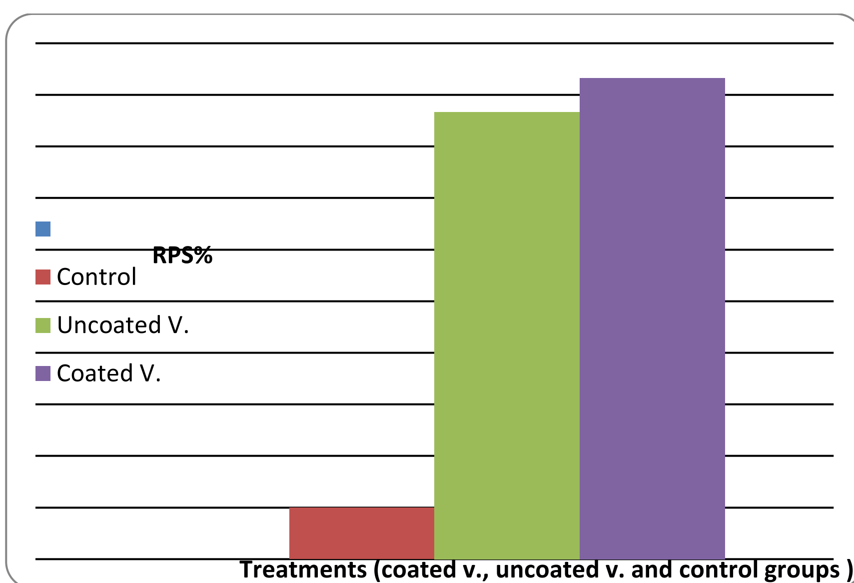

Figure (1). Relative percent survival (RPS) of coated, uncoated vaccine and control groups of Nile tilapia post-challenge with *Aeromonas hydrophila*

Conclusion

In conclusion,formalized killed *A.hydrophila* vaccine coated with gelatin gave an excellent protection against *Aeromonas hydrophila*. Therefore, coated *A.hydrophila* vaccine with gelatin is recommended to be given orally (mixed with feed) to tilapia as it induces significantly higher antibody titres and stimulates non-specific immune response of Nile tilapia.

This work was designed for vaccination of *Aeromonas hydrophila* is a trial and can be used as a model for other bacterial fish pathogen.

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إستخدام اللقاح الميت لميكروب الإيروموناس هيدروفيلا المغلف أو غير المغلف بالجيلاتين وتأثيره على مناعة أسماك البلطى النيلى

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الملخـص العربـى

أجريت هذه الدراسة لتحديد مستوى الأجسام المضادة والليزوزيم ونسبة الإعاشة للبلطى النيلى باستخدام لقاح مغلف بالجلاتين أو بدونه عن طريق الفم ضد ميكروب الايروموناس هيدروفيلا وتمت هذه التجربة على عدد ١٣٥ سمكه (١٥ سمكة لكل حوض) فى ٣ معاملات أو مجموعات المجموعه الاولى تغذت على لقاح الايروموناس المغلف بالجلاتين والمجموعه الثانيه تغذت على لقاح الايروموناس هيدروفيلا بدون جلاتين ومجموعه ضابطه بدون تحصين واستمرت التجربه لمدة ٨ أسابيع. تم تغذيه الأسماك باللقاحات والمعاملات المذكورة بجرعتين مقويتين فى الأسبوع الثانى والرابع على التوالى بعد الجرعة الإبتدائية. تم فحص عينات الدم فى بداية التجربة ثم كل أسبوعين من الجرعة الإبتدائية لقياس مستوى الأجسام المضادة فى السيرم وايضا نشاط الليزوزيم. وتم اجراء اختبار التحدى التجريبى لقياس مدى كفاءة اللقاح ومستوى حماية الأسماك.قد تم تسجيل عدد الأسماك النافقة يوميا لمدة اسبوعين لتقدير النفوق التراكمى والإعاشة النسبية للأسماك فى المعاملات المختلفة. وقد تبين من النتائج أن مستوى الأجسام المضادة والإعاشة النسبية قد ارتفع للأسماك فى المعامله باللقاح بالجلاتين.