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Trials for Vaccination of *Tilapia* Fish Against *Aeromonas* and *Pseudomonas* Infections Using Monovalent, Bivalent and Polyvalent Vaccines

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Abstract: Different vaccine preparations and formulations for vaccination of Tilapia species were tried by adding formalin to the bacterial culture (bacterin) and used by immersion and oral routes. Fish were vaccinated by using monovalent,bivalent and polyvalent vaccines and the efficacy of these vaccines were tested by using the challenge test with the detection of RPS (Relative Percent Survival) and by using indirect ELISA for estimation of the immune response of fish during and after vaccination. The results of fish vaccination showed that the polyvalent vaccine when used in Tilapia fish through the immersion route was of easier administration and of higher efficacy (RPS) and it was effective against more than one type of bacteria.

Key words: Fish vaccine % Bacterin % Aeromonas % Pseudomonas % Immersion % Oral % Vaccine % Monovalent % Bivalent % Polyvalent % RPS

INTRODUCTION

Fish diseases are among the most important problems and challenges confronting fish culturing. Fish diseases do not occur as a single caused event, but are the end result of interactions of the disease, fish and environment. Fish in intensive culture are continuously affected by environmental fluctuations and managemental practices such as handling, crowding, transporting, drug treatments, undernourishment, fluctuating temperatures and poor water quality. All of these factors can impose considerable stress on the homeostatic mechanisms of fish rendering them susceptible to a wide variety of pathogens.

It is well known that disease threats can pose serious economic problems to any fish farming operation. Disease outbreaks may cause loss of fish, poor feed conversions or product down – grading, which can mean a loss in revenue. As with the growth and intensification of any husbandry system, farmed tilapia and Catfish suffered from increasing disease problems in Egypt [1, 2].

These disease problems are treated with antibiotics. However, the expense of antibiotics, the short period of protection they offered in addition to the need for repeated treatments in extended outbreaks of disease and the difficulties caused by resistant strains as well as increased controls on residues in carcasses lead to application of more investigations to found alternative mean. Also, the limited range of antibiotics available to treat fish in many areas of the world is a further problem.

The use of vaccines, combined with good health management techniques, may result in substantial disease prevention and so production becomes more predictable. Vaccines are a preventative measure as opposed to antibiotic treatment which is used after a disease outbreak. 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. However, vaccines are not impenetrable shields and the resistance they impart can be beaten if other risk factors are not considered.

In assessing the most important bacteria, for Egyptian aquaculture, more consideration have 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 [1, 3]. There are a wide variety of pathogenic bacteria that can infect pond. By far the most common are Aeromonas and Pseudomonas.

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The Aim of this Work Was to Elucidate the Following Points:

- Vaccination trials with bacterins prepared from the local isolated strains through different routes of application (a. Direct immersion. b. Oral vaccination)
- Detecting the efficacy of the prepared vaccines by:
- Challenge test and estimating the Relative Percent Survival (RPS)
- Estimation of the Humoral Immune Response using the ELISA technique.

MATERIALS AND MEHTODS

Materials

Fish: Fingerlings of Tilapia species were obtained from El-Wafaa farm with an average body weight of 5-10 g for the purpose of immersion and oral vaccination (300 fish for each route). Fish were managed as outlined by

Glass Aquaria: (1x1x0.5m) used for experimental purpose. They were supplied with air conductors and dechlorinated tap water.

Culture Media Used: Tryptic soy broth: (Difco)

Chemicals and Buffers: Formalin (37%) -Ethyl alcohol a (70%) - Incomplete Freund's adjuvant (IFA) *(Oxoid)* - Phosphate buffer saline (PBS) pH 7.4-Sodium citrate: Heparin: sodium salt - Elisa Buffers:

Apparatuses and Equipments: McFarland standard tubes - Cooling centrifuge: (15, 000 rpm) - Centrifuge: (4000 rpm) - ELISA reader -Spectrophotometer -Incubator - Hot air oven - Sartorius electronic balance - Microtitre pipets - ELISA plates -96-well microtitre plates - Eppendorf tubes.

Commercial Pellets: (Zoo control company, Egypt) used for fish feeding during the experimental vaccination period.

Gelatin Flakes: (International company for gelatin manufacture, Egypt) used for oral vaccine preparation.

Anaesthesia Used: 0.06 g/l of MS- 222 (Sigma) (Tricainemethane sulphate): used before manipulation of fish during vaccine application or taking blood samples.

Methods: Preparation of *A. hydrophila*, *A. sobria*, *A. caviae* and *P. fluorescens* bacterins: [4,5].

For preparation of bacterins, each bacterial isolate was inoculated separately into tryptic soy broth (TSB) and incubated for 24h at 25°C. Formalin (40% 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 xg for 10 min., then washed twice in 0.3% formalized PBS and resuspended to the density of McFarland standard tube No3 (1 x 10^9 cells / ml) for all isolates. After this, the bacterins were tested for their sterility (free from living cells) by streaking it onto tryptic soy agar which showed no growth.

Vaccination of Tilapia species with formalized whole culture of Aeromonas spp. (*A. hydrophila*, *A. sobria and A. caviae*) and *Pseudomonas fluorescens* by direct immersion route [6]:

Groups of 50 fingerlings of Tilapia fish (5-10 g) were used for each type of vaccination (total 300 fish) in addition to the control group represented by 50 fish per group for each glass aquarium constituting 6 groups for both vaccinated and control fish as shown in Table 1.

Fish were immersed for 30 min in diluted vaccine in a separate vaccine tanks (1 volume of vaccine to 10 volumes of tank water = 10^8 cells / ml). The fish were drained carefully maintaining the vaccine solution in the vaccinating tank and then returned to their original aquaria (holding tank) after vaccination. In case of bivalent and polyvalent vaccines formulations, equal portions of bacterins were added to constitute one volume of the vaccine. Booster dose was applied after 2 weeks with the same technique. The process of vaccination was repeated until the vaccination of all fish groups was completed.

The control fish groups were kept untreated until the end of the vaccination process. Blood samples from vaccinated and control fish were taken from the caudal vein by plastic syringes containing 0.1 ml of sodium citrate as an anticoagulant just before immunization, at the end of vaccination process (after 4 weeks) and after challenge (after 5 and 6 weeks from the beginning of vaccination process). Challenge was applied by the technique called “bath challenge” as outlined by Lillehaug [7] in which each bacterial strain was inoculated in 500 ml of tryptic soy broth for 24 h at 25°C. The cultures (1 volume) were added to 10 volumes of the tank water in a separate aquarium for each group. The challenge process persisted for one hour, for both vaccinated and control fish group. Fish were transferred to their original aquaria and observed for one week post challenge for any clinical abnormalities and mortalities.

Table 1: Vaccination groups of Tilapia species using immersion route

Fish groups	Vaccines formulations	No of vaccinated fish	No of control fish	Dose	Route and period of vaccination
1	Monovalent *A. hydrophila*	50	50	1 volume of	immersion for 30 min
2	Monovalent *A. sobria*	50	50	the vaccine	immersion for 30 min
3	Monovalent *A. caviae*	50	50	to 10 volume	immersion for 30 min
4	Monovalent *P. fluorescens*	50	50	of tank water = 10^8	immersion for 30 min
5	Bivalent (1+4)	50	50	Cells / ml	immersion for 30 min
6	Polyvalent (1+2+3+4)	50	50		immersion for 30 min

Bivalent = *A. hydrophila* + *P. fluorescens*

Polyvalent = *A. hydrophila* + *A. sobria* + *A. caviae* + *P. fluorescens*

Table 2: Vaccination groups of Tilapia species using oral route

Fish groups	Vaccines formulations	No. of vaccinated fish	No. of control fish	Dose	Route and period of vaccination
1	Monovalent *A. hydrophila*	50	50	1 % of the fish body	Orally for 7 days
2	Monovalent *A. sobria*	50	50	weight / day for 7	Orally for 7 days
3	Monovalent *A. caviae*	50	50	days (1g of pellets	Orally for 7 days
4	Monovalent *P. fluorescens*	50	50	contained 1 ml of	Orally for 7 days
5	Bivalent (1+4)	50	50	bacterin =	Orally for 7 days
6	Polyvalent (1+2+3+4)	50	50	$1.5x10^{10}$ cells/ml	Orally for 7 days

Re-isolation of the pathogen from the kidney tissues and cultivated onto specific media from the moribund and dead fish groups for confirmatory diagnosis by typical colonial appearance. Post challenge mortalities were recorded in both vaccinated and control fish groups. The level of protection was calculated according to Amend [8].

Relative level of protection or Relative Percent Survival (RPS) =

$$1-\left(\frac{percent\ \ of\ \ immunized\ \ \ mortlity}{percent\ \ \ of\ \ \ control\ \ \ mortaility}\right) x100\%$$

The evaluation of Tilapia fish immune response after vaccination with Aeromonas and Pseudomonas bacterins by immersion and bath challenge route was done using the indirect-ELISA according to Loghothetis and Austin [9]. The interpretation of ELISA readings (mean of OD values) in triplicate manner was done according to Esteve *et al*. [10].

C Oral vaccination of Tilapia species with formalized whole culture of Aeromonas spp. (*A. hydrophila, A. sobria and A. caviae*) and *Pseudomonas fluorescens* (bacterin) mixed with feeding pellets:

The oral vaccine was given in the form of coated pellets which had been manufactured according to Lillehaug [7], Midtlyng *et al.* [11], Azad *et al*. [12] and Stefaan *et al*. [13] as follows: the bacterial isolates were grown in tryptic soy broth at 25°C for 24-48 h. The bacteria were inactivated with formalin (0.5% v/v) and used for vaccine formulation. The resulting bacterin suspension had a cell density of $1.5x10^{10}$ cells/ml. Commercial food pellets were used as a carrier for vaccine application as shown in Table 2.

Blood samples were taken from the caudal vein from the fish groups before immunization and after vaccination by 4 weeks. The first day of vaccination started at the last day of feeding the coated pellets (after one week). Blood samples were taken also after challenge (after 5 and 6 weeks of vaccination)

Challenge was done after 4 weeks of vaccination by bath challenge method for one hour in separate challenge tanks. The formation of coated pellets in both bivalent and polyvalent formulations was made by the same technique in the monovalent bacterin, but the pellets were immersed in the bacterins suspension of equal volumes.

Fish groups were observed for one week post challenge after transfer to their original aquaria for observation of any clinical abnormalities, lesions or mortalities. Reisolation of the inoculated bacteria from the kidney of moribund and dead fish onto their media for more confirmation. Mortality was recorded in both vaccinated and control fish groups and the level of protection (RPS) was calculated for each group according to Amend [8].

$$RPS = 1-\left(\frac{percent\ \ of\ \ immunized\ \ \ mortlity}{percent\ \ \ of\ \ \ control\ \ \ mortaility}\right) x100\%$$

The antibody titre in fish groups vaccinated by the oral route through the vaccination period and after challenge was measured by indirect ELISA according to Loghothetis and Austin [9] and the interpretation of the ELISA readings was done according to Esteve *et al*. [13].

RESULTS

Table 3 and Fig. 1 record the relative percent of survival in Tilapia fish groups vaccinated by immersion route with different types of formalized whole culture vaccine and challenged with bath route. Fish vaccinated with monovalent vaccine of *A. hydrophila* and *A. sobria* bacterin showed high percentage of RPS (89 and 81%, respectively) and for the polyvalent bacterin, the RPS was 81%. In fish group vaccinated with *A. caviae* bacterin, the RPS was 77%. In fish groups vaccinated with bivalent and *P. fluorescens* bacterins the RPS was found to be 74 and 73 %, respectively.

Antibody titre was measured using the ELISA, four weeks post vaccination and after challenge. As shown in Fig. 2, the antibody titre reached maximum level in all fish groups vaccinated by the immersion route at the 4th week. This level gradually decreased after challenge and then reached its basal level at 6 weeks post vaccination.

In the present study groups of Tilapia fish were vaccinated by immersion route with different vaccine formulations either as monovalent, bivalent or polyvalent vaccines prepared from the bacterins of *A. hydrophila, A. sobria, A. caviae* and *P. fluorescens* and the efficacy of each type of vaccination was determined by the relative percent survival (RPS) through the application of challenge test (Bath challenge route) with the virulent strains of the same bacteria. The results of immersion vaccination showed that the protection was very high as shown by the low mortality percent in the vaccinated fish group and the RPS values reached 89, 74 and 81% with the monovalent, bivalent or polyvalent vaccines, respectively. The humoral immune response or the antibody titre of immersed vaccinated Tilapia fish groups as measured by the ELISA representing the antibody titres of fish serum during the vaccination period showed high antibody titre when compared with the control fish serum or zero day fish serum and this indicated that the antibody titres were directly proportional to the RPS values (level of protection). This antibody level gradually decreased after challenge reaching its basal level at the 6 th weeks postvaccination.

As shown in table 4 and Fig. 3, the RPS in the orally vaccinated fish with *A. sobria* bacterin, bivalent bacterin

Table 3: Mortality and survival percentage with RPS of Tilapia fish vaccinated by immersion route with different types of formalized whole culture vaccines with consequent challenge

			% of mortality		% of survival		
Type of vaccine	Route of vaccinate	Route of challenge	Vaccinated	Control	Vaccinated	Control	RPS
Formalized whole culture of							
A. hydrophila	Immersion	Bath	10	86	90	14	89%
A. sobria	Immersion	Bath	14	74	86	26	81%
A. caviae	Immersion	Bath	16	70	84	30	77%
P. fluorescens	Immersion	Bath	20	72	80	28	73%
Bivalent	Immersion	Bath	20	76	80	24	74%
Polyvalent	Immersion	Bath	18	92	82	8	81%

Table 4. Mortality and survival percentage with RPS of Tilapia fish vaccinated orally with different types of formalized whole culture vaccine

			% of mortality		% of survival		
Type of vaccine	Route of vaccinate	Route of challenge	Vaccinated	Control	Vaccinated	Control	RPS
Formalized whole culture of							
A. hydrophila	oral	Bath	32	86	68	14	63%
A. sobria	oral	Bath	10	74	90	26	86%
A. caviae	oral	Bath	24	70	76	30	66%
P. fluorescens	oral	Bath	22	72	78	28	70%
Bivalent	oral	Bath	12	76	88	24	85%
Polyvalent	oral	Bath	26	92	74	8	72%

Ah= A.hydrophila, As =*A. sobria*, Ac= *A.caviae* and Pf = *P.fluorescens*

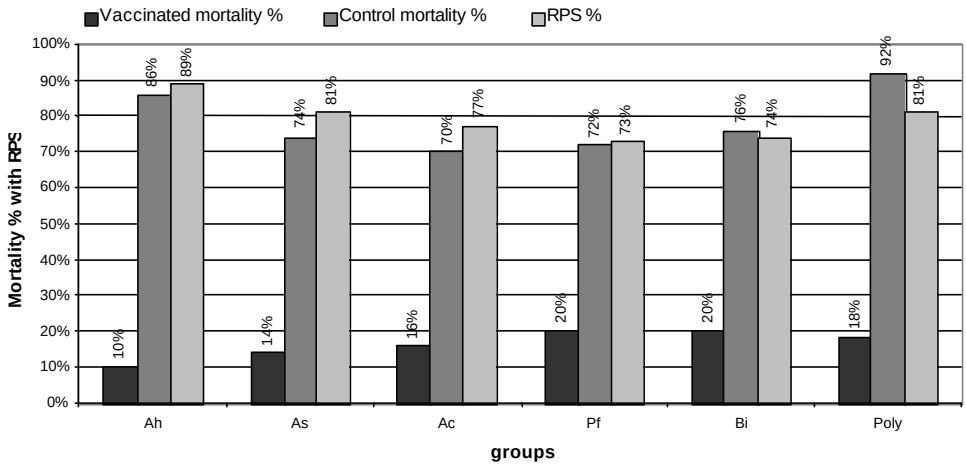

Fig. 1: Mortality % and RPS of Tilapia fish vaccinated by immersion route with different types of formalized whole culture vaccine

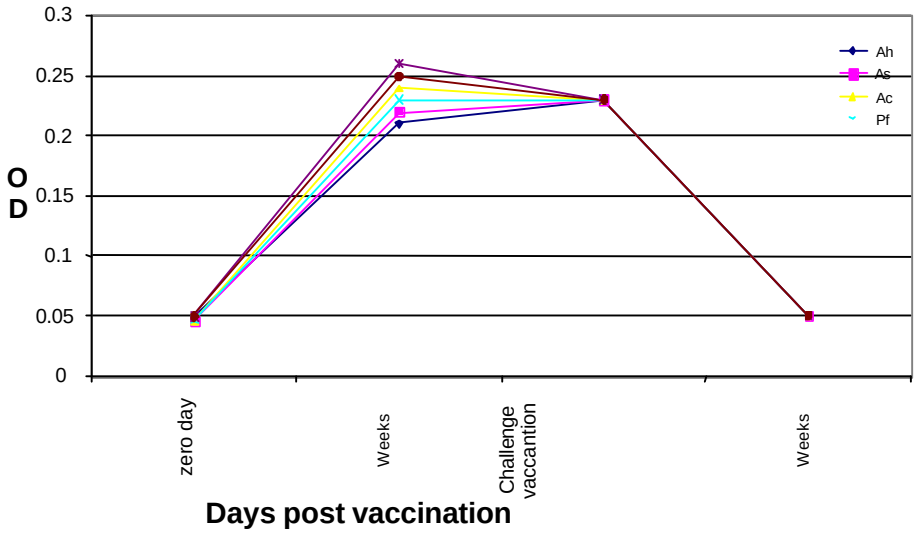

Fig. 2: Antibody titre of immersed vaccinated Tilapia fish groups vaccination and post challenge period measured by during the ELISA

and polyvalent bacterin was very high (86, 85 and 72 %, respectively) while it was moderate in fish vaccinated with *P. fluoresces*, *A. caviae* and *A. hydorphila* bacterin (70, 66 and 63%, respectively).

The antibody titre of the orally vaccinated fish groups with different bacterin formulations at the end of vaccination period and after challenge was measured by ELISA. As shown in Fig. 4, the antibody titre reached its maximum level at 4 weeks of vaccination, then gradually decreased till the 5th week (challenge), then reached basal level at 6 week post vaccination.

In the present study, Tilapia species were vaccinated orally with monovalent, bivalent and polyvalent vaccines of *A. hydrophila*, *A. sobria*, *A. caviae* and *P. fluorescens* bacterin in the form of coated pellets with the vaccine and gelatin flakes. It was clear that the oral vaccination by this methodology of pellet preparation and coating was capable of inducing a relatively high level of protection (RPS) and antibody response.

The results of RPS in the present investigation showed that it ranges from 63 to 86% due to the variation in the mortality percent in each group of vaccinated fish and according to the difference in the mortality ratio between the vaccinated and control groups, but significantly the level of protection was very high in this type of vaccination regardless the formulation of vaccine either monovalent, bivalent or polyvalent. It was clear that the antibody titre of orally vaccinated fish in the present

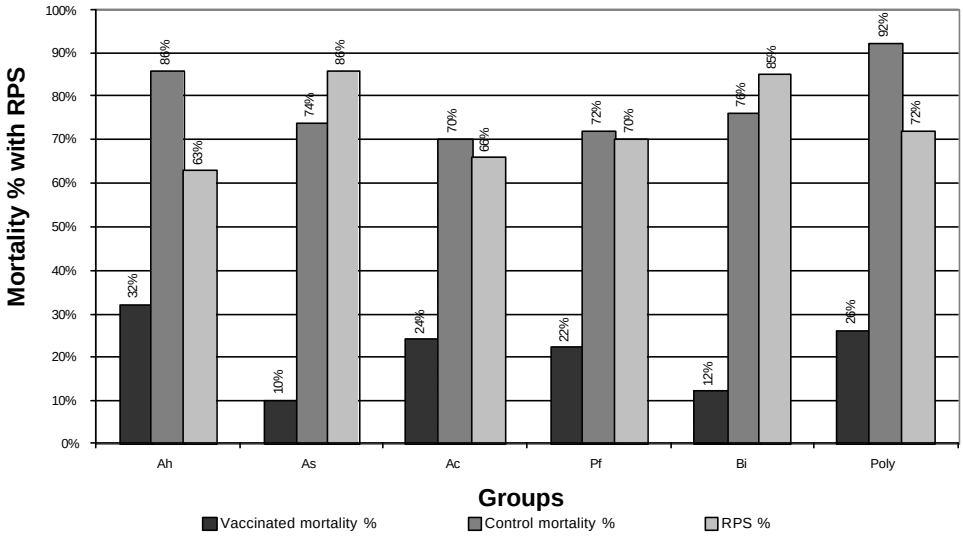

Fig. 3: Mortality % and RPS of Tilapia fish vaccinated orally with different types of formalized whole culture vaccine

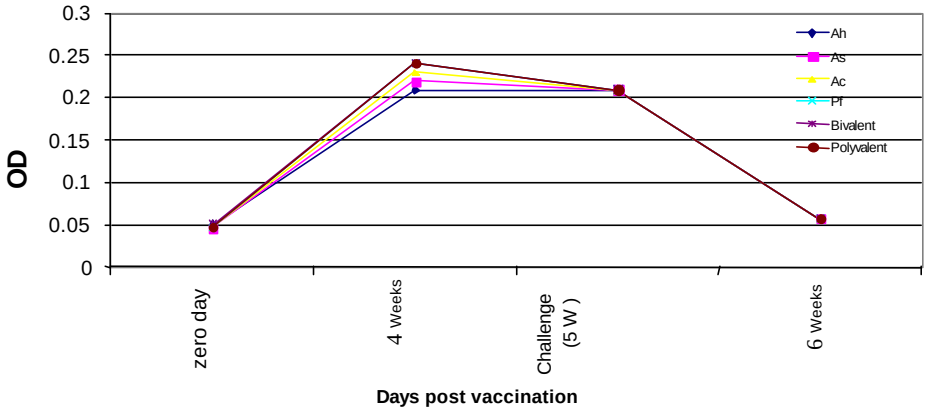

Fig. 4: Antibody titre of orally vaccinated Tilapia fish groups during the vaccination and post challenge period measured by ELISA

work with different bacterin formulations at the end of vaccination period is directly proportional to the RPS values where it reached maximum level 4 weeks after vaccination and after challenge it decreased gradually and then reached basal level 6 week post vaccination.

DISCUSSION

Immersion immunization methods are associated with variable efficacy, yet they offer the benefits of low labour input, minimal handling stress and stimulation of the immune system via the natural route of the pathogen entry. A variety of immersion methods have been used ranging from briefly dipping fish in a highly concentrated suspension to the addition of the bacterin to the rearing environment. The bacterin typically consists of a formalin killed suspension culture of the pathogen. The efficacy of immersion vaccination is highly dependent upon fish species, pathogen type and specific method employed [14].

The humoral immune response of Tilapia fish was investigated following injection, immersion and oral administration of the formalin killed vaccine *A. hydrophila, A. caviae, A. sobria* and *P. fluorescens*. Humoral antibody as measured by ELISA was detected with all antigen preparations.

Among the various methods of vaccination, the oral and immersion routes are 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 parental administration and the possibility of quickly vaccinating large number of fish with reduced costs. However, the effectiveness of this approach is limited. The step of antigen entry is likely to be a critical

factor limiting the success of this delivery method. Qualitative and some quantitative evidence has shown that antigen enters through the skin [15], the gills[16] and in the gastrointestinal tract as a result of swallowing during exposure[17].

Attempts of oral vaccination of fish against motile aeromonad septicaemia, vibriosis, yersiniosis and furunculosis have either yielded mild and short lived or inadequate responses. 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 [18]. Strategies that have been explored for improving the oral vaccination have included protected antigens such as encapsulated antigens [7] and a biofilm of *A. hydrophila* for oral vaccination of carp which induced significantly higher antibody titres and protection[19]. Stefaan *et al.* [13]used the commercial feed pellets as a carrier for vaccine application whereas, the feed pellets were first coated with *V. anguillarum* bacterin suspension.

Data from other workers revealed that the oral route of fish immunization conferred full protection [20]. Also, the oral route of administration of antigens induced higher immune response than the other routes of administration [21, 22]. Esteve *et al*. [23] demonstrated that the oral route of vaccine administration induced protection level higher than 80% and induced significant systemic and mucosal immune response. The skin mucous antibody level was higher after oral vaccination compared to the i/p route [13]. The oral vaccination of fish could be successful when the antigens reach the second gut of the intestine in sufficient quantities [24].

The results of Esteve *et al*. [13] suggested that the stimulation of antibody production after oral vaccination is produced firstly at the mucosal level and secondly at the kidney and spleen level. Thus, the mucosal lymphocytes would be responsible for the low levels of secreted antibodies detected in mucous mainly during the first day, while the kidney and spleen lymphocytes for the high levels of circulating antibodies detected in plasma along the overall period. The levels of systemic antibodies on the day at which the experimental challenge was performed were theoretically high enough to protect fish against the challenged bacteria. Several authors have pointed out that the oral immunization may protect against different pathogens like *A. hydrophila* [12, 19]. *A. Veronii* [25], *V. anguillarum* [20, 26] and Y. *Ruckerii* [27].

The degree of protection achieved in the present trial by oral administration of a single dose (with no booster dose) of the protected vaccine was relatively high compared to the conventional vaccination methods of injection and immersion. The encapsulation of antigen in the oral vaccination of fish were found to be a promising method [26] due to the development of systemic together with the induction of mucosal response (production of IgM) implying that it can be applied to achieve a better protection against fish bacterial disease.

Purely on the basis of cost per volume, vaccines are expensive. However, it can be cost effective as a small volume can be diluted and used to dip a large number of small fish. Good vaccine should offer an acceptable return on investment.

In conclusion, the current trial for fish vaccination showed that the polyvalent vaccine when used in Tilapia fish through the immersion route was of easier administration and of higher efficacy (RPS) and it was effective against more than one type of bacteria.

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