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Efficacy of Formalin-killed *Aeromonas hydrophila* and *Streptococcus* sp. Vaccine in Red Tilapia

S. Prasad[1] and N. Areechon[2]

[1]*Fisheries Research Center, Kaski, Pokhara, Nepal*
[2]*Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Thailand*
[1]E-mail: *g43sdp@yahoo.com*

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Abstract

Humoral response in red tilapia against formalin-killed *Aeromonas hydrophila* and *Streptococ cus* sp. vaccine administered by intraperitoneal injection was evaluated.The result indicated that *A. hydrophila* vaccine induced significantly differed ($P<0.05$) high mean peak antibody titers of 925.87±467.92 and 4983.47±1832.74 in both primary and secondary immune response, respectively. However specific antibody produced by red tilapia in response to administration of *Streptococcus* sp. vaccine revealed only weak secondary response of 101.33±45.38.

In separate experiment, relative protection in red tilapia immunized with *A. hydrophila* and *Streptococcus* sp. vaccine was conducted. Immunization were done by direct immersion for 1 hr in vaccine suspension and then challenged 2 weeks after by immersing fingerlings for 6 hr with virulent *A. hydrophila* and *Streptococcus* sp. Percent cumulative mortality in vaccinated and unvaccinated groups was compared after 14 days of post challenge. Red tilapia immunized by *A. hydrophila* vaccine demonstrated a particularly high level of immunity (76.67%) compared with unvaccinated (43.33%). *Streptococcus* sp. vaccine greatly reduced the mortality in vaccinated (31.67%) compared with unvaccinated fish (55%) but these differences in mortality were insignificant ($P>0.05$).

Results from this study indicated the importance of vaccine for increasing disease resistance against *A. hydrophila* and *Streptococcus* sp infection by stimulation of specific humoral immunity. However the most important factor must be the method of vaccine administration which should be effective and applicable to farm scale.

***Key words*:** Red tilapia, *Aeromonas hydrophila*, *Streptococcus* sp., antibody response, immersion immunization

Introduction

The culture of aquatic animal has experienced a rapid growth in recent years. Tilapia makes a majority share in today's world aquaculture production. Various tilapia species have been cultured in fresh and saline water. The species of tilapia that are of interest to an aquaculturist includes *Tilapia aurea, T. nilotica, T. mossambicus* and red hybrids that have been produced by crossing them with other species (Ridmontri, 2001). Red tilapia strains are considered important in aquaculture (Pullin, 1983) due mainly to market preferences over wild type. The technical advancement of red tilapia farming in the Southeast Asia over the past decade has been adopted by a variety of local commercial production systems. As a result, the culture of red

tilapia has a profound impact on the economy of a large number of fisheries communities. Red tilapia is a common species of cage aquaculture in Thailand (Ridmontri, 2001).

Despite the success in tilapia farming, mass mortality due to different diseases normally occurs in culture with high stocking density. The loss of crop has not only shaken the individual tilapia farmers but also cast a gloomy shadow over the golden economy. The most common diseases of tilapia are protozoan *Trichodina* and bacterial infection caused by *Aeromonas hydrophila*, *Flexibacter columnaris* and *Streptococcus* sp. (Areechon *et al*., 1992; Shoemaker *et al*., 2000). The physical appearance of infected and uninfected fish in the market place can be vastly different and external signs of the affected fish make them unmarketable (Nieto *et al*., 1995).

As the severity of these diseases has increased proportionally with the development and expansion of red tilapia farming, there is an urgent requirement for more effective methods for the control of these pathogens. *Aeromonas* and *Streptococcus* can be controlled at present by effective management practices and chemotherapy. In many cases, control of disease by management practices has not proven practical. Moreover extensive uses of antibiotics are undesirable because of the risk of antibiotic residues occurring in fish products, development of resistant strains of bacteria and possible adverse effects on the aquatic environment. Therefore researches are underway to investigate the feasibility of vaccination against these diseases in many countries.

At the moment although conclusive experimental evidence is lacking, some studies have provided encouraging results which suggest that vaccination against *Streptococcus* is possible in some species like tilapia (Klesius *et al*., 1999) and rainbow trout (Eldar *et al*., 1997). Similarly vaccination work with *Aeromonas hydrophila* in Nile tilapia also provided encouraging results (Ruangpan *et al*., 1986). However, the literature indicates a lack of studies on vaccine where protection against *Streptococcus* sp. and *Aeromonas hydrophila* are experimentally investigated in economically important strain of hybrid red tilapia (*O. niloticus* X *O. mossambicus*). The significant variations in disease resistance have been reported from different fish species (Chevassus and Dorson, 1990). Therefore information concerning the response of vaccination against aforesaid diseases in hybrid tilapia (*O. niloticus* X *O. mossambicus*) is essential.

The purpose of this study was to assess whether *Aeromonas hydrophila* and *Streptococus* sp. vaccine vaccinated by immersion method can confer protection in red tilapia against infection from their respective disease to contribute to the development of vaccine for controlling these diseases in aquaculture.

Materials and methods

Bacterium

A stock of *A. hydrophila* and *Streptococcus* sp. isolates were obtained from Department of Aquaculture, Faculty of Fisheries, Kasetsart University. Bacterial isolates were initially distinguished on the basis of colony morphology and shape by growth on brain heart infusion (BHI) agar media (Merk) for 24 hr at 30°C. The predominant types of bacterial colonies were purified on fresh medium. Further pathogens were identified by examination of Gram-staining and

various biochemical tests. The result of biochemical tests were compared with previously identified species following diagnostic table of BMSB (1984, 1986).

Vaccine preparation

Isolates were injected to fish and re-isolated twice to enhance the virulence. *Aeromonas hydrophila* from kidney of hybrid catfish and *Streptococcus* sp. from liver of Nile tilapia were isolated to prepare vaccine. The isolated bacteria were grown for 24 hr in incubator at 30°C. Grown bacteria were washed two times with 0.85% saline and harvested by centrifugation (Dynac II centrifuge) at 2500-3000 rpm for 15 min. The cells were killed by adding 1% formalin and growth observation for 24 hr at 4°C. The culture determined to be killed by lack of growth on BHI agar after 24 hr at 30°C. Formalin treated cultures were again washed two times with 0.85% saline and adjusted to an optical density of 1.000 absorbency at wavelength of 540 nm using spectrophotometer (Milton Roy, Spectronic 401) to give a concentration of 10^9 cells/ml which were pre-determined by pour plate method. The vaccine was preserved in 0.1% formalin and refrigerated before use. The same vaccine was used as antigen also.

1. *Humoral response study*

Fish

Humoral response study was performed on red tilapia with average weight of 156.32±60.24 g stock maintained at Aquaculture Department, Faculty of Fisheries, Kasetsart University. The fish were divided into 3 groups of 20 fish each with three replicates. Each of two groups was vaccinated with one of the vaccine and third group serving as a control group. The fish were acclimatize for 2 weeks and maintained in flow through 500L fiberglass tanks. Fish were fed twice with commercially prepared pellet feed at satiation. The water temperature averaged 26.7±1.5 during experimental period.

Vaccination protocol

Fish were vaccinated by intraperitoneal injection (i.p.) with 0.2 ml of respective vaccine through abdominal wall. Control fish received equal volume of 0.85% saline. When the initial antibody titers began to decline at the 4th week in *Aeromonas hydrophila* and the 2nd week in *Streptococcus* sp. vaccinated fish a second dose of vaccine was administered in same way. Control fish were also injected with saline at the time of *A. hydrophila* booster injection.

Blood collection and antibody titration

Blood was collected weekly from a random sample of 5 fish from each replicated tank through caudal vein. Blood samples were allowed to clot for 1 hr at room temperature and then refrigerated. Serum was collected after 24 hr and immediately used for antibody measurement. Antibody titer in serum was determined by use of micro titration agglutination test in 96-well plates using serial two-fold dilution of each serum pool. When the antibody titer after the first vaccination declined, then second injection was performed and titers were determined until it dropped.

2. *Challenge experiment*

Fish

The degree of protection was tested in red tilapia of average weight 1.46±0.53 g maintained in 50L glass aquaria with continuous aeration. Two vaccinated and two control groups separately for each

vaccine were established and stocked with 20 fish each in triplicates aquaria. The fish were fed daily to satiation with commercially prepared feed. The average water temperature was 27.0±1.0 during observation period.

Vaccination protocols

Both vaccinated groups each was vaccinated with *Aeromonas hydrophila* and *Streptococcus* sp. vaccine at concentration of 10^9 cells/ml by immersing 20 fish in 2L of vaccine for 1 hr with proper aeration in glass jar. Both control fish were immersed in 0.85% saline. After vaccination fish were re-stocked in 50L-glass aquaria for rearing until challenge.

Challenging

Virulence was maintained by twice passages of isolates through red tilapia. The challenge dose was standardized to give more than 50% mortality in control fish. The pre-challenge study indicated a challenge dose of 10^8 cells/ml for *A. hydrophila* and 10^9 cells/ml for *Streptococcus* sp. to be used for 6 hr. Prior to challenge fish were starved for 24 hr. Challenges were performed after two weeks post vaccination in 3 replicated glass jar by immersing 20 fish in 1L of virulent bacterial suspension for 6 hr. Arrangement was made to provide continuous and vigorous aeration during challenges. Total bacterial count from final challenge dilution showed that the infectious doses used were 2.75×10^8 cells/ml for *Aeromonas hydrophila* and 1.33×10^9 cells/ml for *Streptococcus* sp. After challenging period, fish were transferred to rearing aquaria and feeding restarted after 3 days of challenge. The fish were monitored for mortality daily for 14 days post-challenge. The cause of mortality was verified by bacterial isolation from kidney, spleen and liver.

Statistics

Statistical differences between primary and secondary immune response and percent cumulative mortality were analyzed by analysis of variance using Duncan's multiple range tests for significance. Probabilities of 0.05 or less were considered statistically different.

Place and duration

The experiments were conducted from May 2002 to August 2002 at Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Thailand.

Results

Humoral response study

Vaccination with *A. hydrophila* resulted in a significantly differed ($P<0.05$) mean peak antibody titers in primary response with value of 925.87±467.92 that peaked in 3 weeks. *Streptococcus* vaccine induced non-significant ($P>0.05$) mean peak antibody titer of 2.00±1.74 at 7 days in primary response. However following second vaccination red tilapia responded better with both vaccine and induced mean peak titer of 4983.47±1832.74 by *A. hydrophila* and titer value of 101.33±45.38 by *Streptococcus* sp. which were significantly different ($P<0.05$) than primary one and control within same immune response. The peak reached at 4 weeks and 1 week respectively. Unvaccinated control fish showed titer of 11.10±10.61 and 6.43±0.38 after first and second injection respectively (Tab. 1). It was noted that antibody titer was declined after secondary peak reached but a titers of 333.9 was maintained even at 13 weeks observation with *A. hydrophila* vaccine.

However titer persisted for only 9 weeks with value of 8.0 vaccinated with *Streptococcus* sp. The weekly antibody responses after first and second vaccination are shown in figure 1.

Protective efficacy

Percent cumulative mortality after immunization and challenge are shown in figure 2. After challenging with virulent *A. hydrophila* a significantly ($P<0.05$) different percent cumulative mortality of 23.3% was recorded in vaccinate compared with 56.7% in unvaccinated control. Fish challenged by *Streptococcus* sp. had non-significant ($P>0.05$) percent cumulative mortality of 31.7% in vaccinates and 55.0% in unvaccinated (Tab. 2). The daily cumulative mortality curve showed that mortality in vaccinates and unvaccinated was continued throughout 14 days observation period in both bacterial challenged fish. However the pattern of mortality was slightly different showing throughout less mortality in vaccinated fish challenged by *A. hydrophila* (Fig. 3). Fish vaccinated with *Streptococcus* sp. had initial mortality high compared with unvaccinated (Fig. 4) but after peak reached at day 4 showed comparatively lower and steady pattern of mortality against its virulent challenge. External signs of disease were not very much distinct in both challenged groups. However; bacterial isolation from dead fish confirmed that the infection was from respective bacteria.

Discussion

The present study shows that red tilapia responded with high serum antibody production and mounted significant protection against challenge with virulent *A. hydrophila.* Different investigators have reported antibody responses and immunity of fish to *A. hydrophila* (Karunasagar *et al*., 1991; Areechon *et al*., 1992). The higher antibody production in response to *A. hydrophila* vaccine agrees with the results obtained by Ruangpan *et al.* (1986) who found highest antibody titer in tilapia injected with formalin-killed *A. hydrophila.* This is an indicative of highly immunogenic nature of *A. hydrophila.* In present study however, the response of individual fish was highly variable as evidence by the large standard deviation about the mean peak titers with some individual exhibiting average titer as high as 16384. This suggests that fish population may be composed of sub-population of high responder and low responders. This would be analogous to the situation in mammals and presumably reflect to the genetic make-up of individual fish (Newman and Tripp, 1986). The immunization efficiency of *A. hydrophila* was also higher in red tilapia challenged by immersion route. This could be attributable to considerable amount of antibody production during course of protection. The correlation between antibody production and level of protection were not determined in this study because this study was conducted separately with different size of fish. However higher level of antibody production noted during humoral response study and significant degree of disease resistance shown during experimental challenge led to postulate that *A. hydrophila* elicited protective antibody during immersion vaccination. The present result was supported by earlier observation with different species (Karunasagar *et al*., 1991; Areechon *et al.*, 1992; Supriyadi and Shariff, 1995) that circulating antibody is produced after immersion vaccination with *A. hydrophila.* However some differences in level of antibody production (Ruangpan *et*

Table 1. Means antibody titer peak after the first and second injection with A. *hydrophila* and *Streptococcus sp*. vaccine in red tilapia

Vaccine	Antibody Titer	
	Primary response	Secondary response
A. hydrophila	925.87 ± 467.92[a]	4983.47±1832.74[a*]
Streptococcus sp.	2.00±1.74[b]	101.33±45.38[b*]
Saline control	11.10±10.61[b]	6.43±0.38[*]

Means with different letters are significantly different (P<0.05) when compared with control within the same immune response. Asterisk indicates significant differences between primary and secondary immune response in each vaccination.

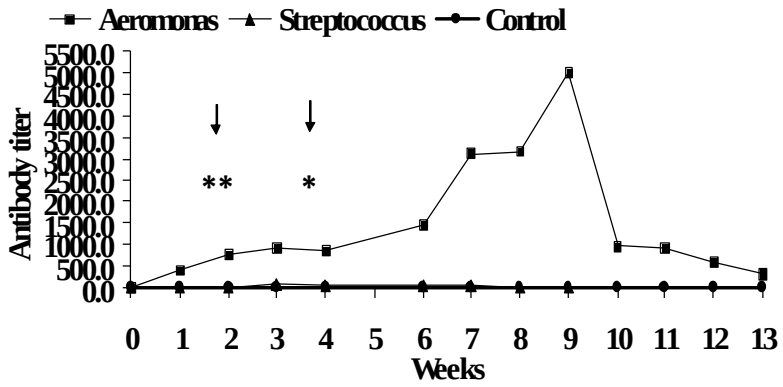

Figure 1. Kinetics of immune response after primary and secondary vaccination
Note: * = 2nd injection *A. hydrophila*; ** = 2nd injection *Streptococcus* sp.

Table 2. Percent cumulative mortality in immersion challenge with *Aeromonas hydrophila* and *Streptococcus* sp. in red tilapia

Virulent bacteria	Challenge dose (CFU/ml)	% cumulative mortality		RPS
		Vaccinate	Non-vaccinate	
A. hydrophila	2.75 X 10^8	23.33	56.67[*]	58.88
Streptococcus sp.	1.33 X 10^9	31.67	55.00[*]	42.55

Means with asterisk are significantly different (P<0.05) when compared with control within same bacterial challenge

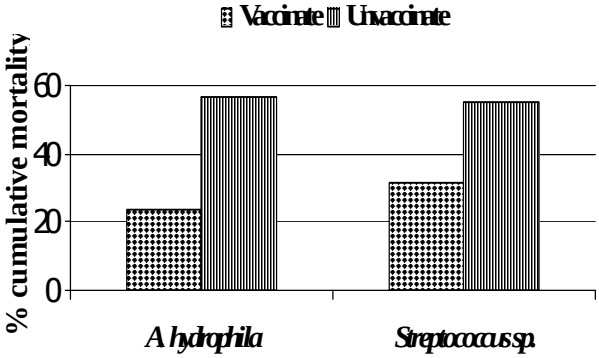

Figure 2. Mortality during immersion challenge experiment

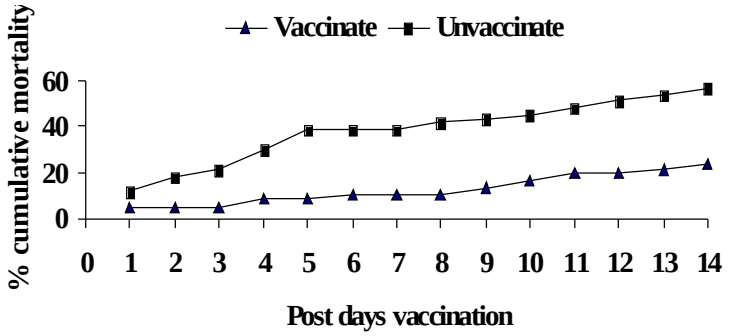

Figure 3. Daily mortality pattern during A. hydrophilla challenge

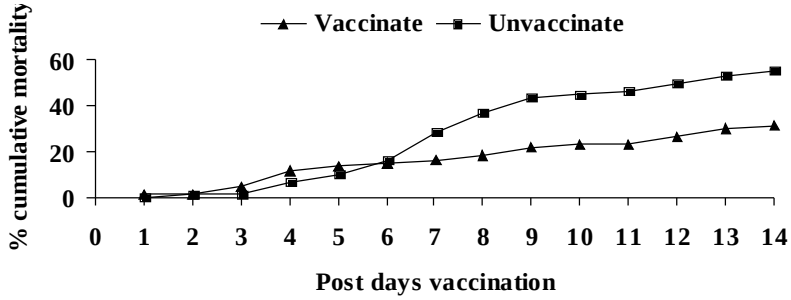

Figure 4. Daily mortality pattern during *Streptococcus* sp. challenge

al., 1986) and degree of protection (Karunasagar *et al*., 1991; Areechon *et al*., 1992; Supriyadi and Shariff, 1995) with these authors investigation could be due to differences in bacterial strain and/or fish species used. Varied responses of fish to *A. hydrophila* (Supriyadi, 1986) and highly heterogeneity among isolates of *A. hydrophila* (Shanker *et al*., 2000) have been documented and pinpointed to be a major problems in the successful development of vaccine for *A. hydrophila*.

On contrary, vaccination with *Streptococcus* sp. did not elucidate appreciable antibody titer in red tilapia however secondary response was significantly higher than primary antibody response. This finding was not surprising and confirms the earlier works by Eldar *et al*. (1995) against formalin-killed *Streptococcus difficile* on tilapia that antibodies were detected at low levels. In similar study Sakai *et al*. (1989) found very low antibody titer against β-haemolytic streptococcal in rainbow trout. The results of recent work concerned with this investigation has also been reported by Shelby *et al*. (2002) who found significantly increased antibody titer only in secondary response in tilapia vaccinated with *S. iniae*. This may suggest that *Streptococcus* sp. could be less immunogenic to induce circulating antibody. Ellis (1988) stated that not all the antigens associated with virulence and pathogenicity of microbial pathogen is effective stimulators of the immune response. Areechon *et al*. (1992) mentioned the degree of responsiveness varies depending upon type of vaccine used.

Our result showed that agglutination reaction in control fish always had negative reaction against *Streptococcus* sp. antigens. Various authors debating that detection of fish antibody against a specific antigen is influenced by the assay procedure selected to measure the response. This allowed arguing that negative agglutination in control fish and probably low level of antibody titer exhibited with *Streptococcus* sp. could be due to antibody assay method was not sensitive enough to detect antibody titer. Shelby et *al*. (2002) reported an enzyme linked immunosorbent assay (ELISA) is a more sensitive and specific assay method than an agglutination assay to measure an antibody response against *S. iniae*. However, conflicting view presented by Schachte (1978 cited by Newman and Tripp, 1986) who stated agglutination assay appear most appropriate for particulate antigens. Toranzo *et al*. (1995) also did not find any increase in circulating specific antibody by ELISA technique compared to microagglutination test against formalin-killed *Enterococcus* sp. Therefore, in the present study microagglutination method used to assay antibody titer seems not likely the factor of low antibody titer and negative agglutination in control against *Streptococcus* antigen. This was also supported by detection of average titer of 6.43 in control fish when assayed with *A. hydrophila* antigen. This difference may reflect the antigenic nature of both vaccines.

During challenge experiment also immunization with *Streptococcus* sp. failed to provide significant protection in vaccinates. Although antibody titer were not detected it would appear that low levels of antibody response detectable in the intraperitoneally vaccinated fish were reflected in protection level also and the lack of protection was due to low level or more probably lack of generating specific anti-*Streptococcus* sp. antibody from immersion immunization. Similar results observed by Sako (1992) who reported absence of protection in yellowtail by immersion vaccination against streptococcal infection. However, the results also suggest despite the percent cumulative mortality was non-significant the survival was higher in vaccinates. This was encouraging and indicates serum antibody may not be solely responsible for protective immunity and it is possible that limited protection it conferred in red tilapia during immersion immunization might be due to some non-specific serum component or collaboration of specific and non-specific mechanism. In spite of protective effect of immersion vaccination against β-haemolytic streptococcal Sakai *et al*. (1989) reported serum antibodies were not detectable in rainbow trout. Kusuda and Salati (1982) showed greater enhancement of secretary (mucus) antibody rather than serum antibody in immersion vaccination with *Enterococcus* sp**.** In contrast with present study, Clark and Smith (1999) found significantly different protection in 1-2 g tilapia by immersion vaccination against *Streptococcus* sp. Although they did not mention the role of protective immunity but their post challenge observation period was 12 weeks. This suggests post challenge observation period kept in this study was short. The present study seems also did not fulfill the criteria of EU guideline (EU CVMP, 1993) as indicated by continued mortality in vaccinates during 14 days observation. It could be postulated that difference between mortality in vaccinates and unvaccinated would reach higher if observation period were extended.

The kinetics of immune response appeared slightly different in both vaccines used. *Streptococcus* sp. vaccine induced peak antibody at 1 week in both primary and secondary response that was rapid than those reported by Shelby *et al*. (2002). They observed primary and secondary antibody peaked at 2 weeks and 3 weeks respectively. This variability in immune response indicates that antigenic heterogeneity exists and is important to development of efficacious streptococcal vaccines (Klesius *et al*., 2000). However vaccination with *A. hydrophila* took little longer time to reach highest levels. This phenomenon might be a common feature in fish, for it has also been described after immunizing carps with *A. hydrophila* cells (Lamers *et al*., 1985). It was noted that antibody titer was started to decline 5 weeks and 2 weeks after second vaccination respectively with *A. hydrophila* and *Streptococcus* sp. however, both bacterial antigens maintained elevated antibody levels for a considerable period of time that may suggest it should be related to specific immune responses.

In summary, our results showed that vaccine prepared from formalin-killed *A. hydrophila* cells can induce humoral immune response and well protect red tilapia against a virulent *A. hydrophila* challenged by water borne route which could be relevant to widely practiced in field conditions. In contrast, *Streptococcus* sp. induced weak secondary response and vaccine preparation was not protective when it was delivered by immersion. However considering the better survival rate in vaccinates there is further scope to put forth effort for use of immersion vaccination against *Streptococcus* sp.

References

Areechon, N., Kitancharoen and K. Tonguthi 1992. Immune response of walking catfish (*Clarias macrocephalus*) to vaccination against *Aeromonas hydrophila* by injection, immersion and oral administeration. In *BIOTROP* (Eds. J.S. Langdon, G.L. Enriquez and S. Sukimin). Spec. Publ. ***48*:** 143-151.

BMSB 1984. Genus III *Aeromonas* Kluyver and van Niel, 1936, 398^{AL}. In *Bergey's Manual of Systemic Bacteriology, Vol 1* (Eds. N.R. Krieg and J.G. Holt). Williams and Wilkins, Baltimore. pp. 545-548.

BMSB 1986. Genus *Streptococcus* Rosenbach 1884, 22^{AL}. In *Bergey's Manual of Systemic Bacteriology, Vol 2*. (Eds. P.H.A. Sneath, N.S. Mair and M.E. Sharpe). Williams and Wilkins, Baltimore. pp. 1043-1071.

Chevasus, B. and M. Dorson 1990. Genetics of diseases in fishes. *Aquaculture* ***85*:** 83-107.

Clark, J.S. and P.D. Smith 1999. Prevention of *Streptococcus* in tilapia by vaccination. Source (www.avl.co.uk.)

Eldar, A., A. Horovitcz and H. Bercovier 1997. Development and efficacy of a vaccine against *Streptococcus inae* infection in farmed rainbow trout. *Vet. Immunol. Immunopathol.* ***56*:** 175-183.

Eldar, A., O. Shapiro, Y. Bajerano and H. Bercovier 1995. Vaccination with whole cell vaccine and bacterial protein extract protects tilapia against *Streptococcus difficile* meningoencephalitis. *Vaccine* ***13*(*9*):** 867-870.

Ellis, A. 1988. Optimizing factors of fish vaccination. In *Fish vaccination* (Ed. A.E. Ellis). Academic Press Ltd. pp. 32-46.

EU CVMP 1993. *Guidance for vaccine intended for fish*. Commission European Communities III/3590/92/EN: 1-9.

Karunasagar, I., G. Rosalind and I. Kasunasagar 1991. Immunological response of the Indian major carps to *Aeromenas hydrophila* vaccine. *J. Fish Dis.* ***14*:** 413-417.

Klesius, P.H., C.A. Shoemaker and J.J. Evans 1999. Efficacy of a killed *Streptococcus iniae*

vaccine in tilapia (*Oreochromis niloticus*). *Bull. Eur. Ass. Fish Pathol.* ***19(1)*****:** 1-3.

Klesius, P.H., C.A. Shoemaker and J.J. Evans 2000. Efficacy of single and combined *Streptococcus inae* isolate vaccine administered by intraperitoneal and intramuscular routes in tilapia (*Oreochromis niloticus*). *Aquaculture* ***188*****:** 237-246.

Kusuda, R. and F. Salati 1982. *Enterococcus seriolicida* and *Streptococcus iniae*. Source (www.cabi-publishing.org/Bookshop/ReadingRoom/0851991947ch8.pdf)

Lamers, C.H.J., M.J.H. DEHaaS. and W.B. van Muiswinkel 1985. The reaction of the immune system of fish to vaccination: development of immunological memory in carp, *Cyprinus carpio* L., following direct immersion in *Aeromenas hydrophila* bacterin. *J. Fish Dis.* ***8*****:** 253-262.

Newman, D.A. and M.R. Tripp 1986. Influence of route of administration on the humoral response of channel catfish (*Ictalusus punctatus*) to *Yersina ruckeri*. *Vet. Immunol. Immunopathol.* ***12*****:** 163-174.

Nieto, J.M., S. Devesa, I. Quiroga and A.E. Toranzo 1995. Histopathology of *Enterococcus* sp. infection in frarmed turbot, *Scophthalmus maximus* L. *J. Fish Dis.* ***18*****:** 21-30.

Pullin, R.S.V. 1983. Choice of tilapia species for aquaculture. In *First International Symposium on Tilapia in Aquaculture* (Eds. L. Fishelson and Z. Yaron). *ICLARM Conf. Proc.* ***15*****:** 64-67.

Ridmontri, C. 2001. Tabtim fish: A healthy and nutritious image. *Asian Aquaculture Magzine* March/April: 12-16.

Ruangpan, L., T. Kitao and T. Yoshida 1986. Protective efficacy of *Aeromonas hydrophila* vaccine in Nile tilapia. *Vet. Immunol. Immunopathol.* ***12*****:** 345-350.

Sakai, M., S. Atsuta and M. Kobayashi 1989. Protective immune response in rainbow trout. *Oncorhynchus mykiss*, vaccinated with β-hemolytic streptococcal bacterin. *Fish Pathol.* ***24*****:** 169-173.

Sako, H. 1992. Efficacy of vaccination against beta-hemolytic streptococcal infection in cultured yellow tail, *Seriola quinqueradiata*. *Suisan Zoshoku* ***40*****:** 393-397.

Schachte, C.J. Jr. 1978. Immunization of channel catfish, *Ictalurus punctatus*, against two bacterial diseases. *Mar. Fish. Rev.* ***40(3)*****:** 18-19.

Shankar, K.M., C.V. Mohan, T.M. Anil and R. Vidhya 2000. Monoclonal antibodies in fish and shelfish health management in India. *Naga*, The ICLARM quarterly ***23*****:** 4

Shelby, R.A., P.H. Klesius, C.A. Shoemaker and J.J. Evans 2002. Passive immunization of tilapia, *Oreochromis niloticus* (L.) with anti-*Streptococcus iniae* whole sera. *J. Fish Dis.* ***25*****:** 1-6.

Shoemaker, C.A., J.J. Evans and P.H. Klesins. 2000. Density and dose: factors affecting mortality of *Streptococcus inae* infected tilapia (*Oreochromis niloticus*). *Aquaculture* ***188*****:** 229-235.

Supriyadi and M. Shariff 1995. Evaluation of the immune response and protection conferred in walking catfish, *Clarias batrachus*, administered inactivated *Aeromonas hydrophila* bacterin by immersion. In *Diseases in Asian Aquaculture II.* (Eds. M. Shariff, J.R. Arthur and R.P. Subasinghe). *Asian Fish. Soc.* pp. 405-412.

Supriyadi, H. 1986. The susceptibility of various fish species to infection by the bacterium *Aeromonas hydrophila*, *In* The First Asian Fisheries Forum. (Eds. J.L. Maclean, L.B. Dizon and L.V. Hosillos). *Asian Fish. Soc.* pp. 240-242.

Toranzo, A.E., S. Devesa, J. Romalde, J. Lamas, A. Raja, L. Leiro and J. Barja 1995. Efficacy of intraperitoneal and immersion vaccination against *Enterococcus* sp. infection in turbot. *Aquaculture* ***134*****:** 17-27.