Available online at http://www.journalijdr.com

IJDR

International Journal of
DEVELOPMENT RESEARCH

ISSN: 2230-9926

International Journal of Development Research
Vol. 6, Issue, 04, pp. 7319-7325, April, 2016

Full Length Research Article

EFFECT OF WHOLE CELL (WC) AND OUTER MEMBRANE PROTEIN (OMP) VACCINES ON PROTECTION OF CATLA CATLA AGAINST COLUMNARIS DISEASE OF FLAVOBACTERIUM COLUMNARE

***Mumtaj, S. Brindha Devi, G.B. and Rajalakshmi, P.**

Department of Zoology, Queen Mary's College, Chennai - 600 004, Tamil Nadu, India

ARTICLE INFO

Article History:

Received 19th January, 2016
Received in revised form
27th February, 2016
Accepted 17th March, 2016
Published online 27th April, 2016

Key Words:

Flavobacterium Columnare,
Catla catla,
Asparagus racemosus,
WC and OMP.

ABSTRACT

Recent studies evaluated that immunoproteomic vaccines provide protection against bacterial infections in fish aquaculture and the vaccines are popular due to its long lasting immunity, safety and low cost versatile characteristics. In this study we used Whole cell – Formalin Killed (WC) and Outer Membrane Protein (OMP) of *F. Columnare* as a vaccine to provide protection against columnaris disease in fish *Catla catla*. We used the extract of *Asparagus racemosus* as an adjuvant in the vaccine preparation. Survival of the vaccinated fishes (30 and 60 days' post vaccination (dpv)), were evaluated after challenge with virulent *F. columnare*. The results showed that the relative percentage of survival in the catla fish groups vaccinated by Outer Membrane Protein (OMP) vaccine with adjuvtant were significantly higher than that the other types of vaccines (92%). The vaccine treated experimental groups significantly improved ($P<0.05$) the survival at 50% compared to the controls.

INTRODUCTION

Bacterial fish diseases caused by a variety of pathogens are a significant problem in commercial aquaculture. Fish vaccination was employed successfully to protect the farmed fish from the common pathogens. Although the vaccinations are efficient, the vaccination mode remains a critical point to determine the efficiency of a vaccine. On the other hand, the vaccination has to be quick, from statement with low labour and not to be stressful for the fish. At the same time, the minimum amount of the vaccine has to be used, such a way is the immersion immunization works. *F. columnare* is a Gram-negative bacterium, with long, slender rods, and gliding mobility. It was first discovered in 1917 in 16 warm water fish in the Mississippi River and has since been found worldwide in a large variety of fish (Pillay and Knutty, 2005; Figueiredo, *et al*, 2005; Grabowski, et. al. 2004; Woo and Bruno, 1999; Altinok, 2004). Bacterial outer membrane proteins (OMPs) play an important significant role in virulence as they comprise the outermost surface in contact with host cells and are also involved in induction of immune defence factors. Recently, attention has been given to OMPs as a potentially important vaccine component. OMPs are located at host–bacterial interface and are important for host immune responses and as targets for drug therapy. In this study, the effectiveness of newly developed vaccine was tested in *Catla catla* fish. Additionally tests were performed to evaluate the possible effect of booster vaccination to extend the protection against the columnaris disease. In most vaccines, adjuvants are a crucial and vital ingredient for vaccine efficacy. Various adjuvants have been used in fishery and they induce better and more long-lasting protection than non-adjuvant vaccine. Recently the herbal immuno adjuvant *Asparagus racemosus* has been shown to improve vaccine delivery against aquatic pathogens. *A. racemosus*, demonstrated significant immuno stimulatory activity particularly at the humoral level in experimental systems. Saponin is the major active immuno adjuvant compounds of *A. racemosus* and they elevate peripheral lymphocyte proliferation, enhance serum antibody titer and offer safer advantages than chemical adjuvants. In the present study we demonstrated that the immunoproteomic *Flavobacteriumcolumnare* WC and OMP vaccine in the presence of the herbal adjuvant *A. racemosus* extract that could provide protection against *Flavobacteriumcolumnare* infections in Indian Major Carp catla (*Catlacatla*). Bacterial infections are the leading cause of losses in the commercial production of channel catfish. The losses from columnaris disease are second only to enteric septicemia of catfish (ESC) caused by Edwardsiella ictaluri, which account for 60% of all

****Corresponding author: Mumtaja, S.,***
Department of Zoology, Queen Mary's College, Chennai - 600 004, Tamil Nadu, India

reported fish diseases from 1996 to 2001 (Bader *et al*, 2003). Columnaris is a known problem in hatcheries, pen, and cage and pond cultures and is responsible for serious fish kills the world over (Eimers and Cardella, 1990).

Aim

Vaccination trials with bacterins prepared from the *Flavobacterium columnare* strains through direct immersion. Detecting the efficacy of the prepared vaccines by estimating the Relative Percent Survival (RPS).

MATERIALS AND METHODS

Fish

300 Fish fingerlings of *Catla catla* were purchased from Fish Seed farm, Tiruvallur District, Tiruvallur, Near Chennai, Tamil Nadu State in India. Fishes were brought to the laboratory with aerated bags. Fish fingerlings were very active, healthy and weighed 30 ± 5.0 g averagely. They were used for the purpose of immersion vaccination. (Figure 3)

Source of virulent *Flavobacterium Columnare* strain

Virulent strain of *Flavobacterium columnare* sub culture was collected from CMC (Christian Medical College) Vellore. After Biochemical, Phenotypic and Antimicrobial susceptibility confirmation studies, they were maintained in -80˚C as glycerol stock for further studies.

Preparation of whole cell - formalin – killed [Wc] vaccine

F. columnare was inoculated into TYEB broth and incubated at 26ºC for 24 hr. For Formalin-killed *F. columnare* (FKC) preparation, formalin was added to the culture at a final concentration of 0.3%. After 24 h incubation at 26ºC, bacteria were washed three times with PBS and resuspended in PBS to a bacterial concentration of 1×10^8 cells/ml. A viability test of 1% of the resulting volume was conducted on TYEB broth medium and determined to be culture negative. FKC preparation was frozen at -20ºC until use.

Preparation of omp vaccine

F. columnare was grown in TYEB medium and incubated for 48 hours at 26°C under constant shaking conditions. Once the culture was confluent, bacteria in media were centrifuged to collect pellets. Cell pellets were either used directly to obtain outer membrane proteins (OMP), or they were resuspended in media containing 20% glycerol and stored at -80°C for further use. Outer membrane proteins were isolated as described previously by Srisopaporn (2002). Bacterial cell pellets were resuspended in 10 ml of 10 mM HEPES buffer (pH 7.4) supplemented with the following three protease inhibitors – pepstatin (12.5 µg/ml), aprotinin (80 µl/ml), and PMSF (Phenyl methyl sufonyl fluoride 10 µM). Samples were then sonicated for 15 seconds and allowed to rest for 15 seconds. This process was repeated 3 more times. Broken cells were collected by centrifugation for 20 minutes at 1750 x gravity (g) and centrifuged again at 100,000 x g for 1 hour at 4°C. Cell pellets were then treated with equal volumes of 2% (w/v) lauryl sulfonate (Sodium Dodecyl Sulfate, SDS) and 10 mM HEPES buffer (pH 7.4) before incubating for 30 minutes at room 16 temperature, followed by another 100,000 x g centrifugation for 1 hour at 4°C. The pellet of insoluble outer membrane proteins was collected and resuspended in deionized water and stored at -20°C.

SDS page – Qualitative analysis of WC & OMP proteins

SDS-PAGE results showed that the WC vaccines had six polypeptide bands with molecular weight of 66.4, 42.5, 19.6, 14.0, 10.3 and 6.4KDa and OMP vaccines had 7 Polypeptide band with molecular weight of 66.0, 59.5, 42.8, 34.9, 18.9, 14.6 and 9.8 KDa. (Refer Figure.2)

Lowery's method – quantitative analysis of protein

The Protein concentration for WC and OMP vaccines was estimated as **54µg/ml** and **100µg/ml.**

Immuno Adjuvant

Asparagus racemosus tubers were extracted with hot water at 100˚C for two hours. The extracts were filtered and the supernatant were condensed by rotary evaporator at 55˚C, lyophilized and stored at 4˚C. The extracts contain steroidal saponins having immunoadjuvant properties. (Figure 6) Immunostimulants and adjuvants can be administered before, with, or after vaccines to amplify the specific immune response generating elevations of circulating antibody titres and numbers of plaque-forming cells.

Vaccine delivery and immunization

Experimental set-up: Healthy fish, *Catla catla* having the mean weight of 30 ± 5.0 g were used for immunization study. They were acclimatized and kept in plastic tanks for a period of 10 days to assess their disease-free healthy status and fed with commercial feeds. After acclimatizing (Figure 4), six tanks containing a total of fifty fishes (50 X 6 = 300) were maintained in each group. The fishes were kept in plastic tanks of 500 l capacity, flow-through water and they were maintained under constant photoperiod conditions (12hr light / 12hr darkness).

Immersion immunization procedures

Prior to immersion in the diluted bacterin the fishes were immersed in a hyper osmotic solution of NaCl (2% w/v). The fishes were immersed for 5 min, which were aerated during the treatment. (Figure 5) Fishes were immersed for 30 min in diluted vaccine in a separate vaccine tanks (1 volume of vaccine to 9 volumes of tank water = 10 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. Booster dose was applied after 30 days with the same technique. The process of vaccination was repeated until the vaccination of all fish groups was completed (Figure 7). For immune adjuvant treated groups, 500 µg of *A. racemosus* extracts was added to the antigenic proteins. The blank control groups were unvaccinated fishes without bacterial challenge.

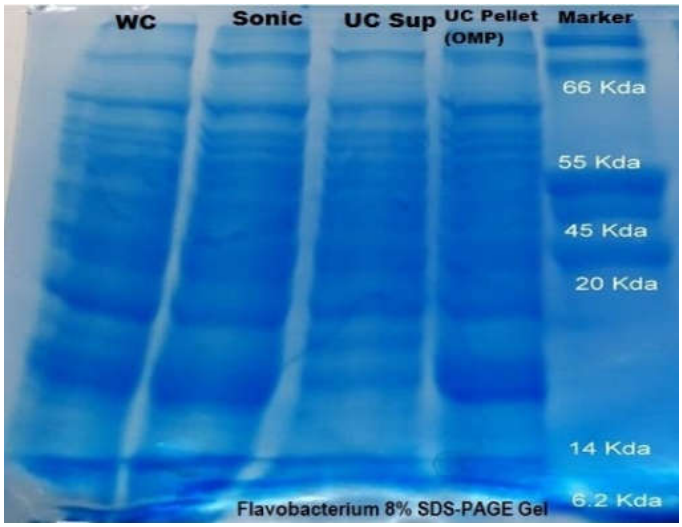

Figure 2. Sodium dodecyl sulphate - polyacrlamide gel electrophoresis Products of *Flavobacteriumcolumnare*. Lane 1, 2, 3, 4 and 5 were loaded with WC, Sonic, UC supernatant, OMP (UC Pellet) and Marker respectively

Figure 3. Fish Samples used for Vaccination

Figure 4. Died Fishes during Acclimatization Process

Figure 5. 2%Nacl (Prior Treatment)

Figure 6. *Asparagus racemosus*

Figure 7. Immunization

Mouth and Eye Ulceration

Skin Ulceration (darkened)

Figure 8. Negative Challenge Fishes – Shows Columnaris Symptoms

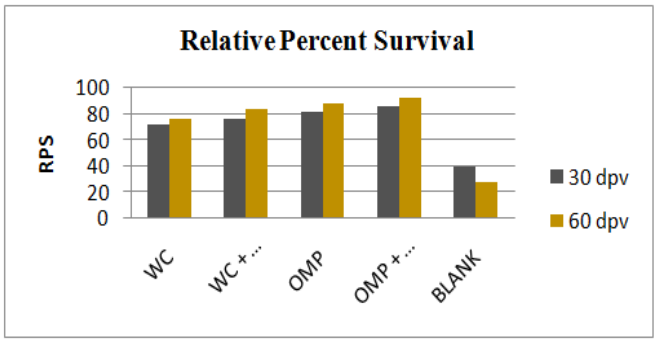

Figure 9. Relative Percent survival of the catla fish vaccinated with different types of vaccines after 30 and 60 dpv

The control groups consisted of unvaccinated fishes subjected to bacterial challenge. The experimental as well as control fishes were fed with commercial feed twice per day. The detailed vaccine protocols are given in the Figure 1. On 30 and 60 days post vaccination (dpv), group of fishes were challenged with a lethal dose (LD_{50}) of *F. Columnare* (1 X 10^8) by 1 volume of culture were added to 9 volume of the tank water in a separate aquarium for each group.

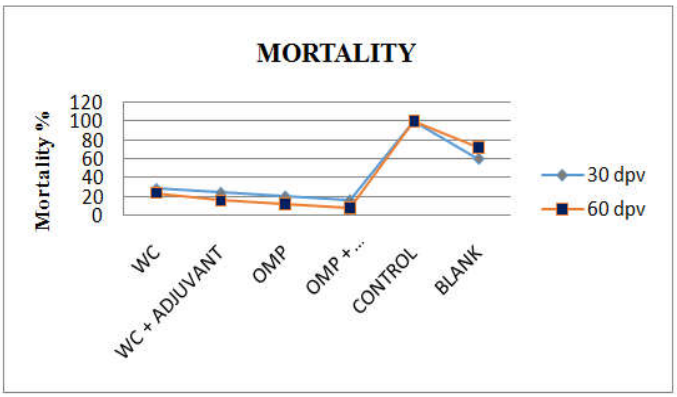

Figure 10. Percentage of Mortality of catla fish vaccinated with different types of vaccines after 30 and 60 dpv

Challenge with virulent *Flavobacteriumcolumnare*

The challenge process persisted for half an hour, for both vaccinated and control fish group. Fishes were transferred to their original aquaria and observed for the cumulative mortality and other pathological signs for 10 days. Figure 8 shows the pathological signs developed in control groups.

RESULTS & DISCUSSION

SDS-PAGE results showed that the WC vaccines had six polypeptide bands with molecular weight of 66.4, 42.5, 19.6, 14.0, 10.3 and 6.4KDa and OMP vaccines had 7 Polypeptide band with molecular weight of 66.0, 59.5, 42.8, 34.9, 18.9, 14.6 and 9.8 KDa (refer Figure 2). The Protein concentration for WC and OMP vaccines was estimated by Lowery method as 54µg/ml and 100µg/ml. Vaccine potency was estimated by calculating the relative percent survival (RPS) according to Amend (1981) as follows: RPS = 1 (% vaccinates mortality / % control mortality)* 100%. Table 1 showed the relative percent of survival in *Catla catla* fish groups vaccinated by immersion route with different types of vaccines and challenged with immersion route. After 30dpv Fishes vaccinated with OMP with adjuvant showed high percentage of RPS as 84%. In fish group vaccinated with OMP records 80% RPS.

For fish groups vaccinated with WC and WC + Adjuvant, the result of RPS was found to be 72% and 76 %, respectively (Refer Table 1 & Figure 9). Similarly, after 60dpv Fishes vaccinated with OMP with adjuvant showed high percentage of RPS as 92% and OMP without adjuvant recorded 88% RPS. For fish groups vaccinated with WC and WC + Adjuvant, the result of RPS was found to be 76% and 84%, respectively (Refer Table 2 & Figure 9). 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

Table 1. RPS Results after 30 dpv Challenge

Type of Vaccine	Route of Vaccinate	Route of Challenge	Number of Challenged Fishes	Number of Died Fishes	% of Mortality	RPS
WC Vaccine	Immersion	Immersion	25	7	28	72
WC + Adjuvant	Immersion	Immersion	25	6	24	76
OMP Vaccine	Immersion	Immersion	25	5	20	80
OMP + Adjuvant	Immersion	Immersion	25	4	16	84
Control	Immersion	Immersion	0	25	100	0

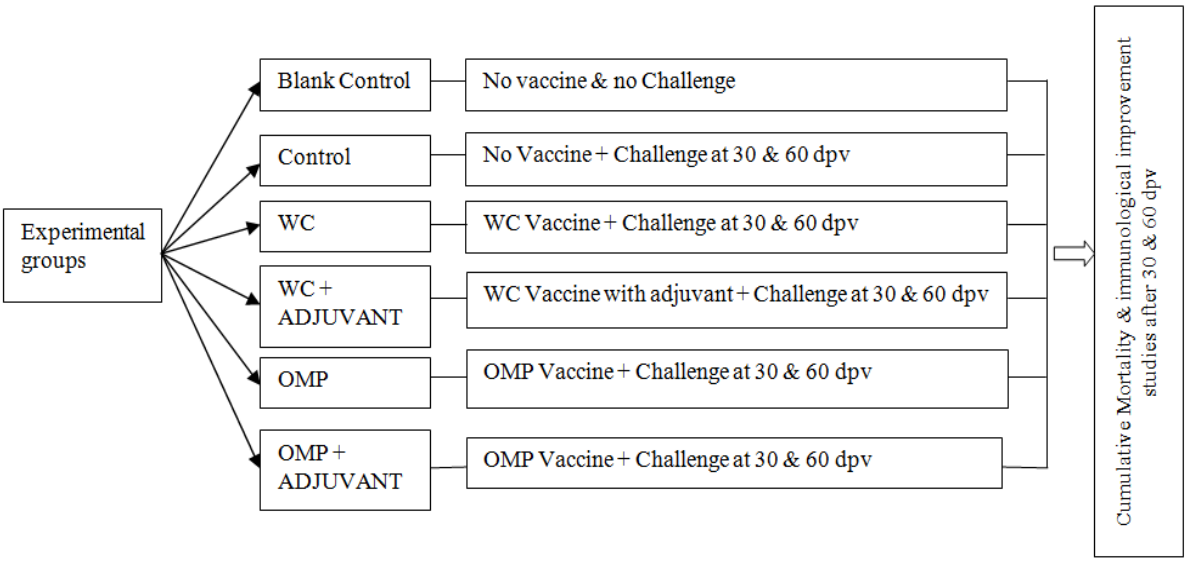

Figure 1. Experimental Design for WC and OMP Vaccine Preparation and Delivery Methods to Indian Major Carp Catla *(Catla catla)*

Table 2. RPS Results after 60 dpv Challenge

Type of Vaccine	Route of Vaccinate	Route of Challenge	Number of Challenged Fishes	Number of Died Fishes	% of Mortality	RPS
WC Vaccine	Immersion	Immersion	25	6	24	76
WC + Adjuvant	Immersion	Immersion	25	4	16	84
OMP Vaccine	Immersion	Immersion	25	3	12	88
OMP + Adjuvant	Immersion	Immersion	25	2	8	92
Control	Immersion	Immersion	0	25	100	0

environment. Anbarasu *et al*., found that formalin inactivated vaccines were superior to heat killed preparations, especially when the vaccines were injected with adjuvants. Shoemaker CA *et al.,* (2011), Results demonstrated safety of the vaccine and significant protection following challenge with RPS values between 74-94%, depending on vaccine dose. 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. Wenxing Zhu *et al*., (2012) found that Fish grass carp (*Ctenopharyngodon idellus*) FCG vaccine potency was observed by intraperitoneal route, showed significantly higher serum agglutination titers and bactericidal activity than fish immunized with FKC or PBS. Most importantly, after challenge with the parent strain G4, the relative percent survival (RPS) of fish in FCG group (70.9%) was significantly higher than FKC group (41.9%). These results showed that FCG could confer immune protection against *F. columnare* infection. As a nonliving whole cell envelope preparation, FCG may provide an ideal alternative to pathogen-based vaccines against columnaris in aquaculture.

A lower mortality percentage was achieved in all vaccinated groups in comparison with the control group. (Figure10) Shoemaker CA *et al.,* (2006), Efficacy of monovalent modified live *F. columnare* vaccine administered singly or with a booster vaccination was shown to be protective with relative percent survival (RPS) values ranging from 50.0 to 76.8. Some variation was seen in RPS values following bivalent immunization, ranging from 33.0 to 59.7 in the fish challenged with *F. columnare* and 44.5 to 66.7 in fish challenged with *E. ictaluri*. The booster vaccination significantly enhanced the efficacy of WC and OMP, achieving RPS values higher for in *Catla catla*. Thus, this result confirms the need for a booster after the initial immersion. Good vaccine should offer an acceptable return on investment. In conclusion, fish vaccination showed that the OMP with Adjuvant vaccine used in *Catla catla* fish through the immersion route showed higher efficacy (RPS) and it was effective against columnaris disease in fishes. Table 1.Mortality and Relative Percent Survival (RPS) after Challenging of *Catla catla* fish Vaccinated by immersion route with *Flavobacterium columnare* Formalin-Killed Whole cell vaccines and OMP Vaccines.

Relative Level of protection or Relative Percent Survival (RPS)

$$RPS = 1 - \left(\frac{\text{Percent of immunized mortality}}{\text{Percent of control mortality}} \right) * 100$$

A specific diagnosis of *F. columnare* as the cause of the disease is to take a scraping or smear from the infected gill tissue or mucus and observe the bacteria under the microscope (Woo and Bruno, 1999, Pillay and Knutty, 2005). Healthy fishes are found to not be affected by *F. columnare* and may even serve as carriers of the disease in some populations (Suomalaninen, *et al*, 2005; Woo and Bruno, 1999). Fish with any type of lesions can be a source of infection to an entire tank, and it is uncertain if carriers of *F. columnare* can be stimulated to become infectious to other fish though it has been shown to be inducible with other *Flavobacterium* species (Pillay and Knutty, 2005; Suomalainen, *et al*, 2005).

Conclusion

We conclude that immunostimulants enhancing the *Catla catla* fish innate immune response and disease resistance against columnaris disease. The herbal adjuvant *Asparagus racemosusis* efficiently help to improve immunity in *Catla catla* throughout the vaccination process. OMP Vaccine combined with herbal adjuvant from *Asparagus racemosus*is much effective in providing protection against columnaris disease in Indian Major Carp Catla (*Catla catla)*. The *Flavobacterium columnare* OMP antigenic protein could be used as a potential vaccine to control columnaris infections in fishes. OMP vaccines have been shown to induce stronger protective immunity than WC vaccines in *Catla catla.* At the same time cumulative mortality of the vaccinated fishes shown to decrease when compare with untreated, blank group fishes. Disease can decrease harvest yields quality, with reduced growth and malnutrition of the fish (Thoratinsson and Powell, 2006). Increased density of animals and increased stress exposure all lead to greater incidence of disease. These results showed that OMP could confer immune protection against *F. columnare* infections and provide an ideal alternative to pathogen-based vaccines against columnaris in aquaculture. Environmental changes such as water temperature alterations, low dissolved oxygen, pH, or salinity changes all increase the susceptibility of disease outbreaks. For some pathogens, overcrowding and handling stress are important stressors to avoid (Stickney, 2005).

Acknowledgments

The author would like to thank the Almighty God for giving strength and knowledge throughout the project. Mumtaj like to thank Mr. S.A. Mohamed Abdullah, for his physical and moral support.

REFERENCES

Amend, D.F. 1981. Potency testing of fish vaccines. Developments in Biological Standardization. 49: 447–454.

Amend, D.F. and Fender, D.C., 1976. Uptake of bovine serum albumin by rainbow trout from hyperosmotic solutions, a model for vaccinating fish. Science (N.Y.) 192: 793-794.

Bootsma, R. and Clerx, J.P.M., 1976. Columnaris disease of cultured carp (*Cyprinuscarpio L*); Characterization of the causative agent. Aquaculture 7: 731-384.

Bootsma, R., 1976. Studies on two infectious diseases of cultured freshwater fish. PhD-thesis, Utrecht. University, Utrecht, The Netherlands.

Chase, J.M., 1965. Nutrition of some aquatic Myxobacteria. MSc-thesis, University of Washington, U.S.A,

Chilmonczyk, S., 1978 . In vitro stimulation by mitogens of peripheral blood lymphocytes from rainbow trout (*Salmo gairdneri*). Ann. Immunol. 129 (I): 3-12.

Cuchens, M.A. and Clem, L.W., 1977. Phylogeny of lymphocyte heterogeneity 2) Differential effects of temperature on fish T-like and B-like cells. Cell. Immunol. 34: 219-230.

Davina, J.H.M; Rijkers, G.T.; Rombout, J.H.W.M.; Timmermans, L.P.M. and Muiswinkel, W.B. van, 1979. Lymphoid and non-lymphoid cells in the intestine of cyprinid fish. In: J.B. Horton (Editor), Development and differentiation of vertebrate lymphocytes. Elsevier, Amsterdam.

Davis, H.S., 1973 . Culture and diseases of game fishes. University of California Press, Berkely, Los Angeles and London (7th printing).

Egidius, E.C. and Andersen, K., 1979. Bath immunization - a practical and nonstressing method of vaccinating sea farmed rainbow trout (*Salmogairdneri*Richardson) against vibriosis. J. Fish. Dis. 2: 405-410.

Etlinger, H.M.; Hodgins, H.O. and Chiller, J.P., 1976. Evolution of the lymphoid system. 1) Evidence for lymphocyte heterogeneity in rainbow trout revealed by the organ distribution of mitogenic responses. J. Immunol. 116 (6): 1547-1553.

Fletcher, T.C. and White, A., 1973. Antibody production in the plaice after oral and parenteral immunization with *Vibrio anguillarum*antigens. Aquaculture 1: 417- 428.

Fryer, J.L.; Rohovec, J.S. and Garrison, R.L., 1978. Immunization of Salmonids for control of Vibriosis. Mar. Fish. Rev. 40 (3): 20-23.

Gould, R.W.; O'Learly, P.J.; Garrison, R.L.; Rohovec, J.S. and Fryer, J.L., 1978. Spray vaccination; a method for the immunization of fish. Fish Pathology 13 (1) 63-68.

Lorenzen, E. and Olesen, N.J., 1997. Characterization of isolates of Flavobacteriumpsychrophilum associated with coldwater disease or rainbow trout fry syndrome II: serological studies. Diseases of Aquatic Organisms. 31: 209–220.

Madetoja, J., Lönnström, L.G., Björkblom, C., Uluköy, G., Bylund, G., Syvertsen, C., Gravningen, K., Norderhus, E.A. and Wiklund, T., 2006. Efficacy of injection vaccines against Flavobacteriumpsychrophilum in rainbow trout, Oncorhynchus mykiss (Walbaum). J. Fish Dis. 29: 9–20.

Roberts, R.J., 1978. Fish Pathology, Bailliere - Tindall, London.

Rijkers, G.T.; Fredrix-Wolters, E.M.H. and Muiswinkel, W.B, van, 1980. The hemolytic plaque assay in carp (*Cyprinuscarpio* L) J. Immunol. Meth. 33: 79-86.

Shoemaker, C.A., Klesius, P.H. and Bricker, J. M., 1999. Efficacy of a modified live Edwardsiellaictaluri vaccine in channel catfish as young as seven days' post hatch. Aquaculture. 176: 189–193.

Van Dam, R.H.; Van Kooten, P.J.S. and Van der Donk, J.A., 1978. In vitro stimulation of goat peripheral blood lymphocytes; optimization and kinetics of the response to mitogens and to allogeneic lymphocytes. *J. Immunol.* 116 (6): 1547-1553.

Yin, Z., T.J. Lam and Y.M. Sin, 1996. The role of specific antiserum of catfish as a defense against Aeromonashydrophila. *Fish Shellfish Immunol.,* 6: 57-69.

****