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Effect of using bivalent vaccines (Whole cell and Outer membrane protein) against the infections of *Edwardsiella tarda* and *Pseudomonas fluorescens* in *Cirrhinus mrigala*

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Abstract

The bivalent(or)mixed whole cell vaccines and outer membrane protein vaccines of *Edwardsiella tarda* and *Pseudomonas fluorescens* is the common Gram negative bacterial pathogens associated with diseases of Indian major carps were evaluated for their efficacy in *Cirrhinus mrigala* (Ham.). The *Cirrhinus mrigala* fingerlings were vaccinated with bivalent vaccines from *E. tarda* and *P. fluorescens* with or without immunoadjuvant (*Asparagus racemosus* tuber powder extract)by immersion method. On various days of Post Vaccination, [30 & 60 dpv] the treated fish were challenged with virulent strains (*Edwardsiella tarda* and *Pseudomonas fluorescens*) and fingerlings were monitored for RPS, cumulative mortality and other pathological symptoms for 10 days. The fishes were attained 100% mortality within 7,days in non-vaccinated groups, whereas the vaccines helped to increase the survival rate after 30&60 days of post vaccination [dpv]. Upon challenge with mixed pathogen, a high relative percent survival was recorded in the OMP vaccinated groups with immunoadjuvant (92%), when compare to other vaccinated and control groups.

Keywords: *Cirrhinus mrigala*, *Edwardsiella tarda*, *Pseudomonas fluorescens*, Whole Cell, Outer membrane Protein Vaccine, Immunoadjuvant, *Asparagus racemosus.*

Introduction

Aquaculture is one of the most economically important applied strategies all over the world and fishes are one of the most beneficial and nutritional resources of human beings. India is the second largest producer with about 9% share of the world's total fish production next to China with a total production of 57%. Among the fish culture about 80% are carps. Some of the Indian major carps such as *Cirrhinus mrigala, Labeo rohita and Catla catla* are mostly cultured in freshwater in India. *Cirrhinus mrigala* is an endemic species to Indo-Gangetic river systems and is considered as one of the 3 major Indian carp species. The mrigal carp is a species of ray- finned fish in the carp family. It is popular as a food fish and an important aqua cultured freshwater species throughout South Asia. It is widely farmed as a component of polyculture system of three Indian major carps along with rohu and catla.

Infectious diseases are the major cause of economic losses in aquaculture industries which is negatively impacted by various pathogenic organisms (plumb, 1997). Among different types of infectious agents, bacterial pathogens are often responsible for severe mortalities in a wide range of fishes at different stages of growth (Grisez and Ollevier, 1995; Swain *et al*., 2002). Among these, the bacterial pathogens belonging to the genus *Aeromonas, Pseudomonas, Edwardsiella, Flavobacterium* etc, are mainly responsible for severe mortality and morbidity of Indian major carps (Bootsma *etal.*, 1977; Kumar *et al.*, 1986; Shome *et al.,* 1996).

Edwardsiella tarda is ubiquitous to the aquatic environment and infection by this organism can lead to mass mortality in commercially important fishes,

including eels, channel cat fish, mullet, Chinook salmon, flounder, carp, tilapia and striped bass (Thune *et al.,* 1993). *E. tarda* is known to be the causative agent of Edwardsiellosis. Edwardsiellosis is a septicemic disease characterized by extensive lesions in the skin, muscle and internal organs and leads to mortality.

Pseudomonas fluorescens is a Gram – negative, obligate aerobic, motile, rod-shaped ubiquitous bacterium belonging to the Family of *Pseudomonadaceae*. It is the recognized bacterial pathogen that is commonly associated with reared aquaculture species. It is considered as a secondary invader of damaged fish tissues, but also be a primary pathogen of fish (Roberts and Horne, 1978). It can cause Septicemic disease, which is characterized by haemorrhage, scale falling off, Fin rot and tail rot.

During the last decade, there was a major increase in the freshwater aquaculture fish production. Due to various factors like residual effect and other environmental degradation/ pollution lead to the production of antibiotic resistant strains and more attention were shown to protect the aquaculture species by various vaccination methods (Evelyn, 1997). Vaccination is becoming an increasingly important part of aquaculture, since it is considered an easy, low cost and preventive method of protecting fish from diseases. Most bacterial vaccines used in aquaculture to date have been inactivated vaccines obtained from a broth culture of a specific strain(s) subjected to subsequent formalin inactivation. The outer membrane proteins (OMP-components of bacteria) of bacteria function as the dynamic interface between the bacterium and its surroundings and are involved in maintenance of cell structure, binding a variety of substances, adhesion to other cells and regulation of transport of both nutrients and bactericidal agents. OMP has been considered to be the Noval vaccine due to its ability to play a role as molecular adhesion molecule and also their exposed epitopes on the surface.

Recently there were many progresses in the field of developing appropriate vaccines and also the method of vaccination to effectively control the infections and diseases (Vinitnantharat *et al*., 1999; Hastein, *et al*., 2005). Till date there are only a few vaccines to treat diseases like furunculosis, vibriosis, enteric red mouth, yersiniosis, pasteurellosis etc. (Vinitnantharat *et al*., 1999; Gudding and Evesen, 2005). However these vaccines were mono-valent vaccines which contain a single pathogen or whole of a pathogen. The major concern associated with that of vaccination against a single pathogen is that, the aquaculture species lives in an environment where there are various infectious agents and other pathogens are present, and a specific type of vaccination is might protect from that pathogen only and not against other pathogens. So that in the present study was used to prepare the mixed (bivalent) whole cell and outer membrane protein vaccines against *Edwardsiella tarda* and *Pseudomonas fluorescens* infection in *Cirrhinus mrigala*.

In most vaccines, adjuvants are a vital ingredient for efficacy. Recently, the herbal immunoadjuvant (*Asparagus racemosus)* has been shown to improve vaccine delivery against aquatic pathogens. Vaccination is becoming an increasingly important part of aquaculture, since it is considered a cost effective method of controlling different threatening diseases. The efficacy of vaccines in stimulating the fish immune system is related to the routes of administration, dose and nature of antigens, adjuvant addition, environment and water temperature (Bly *et al.,* 1997).Among the various methods of vaccination, the immersion method are simple, cheap, and ideal for mass administration to fish of all sizes and for larger scale of aquaculture. In the present study, Mrigal carp fingerlings (*Cirrhinus mrigala*) were vaccinated by mixed(bivalent) OMP and WC vaccines of *Edwardsiella tarda* and *Pseudomonas fluorescens* with or without immunoadjuvant (*Asparagus racemosus* tuber powder extract) by immersion method. The efficacy of each type of vaccination was determined by estimates the relative percent survival, mortality rate and other pathological symptoms through the application of challenge test.

Objectives:

The main objective of the present study was to analyze the effect of bivalent WC and OMP vaccines against *Edwardsiella tarda* and *Pseudomonas fluorescens* infections in *Cirrhinus mrigala* fingerlings through immersion method.

Materials and Methods

Experimental fish:

Cirrhinus mrigala fingerlings of average weight 5.0±2.0 were purchased from Poondi Lake, Thiruvallur District, Tamil Nadu. The fingerlings were maintained in 200 L Fiber reinforced plastic tanks. The water was replaced daily to maintain the quality of the water. Various environmental factors like temperature, pH, oxygen, were maintained properly throughout the experimental process and also the feed were given at proper intervals. Fishes were allowed to acclimatize for 15 days to meet the laboratory conditions and to assess their disease free health status.

Bacterial strain:

The virulent strain of *Edwardsiella tarda* (ATCC 15947) subculture was obtained from Christian Medical College, Vellore, Tamil Nadu and *Pseudomonas fluorescens* (ATCC 13525) obtained from Government Medical College, Theni, Tamil Nadu were used in the present study.

Bacterial cultures:

The two Bacterial isolates were inoculated separately on respective *(Edwardsiella tarda* in Tryptic soy broth and *Pseudomonas fluorescens* in Nutrient broth) medium at temperatures of 28^{o}C to 30^{o}C overnight and pH 7.5 was maintained for the growth. After 24 hours, bacterial culture were characterized and used for vaccine preparation.

Methods of vaccine preparation:

The whole cell vaccine and outer membrane protein vaccine were prepared by the method of Thangaviji *et al.,* (2013).

Whole cell vaccine (formalin inactivated):

The Whole cell vaccine is prepared from the harvested bacterial cultures in Tryptic soy broth (*Edwardsiella tarda*) and Nutrient broth medium (*Pseudomonas fluorescens*) at 28°c to 30°c for 24 hours followed by inactivation of using 0.6 % Formalin. The Formalin was removed by spinning the culture at 1500xg (20 min) at room temperature and washing with 0.85% saline and finally re suspending in saline to a concentration of 1x10^{8} cells/ml. This is collected and stored at 20°C until it used for the experimental work

Outer membrane protein vaccine:

The 24hours bacterial culture were harvested by centrifugation from Tryptic soy broth (*Edwardsiella tarda*) and Nutrient broth medium (*Pseudomonas fluorescens*) at 3000 x g [20min at 25°C]. The cell pellets were washed twice in phosphate buffer saline (PBS) and once in 10mM Tris-hydrochloride [pH 7.5]. The cells were re-suspended in Tris -HCl and sonicated at 50w for 30s (4 times on ice). After sonication, the suspension was mixed with sarkosyl for solubilization of the outer membrane protein and incubated at 25°C for 30mins. After incubation, the suspension was centrifuged at 4000 x g (20 min) and the supernatant were collected and again centrifuged at 45,000 x g (45 min) for collection of the pellets and stored at 20°C until it used for the experimental work.

Bivalent vaccines: (Mixed vaccines)

The two monovalent vaccines (*Edwardsiella tarda* and *Pseudomonas fluorescens*) were mixed with equal volume to form the final volume (1:1) of bivalent vaccines.

Quantitative and Qualitative protein analysis of prepared vaccines:

The prepared WC and OMP vaccines were re-suspended in PBS and the protein concentration was estimated by using Lowry's method. Further the above proteins were resolved by 10% SDS -PAGE [LAEMMLI 1970] to generate protein profiles.

Preparation of immuno adjuvant:

Recently the herbal Immunoadjuvant (*Asparagus racemosus*) has been shown to improve the vaccine delivery against aquatic pathogens. *Asparagus racemosus* tuber powders were extracted with hot water at 100°C for 2 hours. The extracts are filtered and supernatants were condensed (by using a rotary evaporator at 55°C) lyophilized and stored at 4°C. The extracts contained saponins possessing immune adjuvant properties (Gautam *et al.,* 2004).

Method of vaccination:

Immersion method (Bath vaccination):

In Bath vaccination, fingerlings were exposed for a longer period usually several hours in a lower concentration of vaccine. Fingerlings were immersed for 30 min in diluted vaccine in separate vaccine tanks in the concentration of 1 volume of vaccine in 10 volume of tank water (1:10) = 10^{8} cells/ml according to Macintosh and Austin (1993).

Experimental design:

Fishes are divided into six groups and each tank contains 50 fishes(One blank group, one control group and four experimental groups). Fingerlings were vaccinated twice by using immersion method during the experimental period. The first dose (or) primer dose is delivered on day 1 and the second dose (or) booster dose is delivered on day 30 of the experimental period. The dosage of outer membrane protein vaccine is 33ug/ ml and whole cell vaccine is at a concentration of 1 x 10^{8}cells/ml. The experimental groups were treated by immunoadjuvant with antigens to form a concentration of 1:1 ratio. The blank control groups have unvaccinated fishes without bacterial challenge. The control groups comprises of unvaccinated fishes with bacterial challenge. The experimental groups received both vaccine delivery and bacterial challenge. The process of vaccination was repeated for all groups.

Bacterial challenge:

After 30 and 60 days of post vaccination (dpv),25 fingerlings from each group were challenged or experimentally infected by bath challenge method (1:10) lethal concentration of 10^8cells/ml outlined by Lillihaug, (1989). The fingerlings were observed for Relative percent survival, mortality rate and other pathological signs for 10 days. The relative percent survival (RPS) in each group is calculated by the following formulae (Amend, 1981).

$$RPS = 1 - \frac{\% \text{ of mortality in vaccinated group}}{\% \text{ of mortality in unvaccinated group}} \times 100$$

Results and Discussion

Edwardsiella tarda (ATCC 15947) is a gram negative, motile, short, rod shaped, and anaerobic bacterium. This bacterium can survive 0-4% sodium chloride, 4.0-10.0 pH, and temperature of 14-45^{o}C. The biochemical characteristics of *Edwardsiella tarda* were catalase positive, production of indole and hydrogen sulfide, fermentation of glucose and reduction of nitrate to nitrite. Organism shows negative results for Cytochrome oxidase, sucrose and Lactose fermentation.

Pseudomonas fluorescens is a gram negative, motile, rod shaped, aerobic bacterium. This bacterium can survive 0-7% sodium chloride, 5.0 -7.5 pH, and temperature of 25-30^{o}C. The biochemical characteristics of *Pseudomonas fluorescens* were positive for catalase, hydrogen sulfide, cytochrome oxidase, and simmon citrate. Organism shows negative reaction for vogus proskar, nitrate to nitrite and Lactose fermentation. After Biochemical conformation the Bacterial cultures were used for vaccine Preparation.

The protein concentration of prepared WC and OMP vaccines were estimated by Lowry's method. Further the above proteins were resolved by 10% SDS -PAGE [LAEMMLI 1970] to generate protein profiles. The total protein concentration of OMP vaccine was estimated as 58 µg/ml and WC vaccine was 33µg/ml. The SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) profile of *Edwardsiella tarda* (ATCC 15947) OMP vaccine had 7 poly peptide bands with the molecular weight of 210.64, 138.53,89.12,65.45, 42.57, 31.96 and 21.49 KDa and WC vaccine had 8 polypeptide bands with the molecular weight of 220.14, 172.71, 104.00, 50.79, 38.98, 28.62, 22.96 and 18.01 KDa.

The total protein concentration of OMP vaccine is estimated as 100µg/ml and WC vaccine was 66µg/ml. The SDS-PAGE profile of *Pseudomonas fluorescens* (ATCC 13525) OMP vaccine had 8 poly peptide bands with the molecular weight of 121.80, 76.49, 59.30, 31.52, 25.31, 18.45, 15.08, 3.70 KDa and WC vaccine had11 poly peptide bands with the molecular weight of 248.00, 188.92, 142.65, 99.53,72.56, 53.84, 40.65, 22.98, 17.05, 11.79 and 9.89 KDa.

Increased incidence of microbial diseases in aquaculture system is the major obstacle in the success of the industry. Use of antibiotics has attracted lot of criticism due to the issues like antibiotic residues, bacterial drug resistance and toxicity. In this present scenario, vaccination would be the best alternative to combat bacterial and viral disease for the sustainable aquaculture. Among the various bacterial vaccines, WC and OMP vaccines trigger the antibody response of the fish. Vaccines using whole cells or cell components of bacteria as immunogens can prevent outbreaks of bacterial diseases in aquaculture (Chandran *et al.,* 2002). Bacterial OMPs play a significant role in virulence as they compromise the outer most surface in contact with host cells and are also involved in induction of immune defense factors (Ebanks R.O *et al.,* 2005). In the present study was used to analyze the effect of bivalent WC and OMP vaccines against *Edwardsiella tarda* and *Pseudomonas fluorescens* infections in *Cirrhinus mrigala* fingerlings. Mrigal Fingerlings were vaccinated twice (30 &60 dpv) by immersion method and after 30 & 60 dpv period analyze the protective efficacy, mortality rate and pathological symptoms.

Tab-1 Mortality & RPS rate of C*irrhinus mrigala* fish after 30dpv mixed bacterial challenge.

Types of vaccine used	Route of vaccination	Route of challenge	Total no of fish	Mortality		RPS%
				No	%	
OMP +A and OMP+A	Immersion	Bath	25	5	20	80
OMP and OMP	Immersion	Bath	25	7	28	72
WC +A and WC+A	Immersion	Bath	25	8	32	68
WC and WC	Immersion	Bath	25	9	36	64
Control	-	Bath	25	25	100	0
Blank	-	-	50	0	0	100

Based on the results, after 30dpv challenge with mixed bacterial pathogens high protection was recorded in groups vaccinated with bivalent OMP vaccines with adjuvant (80%), bivalent OMP vaccines (72%), bivalent WC vaccines with adjuvant (68%), bivalent WC vaccines (64%) respectively when compared to control groups (Fig-1,Table-1).The Mortality was found low in OMP vaccines with adjuvant (20%) and high (36%) in WC vaccine without adjuvant when compared to other groups. Control groups attained 100% mortality within 7 days. Similarly the Booster dose [2nddose] helped to reduce the cumulative mortality rate to 8% in OMP with adjuvant and 16% in OMP without adjuvant (Fig-2, Table-2). There is an increase in the RPS rate in bivalent OMP vaccines with adjuvant [92%] and bivalent OMP vaccines without adjuvant [84%], bivalent WC vaccines with adjuvant (76%), bivalent WC vaccines without adjuvant (72%) respectively. The adjuvant could enhance the immune response by increasing the uptake of antigens and provide protection against the bacterial pathogen in aquaculture. The results showed that the WC and OMP vaccines were confirmed to provide immune protection against *Edwardsiella tarda* (Edwardsiellosis) and *Pseudomonas fluorescens* infection (*Pseudomonas* Septicemia) inmrigal fingerlings.

Tab-2 Mortality & RPS rate of C*irrhinus mrigala* fish after 60dpv Mixed bacterial challenge.

Types of vaccine used	Route of vaccination	Route of challenge	Total no of fish	Mortality		RPS%
				No	%	
OMP +A and OMP+A	Immersion	Bath	25	2	8	92
OMP and OMP	Immersion	Bath	25	4	16	84
WC+A andWC+A	Immersion	Bath	25	6	24	76
WC and WC	Immersion	Bath	25	7	28	72
Control	-	Bath	25	25	100	0
Blank	-	-	50	0	0	100

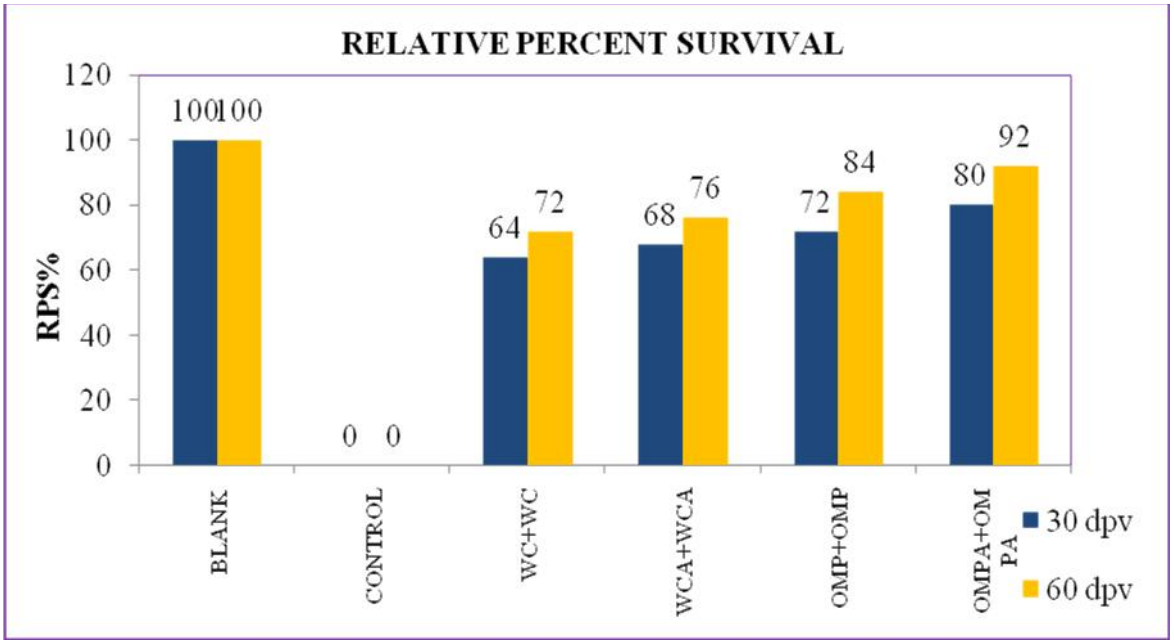


Fig – 1 RPS % of C*irrhinus mrigala* fish vaccinated with different types of vaccines after 30 and 60dpv bacterial challenge.

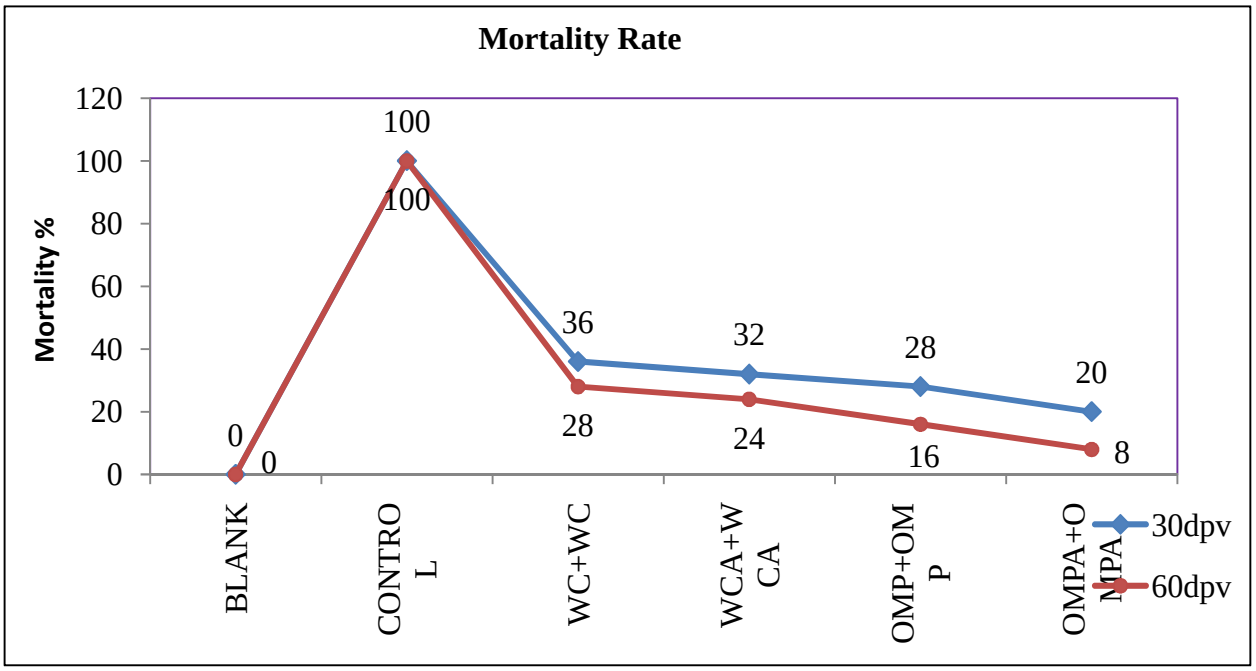
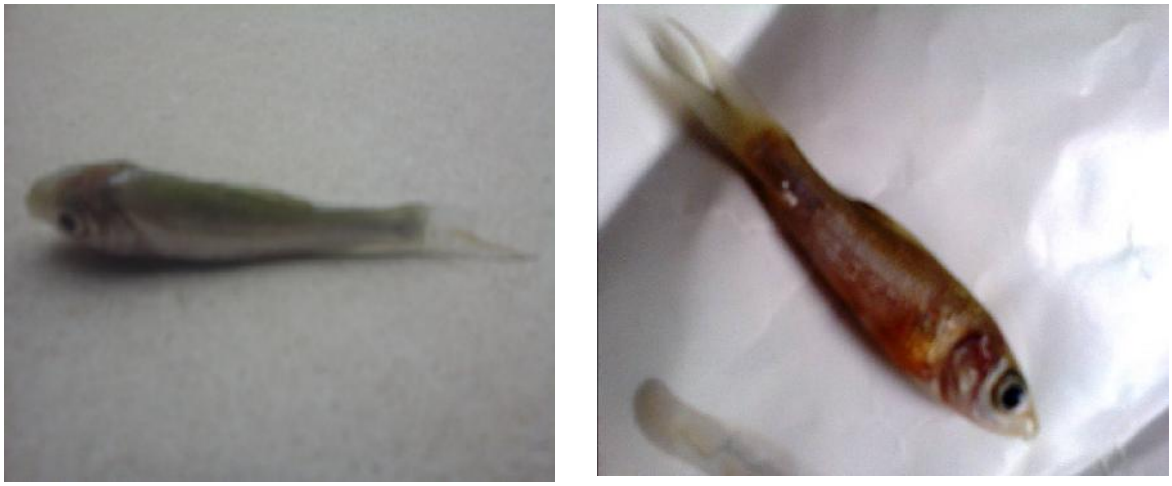


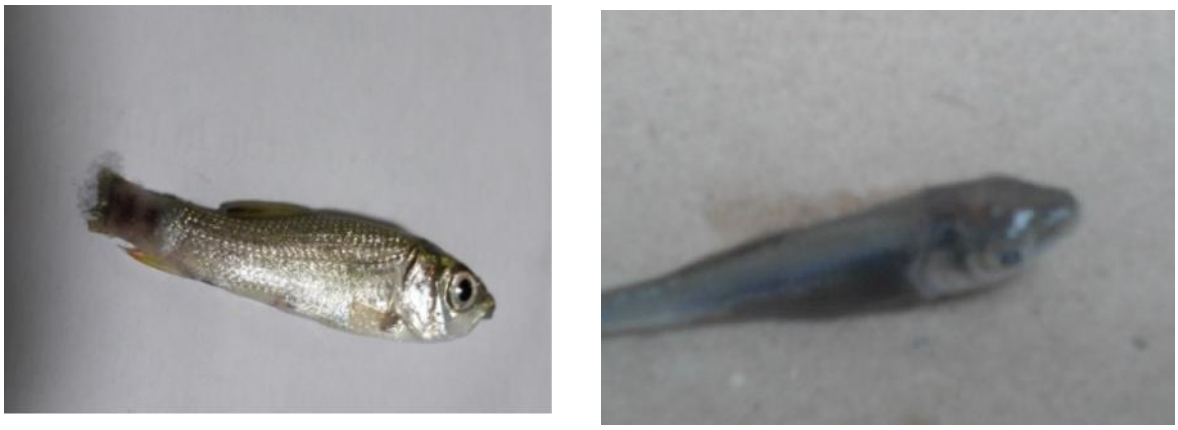
Fig – 2 Mortality % of C*irrhinus mrigala* fish vaccinated with different types of vaccines after 30 and 60dpv bacterial challenge.

The intensive farming of fin fishes and shell fishes has led to an imbalance of optimal culture conditions, which shows increased susceptibility to infectious diseases. Stress from low dissolved oxygen, high stocking density, physical trauma and poor nutrition are pre disposing factors in the development of bacterial diseases (Post, 1983).The resistance of the fish can be evaluated by analyzing the survival rate after experimental infection (Wassom and Kelly, 1990). Symptoms of Edwardsiellosis (Fig-3) and *Pseudomonas* septicemia (Fig-4) were observed after the experimental infections. Fish affected by edwardsiellosis shows signs of spiralling movement and die with the mouth agape and opercula flared, which may be due to the development of anaemia leading to oxygen insufficiency. Lesions on the skin, rectal prolapse, ocular disease, oedema, abnormal behavior, swollen areas, loss of pigmentation, and haemorrhage throughout the body. The common symptoms were swollen areas, loss of pigmentation and lesions on the skin were similar to the result of Sahoo *et al.,* 1998. Hemorrhagic septicemia caused by *P. fluorescens* may be acute or chronic, with the most common clinical signs being large hemorrhagic skin lesions and fin or tail rot. Hemorrhagic septicemia caused by *P. fluorescens* may be acute or chronic, with the most common clinical signs being large hemorrhagic skin lesions and/or fin or tail rot (Austin and Austin 1987).



(A)Lesions on the head B) Haemorrhage throughout the body

Fig – 3 Symptoms of Edwardsiellosis



(A) Scale erosion and Tailrot (B) Darkening of the skin

Fig – 4 *Pseudomonas* septicemia

In the present study, RPS of vaccinated group (after challenge infection) was higher in 30 and 60dpv when compared to unvaccinated groups. In earlier reports, Swain *et al.,* 2007 have studied the immune response to mixed whole cell antigens of *Aeromonas hydrophila*, *Edwardsiella tarda* and *Pseudomonas fluorescens*, the common Gram negative bacterial pathogens associated with diseases of Indian major carps. Booster dose helped to increase the efficacy (RPS) in vaccinated groups compared to prime or first dose. In the previous study of Thangaviji *et al*., 2013 the immuno adjuvant *Asparagus racemosus* was found to improve the activity of different types of *Aeromonas hydrophila* vaccines [WC, ECP, OMP, and BF] and also improved the immunological enhancement, RPS rate, and decrease the mortality rate in *Carassius auratus* [Goldfish]. Likewise, in the present study, WC and OMP vaccines when combined with the immunoadjuvant *Asparagus racemosus* enhanced the immune response by increasing the uptake of antigens after 30 and 60 days of post vaccination. Saponins is the major active immuno adjuvant compounds of *Asparagus racemosus* and they promote peripheral lymphocyte proliferation, enhance serum antibody titer and offer safe advantages than chemical adjuvants (Patwardhan, 2000).

Shoemaker, *et al.,* (2011) revealed that the safety of the vaccine and significant protection following challenge with RPS values between 74-94%, depending on the vaccine dose. Among the various methods of vaccination, the immersion method was simple, cheap and ideal for mass administration to fish of all sizes and large scale aquaculture. In most vaccines, adjuvants are a crucial ingredient for efficacy. Therefore, strategies may be made to develop a bivalent vaccine commercially for protecting mrigal carps, so that they can be saved from these common infections and the cost of vaccine and vaccination can be reduced significantly with less stress to cultured fish.

Conclusion

Edwardsiella tarda and *Pseudomonas fluorescens* are ubiquitous parasites and a potential pathogen posing a serious threat to freshwater aquaculture and fish industry. The use of vaccines, combined with good health management techniques, may result in substantial disease prevention and production becomes more predictable. Vaccines stimulate the fish immune system to produce antibodies that help to protect the fish from diseases. Therefore, the present study was used for the development of immunoproteomic vaccines and these potential effective vaccines could be used to prevent the infection of *Edwardsiella tarda* (Edwardsiellosis) and *Pseudomonas fluorescens* (*Pseudomonas* Septicemia) in aquaculture Industry.

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