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EFFECT OF WHOLE CELL (WC) AND OUTER MEMBRANE PROTEIN (OMP) VACCINES ON PROTECTION OF CATLA CATLA AGAINST STAPHYLOCOCCOSIS OF STAPHYLOCOCCUS AUREUS

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ABSTRACT

Immunoproteomic vaccines provide protection against bacterial infections in fish aquaculture and the vaccines are getting popular due to its long lasting immunity, safety and low cost. In this study we used Whole cell – Formalin Killed (WC) and Outer Membrane Protein (OMP) of *S.aureus* as a vaccine to provide protection against staphylococcosis 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 *S.aureus*. The results showed that the relative percentage of survival in the catla fish groups vaccinated by Outer Membrane Protein (OMP) vaccine with adjuvant were significantly higher than that the other types of vaccines (84%).

KEYWORDS: *Staphylococcus aureus, Catla catla, Asparagus racemosus*, WC and OMP.

INTRODUCTION

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.

Bacterial outer membrane proteins (OMPs) play a crucial 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 staphylococcosis disease.

Staphylococcus aureus has a wide range of potential virulence factors, either surface associated or secreted. The proteins present in the outer membrane of bacteria belong to one of two major classes, viz., lipoproteins, which are anchored to the outer membrane with N-terminal lipid tails, and integral proteins, which are referred to as outer membrane proteins (OMPs), containing membrane-spanning regions (Bos and Tommassen, 2004).

In most vaccines, adjuvants are a crucial and vital ingredient for vaccine efficacy. 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 titre and offer safer advantages than chemical adjuvants.

In the present study we demonstrated that the immunoproteomic *Staphylococcus aureus* WC and OMP vaccine in the presence of the herbal adjuvant *A. racemosus* extract that could provide protection against staphylococcosis infections in Indian Major Carp catla (*Catla catla*).

AIM

- Vaccination trials with bacterins prepared from the *Staphylococcus aureus* 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.

Source of Virulent *Staphylococcus aureus* strain

MTCC 96 virulent strain of *Staphylococcus aureus were used in this study.*

Preparation of Whole Cell – Formalin – Killed (WC)

In order to prepare the bacterins, bacterial isolate was inoculated separately into tryptic soy broth (TSB) and incubated for 24 h at 37°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 4000xg for 10 min., then washed twice in 0.3% formalized PBS and resuspended in PBS to the bacterial concentration of 1 x 10^8cells/ml. After that, the bacterins were tested for their sterility (free from the living cells) by streaking them onto trypticase soy agar which showed no growth.

Preparation of OMP Vaccine

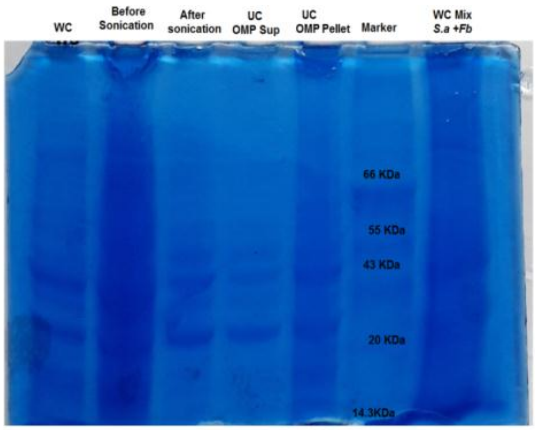
Outer membrane protein (OMP) was extracted using the protocol of Austin & Rodgers (1981) with little modification. Briefly, *S.aureus* was grown in TSB broth at 37°C for 24h and harvested by centrifugation at 8500 rpm for 10 minutes.

After centrifuge supernatant was discarded and pellet was washed with 20mM Tris buffer at pH 7.2 and finally pellet was resuspended in 10mM EDTA buffer. The bacterial cell suspensions were then subjected to sonication at 50hz for 10 mins in a sonicator to disrupt the cell wall. The unbroken cells were sedimented by centrifugation at 8500rpm and supernatant was collected. Ultracentrifugation of collected supernatant was carried out at a speed of 27400 rpm for 45 mins.

The supernatant was discarded and the sediment was resuspended in Tris buffer containing 0.5% sarkosyl. Further centrifugation at 27400 rpm for 45 mins was repeated. The sediment collected after ultracentrifugation was OMP and supernatant obtained was the CMP. OMP was kept in-20°C for further use.

Protein estimation was done by Lowery's method (1951).

SDS – PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophorosis) of Whole cell and OMP Proteins



S.aureus in 10% SDS-PAGE

Fig.1 Sodium dodecyl sulphate-Polyacrlamide Gel Electrophoresis Products of *Flavobacterium columnare*. Lane 1, 2, 3, 4, 5 and 6 were WC, before Sonic, before Sonic, UC supernatant, OMP (UC Pellet) and Marker respectively.

Results of SDS PAGE – Qualitative Analysis of WC& OMP Proteins

SDS-PAGE results revealed that the WC vaccines had ten polypeptide bands with molecular weight of 161.2, 137.1, 112.1, 85.8, 73.4, 63.1, 55.8, 46.2, 36.1, and 33.1KDa and OMP vaccines had four Polypeptide band with molecular weight of 84.9, 72.2, 60.5 and 46.9 KDa.

Lowery's Method – Quantitative Analysis of Proteins

The amount of Protein content in whole cell and OMP proteins were **88µg/ml** and **55µg/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. 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, 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.

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.

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. 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 Fig.2.

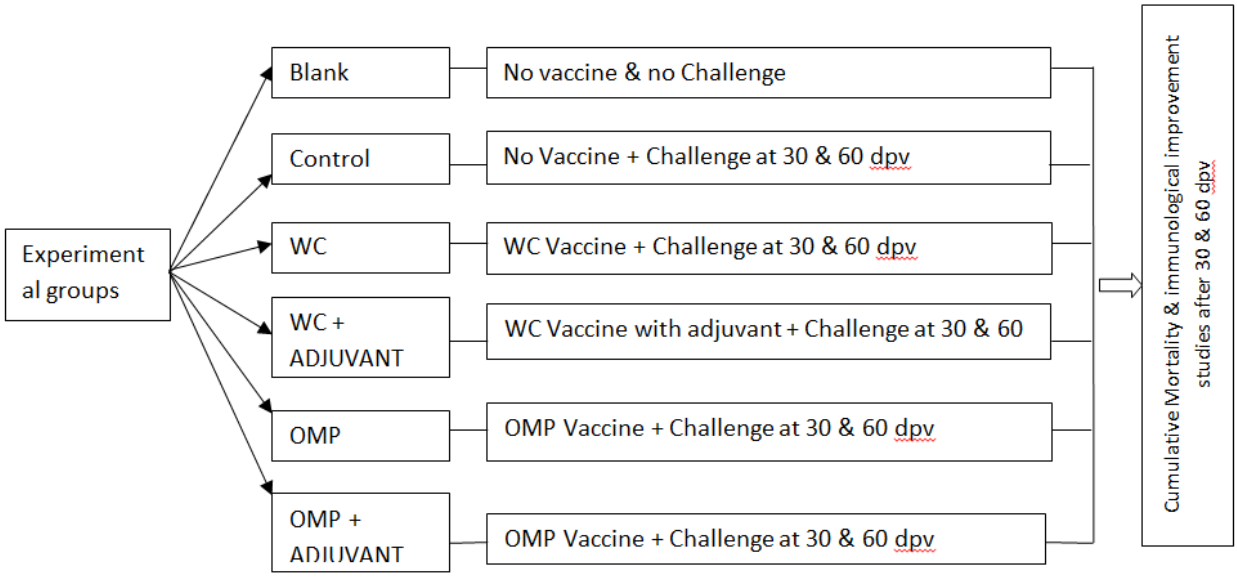


Fig. 2 Experimental Design for WC and OMP Vaccine Preparation and Delivery Methods to Indian Major Carp Catla *(Catla catla)*.

CHALLENGE WITH VIRULENT STAPHYLOCOCCUS AUREUS

On 30 and 60 days post vaccination (dpv), group of fishes were challenged with a lethal dose (LD_{50}) of *S.aureus* (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. 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.

RESULTS AND DISCUSSION

SDS-PAGE results revealed that the WC vaccines had ten polypeptide bands with molecular weight of 161.2, 137.1, 112.1, 85.8, 73.4, 63.1, 55.8, 46.2, 36.1, and 33.1KDa and OMP vaccines had four Polypeptide band with molecular weight of 84.9, 72.2, 60.5 and 46.9 KDa.

Estimated Protein content of whole cell and OMP proteins were **88µg/ml** and **55µg/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%.

After 30dpv Fishes vaccinated with OMP with adjuvant showed high percentage of RPS as 76%. In fish group vaccinated with OMP records 72% RPS. For fish groups vaccinated with WC and WC + Adjuvant, the result of

RPS was found to be 64% and 68 %, respectively. (Refer Table 1).

Similarly, after 60dpv Fishes vaccinated with OMP with adjuvant showed high percentage of RPS as 84% and OMP without adjuvant recorded 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 2).

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. 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.

A lower mortality percentage was achieved in all vaccinated groups in comparison with the control group. (Refer Table 1 & 2).

J. Michael *et al.*, (2011) found that fish had significantly higher relative percentage survival with the extracellular protein 5 weeks post – vaccination by bath, in comparison with the unvaccinated fish. At 5 weeks post – vaccination the unvaccinated fish had high mortality and the fish vaccinated with the extracellular protein achieved high protection.
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 staphylococcosis disease in fishes.

Table 1.Mortality and Relative Percent Survival (RPS) after Challenging of *Catla catla* fish Vaccinated by immersion route with *Staphylococcus aureus* Formalin-Killed Whole cell vaccines and OMP Vaccines.

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	9	36	64
WC + Adjuvant	Immersion	Immersion	25	8	32	68
OMP Vaccine	Immersion	Immersion	25	7	28	72
OMP + Adjuvant	Immersion	Immersion	25	6	24	76
Control	Immersion	Immersion	0	25	100	0

Relative Level of protection or Relative Percent Survival (RPS)

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

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	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

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).

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). 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).

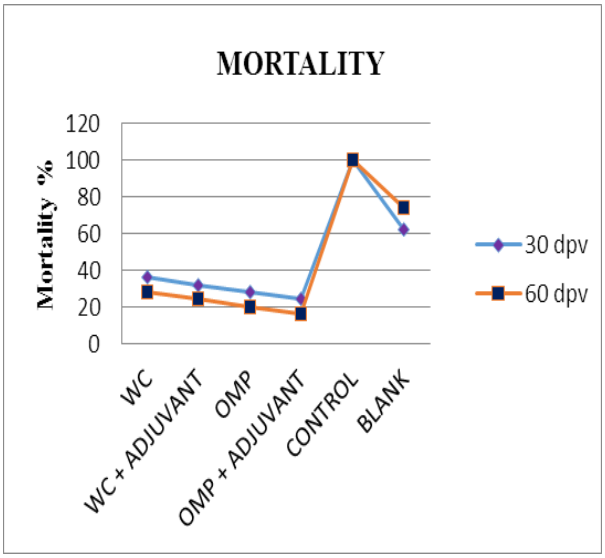


Fig. 3 Percentage of Mortality of catla fish vaccinated with different types of vaccines after 30 and 60 dpv.

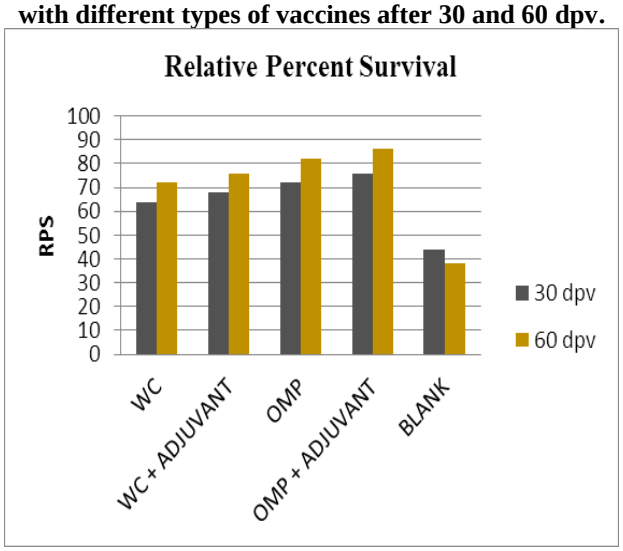


Fig. 4 Relative Percent survival of the catla fish vaccinated with different types of vaccines after 30 and 60 dpv.

CONCLUSION

We conclude that immunoproteomic vaccines enhancing the *Catla catla* fish innate immunity and disease resistance against staphylococcosis disease.

The herbal adjuvant *Asparagus racemosus* 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 staphylococcosis disease in Indian Major Carp Catla (*Catla catla).*

The *Staphylococcus aureus* OMP antigenic protein could be used as a potential vaccine to control staphylococcosis 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 and 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 *S.aureus* infections and provide an ideal alternative to pathogen-based vaccines against staphylococcosis in aquaculture.

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