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IMMERSION VACCINATION OF WHOLE CELL (WC) AND OUTER MEMBRANE PROTEIN (OMP) VACCINES IN *LABEO ROHITA* FINGERLINGS AGAINST STAPHYLOCOCCOSIS DISEASE

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ABSTRACT

Immersion vaccination is a widely accepted and sensible technique for mass vaccination in small fishes (fry and fingerlings). Whole cell (WC) and Outer membrane protein (OMP) vaccines were prepared from the *Staphylococcus aureus* culture. Qualitative analysis by Lowry's method and Quantitative analysis by SDS-PAGE of proteins were performed for the prepared vaccines. Fingerlings were immunized after pre-treatment with hyper-osmotic solution of 2% NaCl for 5 minutes by immersion (bath) vaccination of prepared vaccines for about 30 minutes. Immunization was carried out twice as booster dose on day 1 and second dose on day 30. After 30 and 60 dpv fingerlings from each group were bath challenged with virulent *Staphylococcus aureus* in lethal dose concentration of $1x10^8$ cells/ml. Fingerlings were observed for pathological signs and symptoms. Mortality rate and relative percent survival were recorded for up-to 10 days. After 30 dpv control group showed 100% mortality within 10 days of bacterial challenge, whereas significant relative percent survival (RPS) was recorded in vaccinated groups with 68% in WC group and 64% in OMP group. After 60 dpv there was a significant increase in the RPS rate in OMP group with 84% and 76% in WC.

KEYWORDS: *Labeo rohita* (rohu), Whole cell vaccine (WC), Outer membrane protein vaccine (OMP), *Staphylococcus aureus*, Mortality and Relative percent survival (RPS).

INTRODUCTION

In India, freshwater aquaculture mainly depends on the three Indian major carps Catla (*Catla catla),* Rohu (*Labeo rohita)* and Mrigal (*Cirrhinus mrigala).* Indian major Carps contributes

about 80% of the total fish production (Swapna *et al.*, 2010), of which *Labeo rohita* (rohu), is the most important species among the three Indian major carps and one of the widely cultured and most preferred species in the Indian sub-continent. Rohu gains high consumer preference for its fast growth rate and good market value and contributes about 45% of the total carp production in India (FAO,2010). In aquaculture, *Labeo rohita* serves as an ideal candidate for carp polyculture systems for its compatibility with other carps like Catla and Mrigal. Bacterial diseases are one of the major problems in freshwater aquaculture which confronts high density fish culture systems. It is well known that bacteria can pose serious threats in aquaculture industries by declining the water quality.

Staphylococcus aureus is an opportunistic pathogen, capable of causing a wide variety of diseases worldwide and one of the most commonly occurring infectious pathogen in freshwater fishes. Staphylococcal infections arise under a variety of pre-disposing conditions like poor water quality, fluctuating temperatures, pH and other environmental stressors. *Staphylococcus aureus* has been identified as the most commonly occurring bacterial pathogens in fish farms (Dey *et al.*, 1995). Staphylococci is one of the important human and animal pathogen, responsible for causing nosocomial infection. In fishes, the typical symptoms of *Staphylococcus aureus* were Exophthalmia, Septicaemia and eyes become vascularised leading to opacity which affects the brain and optic nerves.

In aquaculture, the most commonly used vaccination methods are of three types; 1) Inactivated whole cell vaccine 2) Live-attenuated vaccine and 3) Sub-unit vaccine, of which inactivated whole cell and live attenuated vaccines are gaining wide importance because of their convenience (Caipang *et al.*, 2014). Inactivated whole cell vaccine is the modified heat killed vaccine using formaldehyde, formalin completely kills and inactivate the bacteria, in which it can no longer be able to replicate inside the host (Petrovsky *et al.*, 2004). Vaccines that were prepared from whole cell inactivated extracellular bacteria provides sufficient protection, resulting in significant reduction in mortalities and antibiotic usage in the aquaculture industry (Sommerset *et al.*, 2005).

Vaccines made using outer membrane protein (OMP) bacteria, significantly prevent disease outbreaks and reduce mortalities in aquaculture when administered in proper dose concentration. Outer membrane proteins possess immunogens which possess immunogenicvproperties to fight against bacterial diseases in fish (Hirst and Ellis *et al.*, 1994). Although all vaccines have their own advantages and disadvantages with respect to the

level of protection, side-effects and cost-efficiency, the widely accepted method is immersion vaccination and injection vaccination as it gives enough protection to the fishes against bacterial diseases (Horne *et al.*, 1984).

OBJECTIVE

Thus, the present study is aimed to assess the efficacy of the prepared whole cell (WC) and outer membrane protein (OMP) vaccines against *Staphylococcus aureus* in *Labeo rohita* fingerlings.

MATERIALS AND METHODS

Experimental Fingerlings

Fingerlings of average weight 5g to 10g were procured from Poondi fish farm in Thiruvallur. Fingerlings were acclimatized for 15 days to meet the laboratory condition. During the experimental period, fingerlings were fed with natural feed of ground nut oil cake once in a day. Water quality parameters such as pH, temperature and salinity were checked periodically.

Bacterial Strain

Virulent bacterial strain MTCC 2940-*Staphylococcus aureus*, sub-culture was obtained from King Institute of Preventive Medicine and Research, Guindy.

Preparation of Whole Cell (WC) Vaccine (Formalin-Killed)

The whole cell vaccine was prepared from the isolate of *Staphylococcus aureus*, which was inoculated in tryptic soy broth (TSB) and incubated for 24 hours at the temperature of 37°C. Formalin (40% w/v) was added to the broth culture at a final concentration of 0.5% (V/V) and left for up-to 48 hrs in room temperature. The cells were then washed twice in 0.3% formalized PBS and resuspended in PBS (phosphate buffered saline) to the bacterial concentration of 1 x 108cells/ml. The prepared vaccine was tested for their sterility (free from the living cells) by streaking them onto trypticase soy agar which showed no growth.

Preparation of Outer Membrane Protein (OMP) Vaccine

Outer membrane protein (OMP) vaccine was prepared following the protocol of Austin & Rodgers (1981) with little modification. Bacterial culture, *Staphylococcus aureus* was grown in TSB (tryptic soy broth) at 37^{o}C for 24hours. The cells were harvested by centrifugation at 8500 rpm for about 10 minutes Ultracentrifugation of collected supernatant was carried out at

a speed of 27,400 rpm for about 45 minutes. The supernatant was discarded, and the sediment was resuspended in Tris buffer containing 0.5% sarkosyl (Sodium lauroyl sarcosinate). The sediment collected after ultracentrifugation was OMP and kept in -20ºC for further use.

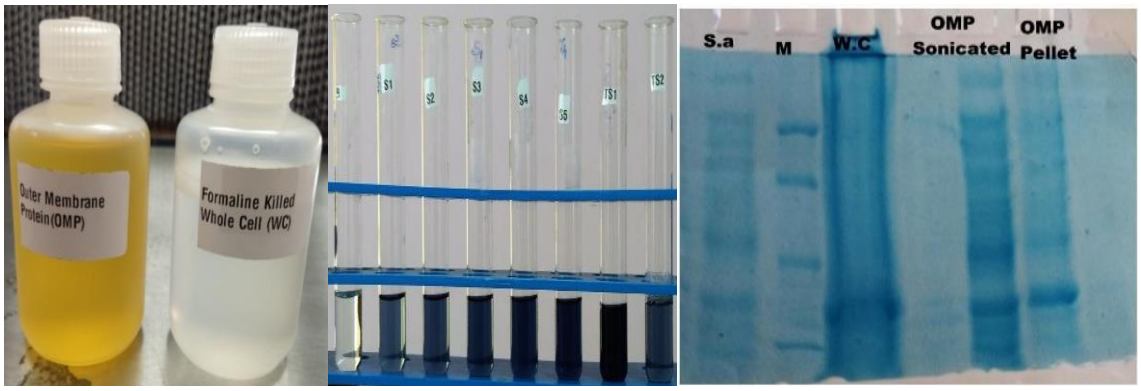


Fig. 1: WC, OMP Estimation of Protein Sds-Page Profile.

Qualitative and Quantitative Analysis of Proteins

Quantitative analysis of proteins for the prepared vaccines of whole cell and outer membrane protein was performed using Lowry's method (Lowry *et al.*, 1951) and qualitative analysis of proteins were performed using SDS-PAGE method developed by Laemmli in 1970.

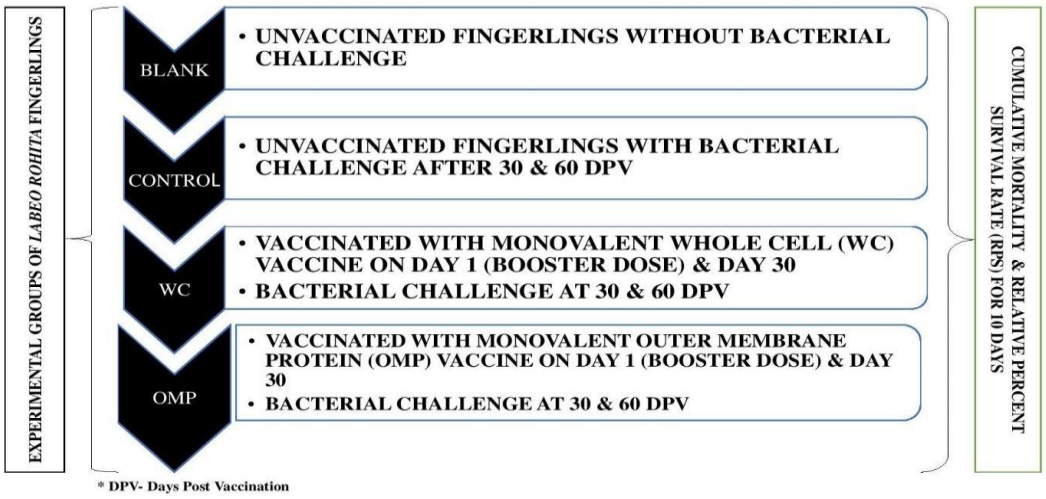
Experimental Design of *Labeo Rohita*

25 fingerlings were randomly selected and divided into groups with blank, control, WC and OMP respectively. Feeding was stopped 24hours prior to the experimental work, for the fingerlings to respond better for the immunisation. Booster dose was given on Day 1 for all the experimental groups, except the control group. Second dose was given on Day 30 and the experiment was repeated as above.

Vaccine Delivery

Fingerlings were immersed in hyper-osmotic solution of 2%Nacl for about 5 minutes, followed by the immersion (bath) vaccination of 30 minutes for the experimental groups (WC and OMP). The vaccine dose concentration was $1x10^8$ cells/ml.

Vaccine Delivery and Bacterial Challenge of Rohu Fingerlings



Bacterial Challenge

Fingerlings from each group were challenged with virulent strain of *Staphylococcus aureus* after 30 and 60 dpv. 25 fingerlings were bath challenged using the virulent strain *Staphylococcus aureus* in the lethal concentration of $1x10^8$ cells/ml. Bacterial challenge persists up-to 1 hour and fingerlings were observed for pathological signs and symptoms for up-to 10 days.

Mortality and Relative Percent Survival (RPS)

Mortality was recorded periodically for up-to 10 days post challenge and relative percent survival was calculated using the formula by Amend (1981),

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

RESULTS AND DISCUSSION

Vaccination plays a vital role in large-scale commercial fish farming and has been a key reason for the success of carp cultivation. In the present study, *Labeo rohita* fingerlings were vaccinated using WC and OMP against *Staphylococcus aureus*, cumulative mortality and relative percent survival were recorded after 30 and 60 dpv.

Qualitative and quantitative analysis of protein from prepared vaccines revealed that, total protein was found to be 34µg/ml in WC and 22µg/ml in OMP. Qualitative analysis of proteins revealed that, the band length of WC vaccine was found to be 27.95 KDa and band length of OMP vaccine was found to be 27.43-35.43 KDa (Fig-1). Similarly, Nikoo *et al.*,

(2008) studied the SDS-PAGE profile of *Aeromonas hydrophila* bacterin with band length 14KDa in *Labeo rohita* fingerlings.

In the present study, fingerlings were observed for the cumulative mortality after 30 and 60 dpv. After 30 dpv, mortality rate was 100% in control group and there was relatively low mortality found in WC with 32% and 36% in OMP groups. Higher RPS was recorded in WC with 68% followed by 64% in OMP group (Tab-1, Fig-2). This result correlated with the study of Kamelia *et al.*, (2009), when fish vaccinated with monovalent vaccine of *Aeromonas hydrophila* showed higher degree of RPS with 89% and 81% respectively.

Table 1: Mortality and RPS of *Labeo Rohita* Fingerlings after 30 DPV.

S. N.	Types of Vaccines	Method of Vaccination	Method of Bacterial Challenge	No. of Fish	Mortality (%)	RPS (%)
1	Blank	-	-	25	-	100
2	Control	-	Immersion (Bath challenge)	25	100	0
3	Whole cell (WC)	Immersion (Bath)	Immersion (Bath challenge)	25	32	68
4	Outer membrane Protein (OMP)	Immersion (Bath)	Immersion (Bath challenge)	25	36	64

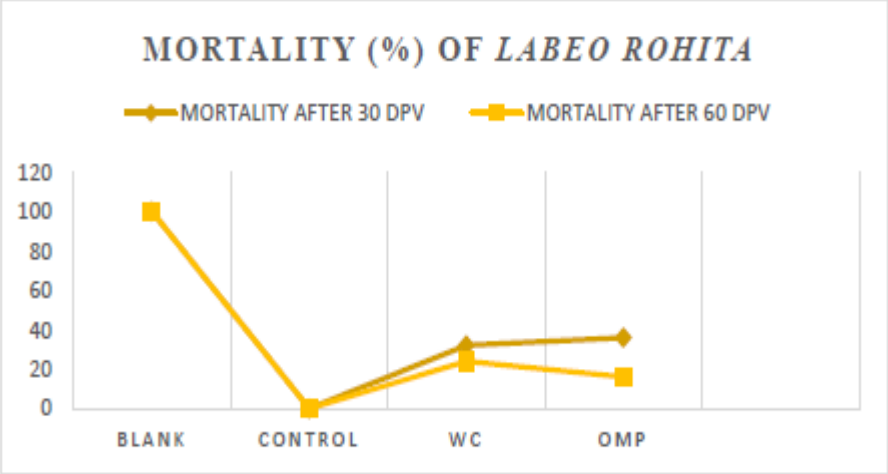


Fig. 2: Mortality of *Labeo Rohita* Fingerlings after 30&60 DPV.

Immersion vaccination greatly influenced the RPS rate and significantly increased the immune response in Indian major carp *Labeo rohita*. After 60dpv, the booster dose significantly elevated the RPS rate and reduced the mortalities in all the experimental groups (Tab-2, Fig-3). Mortality was reduced to 24% in WC and 16% in OMP, thereby increasing the RPS by 76% in WC and 84% in OMP respectively. Similarly, Rajeshwari Shome *et al.*,

(2005) reported that, immersion vaccination in Indian major carps such as *Cirrhinus mrigala* and *Catla catla* when challenged with *Aeromonas hydrophila*, elevated the RPS rate to 83.3% in mrigal and 75% in catla respectively.

Table 2: Mortality And Relative Percent Survival (RPS) Of *Labeo Rohita* Fingerlings, After 60 DPV (days post vaccination).

S. N.	Types of Vaccines	Method of Vaccination	Method Of Bacterial Challenge	No. Of Fish	Mortality (%)	RPS (%)
1	Blank	-	-	25	-	100
2	Control	-	Immersion (Bath challenge)	25	100	0
3	Whole cell (WC)	Immersion (Bath)	Immersion (Bath challenge)	25	24	76
4	Outer membrane Protein (OMP)	Immersion (Bath)	Immersion (Bath challenge)	25	16	84

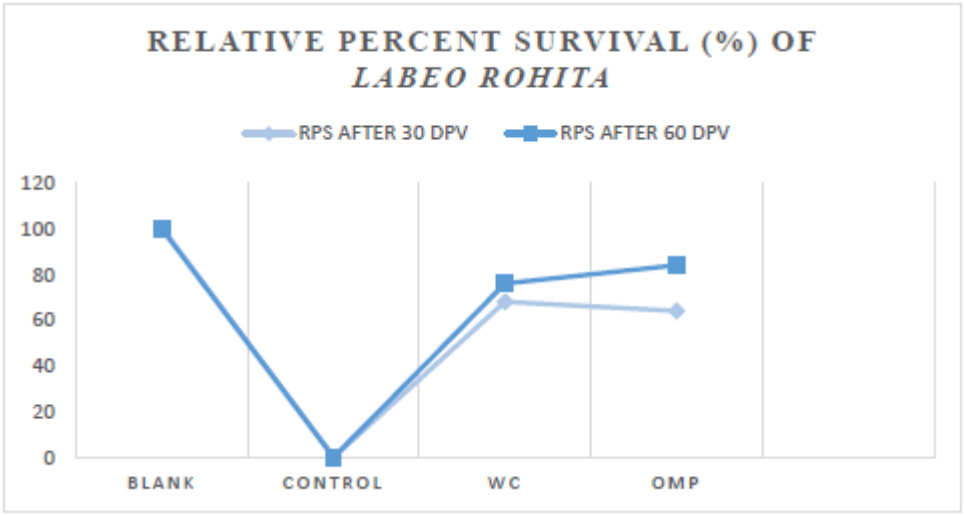


Fig. 3: Relative Percent Survival Rate (RPS) of *Labeo Rohita* Fingerlings, AFTER 30&60 DPV.

Eyes vascularised (opacity) **Exophthalmia** **Septicaemia**

Fig. 4: Pathological Signs and Symptoms of *Staphylococcosis* in Labeo Rohita Fingerlings.

Staphylococcosis caused by *Staphylococcus aureus* leads to the morbidity and mortality to a considerable extent globally Diekema *et al.*, (2001). In the present study, fingerlings were bath challenged after 30 and 60 dpv in the lethal dose of $1x10^8$ cells/ml. Fingerlings were observed for the pathological signs and symptoms for 10 days. As a result of post-challenge, dead fishes pronounced the typical symptoms of Staphylococcosis disease resulting in exophthalmia, where eyes become vascularised leading to opacity as a result of bacterial invasion (Fig-4). Fingerlings become lethargic and feeding was greatly reduced after 48 hours of bacterial challenge. The first mortality was observed after 24 hours of challenge. Fingerlings were severely affected with septicaemia. Shah and Tyagi, (1986) reported that, *Staphylococcus aureus* is the causative agent of an eye disease in carp fishes. Shah *et al.*, (1986) first reported the occurrence of Staphylococcosis disease in silver carp (*Hypophthalmichthyes molitrix*). Thus, the above results revealed that immersion vaccination of whole cell and outer membrane protein could be an ideal vaccine candidate on protection against Staphylococcosis disease in *Labeo rohita* fingerlings.

CONCLUSION

It could be conferred that, the prepared vaccines of whole cell and outer membrane protein provided sufficient protection when bath challenged with virulent *Staphylococcus aureus.* Immersion vaccination of 30 minutes with pre-treatment in hyper-osmotic solution of 2% NaCl to all the experimental groups showed higher degree of protection in *Labeo rohita* fingerlings and significantly reduced the mortality. Booster dose greatly enhanced the vaccine uptake in OMP groups with better immune response and RPS after 60 dpv when compared to WC respectivey. It is concluded that, immunization through immersion vaccination could be an ideal method of vaccinating fingerlings and provides sufficient protection against various bacterial diseases in aquaculture.

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