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Evaluation of Immunostimulant and Vaccine on *Labeo rohita* (Rohu)

Sujata A. Mankar*

Dada Ramchand Bakhru Sindhu Mahavidhyalaya, Panchpaoli, Nagpur, India
**Corresponding author*

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A B S T R A C T

Immunostimulant is used in combination with formalin inactivated vaccines which gives early activation to non-specific defense mechanisms. However, it also elevates the specific immune response and enhances protection against bacterial infection in fish. The present study demonstrates an effect of immunostimulant viz. vitamin C along with formalin inactivated polyvalent vaccine on fingerlings of *Labeo rohita* (rohu). The statistical significance of difference in the number of survivals in four vaccines and vitamin C groups, for before and after challenge was obtained using *Fisher's exact test*. The relative percent survival (RPS) was found to be higher for the group treated with immunostimulant vitamin C supplement; while lower for group treated with monovalent and polyvalent without vitamin C. The difference in the proportion of survivals in vaccine (T4/T5/T6) and control group after challenge was found statistically significant at 36th day.

Introduction

Aeromonas sp. is associated with disease in aquatic animals, humans and domestic animals including sheep, dogs and cats, especially when exposed to periods of stress conditions (Cipriano *et al.*, 2001; Groff & Lapatra, 2000; Ibrahem *et al.*, 2008; Janda & Duffey, 1988; Jeney & Anderson, 1993). The Motile Aeromonas Septicemia (MAS), caused by member of *Aeromonas sp.* is among the dangerous and most infectious diseases encountered in freshwater fish culture (Guimaraes *et al.*, 2002; Mulero *et al.*, 1998). Aeromoniasis responsible for primary or stress-associated pathogenicity in warm and cold water fish (e.g. carps, catfish and salmonids) and commonly associated with bacterial hemorrhagic septicemia, infectious dropsy, red mouth disease, dropsy, exophthalmia, and fin and tail rot and ulcerative conditions (Austin & Austin, 1999; Sahoo *et al.*, 1998; Sakai, 1999). Many factors have been associated with virulence of the *Aeromonas sp.* infection including hemolysins, proteases, surface array proteins and acetyl cholinesterase (Karunasagar *et al.*, 2003). It is also reported to contribute to intestinal and extra-intestinal infections including diarrhea in humans and other animals (Hamid, 2003).

No effective vaccine is currently available to prevent or control above bacterial infection life long, and extensive use of chemotherapeutic agents such as antibiotics to control of *Aeromonas sp.* may become an

ecological threat and is not desirable since it may lead to emergence of resistant strains. Since it affects the bacteria but not the toxin, and it creates a public health hazard if treated fish is used for human consumption (Gado, 1994). The preventive measure of endemic diseases imposes severe costs on fish farmers, and these measures made in the development of vaccines for fish bacterial diseases. Various vaccination strategies have been employed with different bacterin preparations against *A. hydrophila* in carp and catfish species but with limited success (Poobalane, 2007; Yin *et al.,* 1996). The efficiency of vaccination is largely dependent on the immune status of the fish and the conditions under which the fish were kept (Sahoo *et al.,* 1998). Use of immunotherapy is an approach in fish immunology that has been actively practiced in recent years as a method for bacterial infection. It does not involve recognition of a specific antigen or targeting the immune response towards a specific pathogen, but causes an overall immune response that hastens recognition of foreign proteins (Flores *et al.,* 2003; Soliman, *et al.,* 1989; Stevenson, 1988). So the use of immunostimulants for prevention of bacterial diseases in fish is considered as good alternative and promising area (Abdelkhalek *et al.,* 2008; Secombes, 1994). Immunization against *Aeromonas sp.* is difficult because of its heterogeneity as well as its stereotyping. With the exception of perhaps two or three species, vitamin C biosynthesis does not occur in fish due to the lack of the last enzyme i.e. L-gluconolactone oxidase of the ascorbic acid biosynthesis pathway. Vitamin C must be necessarily supplied via the feed and in the feed it can inhibit serum cortisol levels. Major signs of ascorbate deficiency include reduced growth, scoliosis, lordosis, internal and fin haemorrhage, distorted gill filaments, fin erosion, anorexia and increased mortality To cope with this problem, Hardie *et al*. (1991) had already reported that a high level of ascorbic acid is essential for reducing the effects of physiological stress as well as wound healing in fishes, and also its influence on fish macrophage functions such as engulfment and destruction of bacteria. Hence, during intensive aquaculture the addition of ascorbic acid can act at a number of levels to be highly effective (Anbarasu & Chandran, 2001).

The role of vitamin C to enhance inflammatory response and disease resistance has been recently demonstrated in Indian major carps. Vitamin E also has shown to influence non-specific and specific defense mechanism in rainbow trout. It also plays an important role in cell membrane structure, stability and function. Several researchers planned to produce different types of vaccine such as the formalized whole culture vaccine (Ghenghesh *et al.,* 1999), the hyper - osmotic infiltration vaccine (AQUIGRUP. 1980), the toxoids (Baba *et al.,* 1988) and the genetically engineered live bacteria with removal of one of the aerolysin genes (Ilhan *et al.,* 2006; Sordello *et al.,* 1997). The aim of this study was to determine the efficiency of formalin inactivated monovalent or polyvalent vaccine and to evaluate the RPS value of vaccine administered with and without immunostimulant.

Materials and Methods

Bacterial strain: *Aeromonas sp. and Streptococcus sp.* used in the present study was obtained from a diseased, infected fish during field survey in and around Nagpur region . Culture Media brain heart infusion Agar, Tryptone soya broth were used.

Fish: Fingerlings of Rohu species were obtained from Vidharbha Macchimar Sangh

with an average body weight of 35-40 g for experiment. Glass Aquaria used for experimental purpose. They were supplied with air conductors and dechlorinated well water. Commercial Pellets (Jalaram Feed Company, Nagpur, India) Food in ratio of 5% body weight per day was considered to be the optimal maintenance amount required for fingerling used for fish feeding during the experimental vaccination period.

Determination of Fifty percent median lethal dose (LD50):LD_{50} values were calculated according to the method of Reed and Muench (1938).

Preparation of Polyvalent Bacterin:For preparation of polyvalent bacterin, each bacterial isolates was inoculated separately into Brain heart infusion broth and incubated at 30ºC for 24h under continuous agitation. Equal volumes of each bacterial culture were taken and formalin (0.5% V/V) was added to the broth culture at a final concentration and left for 48 hrs at room temperature with continuous agitation. Formalin inactivated bacterial cultures were centrifuged at 4,000g for 30min, and supernatant were discarded and re-suspended in PBS. The bacterin was tested for their sterility in brain heart infusion agar medium at 30ºC for 24h.For the treatment T3, T4, T5; formalin inactivated vaccine 1:10 part dissolved in decholrinated water for bath immersion while for treatment T6 formalized inactivated polyvalent vaccine (vitamin C along with feed 150mg per kg) vaccination doses were used and fish were kept in bath for 20 mins. Groups of 10 fingerlings of *Labeo rohita* (rohu) (35-40 g) were used for vaccination in addition to the control group represented by 10 fish per group for each glass aquarium constituting both vaccinated and control fish as shown in Table1.

Challenge: For challenge study, 24h TSB culture was used. The culture was diluted 1:10 in PBS. Twenty eighth days after immunization each group were challenged with culture containing 5×10^6 viable cells per ml. Mortalities were monitored and any clinical signs in survivors were noted. Post challenge mortalities were recorded in both vaccinated and control fish groups. The level of protection was calculated according to Amend (1981).

Relative level of protection = 1- $\frac{\text{percent of immunized mortality}}{\text{percent of control mortality}} \times 100\ \%$

Relative percent Survival (RPS)

Statistical Analysis

The statistical significance of difference in the number of survivals for each vaccine and the corresponding control group was carried out using *Fisher's exact test*. The significance was tested at 28th day after administering the booster dose and at 36th day after challenge. Further, the association between the survival and the type of vaccines used was studied using *chi-square test*. Also, a pair wise comparison of T6 with each vaccine type was performed using *Fisher's exact test* to evaluate the statistical significance of difference in the number of survivals. The analysis was carried out for 28th and 36th day.

Results and Discussion

The all prepared formalin inactivated vaccines were tested for their sterility (free from living cells) by streaking it onto brain heart infusion agar, which showed no growth after 24 hrs. After sterility test all vaccines were kept at 4ºC for further use.

Fingerling groups of rohu were vaccinated

by bath immersion route with different formalin inactivated vaccine either as monovalent or polyvalent. The efficiency of each type of vaccination was determined by relative percent survival (RPS), through the application of challenge test (bath immersion route) in respect to mortality and survival with the virulent strains of relevant bacteria after vaccination (28^{th} day).

Figure 1 shows the survival rate for different treatment modalities against control in 36h of observation time. For all treatments, survival was better than the corresponding control; and treatment T6 yielded 100% survivals after 36h. The statistical significance of difference in the number of survivals for each treatment and respective control was obtained as shown in Table 2. Non-parametric *Fisher's exact test* revealed that except T6, no other treatment had significantly different number of survivals after 28^{th} hr. While after challenge i.e. 36^{th} hr, treatments T4 and T5 along with T6 showed significant difference in the number of survivals ($p < 0.05$).

Further, to evaluate the significance of difference in the number of survivals across all treatment groups for 28^{th} hr, *chi-square test* was used which resulted into *p*-value of 0.6127, indicating insignificant difference due to treatments. Similarly, insignificance was observed after 36^{th} hr with *p*-value of 0.1833 ($p > 0.05$).

Table.1 Formalin Inactivated Vaccination and Control Groups of Rohu Fingerlings using Bath Immersion Route

S.No.	Type of vaccine	Group	No. of fingerlings	No. of controls
1	Tc	Vitamin C	10	10
2	T3	Monovalent *Aeromonas sp.*	10	10
3	T4	Monovalent *Streptococcus sp.*	10	10
4	T5	Polyvalent [*Aeromonas sp.*+ *Streptococcus sp]*	10	10
5	T6	T5 +Ascorbic acid	10	10

Table.2 Significance of Difference in the Number of Survivals in Four Vaccine Groups before and After Challenge

	T3		T4		T5		T6		Vitamin C	
	Vaccine	Control	Vaccine	Control	Vaccine	Control	Vaccine	Control	Vaccine	Control
28th day	8	4	8	3	9	4	10	3	8	4
Fisher's exact test (p-value)	0.1698		0.0698		0.0573		0.0031		0.1698	
36th day	8	3	7	1	9	0	10	0	4	0
Fisher's exact test (p-value)	0.0698		0.0198		0.00012		1.08E-05		0.0866	

Indicate statistically significant difference in treated and control

Table.3 Statistical Significance of Difference in Number of Survivals between T6 and other

Treatment Modalities (Before and After Challenge)

After 28 days	
Comparison	p-value
T3 & T6	0.4737
T4 & T6	0.4737
T5 & T6	0.9999
Vit C & T6	0.4737

After 36 days	
Comparison	p-value
T3 & T6	0.4737
T4 & T6	0.2105
T5 & T6	0.9999
Vit C & T6	0.0108

Fig.1 Graphical Representation of Survival Rate of Tc (vit. C),T3, T4, T5 and T6 Compared with their Respective Controls

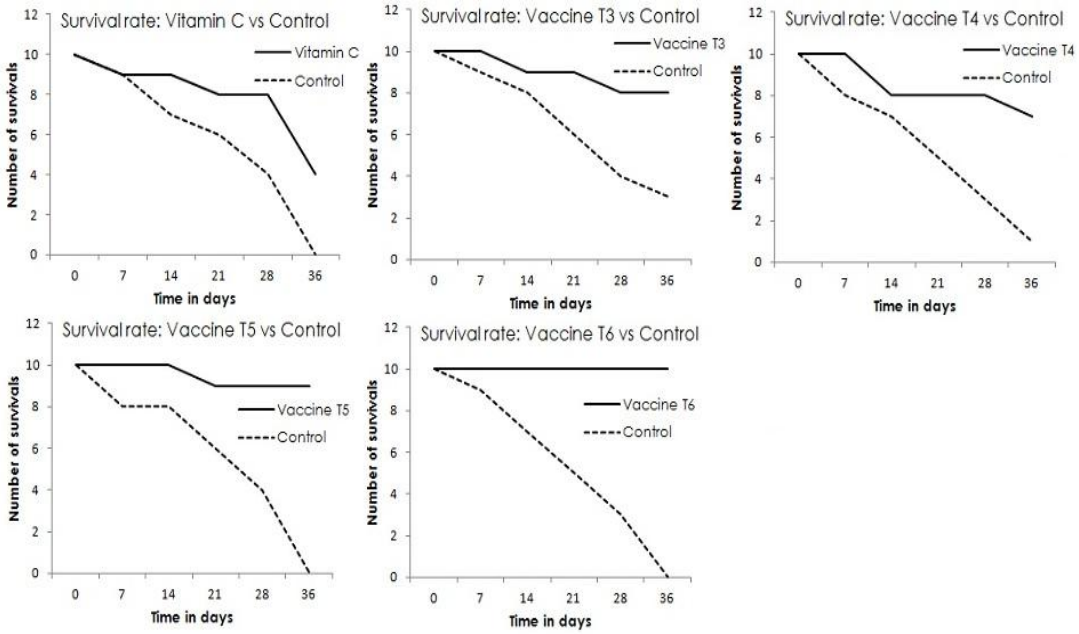
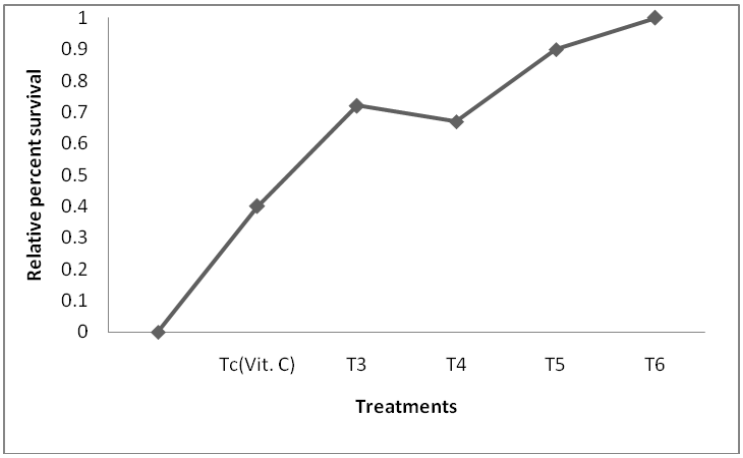


Fig.2 Relative Percent Survival (RPS) % Performance of Fish Vaccinated by Bath Immersion Route with Different Types of Formalin Inactivated Vaccine by the End of Experiment



Comparison between T6 and other Vaccines based on Survivals

The difference in the number of survivals in T3/T4/T5 group and T6 group was found insignificant ($p > 0.05$) after 28 days and after 36 days; while the comparison between vitamin C and T6 is significant with ($p < 0.05$) after challenge study as shown in Table 3.

The result of relative percent survival (RPS) in the present study showed that vitamin C supplemented group i.e. T6 has high values of RPS as compared to non-supplemented groups (T3, T4, T5). The level of protection is also high in T6 (polyvalent along with vitamin C) after challenge study. Post challenge mortalities were recorded in both vaccinated and control fish groups. The level of protection as RPS % was calculated (Fig 2).

Formalin killed vaccine is applied externally to fish using spray or direct immersion technique (Anderson, D.P. (1997) and this technique is useful for mass vaccination especially for small fish as the antigen then enters the fish body through the skin or the gills. (Horne & Ellis, 1988). The concentration and the duration of exposure are important factors, which affect the conclusive results regarding the protection using this method (Hamid, 2003). In above experiment trials using immunostimulants as vitamin C in combination with formalin inactivated polyvalent vaccine by bath immersion technique is an effective method for increasing the protective capabilities of fish, and boosting the potency of the vaccine smaller doses (Janda, 1991). As per Anbarasu *et al*. (2001), an optimal level of vitamin C (150 mg kg^{-1}) certainly enhances both the specific and non-specific immunity of the catfish, *M. gulio*. The observed RPS values of 82-100 % in common carp immunised with an A. *hydrophila biofilm* (heat inactivated) vaccine, while an RPS value of 76-81 % was seen in fish immunised with heat inactivated free-cell suspension of *A. hydrophila* (Burke *et al.*, 1984). An RPS value of 90.8-100 % was obtained with three batches of catfish, *Clarias batrachus* vaccinated with heat inactivated *A. hydrophila* biofilm and challenged with same *A. hydrophila* isolate compared with RPS values of 28.8-42.1 % for fish immunised with heat inactivated free cell suspension of *A. hydrophila* (Horne & Ellis, 1988) while low doses of vitamin C (101.2 mg\kg diet) and E (150mg/kg diet) present in the supplemented commercial diet and so it could not induce maximum immunity but induce enhanced immune response through leukopoiesis and enhanced lymphocytes proliferation (Abdelkhalek *et al.*, 2008). Similar results on rainbow trout obtained by Wahli *et al.*, (1997) who reported that, combination of vitamin C and E significantly increase lymphocyte proliferation. In contrast, Mulero *et al.* (1998a) found that in vitro addition of either vitamin C or E individually had no effect on the phagocytic activity of gilt head sea bream leukocytes and even combination of both vitamins failed to further increase such activity at any of the tested concentrations vitamin C (1-100 µg / ml) or vitamin E (0.01- 10 µg / ml) for 48 hrs (Abdelkhalek *et al.*, 2008).

In case of present finding, as T3 and T4 are monovalent vaccines, while T5 is polyvalent without vitamin C and T6 is a combination of T5 + vitamin C, it is found that T6 is more effective than all other three trials. The effect of immunostimulants via bath immersion technique and subsequent relative challenge study, the relative percent survival (RPS) % was found to be the high for immunostimulant as vitamin C supplemented treated groups, while lower

for formalin-killed monovalent and polyvalent vaccine without immunostimulant immunized group. The significance of difference in the number of survivals using *Fisher's exact test* reveals that the vaccine groups T4/T5/T6 with respective control after challenge (36^{th} days) is significant ($p < 0.05$). Immunostimulants along with vaccination is one of the most reliable method to control fish diseases and prevent looses from fish bacterial disease. Also, the method of immersion vaccination is very attractive since it's suitable for mass administration to fish of all size, imposes on stress on the fish because handling is not required and therefore dose not interfere with routine husbanding practices. Furthermore, these findings are also expected to assist aqua industry and farmers while significance of this study will increase the relevant knowledge of immunoprophylaxis using immunostimulants as vitamin C in combination with formalin inactivated polyvalent bacterin in carp.

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