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Survival studies on genetically modified bacterins and their immunization effect on gold fish (*Carassius auratus*)

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ABSTRACT *Aeromonas hydrophila was known to produce hemorrhagic ulcerative disease in gold fish and causing serious losses in ornamental fish farming. The pathogenic A. hydrophila was isolated from infected gold fish (Carassius auratus) and the LD50 was determined. Four ampicillin sensitive bacterins were developed by treating the organism with heat, UV, pH and formalin. The immune responses of these four bacterins were determined by injecting in fish individually and also by mixing it with Freund's complete adjuvant. High levels of agglutinating antibody titers were formed in adjuvant mixed vaccinated fish and significant protection was observed in the fish when they were experimentally challenged. The survival study of these mutant strains in aquatic environment clearly indicate that the mutant strains decline rapidly suggesting that nutrient supplementation was needed for the survival of those genetically modified ampicillin mutants.*

Introduction

Ulcerative skin disease of gold fish is of special concern because such disease may result in death or permanent disfigurement. Loss of market value of diseased fish is obvious and may be a cause for considerable economic concern. Many pathogens such as parasites, fungi, viruses and bacteria can cause skin disease in fish, but bacteria are of particular interest because infections are common and often deadly if untreated. Most of the bacteria that are commonly isolated from fish are gram negative bacilli.

Aeromonas hydrophila is a motile, gram negative bacillus. This bacterium is free living and is always present in the water. As an opportunist, this bacterium may infect many species of fresh water and brackish water fishes (Elliot & Shotts, 1980). Systemic infections of *A. hydrophila* in gold fish (*Carassius auratus*) resulted in the involvement of the hemopoietic system, degeneration of the liver and mild necrosis of the kidney (Brenden & Huizinga, 1986).

Treatment of bacterial skin disease depends upon the species of bacteria involved and the severity of the disease process. Antibiotics, 8% salt dip, and cleansing wounds with 7.5% betadine solution (an organic iodine preparation) have been used to successfully treat skin ulcers and erosions in fish. Selection of antibiotics should always be made with knowledge of the culture and sensitivity data. The long term use of antibiotics is also likely to affect the growth of the fish negatively (Wishkovsky *et al*., 1987) and another counter measure is needed to improve the resistance of the fish to infection.

Immunostimulation may be such counter measure and include methods that increased the capacity of the specific and/or the non-specific immune system. The immune system of fish exhibits all the characteristic feature of the immune system as known in mammals according to Ellis (1982). Successful immunostimulation against specific agents had been obtained by live vaccination using genetically modified mutants. To promote the efficiency of antigen stimulation of antibody production, the antigen is mixed with Freund's complete adjuvant, which enhances the rate of quantity of antibody produced. The use of genetically modified mutant strains in fish may facilitate the spread of these bacteria into aquatic environments. Because licensing regulations require assessing the risks associated with the release of genetically modified microorganisms, the persistence and behavior of mutant strains in natural environments need to be carefully studied.

The objective of this study is to isolate *A. hydrophila* and to estimate the degree of virulence and also to determine the immune response of *C. auratus* to Non-adjuvanted and adjuvanted *A. hydrophila* bacterins. This study also focuses on measurement of specific immune response of vaccinated fish. Finally, the genetically modified mutant of *A. hydrophila* was used as live vaccine and the survival of these strains in aquatic environment was studied.

Materials and methods

Isolation and identification of A. hydrophila from diseased gold fish (C. auratus): Infected gold fish (*C. auratus*) were collected from a private aquarium in Nagercoil, Kanyakumari District and transferred to the laboratory. Samples were taken from infected tissues and organs of fish and *A. hydrophila* strain was isolated by pour plate technique using Aeromonas Isolation Basal Agar Medium supplemented with Ampicillin. The colonies were identified based on Joseph and Carnahan (1994) and were maintained on TSA slants as stock cultures.

Preparation of bacterial antigen: The test culture was allowed to grow in 100ml nutrient broth for 24 hours at 37^0C. The entire culture was centrifuged at 10,000 rpm for 15 minutes. The bacterial pellet was washed in phosphate buffer saline (PBS) and again the pellet was resuspended in the same buffer saline.

Estimation of the degree of virulence (LD_{50}): The antigen was prepared and tenfold serial dilution was made. 0.1 ml

Volume : 3 | Issue : 11 | Nov 2013 | ISSN - 2249-555X

of each dilution was injected intraperitoneally to 7 sets of fish. The total viable count (TVC) in the injected samples was enumerated by plating into nutrient agar. Mortality rate was observed from 18 hours to 7 days. LD_{50} was estimated using the formula of Reed and Muench (1938).

Antibiotic sensitivity test: Sterile Muller Hinton agar plates were prepared. The test isolates were spread on sterile Muller Hinton plates and selected antibiotic discs were kept on the media. The plates were incubated at 37^{0}C for 24 hours. The zone of inhibition was measured in mm and the sensitivity pattern for the test isolate was recorded.

Preparations of genetically modified live vaccines: 100ml broth culture was used for preparing different types of bacterins. The prepared bacterins were genetically modified ampicillin sensitive mutants of *A. hydrophila* obtained by treatment with formalin, heat, ultra violet and pH. The mutated strains were cultured on TSA plates for 24 hours at 37^{0}C. The next day the cultures were transferred to Aeromonas Isolation Basal Agar Medium supplemented with ampicillin with the help of sterile velvet pad. The strain which fails to grow on the ampicillin medium was selected for further study. The bacterins were further mixed with equal volume of adjuvant to obtain four different adjuvanted vaccines.

Vaccine administration: 0.1ml (undiluted) of each vaccine was intraperitoneally injected to a set of six fishes. Another set was injected with 0.1ml of saline and treated as control.
Collection of blood and serum separation: Blood was collected at 7th, 14th, and 28th days after vaccination by caudal vein puncture. About 0.2ml of blood was collected in a sterile eppendrof tube and gently tapped to ensure better cell retraction and release of serum. The tubes were centrifuged at 1500 rpm for 15 minutes and the serum was separated. Decomplementation was done by placing the eppendrof tubes containing serum in a water bath at 40^{0}C for15 minutes.

Bacterial agglutination assay: Titres of specific antibody were determined by agglutination tests performed, in duplicate, in a microtitre plate as described by Barnes *et al*. (2003). Specific antiserum (100µ) was serially 2 fold diluted in PBS, and a suspension of *A. hydrophila* was added to each well (50µl). As a control, heat inactivated normal serum was also tested. Plates were incubated for 1 hour at room temperature and then overnight at 4^{0}C. Titres were taken as the highest dilution of serum to give a positive agglutination reaction.
Experimental challenge: After 30 days of immunization, the fish were challenged by intraperitonial inoculation with 0.1ml of bacterial isolate (10^2 CFU/ml). Fish were checked at least twice daily for the subsequent 14 days. All dead fish were removed and examined to confirm the re-isolation of the inoculated strain from the internal organs by streaking directly onto Aeromonas Isolation Basal Agar Medium supplemented with ampicillin. The potency of the vaccines was calculated as relative percent survival (RPS) (Amend, 1981) using the formula: RPS = {1-(% vaccinated mortality/ % non-vaccinated mortality)} x 100.

Survival assays in tap water and bacterial counts: The genetically modified ampicillin sensitive mutants and wild strains were grown on 100ml TSB with orbital shaking at 25^{0}C for 24 hours. The cells were harvested by centrifugation (2000xg, 30 min, 4^{0}C) and washed three times in phosphate-buffered saline (PBS). The pellets were resuspended in the sterile tap water. 1000ml of sterile tap water was inoculated with the mutants and wild strains respectively and the survival rate were determined from the first day of inoculation to 7 days by incubation at room temperature. Populations were directly enumerated on TSA plates.

Result and discussion
A. hydrophila is a gram negative, oxidase positive, facultatively anaerobic, polarly flagellated, rod shaped bacteria. The disease sign observed in gold fish (*C. auratus*) was similar to the signs caused by bacteria such as Aeromonads and Pseudomonads (Srieszko & Axelrod, 1971). The isolate grows well on Aeromonas Isolation Basal Agar Medium supplemented with ampicillin and suggested that the pathogen in gold fish belongs to *A. hydrophila* based on biochemical characterization.

From the LD_{50} value, the degree of virulence showed *A. hydrophila* induces hemorrhage in more than 50% of the individuals tested (table 1). The virulence dose for *A. hydrophila* was found to be 326 x 10^4 CFU/ml. Lio-po *et al*. (1996) suggested that the localization of *A. hydrophila* to a level of 10^6 CFU/ml in the musculature must be established for dermal lesions to develop, and the virulence dose varies with the portals of entry and types of fish.

Table 1: Percentage mortality in *Carassius auratus* **injected with 0.1ml of** *Aeromonas hydrophyila* **at different dilutions**

S.no	Pathogen Dilution	% Mortality in injected fish
1	10^{-1}	100
2	10^{-2}	100
3	10^{-3}	66.7
4	10^{-4}	50.0
5	10^{-5}	16.67
6	10^{-6}	Nil
7	10^{-7}	Nil

A. hydrophila exhibited significantly wide range of resistance to antibiotics (table 2). The maximum resistance was observed for ampicillin followed by bacitracin, amphotericin and cephalexin. The isolate was moderately sensitive to erythromycin and kanamycin, as well as exhibited susceptibility to neomycin and streptomycin. Similar observations were made by Pathak *et al*. (1993) and Rahim *et al*. (1984) who showed maximum resistance to ampicillin in 50% of *A. hydrophila* from fresh water fishes. Moreover the use of antibiotics led to increased bacterial resistance (Stoffregen *et al*., 1996).

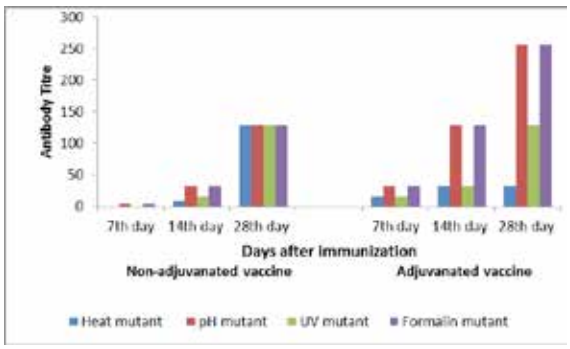
Table 2: Antibiotic test for *Aeromonas hydrophila*

S.no	Antibiotics	Nature
1	Ampicillin	Resistant
2	Amphotericin	Resistant
3	Bacitracin	Resistant
4	Cephalexin	Resistant
5	Neomycin	Sensitive
6	Erythromycin	Moderately sensitive
7	Kanamycin	Moderately sensitive
8	Streptomycin	Sensitive

Under laboratory conditions, gold fish can be effectively vaccinated against *A. hydrophila* (Figure 1). The present study clearly revealed an increase in the antibody titres during the course of immunization and this strongly suggested the protection against infection. The antibody titre of gold fish vaccinated with genetically modified mutants such as UV mutant, pH mutant and formalin mutant significantly increased (128) when compared to heat mutant. These results confirmed earlier findings of Sakai *et al*. (1993). The maximal antibody response was achieved with a single inoculation in the presence of adjuvant, where the peak value was increased (256) with pH and formalin mutants, confirming the effectiveness of Freund's complete adjuvant as described by Estevez *et al*. (1994).

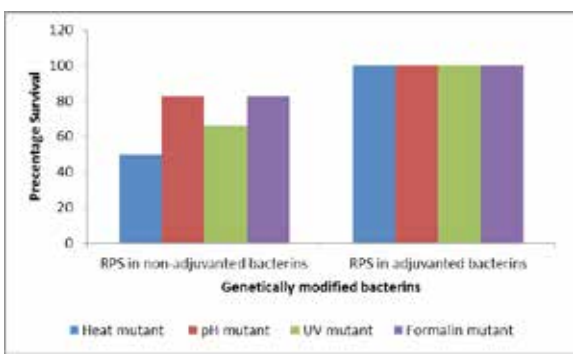
Volume : 3 | Issue : 11 | Nov 2013 | ISSN - 2249-555X

Figure 1: Serum agglutinating antibody titres in gold fish vaccinated with various genetically modified mutant strains of Aeromonas hydrophila (Non-adjuvanted and adjuvanted bacterins)



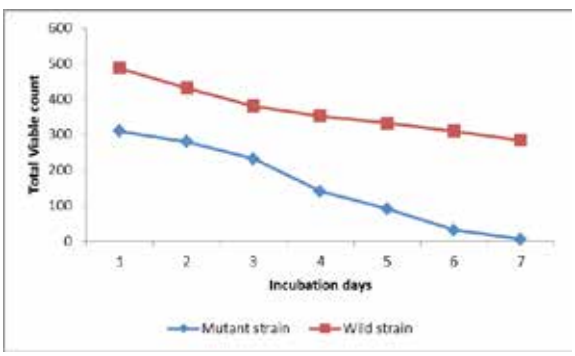
The bacterins were examined for their efficacy using the challenge technique. Challenges were accomplished using an intraperitonial injection of live microorganisms. The vaccinated fish with adjuvanted mutants showed 100% RPS value (figure 2). The mortality was 100% in the unvaccinated controls. The larger differences in mortality between vaccinated and unvaccinated fish provided strong evidence of the protective efficacy of the vaccine against *A. hydrophila*. Eggset *et al*. (1999) suggested that the protection by vaccination was 100% at the 28th week after vaccination, but may decline with time (Eggset *et al*., 1997).

Figure 2: Related percentage survival (RPS) in gold fish vaccinated with non – adjuvanted and adjuvanted bacterins following intraperitoneal challenge at 30th day of immunization



When compared to wild strain the genetically modified ampicillin sensitive mutants showed a rapid decline from the natural environment (figure 3) which agrees with the findings of Vivas *et al*. (2004). From this study, we conclude that the genetically modified live vaccine has lower survival potential in water than wild type strain, and it is of particular interest that the mutant disappeared rapidly from the sterile tap water indicating the disability of these mutants to survive in the natural environment.

Figure 3: Survival rate (TVC) of mutant and wild strains of *Aeromonas hydrophila* at 10^{-4} dilution



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