Immunogenicity of Hemolysin, Protease and Lipopolysaccharide Extracted from *Aeromonas hydrophila* in Common Carp (*Cyprinus carpio* L.)

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Summary

In order to detect the normal antibody response of common carp against *Aeromonas hydrophila*, the aetiological agent of haemorrhagic septicemia in fish farm of Fars province, three different preparations of antigen including hemolysin, protease and lipopolysaccharide were examined. Sixty common carp in 12 groups were selected for 3 routes of administration and 3 antigens; hemolysin 0.5, 5, 4, protease 1, 10, 5 and lipopolysaccha[illegible] 0.2, 2, 2 mg/ml by injection, oral administration and immersion routes respecti[illegible]. Similarly 15 fish in 3 groups were exposed to phosphate buffer saline as contro[illegible] were bled 21 days post-immunization and serum sample from each fish was collected. The fish humoral response was detected using Elisa. Results showed that the lipolysaccharide and hemolysin had the best response by intraperitoneal, immersion and oral administrations. Protease significantly induced the immune response of the fish by intraperitoneal injection when compared with other antigen preparations. A weak immune response of the fish to immersion and oral route of protease antigen may be dose dependent.

Key words: immunogenicity, hemolysin, protease, lipopolysaccharide, *Aeromonas hydrophila,* common carp

Introduction

Motile aeromonad of the *Aeromonas hydrophila* (*A.hydrophila*) complex cause a heamorrhagic septicemia in fish. This bacterium has been observed in numerous

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species of freshwater fish and occasionally in marine fish and in amphibians, reptiles, cattle and humans throughout the world (Bullock *et al* 1971). The bacterium is distributed widely in freshwater and bottom sediments containing organic material as well as in the intestinal tract of fish (Sugita *et al* 1995). *A. hydrophila* is typically recognized as an opportunistic pathogen or secondary invader, however, there have been some reports on acting of *A.hydrophila* as a primary pathogen (Austin & Austin 1999). According to the pathogenicity some strains being highly virulent and others non-virulent. Most cultured and wild freshwater fish are susceptible to the infection and display cutaneous haemorrhages of the fins and trunk and a haemorrhagic septicemia disease.

Fish immunized either intramuscularly or intraperitoneally with vaccine showed protection against challenge. The agglutinin antibody titer increased in the serum of immunized fish (Karunasagar *et al* 1991). Agglutinating antibody was recognized in the serum of carp immunized with *A.hydrophila* bacterine following a second immersion with this vaccine. However, fish vaccinated by immersion or orally showed questionable protection (Lamers *et al* 1985). Catfish immunized intraperitoneally by injection with the acid extract of the S-layer protein of *A.hydrophila* were protected from the homologus virulent strain (Ford & Thune 1992). The purpose of this study was to demonstrate the humoral antibody response of common carp to different *A.hydrophila* antigens.

Materials and Methods

Bacteria. *A.hydrophila* isolated from kidney of affected carp showing a typical haemorrhagic septicemia and confirmed by Dr. Dalsgard (Fish disease reference laboratory, Denmark). The bacterium was sub-cultured on brain heart agar (Merck) several times before preparation of antigens.

Antigen preparation. Protease and hemolysin were prepared from a 48h culture of *A.hydrophila* in tryptone soy broth (TSB, Merck) after centrifugation at 3000*g* for

30min at 4°C. After decantation and evaporation to remove excess acetone, protease was prepared by resuspention of the pellet in small volume of phosphate buffered saline (PBS) pH7.4, dialyzed against the same buffer for several hours. Protein content was determined by method of Lowry (1951). Hemolysin was prepared from the supernatant after filtration through sterile 0.45μm micropore membrane filter and salted out with 35% saturated ammonium sulfate. After centrifugation at 3000*g* for 30min the precipitate was resuspended in 0.05μm Tris-HCL buffer pH8.4, and precipitated again with cold ethanol. It was collected by centrifugation as above, dissolved in Tris-HCL buffer, filtered through Sephadex G-25 (Gray & Kreger 1985), and stored at -20°C until use. Protein content of the solution was determined as above.

Crude lipopolysaccharide (LPS) was isolated by phenol-water extraction procedure according to the method of Westphal & Jann (1965). The antigen was freeze-dried and its dry weight was determined. A total of 65mg LPS was collected.

Immunization procedure. Sixty common carp (mean weight 80g) were transferred and divided in four 65L aquarium continuously filtered and aerated freshwater. Each aquarium was partitioned to hold three parts for keeping five fish. The fish were fed a commercial pellet daily and 10% of the aquarium water was changed every day. The mean water temperature was at 25°C throughout the experiment. They were examined clinically for their health. Each fish was administrated with the prepared antigens or with PBS as control after they were starved for 24h. Immunization schedule of common carp with the prepared *A.hydrophila* antigens in different routs was shown in table 1. The fish were kept for 21 days after immunization.

Rabbit anti-IgM production. Ammonium sulphate purified common carp IgM was prepared and its protein content was measured using Lowry method. Two adult male rabbits were injected intramuscularly by 1ml of 100mg the preparation in 50%w/v Freund's complete adjuvant. Second injection was carried out after 21 days

with the same manner using Freund's incomplete adjuvant. Rabbits were bled on day 28 post-immunization and the sera were collected. Individual as well as pooled sera were stored at -20°C until used.

Table 1. Immunization schedule of common carp with *A. hydrophila* antigens

Aquarium No.	Antigens	Dose	Route of administration
1	Hemolysin	4mg/ml for 30s 5mg 0.5mg/0.25ml	Immersion Oral Injection
2	Protease	5mg/ml for 30s 10mg 1mg/0.25ml	Immersion Oral Injection
3	LPS	2mg/ml for 30s 2mg 0.2mg/0.25ml	Immersion Oral Injection
4	PBS	in PBS for 30s 0.5ml PBS 0.25ml PBS	Immersion Oral Injection

ELISA. Polystyrene 96-well plate (Linbro) was coated with 100µl of the antigens in coating buffer and left overnight at 4°C. Antigens were flicked off and remaining free binding sites were blocked with 100µl of 1% (w/v) gelatin in PBS and 0.05% Tween20 (PBST) for 30min at room temperature (RT) then washed five times in distilled water and 0.05% Tween (DWT). 100µl diluted test sera were added to the wells and the plates were incubated for 90min at RT. Duplicate wells were used for each serum sample. After washing with DWT rabbit anti-carp immunoglobulin diluted in PBS containing 0.5% Tween and 0.1% (w/v) gelatin was added and incubated for another 90min at RT. After washing swine anti-rabbit-HRP (Dako Patts ®) was added. The Plate was washed after 90 min and 100µl of 2,2'-azino-bis (3-ethyl benzen thiazoline-6-suphonic acid) diammonium salt (ABTS) in 100mM citiric phosphate, pH 4.2, 2.5mM hydrogen peroxide was added. The

reaction was stopped after 20min at RT by addition of 50ml 0.015M sodium azide in 0.1M acetic acid and optical density (O.D) was measured at 490nm using an automated microplate reader (Bio-Tek). Pooled positive and negative control sera were included. The positive control serum was diluted by negative serum to yield lightly positive, medium positive, low positive and negative controls.

Using the controls, ELISA O.Ds were corrected by the method of Whittington (1992) and the data were statistically analyzed using one-way ANOVA in Spss-win software.

Results

Antigens protein content. Using Lowry method protein contents of the prepared protease and hemolysin were estimated 32 and 51mg/ml, respectively.

Evaluation of immune response. Serum samples from treated and control groups were analyzed by ELISA for antibodies reactive with protease, hemolysin and LPS. Figure 1 shows the antibody levels in sera from fish administrated with the prepared antigens in three manners. Injection of *A.hydrophila* protease antigen caused a significant elevation of anti-*A.hydrophila* antibodies after 21 days post-immunization (O.D=0.383). A weak immune response induced by other immunization methods in which their O.Ds were significantly lowers ($P<0.05$) than that of the injection route. Hemolysin could induce humoral immune response by different administration routes. There was not a significantly difference ($P<0.05$) among the responses. LPS as an immunogen resembled a significant raise of the specific antibody production by the administration methods after 21 days post-immunization. O.Ds for injection and immersion routes were read 0.427 and 0.330 respectively, which were not significantly different ($P<0.05$). The immunization results of LPS in three different routes, hemolysin in immersion and oral routes, and protease antigen by injection route were not significantly different ($P<0.05$). ,

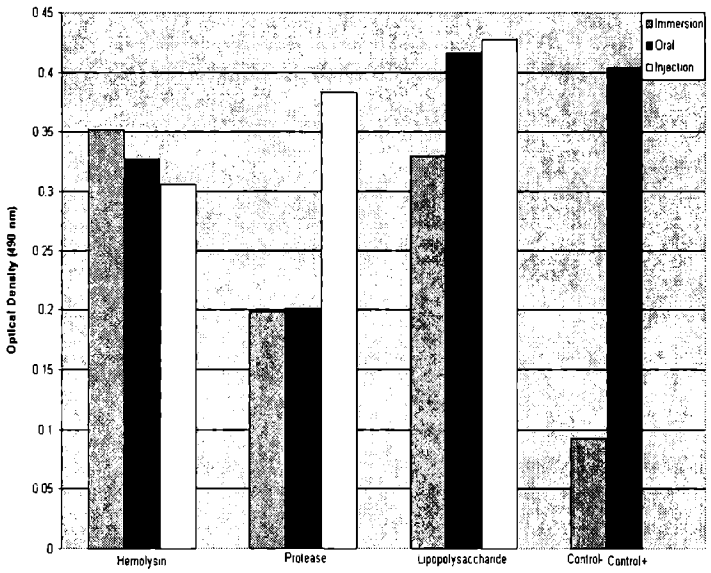

Figure 1. *Humoral immune response of common carp to different A.hydrophila antigens (shown as mean ELISA optical density at 490nm)*

Discussion

During last two decades the interest in protection fish against infectious diseases such as haemorrhagic septicemic disease due to *A.hydrophila* has grown enormously. Initial indications were not optimizing for the development of a vaccine against *A.hydrophila.* Some strains of the bacteria, especially *A.hydrophila* and *A.sobria* are important pathogens of predominantly warmwater fish, including carp (*Cyprinus carpio* L.). The presence of high levels of aeromonads in intensive fish culture, such as the sewage-fed ponds in Hungary and India (Olah *et al* 1986) may present a risk of infection not only for fish, but also for human handlers and consumers. So a preventive strategy for the control of the disease should be designed.

For development of fish vaccination it is important to establish which immunological mechanisms are effective in the defensive process. Results of this

study showed that LPS and hemolysin of *A.hydrophila* have the best immune response both intraperitoneally and orally as well as a raise of antibody level by immersion of fish in hemolysin antigen. LPS induced a significant raise of serum antibody level in both injected and oral route and to some extent in immersion route. Results of the immunogenicity of LPS as antigen was similar to the previous study (Akhlaghi 2000). Vaccination with crude LPS induced better protection against *A.hydrophila* infection in carp than the vaccination with formalin killed vaccine. Dipping fish in vaccine for 2h at 25°C was more effective than intraperitoneal injection of the vaccine in procedural simplicity, lower stress loading and the degree of protection acquired (Baba *et al* 1988).

In spite of LPS, little information is available on the immunogenicity of hemolysin and protease. Boulanger *et al* (1977) isolated two different hemolysins, alpha and beta, which have been implicated in the pathogenesis of infection. Allan and Stvenson (1981) investigated the production of protease and hemolysin in the extracellular product (ECP) of *A.hydrophila* strain and showed a close correlation between the quantity of hemolysin and toxicity to fish. So the selected dose plays an important role to induce the immune response A significant qualitative as well as quantitative difference in the protease components of ECP was produced by *A.hydrophila* and *A.sobria*, which were pathogenic for fish (Nieto & Ellis, 1991). Wakabayashi *et al* (1981) described most of the virulent strains of *A.hydrophila* biovar hydrophila as having a high proteolytic activity.

In this study it has been shown that LPS and hemolysin resulted in a distinct rise in serum antibody when were administrated by the different methods while protease antigen was successful when injected and showed a weak immune response by oral and immersion routes. Low immune response (O.D=0.199) after immersion might be due processing of antigen through gills, skin, gut and alteration in the physicochemical nature of the antigen. The other possibility for the weak immune

response of fish could be a dose dependent effect of the antigen. This needs to be tested by using different doses.

In some warmwater aquaculture operations, after assessment of the environmental conditions, particularly at specific times of the year, vaccine might reduce losses during critical period. A second potential opportunity is the use of vaccine as inexpensive replacement for antibiotics in intensive culture. A vaccine against *A.hydrophila* would be most useful where the options to improve management or environment are limited, when outbreaks are regular occurrences and when antibiotic use is restricted by cost or regulation (Stevenson 1988).

An important finding of this study was the significant immunogencity of the isolated *A. hydrophila* hemolysin in common carp both by immersion and oral routes. A protection trail is proposed to elucidate the protective role of hemolysin in common carp.

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