Humoral Immune Response of Cultured Persian Sturgeon (*Acipenser persicus)* to Four Different *Aeromonas hydrophila* Antigens

Kalbassi, M.R.,*[1] Soltani, M.,[2] Pourbakhsh, S.A.[3] and Adams, A.[4]

1.Faculty of Marine Sciences and Natural Resources, Tarbiat Modarres University, Tehran, Iran

2.Faculty of Veterinary Medicine, Tehran University, Tehran, Iran

3.Razi Vaccine & Serum Research Institute, P.O.Box 11365-1558, Tehran, Iran

4.Institute of Aquaculture, Sterling University, Scotland,U.K.

Received 5 Jun 2000; accepted 1 Aug 2000

Summary

Four antigens consisting of heat killed cells, formalin killed cells, lipopolysaccharides and extracellular products were prepared from *Aeromonas hydrophila*. The immune response of about 3-year-old Persian sturgeon (*Acipenser persicus*) that immunized by intraperitoneally injection of the antigens with or without Freund's adjuvant was evaluated. The immune response to the extracellular products was significantly ($P<0.05$) less than that other antigens. The results indicate that whole cell antigens of *A.hydrophila* represent protective antigens against motile aeromonad septicemia.

***Key words*:** immune response, antigen, *Acipenser persicus*, *Aeromonas hydrophila*

Introduction

There is a little information on protective immunity against infectious diseases in Acipenseridae in contrast to the situation in other groups of fish where there have been numerous works on the nature of vaccines and methods of vaccination. This may be in a part, due to only a few data concerning the infectious diseases of these highly valuable species, in particular, Caspian Sea (Vuillaume *et al* 1986, Brun *et al* 1991, Adkinson *et al* 1998, Hedrick *et al* 1985, Hedrick *et al* 1990, Hedrick *et al* 1991, Scott *et al* 1994, Watson *et al*1995). Based on a national plan of Iranian

Fisheries Department, to protect natural resources and offspring of the Caspian Sea sturgeon, about 20 million fingerlings are produced and released into the sea every year. One of the problems which has been recently identified to cause some mortality in *A.presicus*, is a motile aeromonad septicemia caused by *Aeromonas hydrophila* (Soltani & Rabani 1999). As a part of a continuing investigation on *A.persicus*, this study was evaluated and compared the level of antibody titer of the fish against four *A.hydrophila* antigens consisting of formalin killed cells (FKC), heat killed cells (HKC), lipopolysaccharides (LPS) and extracellular products (ECP).

Materials and Methods

Bacterial strain. *A.hydrophila* lyophilized culture no. AH-04 (Ins. Aquaculture, Uni. Sterling, Scotland) isolated from affected *A.percicus* showing a clinical haemorrahgic septicemia in north of Iran, was used. The bacreium was sub-cultured on tryptone soya agar (TSA; Merck) three times before preparation of antigens.

Antigen preparation. FKC and HKC were prepared from *A.hydrophila* by the procedure described by Baxa 1988. Cells were collected from a 48h tryptone soy broth (TSB, Merck) static culture at 25°C by centrifugation at 5000*g* for 15min and washed three times with sterile phosphate buffer (PBS). HKC was obtained by subjecting the harvested cells to 100°C for 15min. FKC was prepared by adding formalin to cell suspension to make a final concentration of 0.5% v/v. Crude LPS was prepared by hot phenol-water extraction method as described by Westphal and Jann (1964) using ultracentrifugation. Namely, 50gr (wet weight) of cells was suspended in 200ml of water at 68°C, then an equal volume of 90% phenol, which was preheated to 68°C added with vigorous stirring, and the mixture kept for 10min at 68°C. The mixture was cooled to about 10°C in an ice bath and the emulsion centrifuged at 700*g* for 30min. The water phase was taken off by suction and the phenol layer and the insoluble residue were extracted with another 200ml of hot water as described above. The combined water extracts were dialyzed for 3-4 days against distilled water to remove phenol and low molecular weight substances. The solution containing the LPS was concentrated with ultracentrifugation at 80,000*g* for 3h. ECP was prepared by the ammonium sulphate precipitation method as described

by Baxa (1988). A 48h TSB culture was centrifuged at 7000*g* for 30min the supernatant was concentrated by precipitation with ammonium sulfate at 560gr/l, and then allowed to settle for 5h at 4°C. After centrifugation at 12000*g* for 30min the precipitate was suspended in PBS, dialyzed overnight at 5°C against PBS and stored at -80°C until used.

SDS-PAGE analysis of antigens. Protein purity and molecular weight of the antigens were determined by sodium dodecyle sulphate polyacrilamide gel electrophoresis (SDS-PAGE) using a 12.5% (w/v) separating gel and a 5% (w/v) stacking gel, according to the procedure of Laemmli (1970) with low molecular weight standards (Bio Rad). Briefly, a 18h TSA culture at 25°C was harvested in microtubes and well mixed with 100ml of solubolizing buffer and boiled for 10min at 100°C. The samples were then cooled on ice and 20µl of the samples were stacked on 5% (w/v) acrylamide and separated in 12.5% (w/v) acrylamide. After electrophoresis, the gels were stained with Coomassie brilliant blue or Silver stain for 1h and destained with 10% acetic acid, 40% methanol overnight until the gel became clear.

Immunisation protocol. *A.persicus*, of 1000g in weight and 3-year-old, were obtained from Sturgeon Research Institute, Rasht. Six groups each containing four fish were injected intraperitoneally (i.p.) with 0.2ml of FKC (2.1×10^8cells/fish), 0.2ml of HKC (2.1×10^8cells/fish), 0.2ml of LPS (20µg/fish) and 0.25ml of ECP (20µg/fish) containing equivalent volume of Freund's completed adjuvant (CFA). Fish were received booster antigens using the same amount of the above antigens with or without incomplete Freund's adjuvant (IFA), at 4-5 and 8-9 weeks post-immunization. Control groups were injected using 0.2ml of sterile PBS. Positive antiserum was obtained using repeatedly injected fish with HKC, as hyperimmune fish. Fish were maintained in 1000L fiberglass tanks containing fresh water at 10-24°C that was changed daily and fed with artificial sturgeon pelet three times per day. The water temperature was measured daily up to six month and correlation between water temperature and the level of antibody production in fish was determined. Blood was collected every other week up to six months post-immunization by obtaining 5ml of blood from caudal vein of anesthetised fish, left at room temperature for one hour,

and antisera were separated following centrifugation at 4000rpm for 6min. Samples were stored at –20°C until used.

Rabbit. Four adult male albino rabbits (Razi Vaccine & Serum Research Ins., Karaj) were intramusculary immunized using the same amount of FKC, HKC, LPS and EPC antigens containing Freund's adjuvant, as mentioned for fish. Negative control was injected using 0.5ml of PBS and positive control was hyperimmunized with several injection of 0.1ml of HKC every other day. Blood was collected from ear marginal vein, on 1,3,5,7,9 and 11 weeks post-immunization and antisera were collected as described for the fish.

Antisera titration. The titration of fish and rabbit antisera were performed using a standard microtechnique for bacterial agglutination and haemaglutination test (Roberson 1990) using HKC (2.5×10^8 HKC/final concentration) in 96-well microtiter plates (BIO RAD). Red blood cells of goat, rabbit, chicken and sheep were used for HA test. The students *t*-test was used to compare means of antibody titers ($P<0.05$).

Results

The molecular weights of whole cell antigens consisting of HKC and FKC were identical and about116kD, while that of LPS was 52kD. The ECP antigen showed a multi bands with a molecular weight about 45-80kD (Figure1).

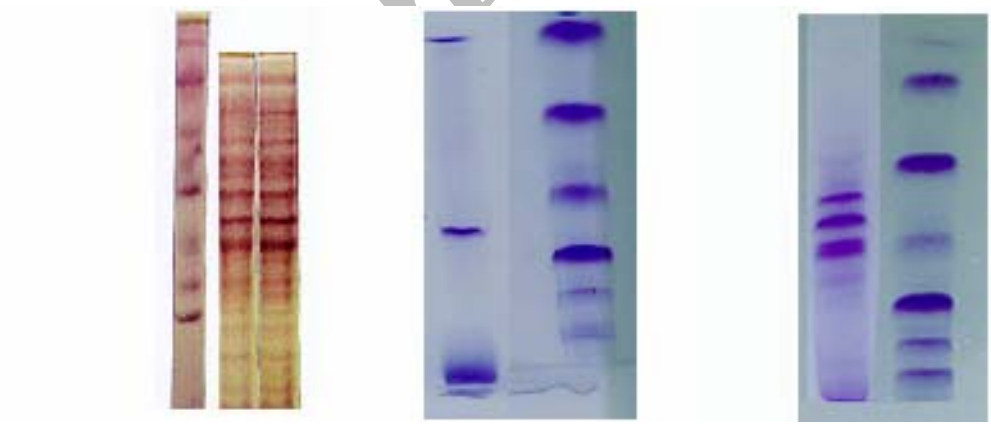

Figure1. *SDS-PAGE analysis of A.hydrophila antigens, A=whole cell, B=LPS, C=ECP, m=standard marker (BIO RAD)*

Results of mean antibody titration in fish against *A.hydrophila* antigens with or without adjuvant are shown in Figures 2 and 3.

Figure 2. *Antibody level in A persicus against 4 different A.hydrophila antigens with adjuvant.*
FKC+adj; HKC+adj; LPS+adj; ECP+adj; C

Figure 3. *Antibody level in A.persicus against 4 different A.hydrophila antigens without adjuvant*
FKC+adj; HKC+adj; LPS+adj; ECP+adj; C

Maximum antibody titer of 1:2560, 1:1920, 1:1280, 1:960 were obtained against HKC, FKC, LPS, ECP antigens with adjuvant in 6, 12, 10 and 16 weeks post-immunization, respectively. Maximum antibody titer of 1:1280, 1:640, 1:640 and 1:480 were obtained against HKC, FKC, LPS and ECP antigens without adjuvant in 10, 8, 14 and 14 weeks post-immunization, respectively.

Resutls of antibody level for rabbit is shown in Figure 4. Response of rabbit to 4 antigens with adjuvant resulted in maximum level of antibody against FKC and HKC (1:640) in 1 and 3 weeks post-immunization while maximum titers of 1:160 and 1:80 were found against ECP and LPS one week post-immunization, respectively.

Figure 4. *Antibody level in rabbit against 4 different A.hydrophila antigens with adjuvant FKC+adj; HKC+adj; LPS+adj; ECP+adj*

Discussion

Mean antibody titer in fish to *A.hydrophila* antigens containing adjuvant was the highest than that fish were immunized using the antigen without adjuvant. Therefore, as many other species of fish, the use of adjuvant resulted in more antibody titration indicating a positive effect of such immunostimulatores in this species. Generally, the progress of *A.persicus* immune response to all *A.hydrophila* antigens shows that the

antibody production by rabbit initiated sooner than fish, but its level was lower. However, future studies involved western blot analysis of the serum to identify which antigens are recognized by fish in comparison the rabbit. To do this it need to produce anti-sturgeon monoclonal antibody. This part of work is under progress with the collaboration of Institute of Aquaculture at Sterling.

Comparison of immunogenicity of HKC, FKC, LPS and ECP in both fish and rabbit showed that whole cell antigens were more immunogenic than those of LPS and ECP antigens. Lamers & de Hass (1983) and Lamers & Van Muiswinkel (1986) found that preparation of *A.hydrophila* antigens using HKC has more immunogenic effect than that of FKC in Carp (*Cyprinus carpio*). In contrast, Loghothetis & Austin (1994) observed better results using FKC than HKC in rainbow trout (*O.mykiss*). One possibility is that the preparation of *A.hydrophila* antigens using FKC may change the antigenic structure of the bacterium to provide antigen presentation for fish macrophages as suggested by Lamers & Van Muiswinkel (1986). Such availability of antigen may not be possible when using heat-killed cells. In this study higher level of antibody was obtained for HKC antigen than FKC. Moreover, similar immunogenicity effects of LPS and ECP antigens found in this study indicating the more effect of LPS and ECP on the cellular immunity than antibody production as shown by Baba *et al* (1988), Imamura & Izawak (1988a). However, the results of this study apparently differ from those of other investigators e.g. Baba *et al* (1988b). This may be attributed to various factors such as fish species, antigen preparation and dose, temperature and degree of stress in fish.

The water temperature during initial immunization stage was about 24°C and reduced to 10°C six months later, according to seasonal changes. The humoral immune response of fish was correlated to the water temperature. Therefore, it seems that a temperature up to 24°C would be a suitable condition for immunization of *A.persicus*.

Although further studies are under progress to assess the antibody levels using an indirect ELISA, so far results obtained here confirm that *A.persicus* is a suitable immunogen species to *A.hydrophila* antigens. However, as heterogenicity variations between *A.hydrophila* strains, a suitable vaccine needed to be firstly aware about

such variations and secondly, to assess the efficacy of vaccines in the *in vivo*. The later part of study is also in progress using HKC as a dip and LPS and ECP as an injection vaccination routes.

Acknowledgement

This study was supported by Tehran University, Tarbiat Modarres University, International Sturgeon Research Center, Razi Vaccine & Serum Research Institute.

References

Adkinson, M.A., Cambre, M. and Hedrick, R.P. (1988). Identification of an iridovirus in Russia sturgeon *Acipenser guldenstadti* from northern Europe. *Bulletin of Europe .Association .Fish Pathology* 18:29-32.

Baba, T., Imamura, J. Izawak. (1988). Immune protection in carp (*Cyprinus carpio*) after immunization with *Aeromonas hydrophila* crude lipopolysaccharide. *Journal of Fish Diseases* 11:237-244.

Baxa, D.V. (1988). In vitro and in vivo activities of flexibacter matrimos toxins. *Report of Usamar Biology Inst. Kochi University* 10:1-8.

Brun, R., Nougayrede, P., Chen, P., Vuillaume, A. and Crespeau, F. (1991). Bilan sanitare des D'Elevage D' Acipenser baeri En pisicultures intensives.Williot P.Ed. Pp:429-437.

Hedrick, R.P., Speas, J., Kent, M.L. and McDowell, T.(19850. Adeno-like virus associated with a disease of cultured white sturgeon *A.transmontanus*. *Canadian Journal of Fisheries Aquatic Science* 42:1321-1326.

Hedrick, R.P., Groff, J., McDowell,T. and Wingfield, W. (1990). An iridovirus infection of the integument of the white sturgeon *A.transmontanus*. *Diseases of Aquatic Organisms* 8:39-44.

Hedrick, R.P., Groff, J., McDowell T. and Wingfield, W. (1991). Isolation of an epitheliotropic herpesvirus from white sturgeon *A.transmontanus. Diseases of Aquatic Organisms* 11:49-56.

Laemlli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lamers, C.H.J., Van Muiswinkel, W.B. (1986). Natural and aquaired agglutination to *Aeromonas hydrophila* in carp (*C.carpio*). *Canadian Journal of Fisheries Aquatic Science* 43:619-624.

Lamers, C.H.J., De hass, M.J. (1983). The development of immunological memory in carp to a bacterial antigen. *Developmental &Comparative Immunology* 7:713-714.

Loghothetis, P.N., Austine, B. (1994). Immune response of rainbow trout (*Oncorhynchus mykiss)* to *Aeromonas hydrophila*. *Fish & Shellfish Immunology* 4:235-254.

Roberson, B.S. (1990). Technological applications of immunochemicals. Biotechnology by open learning. Pp:69-76. B.H. Press.

Scott, L., Groff, J., Jones, G., Munn, B., Paterson, T., Holt, R., Hauck, A. and Hedrick, R. (19940. Occurance of white sturgeon iridovirus infections among cultured white sturgeon in the Pacific Northwest. *Aquaculture* 126:201-210.

Vuillaume, A., Brun, R. Chene, P., Sochon, E. and Lesel, R. (1986). First isolation of *Yersinia rackeri* from sturgeon *Acipenser baeri* ,in south west of France. *EAFP* 7:18-21.

Watson, I.R., Yun, S.C., Groff, J. and Hedrick, R.P. (1995). Characteristics and pathogenicity of a novel herpesvirus isolated from adult and sub-adult white sturgeon *A.transmontanus Diseases of Aquatic Organisms* 22:199-210.

Westphal, O., Jann, k. (1964). Bacterial lipopolysaccharides.in:Methodes. In: *Carbohydrate chemistry*. vol.5, Pp:83-91. Academic Press, NY.