The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (http://www.aquaculturehub.org) members and registered individuals and institutions. Please visit our website (http://siamb.org.il) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB),
University of Hawaii at Manoa Library
and
University of Hawaii Aquaculture Program in association with
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

IMMUNOGENICITY OF *AEROMONAS SALMONICIDA* A-PROTEIN IN GOLDFISH (*CARASSIUS AURATUS* L.)

M. Sinyakov[1], M. Dror[1], S. Margel[2] and R.R. Avtalion[1]*

[1] *Faculty of Life Sciences, Bar Ilan University, Ramat Gan 52900, Israel*

[2] *Department of Chemistry, Bar Ilan University, Ramat Gan 52900, Israel*

(Received 14.10.00, Accepted 8.11.01)

Key words: *Aeromonas salmonicida,* A-layer protein immunogenicity, goldfish

Abstract

Antigenicity of the surface A-layer protein of an atypical *Aeromonas salmonicida* strain was tested in goldfish following immunization with various A-protein (AP) antigenic preparations (soluble form, alum-precipitated, conjugated with polyacrolein nanoparticles, or bacterin). ELISA revealed innate specific anti-AP antibody activity in all fish on day 0, while no significant antibody enhancement was seen on days 21 or 42 following the primary immunization. A second stimulation with soluble AP resulted in a clear response in groups primed with bacterin and alum-precipitated AP but no response in those primed with soluble AP or AP-nanoparticles conjugate. The implication of these results for vaccine design is discussed.

Introduction

A wide range of pathogens is associated with fish diseases. Among the bacteria, *Aeromonas salmonicida*, the causal agent of furunculosis, is one of the oldest known. Due to its economically devastating impact on cultivated fish, particularly salmonids, it is considered one of the most important among fish pathogens (Austin and Austin, 1993). There are a number of bacterial isolates belonging to the species *A. salmonicida* which have distinct biochemical characteristics that are unlike those reported for typical *A. salmonicida* subsp*. salmonicida*. These isolates are considered atypical strains and known to be the causal agents of the cutaneous ulcerative disease of goldfish (Mawdesley-Thomas, 1969) or "atypical furunculosis" in other species. The rising number of severe disease outbreaks associated with

* Corresponding author. Laboratory of Fish Immunology, email: avtalr@mail.biu.ac.il

atypical strains, in both freshwater and marine fish, was the subject of several reports (reviewed in Wiklund and Dalsgaard, 1998).

So far, there is no clear-cut information regarding constituent(s) responsible for the pathogenicity of the atypical strains, and lack of such knowledge prevents design of efficient vaccines. Extracellular A-layer protein (AP) of atypical and typical strains has been suggested to be one of the major virulent factors of *A. salmonicida* (Kay et al., 1981). The aim of the present work was to evaluate the immunogenicity of several soluble and particulate formulations of this antigen prepared from an atypical *A. salmonicida* strain that was isolated in our laboratory from cutaneous ulcers of diseased goldfish (unpublished data).

Materials and Methods

Bacterial strain and growth conditions. An atypical strain (F I 2.1) of *A. salmonicida,* isolated in our laboratory, was used. This strain is virulent, A-protein producing and, according to the majority of biochemical criteria (Popoff, 1984), could be classified as belonging to the subspecies *A. masoucida.* Bacteria were routinely grown at 18ºC for 48 h in complex culture media BHI broth (Difco) supplemented with bovine hemin (Sigma). Cells were harvested by centrifugation (4,100 x g for 15 min at ambient temperature) and washed twice thereafter in normal saline.

A-protein antigenic preparations - bacterin. A bacterial suspension, prepared in a 48 h culture, was washed twice with saline by centrifugation (4,100 x g for 15 min at 4°C). The resulting pellet was suspended in saline to a concentration of $2x10^9$ bacterial cells/ml. Formalin (Bio Lab Ltd., Israel) was added to a final concentration of 0.5% (v/v), and the suspension was incubated at 37°C for 24 h.

A-protein (AP). A soluble monomeric form of the *A. salmonicida* extracellular AP was isolated from whole bacteria cells using the low pH extraction method of McCoy et al. (1975) as modified by Phipps and Kay (1988). The resulting AP acidic extract (pH 2.3) was collected, neutralized and stored at –20°C until used. The purity of the isolated AP was verified by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

A-protein polyacrolein nanoparticles conjugate (AP-paNP). Monodispersed polyacrolein nanoparticles with an average diameter of 200 nm were prepared by γ-irradiation of acrolein in an aqueous solution in the presence of SDS as a surfactant as described by Margel (1984). Covalent binding of the AP isolated from the *A. salmonicida* strain used in this work was performed as detailed by Margel (1989). The concentration of the immobilized AP was estimated by the Lowry assay as modified by Markwell et al. (1978).

A-protein alum precipitate (AP-alum). Two ml of the AP solution (2 mg/ml) were precipitated by dropwise addition of 2 ml aluminum potassium sulfate solution (Sigma; 4 mg/ml) accompanied by gentle stirring and simultaneous neutralization of the formed suspension to pH 7.2 with 0.1 N NaOH. The final precipitate was collected by centrifugation (1,500 x g, 15 min) and resuspended in 4 ml saline.

Goldfish. Six-month goldfish were purchased from a goldfish farm (Gan Shmuel, Israel) and divided into groups of ten fish. Each fish was immunized intraperitoneally (i.p.) with 100 µg AP (usually in a volume of 0.1 ml) of one of the above antigenic preparations. A non-immunized control group of 15 fish was i.p. injected with the same volume of saline. Six weeks after priming, all fish except those in the control group, received a second i.p. injection of 50 µg AP in its soluble form. The fish were bled at three-week intervals, i.e., on days 0, 21, 42, and 63. The resulting sera were kept at –20°C until checked for antibody activity using the enzyme-linked immunosorbent assay (ELISA) performed as reported by Zhong et al. (1999).

Results

OD_{405} levels of specific antibodies increased after both primary and secondary stimulations (Fig. 1). A basal level (0.52±0.05 OD units) of 'pre-existing' anti-A-protein antibodies was detected in all non-immunized control fish. Twenty-one days after priming, the antibody activity level was almost the same in all test groups (0.61±0.05, 0.56±0.05, 0.58±0.07 and 0.58±0.06 in fish injected with AP, AP-alum, AP-paNP and bacterin, respectively). A distinct

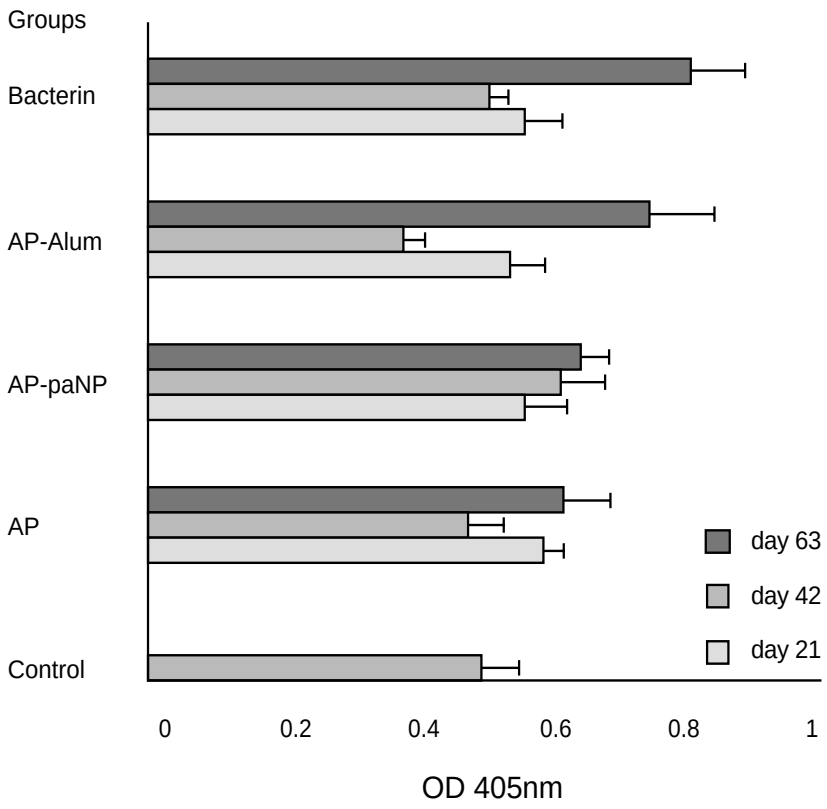

Fig. 1. A-protein antibody response in goldfish 21 and 42 days after immunization with one of several antigenic preparations of A-protein and 21 days (Day 63) after a second immunization with soluble AP. Columns represent means and standard errors in groups of 10-15 fish compared to the non-immunized control.

decrease in antibody activity on day 42 was observed in most of the groups; the level was significantly different ($p<0.032$) in the AP-alum group. Antibody activity on day 63 (21 days after the second injection) revealed a clear response in fish injected with bacterin ($p<0.0067$) and AP-alum ($p<0.014$) but not in fish primed with AP and AP-paNP.

Discussion

The above data reveal: (a) the occurrence of pre-existing (i.e., natural) anti-A-protein antibodies in non-immunized fish, (b) the unresponsiveness of the fish to active immunization with different immunizing forms of A-protein, and (c) evidence of immunological memory following a second stimulation in the AP-alum and bacterin groups.

The pre-existence of specific antibodies against the *A. salmonicida* A-protein in asymptomatic fish populations was reported for Atlantic salmon (*Salmo salar*) by Stromsheim et al. (1994). Smith (1940) suggested that natural antibodies are the result of previous contact of the fish with the pathogen. Our data seem to agree with this suggestion, mainly because most Israeli goldfish hatcheries, including the one from which our fish were obtained, are contaminated with *A. salmonicida*. The failure of fish in all treatments to

respond to primary stimulation by enhancing their level of antibodies suggests that the fish may not have acquired specific memory following their original contact with the antigen. The failure to develop specific memory may be related to the peculiar structure of A-layer protein since (a) normal fish macrophages are unable to process A-layer positive virulent strains of *A. salmonicida* (Olivier et al., 1986; Graham et al., 1988), and (b) macrophages are involved in antigen processing and development of T-helper memory cells (Avtalion, 1981).

AP-alum and bacterin stimuli, but not soluble AP and AP-paNP conjugate, evidently induced immune memory after the second stimulus with soluble AP which, by itself or when conjugated to nanoparticles, was incapable of inducing a distinct primary response. Nanoparticles were used in the present study because they were shown to highly enhance bovine serum albumin immunogenicity in mice when coupled with this protein (data not shown).

The design of improved bacterial vaccines remains an urgent need for effective control of furunculosis in salmonids and goldfish ulcerative disease (reviewed by Austin, 1997). Our work is primarily concerned with assessment of the immunogenicity of the extracellular A-layer protein, which was reported to be directly involved in *A. salmonicida* pathogenicity (Kay et al., 1981). Our results indicate the efficiency of isolated soluble AP as a secondary stimulus but not as a primary, and that efficient priming should be performed with a modified form of the AP antigen. Chemical modification of the AP antigen, or its conjugation to appropriate specific memory-cell stimulating nanoparticles, might be useful in preparing a potent vaccine.

References

Austin B., 1997. Progress in understanding the fish pathogen *Aeromonas salmonicida*. *Trends Biotechnol*., 15:131-134.

Austin B. and D.A. Austin, 1993. Aeromonadaceae representatives (*Aeromonas salmonicida*). pp. 86-170. In: B. Austin and D.A. Austin (eds.). *Bacterial Fish Pathogens Disease in Farmed and Wild Fish*, 2nd ed. Ellis Horwood Limited, Chichester.

Avtalion R.R., 1981. Environmental control of the immune response in fish. *CRC Crit. Rev. Environ. Control*, 11:163-188.

Graham S., Jeffries A.H. and C.J. Secombes, 1988. A novel assay to detect macrophage bactericidal activity in fish: factors influencing the killing of *Aeromonas salmonicida*. *J. Fish Dis*., 11:389-396.

Kay W.W., Buckley J.T., Ishiguro E.E., Phipps B.M., Monette J.P.L. and T.J. Trust, 1981. Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. *J. Bacteriol*., 147: 1077-1084.

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

Margel S., 1984. Characterization and chemistry of polyaldehyde microspheres. *J. Polymer. Sci., Chem. Ed*., 22:3521-3533.

Margel S., 1989. Affinity separation with polyaldehyde microsphere beads. *J. Chromatogr*., 462:177-189.

Markwell M.A.K., Haas S.M., Bieber L.L. and N.E. Tolbert, 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem*., 87:206-210.

Mawdesley-Thomas L.E., 1969. Furunculosis in the goldfish *Carassius auratus* (L.). *J. Fish. Biol*., 1:19-23.

McCoy E.C., Doyle D., Burda K., Corbeil L.B. and A.J. Winter, 1975. Superficial antigens of *Campylobacter* (*Vibrio*) *fetus:* characterization of an antiphagocytic component. *Infect. Immun*., 11:517-525.

Olivier G., Eaton C.A. and N. Campbell, 1986. Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). *Vet. Immunol. Immunopathol*., 12:223-234.

Phipps B.M. and W. Kay, 1988. Immunoglobulin binding by the regular surface array of *Aeromonas salmonicida*. *J. Biol. Chem*., 263:9298-9303.

Popoff M., 1984. Genus III. *Aeromonas* Kluyver and Van Niel 1936. pp. 545-548. In: N.R. Krieg and J.G. Holt (eds.). *Bergey's*

Manual of Systematic Bacteriology, Vol. 1. Williams & Wilkins, Baltimore.

Smith W.W., 1940. Production of anti-bacterial agglutinins by carp and trout at 10°C. *Proc. Soc. Exp. Biol. Med*., 45:726-729.

Stromsheim A., Eide D.M., Fjalestad K.T., Larsen H.J.S. and K.H. Roed, 1994. Genetic variation in the humoral immune response in Atlantic salmon (*Salmo salar*) against *Aeromonas salmonicida* A-layer. *Vet. Immunol. Immunopathol*., 41:341-352.

Wiklund T. and I. Dalsgaard, 1998. Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid fish species: a review. *Dis. Aquat. Org*., 32:49-69.

Zhong M.-C., Mor A. and R.R. Avtalion, 1999. One-step procedure for the purification of goldfish (*Carrasius auratus*) and carp (*Cyprinus carpio,* L.) serum immunoglobulin by precipitation with 9% polyethylene glycol 6000. *Israeli J. Aquacult. - Bamidgeh*, 51:3-9.