BARD

FINAL REPORT

PROJECT NO. IS-2727-96

***Streptococcus iniae* Infection in Trout and Tilapia: Host-Pathogen Interactions, the Immune Response Toward the Pathogen and Vaccine Formulation**

A. Eldar, D.L. Evans

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BARD Final Scientific Report
Cover Page

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Project Title: *Streptococcus iniae* Infection in Trout and Tilapia: Host-Pathogen Interactions, the Immune Response Towards the Pathogen and vaccine Formulation.

Investigators

Principal Investigator (PI):

Dr. Avi Eldar

Co-Principal Investigator (Co-PI):

Dr. Donald L. Evans

Collaborating Investigators:

Institutions

Fish Disease Laboratory
Dept. of Poultry and Fish Diseases
Kimron Vet. Inst., Bet-Dagan, Israel
E-mail: aelda@vs.moag.gov.il

Dept. of Medical Microbiology
College of Veterinary Medicine
Athens, GA, USA
E-mail: devans@calc.vet.uga.edu

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Cellular Cytotoxicity; Streptococci; Gram positive Infections; Antibody Responses;
Adhesion; Vaccines; Lymphocyte Stimulation

Abbreviations commonly used in the report, in alphabetical order:

CPS: capsular material; GC/MS: Gas chromatography/mass spectrophotometry;
NCC: nonspecific cytotoxic cells; RAPD: randomly amplified polymorphic DNA
S. iniae: *Streptococcus iniae*

Budget: IS: \$ 138,600

US: \$ 179,460

Total: \$ 318,060

ELDAR AVI D.V.M.
Lic. No. 726

B.A. Yakobson, D.V.M.
Director
Kimron Veterinary Institute

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

BARD Final Scientific Report Cover Page

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	3	3	3	9
Submitted, in review, in preparation				
Invited review papers				
Book chapters				
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Master theses				
Ph.D. theses				
Abstracts		10		10
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Description Cooperation:

Research was fostered by exchange of reagents and the submission of co-authored manuscripts. Techniques were learned and shared and research information was exchanged. Specifically, Dr. Eldar sent Dr. Evans many isolates of *S. iniae*; Dr. Evans then provided monoclonal antibody to Dr. Eldar such that it was then possible to begin studies on the phenotype of NCC in trout tissue. This allowed a competitive renewal to be completed,

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract

In Israel and in the U.S., *Streptococcus iniae* is responsible for considerable losses in various fish species. Poor understanding of its virulence factors and limited know-how-to of vaccine formulation and administration are the main reasons for the limited efficacy of vaccines. Our strategy was that in order to improve control measures, both aspects should be equally addressed. Our proposal included the following objectives: (i) construction of host-pathogen interaction models; (ii) characterization of virulence factors and immunodominant antigens, with assessment of their relative importance in terms of protection and (iii) genetic identification of virulence factors and genes, with evaluation of the protective effect of recombinant proteins.

We have shown that two different serotypes are involved. Their capsular polysaccharides (CPS) were characterized, and proved to play an important role in immune evasion and in other consequences of the infection. This is an innovative finding in fish bacteriology and resembles what, in other fields, has become apparent in the recent years: *S. iniae* alters surface antigens. By so doing, the pathogen escapes immune destruction. Immunological assays (agar-gel immunodiffusion and antibody titers) confirmed that only limited cross recognition between the two types occurs and, that capsular polysaccharides are immunodominant. Vaccination with purified CPS (as an acellular vaccine) results in protection.

In vitro and *ex-vivo* models have allowed us to unravel additional insights of the host-pathogen interactions. *S. iniae* 173 (type II) produced DNA fragmentation of TMB-8 cells characteristic of cellular necrosis; the same isolate also prevented the development of apoptosis in NCC. This was determined by finding reduced expression of phosphatidylserine (PS) on the outer membrane leaflet of NCC. NCC treated with this isolate had very high levels of cellular necrosis compared to all other isolates. This cellular pathology was confirmed by observing reduced DNA laddering in these same treated cells. Transmission EM also showed characteristic necrotic cellular changes in treated cells. To determine if the (*in vitro*) PCD/apoptosis protective effects of #173 correlated with any *in vivo* activity, tilapia were injected IV with #173 and #164 (an Israeli type I strain). Following injection, purified NCC were tested (*in vitro*) for cytotoxicity against HL-60 target cells. Four significant observations were made: (i) fish injected with #173 had 100-400% increased cytotoxicity compared to #164 (ii) *in vivo* activation occurred within 5 minutes of injection; (iii) activation occurred only within the peripheral blood compartment; and (iv) the isolate that protected NCC from apoptosis *in vitro* caused *in vivo* activation of cytotoxicity. *The levels of in vivo cytotoxicity responses are associated with certain pathogens (pathogen associated molecular patterns/PAMP) and with the tissue of origin of NCC. NCC from different tissue (i.e. PBL, anterior kidney, spleen) exist in different states of differentiation.*

Random amplified polymorphic DNA (RAPD) analysis revealed the "adaptation" of the bacterium to the vaccinated environment, suggesting a "Darwinian-like" evolution of any bacterium. Due to the selective pressure which has occurred in the vaccinated environment, type II strains, able to evade the protective response elicited by the vaccine, have evolved from type I strains. The increased virulence through the appropriation of a novel antigenic composition conforms with pathogenic mechanisms described for other streptococci.

Vaccine efficacy was improved: water-in-oil formulations were found effective in inducing protection that lasted for a period of (at least) 6 months. Protection was evaluated by functional tests - the protective effect, and immunological parameters - elicitation of T- and B-cells proliferation. Vaccinated fish were found to be resistant to the disease for (at least) six months; protection was accompanied by activation of the cellular and the humoral branches.

Major achievements: scientific and agricultural impacts.

1. Development of PCR. For a fast and reliable identification of *S. iniae*, a polymerase chain reaction (PCR) was developed. It is based on the amplification of a specific *S. iniae* sequence using species-specific primers; the resulting specific band is then visualized. The procedure is of 4-5h, allowing screening of a high number of samples with accurate and reproducible results. *The availability of a PCR-based diagnosis greatly improves the reliability of detection and shortens laboratory times without influencing economic expenses.*

***S. iniae* infections in marine species.** Pure colonies *S. iniae* were isolated from diseased marine fish (gilthead sea breams and European sea bass) cultured in a mariculture farm and from wild fish living in the proximity of the farm. This is the first report of *S. iniae* infection in wild populations and in gilthead sea breams and European sea bass, revealing the wide range of potential hosts. Molecular epidemiology suggests that all fish have acquired the infection from the same source. Considering the chronology of the outspread, wild spine-foots were found to be natural forewarns of the disease. *Wild fish, although playing a negative role by disseminating the disease, might be helpful to limit the severity of the event.*

Evolution of *S. iniae* infections in freshwater species. In 1996 reports of new massive outbreaks of *S. iniae* were recorded in trout farms where the entire population was vaccinated against the pathogen. Biochemical schemes revealed that all isolates differed from previous isolates by being ADH negative. New isolates were identified as *S. iniae* type II strains. More details are given later in this report.

Cellular pathology of NCC resulting from *S. iniae* infection. In order to identify virulence factors associated with pathogenic mechanisms of *S. iniae*, several isolates were examined for *in vitro* effects on purified nonspecific cytotoxic cells (NCC). Type II strain (#173) prevented the development of apoptosis in NCC (reduced expression of PS). Treated NCC had very high levels of cellular necrosis compared to all other isolates. This cellular pathology was confirmed by observing reduced DNA laddering in these same treated cells. Transmission EM also showed characteristic necrotic cellular changes in treated cells. *Select S. iniae isolates may augment innate immunity*

by enhancing inflammation, by increasing antigen processing and enhancing cellular infiltration.

To determine if the (*in vitro*) PCD/apoptosis protective effects of #173 correlated with *in vivo* activity, tilapia were injected IV with #173 and #164 (type I strain), and purified NCC were tested (*in vitro*) for cytotoxicity against HL-60 target cells. The significant observations were made : (i) fish injected with #173 had 100-400% increased cytotoxicity compared to #164; (ii) *in vivo* activation, only within the peripheral blood, occurred within 5 minutes of injection; and (iii) the isolate that protected NCC from apoptosis *in vitro* caused *in vivo* activation of cytotoxicity. *The levels of in vivo cytotoxicity responses are associated with certain pathogens (pathogen associated molecular patterns/PAMP) and with the tissue of origin of NCC. NCC from different tissue (i.e. PBL, anterior kidney, spleen) exist in different states of differentiation.*

Construction of host-pathogen interaction models. The tilapia TMB-8 was used to determine the *in vitro* effects of *S. iniae* on cellular pathology (cellular necrosis vs. cellular apoptosis following treatment with killed *S. iniae*. Israeli isolate #173 (serotype II strain) produced DNA fragmentation of TMB-8 cells characteristic of cellular necrosis. This effect was concentration dependent and it occurred after both 4 and 8h treatment with *S. iniae*. *These data confirmed similar experiments examining the in vitro effects of S. iniae on NCC. Certain isolates of S. iniae initiate necrosis of NCC and TMB-8 cells*

Capsular polysaccharides and their immunological role. CPS from "old" (i.e. - before new outbreaks of 1996) and "recent" isolates were characterized. "Old" strains possess a two-component capsule (Inositol and Sorbitol), while "new" strains (e.g. 173) have a more complicated capsular structure, formed of three sugars (Mannitol, Iditol and Sorbitol). The "old" and "recent" isolates are therefore, of two different serotypes. *These data highlight the variability of capsular composition among various S. iniae strains.* Sera against *S. iniae* Dan-1 precipitated only the homologous group-antigen (C-antigen) and that of other type I strains, indicating a complete identity of the C-antigen composition. Similarly, sera raised against *S. iniae* KFP 173 showed a complete identity between all type II strains. But while sera against But while type I antisera failed to recognize type II strains, sera raised against type II

recognized partially type I strains, indicating an incomplete identity of C-Ag. *Therefore, it is concluded that S. iniae polysaccharides are subject to variations, with consequent failure of vaccination program.* We then vaccinated fish with purified CPS. Fish were protected against the disease, indicating that *CPS are not only virulence factors, but also immunodominant.*

Molecular analysis of the two serogroups. Random amplified polymorphic DNA (RAPD) of type II strains produced a 750bp band that was absent in the PCR product of type I strains. Furthermore, while type I strains produced two close bands of roughly 1000bp, strain 173 (the first isolate from diseased vaccinated fish) was the only type II strain to give gave similar bands, demonstrating the evolution of the pathogen. A selective pressure towards more virulent strains has probably resulted in the overcome of *S. iniae* type II strains. *These finding highlight the importance of continuous surveillance which must accompany vaccination.* Contrary to eradication schemes, vaccination does not eliminate the pathogen but reduces mortality while the pathogen still remains in the environment and in some of the animals. *The "adaptation" of the bacterium to the vaccinated environment, is accompanied by increased virulence through the appropriation of a novel antigenic composition.*

Optimization of vaccine. Water-in-oil (W/O) emulsions repeatedly resulted in specific stimulation of the immune response and a straight correlation between the specific immune response and the protective effect was shown.

Potential for application. The PCR detection method which we has been developed in this project is widely used. As a result the availability of the PCR-based diagnosis, bacteriological confirmation of the presence of *S. iniae* is shortened considerably. For prevention of the disease, the serological data generated in this project are being utilized. As a result, bacterin composition has been updated and vaccination is fruitful. Virulence models, improved vaccine formulation and immunological parameters make control of *S. iniae* disease, and other fish diseases more feasible. Eventually, eradication of the infection can be contemplated, particularly when vaccination is combined with refined detection tests that can detect and differentiate the vaccine response to various strains.

LIST OF PUBLICATIONS

1. Zlotkin, A., H. Hershko and, Eldar, A. 1998. Possible transmission of *Streptococcus iniae* to cultured fish. Appl. Env. Microbiol. 64:4065-4067. ✓
2. Eldar, A. and C. Ghittino. 1999. *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases. Dis. Aq. Org. 36:227-231. ✓
3. Eldar, A., Perl, S., Frelie, P. F. and, Bercovier, H. 1999. Red drum (*Sciaenops ocellatus*) mortalities associated with *Streptococcus iniae* infection. Dis. Aq. Org. 36:121-127. ✓
4. Taylor, L. S., Jaso-Friedmann, L., Allison, A. B., Eldar, A., and Evans, D. L. 1999. *Streptococcus iniae* inhibition of apoptosis of nonspecific cytotoxic cells: a mechanism of activation of innate immunity in teleosts. Curr. Microbiology (suggested corrections made and resubmitted). ✓
5. Jaso-Friedmann, L., and Evans, D. L. 1999. Mechanisms of cellular cytotoxic innate resistance in tilapia (*Oreochromis nilotica*). Dev. Comp. Immunol., 23:27-35. ✓
6. Evans, D. L. and Jaso-Friedmann, L. 1999. Nonspecific cytotoxic cells and innate cellular immunity in teleost fish. Recent Advances in Marine Biotechnology (vol. 5). ✓
7. Evans, D. L., Taylor, S. L., Leary, J. H. III, Bishop, G. R., Eldar, A., and Jaso-Friedmann, L. 2000. In vivo activation of tilapia nonspecific cytotoxic cells by *Streptococcus iniae* and amplification with apoptosis regulatory factors (s). Fish and Shellfish Immunol. 10:419-434. ✓
8. Jaso-Friedmann, L., Ruiz, J., Bishop, G. R. and Evans, D. L. 2000. Immunoregulation of innate immunity in tilapia: activation of nonspecific cytotoxic cells by cytokine-like factors. Dev. Comp. Immunol. 24:25-36. ✓
9. Bachrach, G., Zlotkin, A., Hurvitz, A., Evans, D. L., and Eldar, A. 2000. Natural selection of *Streptococcus iniae* virulent strains. App. Env. Microbiol. (suggested corrections made and resubmitted). ✓

APPENDIX

List of publications

1. Zlotkin, A., H. Hershko and, Eldar, A. 1998. Possible transmission of *Streptococcus iniae* to cultured fish. Appl. Env. Microbiol. **64**:4065-4067.
2. Eldar, A. and C. Ghittino. 1999. *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases. Dis. Aq. Org. **36**:227-231.
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6. Evans, D. L. and Jaso-Friedmann, L. 1999. Nonspecific cytotoxic cells and innate cellular immunity in teleost fish. Recent Advances in Marine Biotechnology (vol. 5).
7. Evans, D. L., Taylor, S. L., Leary, J. H. III, Bishop, G. R., Eldar, A., and Jaso-Friedmann, L. 2000. In vivo activation of tilapia nonspecific cytotoxic cells by *Streptococcus iniae* and amplification with apoptosis regulatory factors (s). Fish and Shellfish Immunol. **10**:419-434.
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9. Bachrach, G., Zlotkin, A., Hurvitz, A., Evans, D. L., and Eldar, A. 2000. Natural selection of *Streptococcus iniae* virulent strains. App. Env. Microbiol. (suggested corrections made and resubmitted).

Published Abstracts

1. Taylor, S. L., Jaso-Friedmann, L., and Evans, D. L. Mechanisms of pathogenesis of *Streptococcus iniae* in tilapia nonspecific cytotoxic cells: programmed cell death versus necrosis. 2nd Ann. Mtg. Southern Conf. on Researchers in Aquatic Diseases. Baton Rouge, LA. March 22-24, 1998.
2. Evans, D. L. and Jaso-Friedmann, L. Significance of the cell mediated immune response in fish. First bilateral symposium of "Aquaculture and Fish Health 1998." Russian Academy of Sciences and the Russian Federal Committee on Fisheries and the United States Department of the Interior. Moscow, Russia, July 11-20, 1998.
3. Taylor, S. L., Jaso-Friedmann, L., and Evans, D. L. Programmed cell death in tilapia nonspecific cytotoxic cells produced by *Streptococcus iniae*. Southeastern and South Central Branches, ASM, Gulf Shores, AL., Oct. 29-31, 1998.
4. Jaso-Friedmann, L., Ruiz, J., Bishop, G. R. and Evans, D. L. Immunoregulation of innate immunity in tilapia: activation of nonspecific cytotoxic cells by cytokine-like factors. 24th Ann. Eastern Fish Health Workshop, Atlantic Beach, NC., Mar. 8-11, 1999.
5. Bishop, G. R., Jaso-Friedmann, L. and Evans, D. L. Protection of target cell induced apoptosis in nonspecific cytotoxic cells by serum factors. 24th Ann. Eastern Fish Health Workshop, Atlantic Beach, NC., Mar. 8-11, 1999.
6. Evans, D. L., Taylor, S. L., Bishop, G. R. and Jaso-Friedmann, L. Mechanisms of anti-bacterial immunity in tilapia: role of nonspecific cytotoxic cells. 24th Ann. Eastern Fish Health Workshop, Atlantic Beach, NC., Mar. 8-11, 1999.
7. Taylor, S. L., L. Jaso-Friedmann and D. L. Evans. Nonspecific cytotoxic cell activation and immunoregulation produced by *Streptococcus iniae*. 3rd Annual Mtg. Southern Conference of Researchers in Aquatic Diseases (Animal Disease Research Workers in Southern States, Mississippi State University, Biloxi, MS., 21-23 March, 1999.
8. Ruiz, J., G. R. Bishop, D. L. Evans and L. Jaso-Friedmann. Immunoregulation of innate immunity in tilapia (*Oreochromis niloticus*): activation of nonspecific cytotoxic cells by cytokine-like factors. 3rd Annual Mtg. Southern Conference of Researchers in Aquatic Diseases, Biloxi, MS., 21-23 March, 1999.
9. Jaso-Friedmann, L., Ruiz, J., Bishop, G. R., Taylor, S. L. and Evans, D. L. Regulation of innate immunity: cytokine-like factors in the serum of stressed tilapia

activate nonspecific cytotoxic cells.”, European Association of Fish Pathologists (EAFP) Ninth International Conference on Diseases of Fish and Shellfish, Rhodes, Greece, 19-24 September, 1999.

10. Evans, D. L., Bishop, G. R., Ruiz, J., Leary, J. H. III and Jaso-Friedmann, L. Regulation of innate immunity in tilapia: possible mechanisms of activation of nonspecific cytotoxic cells by apoptosis protection factors. EAFP Ninth International Conference on Diseases of Fish and Shellfish, Rhodes, Greece, September 19-24, 1999.

Unpublished data: The immune response to *S. iniae* water-in-oil emulsion.

Unpublished data: Virulence factors and immunodominant antigens - capsular composition of type I and type II strains.