77

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**Pathogenic Streptococcus in Tilapia: Rapid
Diagnostic, Epidemiology and Pathophysiology**

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TABLE OF CONTENTS

	Page
ABSTRACT.	3
OBJECTIVES OF THE RESEARCH.	4
BODY OF THE REPORT	4
1. Phenotypic and genetic characterization of pathogenic cocci.	4
1.1. Phenotypic and genetic characterization of <i>S. iniae</i> .	4
1.2. Phenotypic and genetic characterization of <i>L. garvieae</i> .	7
1.3. Genetic characterization of <i>S. difficile/agalactiae</i> .	11
2. Restriction fragment length polymorphisms of pathogenic cocci.	12
2. 1. RFLP of 16S rDNA and ribotyping of <i>S. iniae</i> strains from the United States and Israel.	12
2.2. <i>L. garvieae</i> RFLP.	19
3. PCR assays	23
3.1. Species-specific PCR detection of <i>S. iniae</i> .	23
3.2. Field study using PCR detection of <i>S. iniae</i> in tilapia.	24
3.3. <i>L. garvieae</i> PCR assay.	26
4. Experimental pathogenesis.	28
4.1. Materials and Methods	28
4.2. Results and Discussion	30
5. Protective antigens and Specific DNA probes.	35
EVALUATION OF THE RESEARCH ACHIEVEMENTS.	38
COOPERATION	39
CONCLUSIONS	40
LIST OF PUBLICATIONS	41

ABSTRACT

Within the project "Pathogenic Streptococcus in Tilapia", gram positive cocci pathogens of fish in Israel and in the United States were characterized. We showed that *Streptococcus shiloi*, the name for an agent causing septicemic infection in fish, is a junior synonym of *Streptococcus iniae* and that *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningo-encephalitis in fish. Molecular epidemiology studies on these two pathogens, based on 16S rDNA sequences and ribotyping showed that although each country had specific clones, *S. iniae* originated probably from the U.S. and *L. garvieae* from Japan. PCR assays were developed for both pathogens and applied to clinical samples. *S. agalactiae*/ *S. difficile* was also recognized for the first time in the U.S. in tilapia. Our histopathological studies explained the noted paradox (abundant *in vitro* growth often accompanied by scant to small numbers of organisms within the meninges in histologic sections of brain) in diagnostic of fish streptococcus. The greatest concentration of cocci were consistently observed within macrophages infiltrating the extrameningeal fibroadipose tissue surrounding the brain within the calvarium. These results also suggest that the primary route of meningeal infection may be extension from the extrameningeal connective tissue rather than meningeal vascular emigration of cocci-containing macrophages.

Our work has resulted in a cognizance of streptococcus as fish pathogen which goes beyond the pathology observed in tilapia and is already extended to many aquaculture fish species in Israel and in the United States. Finally, our data suggest that vaccines (bivalent or trivalent) could be developed to prevent most of the damages caused by streptococcus in aquaculture.

OBJECTIVES OF THE RESEARCH.

The specific objectives of this research were :

1. To characterize phenotypically and genetically strains of pathogenic cocci (*Streptococcus* and *Lactococcus*) in Israel and in the US. The study of *L. garvieae* was added during this project when an epizootic of *L. garvieae* in Europe threatened to propagate itself in Israel.
2. To identify and compare the 16S ribosomal DNA sequences of pathogenic cocci isolated from tilapia in the US and Israel. For epidemiological reasons (shared water basin in Israel), strains of *S. iniae* and *L. garvieae* isolated from trout were added to the original objectives of this project.
3. To develop a specific 16S rDNA probe to the cocci associated with disease in tilapia. PCR assays were developed for both *S. iniae* and *L. garvieae*.
4. To study the epidemiology of pathogenic cocci (distribution in fish ponds, carrier state of fish) by PCR based on the 16S rDNA sequences.
5. To utilize a specific oligonucleotide DNA probe for in situ hybridization to augment a pathogenesis study of streptococcosis in tilapia. This objective was abandoned after it proved unsuccessful and unnecessary.
6. To chronologically characterize the histologic and ultrastructural changes of the meningoencephalitis in experimental streptococcosis.
7. To identify specific protein antigen which could be the basis of an acellular vaccine. This objective was not attained because of a lack of time due to research time spent on *L. garvieae* studies.

BODY OF THE REPORT.

(METHODOLOGY, RESULTS and DISCUSSION)

1. Phenotypic and genetic characterization of pathogenic cocci.

1.1. Phenotypic and genetic characterization of *S. iniae*.

The data (materials and methods, results and discussion) on *S. iniae* are presented in the following published paper:

Eldar A., P.F. Frelief, L. Assenta, P.W. Varner, S. Lawhon, H. Bercovier. 1995. *Streptococcus shiloi*, the name for an agent causing septicemic infection in fish, is a junior synonym of *Streptococcus iniae*. Int. J. Syst. Bacteriol. 45: 840-842.

Genetic analysis of the 16S rDNA subunit from a representative bacterial isolate from Texas tilapia with meningoencephalitis conclusively demonstrates that the streptococcal species most commonly isolated from Texas tilapia is *S. iniae*. This study represents an initial attempt to compare the ability of species-specific PCR, routine *in vitro* bacterial isolation, and histopathology to specifically detect *S. iniae* in farmed hybrid tilapia. Our recent isolation of a streptococcus which were characterized as *S. difficile* could represent a first isolation of this agent from tilapia in the United States.

5. To utilize a specific oligonucleotide DNA probe for in situ hybridization to augment a pathogenesis study of streptococcosis in tilapia.

This objective was abandoned after it proved unsuccessful and unnecessary.

6. To chronologically characterize the histologic and ultrastructural changes of the meningoencephalitis in experimental streptococcosis.

Our histopathological studies explained the noted paradox (abundant *in vitro* growth often accompanied by scant to small numbers of organisms within the meninges in histologic sections of brain) in diagnostic of fish streptococcus. The greatest concentration of cocci were consistently observed within macrophages infiltrating the extrameningeal fibroadipose tissue surrounding the brain within the calvarium. Our study also suggest that the primary route of meningeal infection may be extension from the extrameningeal connective tissue rather than meningeal vascular emigration of cocci-containing macrophages.

7. To identify specific protein antigen which could be the basis of an acellular vaccine.

This objective was not attained because of a lack of time due to research time spent on *L. garvieae* studies.

DESCRIPTION OF THE COOPERATION

During this project two Veterinarians were able to finalize their Ph.D. studies, Dr. A. Eldar at the Hebrew University and Dr. B. R. Berridge at TAMU. The exchange of strains and data made these two theses complementary and increased the scientific productivity of this project.

Dr. H. Bercovier spend a week at TAMU before starting this grant to plan with Dr. Frelie the work of the first year and to present the latest data on Streptococcosis in Fish. TAMU has sent to HU Texan strains and HU has provided reference strains and DNA to TAMU during the second year of the grant Dr. H. Bercovier visited TAMU and presented data on *S. iniae* and *S. difficile* which allowed the final characterization of *S. difficile* as *S. agalactiae*. Data on *L. garvieae* were also presented. PCR studies were planed during this stay. Methods of DNA extraction from clinical samples for the PCR assays were transferred from Israel to the US team. The Israeli team shared its experience on pathophysiology with the TAMU group especially for methods to the maintain virulence of field isolates (in vivo passages). The collaboration between the two teams resulted in an awareness on cocci pathogen for fish in the US which has been enhanced by the fact

that both *S. iniae* and *L. garvieae* are newly recognized zoonotic agents, is expressed in singular and common articles published.

CONCLUSIONS

This project has contributed to the field of aquaculture by adding new basic data in taxonomy, epidemiology and pathophysiology of gram positive cocci pathogens of fish.

The sudden temporal and geographical emergence of *S. iniae* infections in fish has been a cause of alarm to Israeli and American fish farmers. Taxonomic and epidemiological studies of *S. iniae* strains isolated from infected fish have been based on the study of phenotypic characteristics and on the genetic relatedness of *S. iniae* to other fish Gram positive cocci pathogens, including streptococci, lactococci, vagococci and enterococci. These methods could not differentiate the strains isolated in the US and in Israel, therefore it was necessary to use molecular typing to discriminate between strains of various origins. *S. iniae* Israeli strains except one strain (Dan-1) could be differentiated by ribotyping based on *EcoRI* digests of DNAs from American strains including the *S. iniae* ATCC 29178^T strain isolated in the United States from a freshwater dolphin in 1970. The only Israeli strain similar to the American strains was isolated in 1989 from a trout and not from a tilapia. Since tilapia fry are exported from Israel to the United States on a regular basis, one would expect if *S. iniae* was contaminating these fry that the ribotypes found in both countries would be similar all the time. The Israeli tilapia strains have a ribotype different from that of the American tilapia strains. The possibility that import of Israeli tilapia fry to the United States concomitantly brought *S. iniae* is therefore unlikely. The data on ribotypes of American tilapia strains establish tools to determine the origin of future outbreaks in the United States.

Streptococcus iniae is not the only Gram-positive coccus that can produce clinical illness and histologic lesions in farmed fish. Both *S. difficile* and *Lactococcus garvieae* (*Enterococcus seriolicida*) would appear morphologically similar in histologic sections and produce similar lesions. Species-specific PCR assays were developed and provide a definitive identification of suspect organisms. This study represents an initial attempt to compare the ability of species-specific PCR, routine *in vitro* bacterial isolation, and histopathology to specifically detect *S. iniae* in farmed hybrid tilapia. Genetic analysis of the 16S rDNA subunit from a representative bacterial isolate from Texas tilapia with meningoencephalitis conclusively demonstrates that the streptococcal species most commonly isolated from Texas tilapia is *S. iniae*. A few general observations have been made that might help better define future efforts in defining a rapid, economical, and reliable method of identifying *S. iniae* as an etiologic agent in clinically ill fish, as a potential food fish contaminant, or in clinically normal fish before their introduction into an uninfected production system. The first isolation in tilapia in the United States by the US team, with the help of the Israeli team, of a streptococcus which was characterized as *S. difficile* show the impact of this project. *S. difficile* was further characterized and showed to be identical to *S. agalactiae*, another zoonotic agent. Further study is required to determine the significance of this agent in causing disease in U.S. tilapia.

The PCR assays that we have developed are convenient and simple techniques that can be applied not only to bacterial diagnostic but also to surveillance of Gram positive cocci infections in cultured fish ponds.

The knowledge on the natural history of the infection due to *S. iniae* in tilapia has been clarified during this project. Abundant, morphologically-intact and dividing bacterial cocci within macrophages of the choroid rete and extrameningeal connective tissue coupled with observations made in the macrophage trafficking studies strongly support the role of macrophages in dissemination of *S. iniae* in infected fish. Intracellular dissemination appears to be facilitated by the ability of *S. iniae* to survive and even replicate within macrophage phagosomes and phagolysosomes.

Our work has resulted in a cognizance of streptococcus as fish pathogen which goes beyond the pathology observed in tilapia (both *S. iniae* and *L. garvieae* are newly recognized zoonotic agents) and is already extended to many aquaculture fish species in Israel and in the United States. Finally, our data suggest that vaccines (bivalent or trivalent) could be developed to prevent most of the damages caused by streptococcus in aquaculture.

LIST OF PUBLICATIONS

- Eldar A., **P.F. Frelrier**, L. Assenta, P.W. Varner, S. Lawhon, **H. Bercovier**. 1995. *Streptococcus shiloi*, the name for an agent causing septicemic infection in fish, is a junior synonym of *Streptococcus iniae*. Int. J. Syst. Bacteriol. **45**: 840-842.
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