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Pathobiology of Streptococcus Iniae Infections in Cultured Tilapia.

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PATHOBIOLOGY OF *STREPTOCOCCUS INIAE* INFECTIONS IN CULTURED TILAPIA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences
through the Department of Pathobiological Sciences

by

Alvin C. Camus
D.V.M., Louisiana State University, 1984
August 2001

DEDICATION

In loving memory of my father, Alvin C. Camus, Sr.,
who desired for his son the education he was denied and to my baby daughter,
Caroline Elisabeth, may she have clean earth to till, clear water to drink and
appreciation for the creatures that inhabit both

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ABSTRACT

Streptococcus iniae was first isolated in 1972 from an Amazon River dolphin. It reappeared as a fish pathogen in Japan several years later. Since then it has steadily increased in incidence and host range worldwide, and is a known zoonotic agent. In the United States it is the most significant pathogen affecting growth of tilapia aquaculture. Histopathologic descriptions are incomplete and little is known concerning pathogenesis of the disease or virulence mechanisms of the organism.

A composite of lesions was compiled from diseased tilapia evaluated by the Aquatic Animal Disease Diagnostic Laboratory, Louisiana State University from 1995-2001. In common with certain other streptococci, *S. iniae* causes polyserositis and meningoencephalitis. Previously unreported findings include otitis interna, gastroenteritis, and subcutaneous abscesses. Cocci-laden macrophages traverse olfactory, lateral line, and gastrointestinal epithelia, indicating possible routes of shedding. Gonadal infection suggests possible horizontal and vertical transmission.

Streptococcus iniae isolates of fish and human origin were evaluated for virulence factors identified in other streptococci. No M-like protein was identified using PCR primers designed to amplify entire M protein genes or conserved anchor regions of M-like proteins. Findings were supported by electron microscopy, which revealed a capsule, rather than M protein. Lysine, with ruthenium red, proved superior to glutaraldehyde fixation alone, indicating a capsule composed of acid-polysaccharides. Using a hyaluronidase plate assay and DNase test agar, 79% and 52% of isolates showed activity for the two enzymes, respectively. Streptokinase-like activity was evaluated using a casein overlay containing human plasminogen and a

broth assay using human and tilapia plasma. *Streptococcus iniae* showed no activity with human plasminogen. In tilapia plasma, results were equivocal, delaying but not completely inhibiting clot formation.

The effectiveness of transposon mutagenesis using Tn917 was demonstrated in the production of non-hemolytic mutants. Transposition frequencies, plasmid loss, and frequency of mutant phenotypes were consistent with findings in other streptococci. Single primer PCR, yielded DNA sequence with a high degree of identity to segments of the *S. pyogenes sag* operon, encoding streptolysin S, an important cytolytic virulence factor in that organism. Unexpectedly, in a competitive elimination trial, the non-hemolytic mutant showed greater persistence than the wild-type strain.

INTRODUCTION

The April 2001 edition of National Geographic Magazine included a photograph of a 3500 year old vase in the form of a fish that was recently discovered in an Egyptian tomb along the Nile River. The fish possessed a deep perciform body, long dorsal fin and thick lips, all features that clearly distinguish it as a tilapia. This ancient piece of pottery helps to illustrate the long association that this group of fish has shared with humans, or perhaps more precisely with the human diet. Native to Africa, approximately 100 species of these robust fish have been identified and at least 22 species have been examined in production scale aquaculture experiments, beginning in Kenya over 75 years ago (Jhingran and Gopalakrishan 1974; Huet 1986; Avault 1996).

Tilapia possess a number of traits that make them appear ideally suited for purposes of aquaculture, including hardiness, ease of reproduction in captivity, tolerance of poor water quality conditions, efficient utilization of natural feedstuffs, acceptance of artificial diets, rapid growth in a variety of culture systems, and resistance to stress and disease (Hickling 1960; Lim 1989). They have been grown in almost every type of culture system conceivable, from subsistence level pond culture in Africa, to integrated aquaculture systems in the Philippines, to state of the art indoor recirculating systems in the United States (Pillay 1990; Watanabe 1991; Avault 1996). So called "green water" systems utilizing *Chlorella* algae for denitrification and supplemental oxygen production are popular in Louisiana (Martin 1997).

From a global standpoint, tilapia are second only to carp in the number of total pounds produced (Lim 1989). Sixteen million pounds of tilapia were grown in the Unites States in 1996, while another 60 million pounds were imported to meet domestic

consumer demands for whole fish and processed fillets (Plante 1997). Despite the apparent potential for growth of the industry in this country, competition with foreign markets for lower priced imports has had a significant impact on profitability of the domestic product. Higher operating costs and the inability to control disease in intensive culture systems are cited as major factors affecting competition with imported products (Plante 1997). As in other forms of animal agriculture, increased production under increasingly intensive culture conditions has resulted in an increased incidence of disease. Concomitant with the growth of the American tilapia industry has been the emergence and rapid spread of a previously little known fish pathogen, *Streptococcus iniae* (Perera et al. 1994). The American Tilapia Association now recognizes *S. iniae* as the most important pathogen affecting growth of the tilapia industry in the United States (Bowser et al. 1998).

Streptococcosis was originally described in rainbow trout *Oncorhynchus mykiss* farmed in Japan (Hoshina et al. 1958). Since that time, members of the lactic acid producing bacteria have received increased attention from fish health specialists, due to the increasing incidence of disease caused by pathogenic members of the group in both wild and cultured fish (Austin and Austin 1993). Streptococci are obligate parasites of cutaneous and mucosal surfaces of humans and animals. Some are considered resident flora, causing infection only when introduced into normally sterile sites or in immunocompromised hosts. Other species are true pathogens, which spread between individuals, and cause infections in normal non-immune hosts (Kilian 1998).

Streptococcus iniae was first isolated and described from subcutaneous abscesses in a captive Amazon freshwater dolphin *Inia geoffrensis* in 1976. From 1977 to 1980,

and possibly earlier, large-scale epizootics caused by β-hemolytic streptococci with biochemical profiles compatible with *S. iniae* occurred in tilapia *Tilapia nilotica*, rainbow trout, ayu *Pleyoglossus altivelis*, and amago *Oncorhynchus rhodurus* in Japan (Minami et al. 1976; Kitao et al. 1981; Ohnishi and Jo 1981; Ugajin 1981). Since 1994, *S. iniae* has emerged as the major bacterial pathogen of cultured tilapia (Perera et al. 1994; Eldar et al. 1994) and has steadily increased in incidence and host range worldwide. It is now prevalent throughout the Mediterranean, Middle East, Southeast Asia, and most recently Australia. (Nakatsugawa 1983; Kaige et al. 1984; Foo et al. 1985; Al-Harbi 1994; Stoffregen et al. 1996; Zlotkin et al 1998; Bromage et al. 1999; Eldar et al. 1999; Yuasa et al. 1999). There have also been cases of invasive disease in humans, limited primarily to cellulitis of the hand, following skin injuries incurred while handling fish (Weinstein et al. 1997).

As a β-hemolytic streptococcus, *S. iniae* is unusual in that in does not react with any recognized Lancefield grouping antigens (Pier and Madin 1976). Analysis of 16S rRNA sequences indicate that *S. iniae* belongs to a group of organisms designated as the pyogenic group, including representatives of Lancefield groups A (*S. pyogenes*), group B (*S. agalactiae*), group C (*S. equi, S. dysgalactiae*), group G, groups L and M (*S. canis*), groups E, P, U, and V (*S. porcinus*), and several other nongroupable species including *S. uberis, S. parauberis, S. intestinalis*, and *S. phocae* (Kawamura et al. 1995). All of these bacteria cause suppurative infections either in humans, animals, or both.

Mortalities attributed to *S. iniae* in naïve populations are often described as large scale (Kitao et al. 1981) or massive (Al-Harbi 1994). Eldar et al. (1995b) determined a mortality rate of 30% in tilapia and 50% in rainbow trout. Epizootics in barramundi

have produced mortalities as high as 70% during warm summer months (Bromage et al. 1999). During an outbreak, diseased fish appear darkened and often swim abnormally, many times in a whirling or spiraling fashion (Perera et al. 1994; Eldar et al. 1994). In common with certain other streptococci, *S. iniae* has a tropism for serous membranes and the central nervous system. Microscopically, severe epicarditis, peritonitis, and meningoencephalitis are common findings. Involvement of the inner ear probably accounts for the commonly reported vestibular signs. With the exception of the spleen, and to a lesser extent the head kidney, the parenchymal organs are largely spared. Additional lesions sometimes encountered include the formation of large subcutaneous abscesses, particularly in the caudal peduncle region.

Control of *S. iniae* infections in the United States have been complicated by the fact that there are no antibiotics currently approved by the Food and Drug Administration to treat bacterial diseases in tilapia. When permission has been granted to use Terramycin® or amoxicillin, administration has typically curtailed mortalities during the treatment regimen, but ultimately has failed to eliminate the organism (Stoffregen et al. 1996; Bercovier et al. 1997). Mortalities have begun to rise again once the drug has been withdrawn. Over time, some level of herd immunity appears to develop and mortalities assume a low level or even cease, being exacerbated only during periods of stress. Several killed bacterins have been tested experimentally with limited success (Eldar et al. 1997; Klesius et al. 1999). All suffer from a requirement for parenteral injection and lack of long lasting immunity.

Despite the plethora of case reports relating clinical signs and gross lesions in susceptible fish species, histopathologic descriptions of the disease are limited. Much

remains to be understood concerning the epidemiology and pathogenesis of infections and virtually nothing is known regarding virulence factors utilized by *S. iniae* in the production of disease. Faced with ineffective treatment and preventive measures, unlocking the molecular mechanisms required by the pathogen for invasion, spread, and survival in its fish host may ultimately provide the most effective means of control.

The goal of this dissertation was four-fold. The first chapter begins with a brief introduction to the biology and culture of tilapia. The current literature relating to *S. iniae* is summarized and the chapter ends with an overview of selected streptococcal virulence mechanisms. Chapter II provides a detailed histopathologic description of the disease in tilapia, which provides insight into several aspects of the malady relating to clinical signs, lesion development, and how the organism may be shed into the environment. Using other pyogenic streptococci, primarily *S. pyogenes*, as models, Chapter III examines several potential virulence factors in *S. iniae*, including M protein, capsule production, hyaluronidase, DNase, and streptokinase. The fourth chapter examines the utility of transposon mutagenesis using a temperature conditional delivery vector, pTV1-OK, and Tn917 as a means of creating attenuated strains of *S. iniae*. The chapter concludes by detailing the production of two non-hemolytic insertional mutants. DNA sequence analysis indicates the hemolysin of *S. iniae* to be closely related to streptolysin S of *S. pyogenes*, which is considered to be an important virulence factor in that organism, toxic to a variety of cell types (Betschel et al. 1998; Ginsburg 1999; Nizet et al. 2000).

CHAPTER I. LITERATURE REVIEW

Tilapia

Taxonomy and Natural History

Tilapia, once considered a poor man's protein, have found their way onto upscale restaurant menus under the guise of catchy names like "Saint Peter's fish". This is due to the association of these fish with the Sea of Galilee and the biblical account of Christ's feeding the multitudes following the Sermon on the Mount. The history of these robust perch-like fish and their importance to man, however, is far more ancient, dating to 4500 year old Egyptian tomb paintings, which illustrate the harvest of tilapia from the Nile River (Bardach et al. 1972).

Approximately 100 species of tilapia are found naturally in rivers, lakes, and estuarine areas of Africa (Avault 1996) and at least 22 species have been examined in production scale aquaculture experiments (Jhingran and Gopalakrishnan 1974). All species are recognizable by their deep perciform body conformation, long dorsal fin, and thick lips. Sexual dimorphism exists, with males possessing a pointed urogenital papilla, located caudal to the anus, with a single small terminal orifice. Females have a broader papilla with two openings, a terminal urinary pore and a larger genital pore, which appears as a line perpendicular to the long axis of the body (Huet 1986).

The taxonomy of the tilapine fishes has been traditionally based on their feeding habits and breeding behaviors, but no single classification scheme is universally accepted (Trewavas 1982; Fishelson and Yaron 1983). In the simplest scheme, members of the genus *Tilapia* are recognized as macrophagous substrate spawners. The genus *Oreochromis* was established to include microphagous species that spawn in nests

hollowed from bottom substrates, but incubate their eggs in the female's mouth (Lim 1989). Pillay, however, notes that feeding habits, in both herbivores and omnivores, are highly variable and should not be considered a vital taxonomic feature. Due to the confusion that exists, many authors prefer to group all species under the single genus Tilapia.

General Characteristics and Culture Considerations

Tilapia possess a number of traits that make them appear ideally suited for aquaculture. They are recognized for their hardiness, ease of reproduction in captivity, tolerance of poor water quality conditions, efficient utilization of natural feedstuffs and acceptance of artificial diets, rapid growth in a variety of culture systems, and resistance to disease and handling stress (Hickling 1960; Lim 1989). While low in fecundity, about 2000 eggs per spawn, the mouthbrooding characteristic of most of the cultured species provides for high survivability of fry, making the tilapia a highly prolific group of fish. Additionally, some species are capable of reproducing at 2 to 3 months of age, and up to 5 to 6 times per year, under favorable temperature conditions (Lim 1989; Avault 1996). In the initial attempts at subsistence level pond culture this perceived advantage rapidly led to problems of overpopulation and stunting, which diminished interest in early development of tilapia aquaculture (Pillay 1990).

To overcome the problem of overpopulation and due to the more rapid growth of males, most modern culture facilities utilize mono-sex, all-male culture. Manual sexing is the simplest method used to obtain only males, but it is laborious, requires skill, and is only 80-90% accurate (Hickling 1960; Bardach et al. 1972). In developed countries, a high percentage of males can be obtained through the sex reversal of genotypic females

achieved by feeding methyl- or ethynyltestosterone (Guerrero 1982; Pillay 1990). Dissolving the androgens in ethanol and spraying them onto feed at a dose of 10-60 mg/kg of feed, and feeding at a rate of 10-12% body weight for 18-60 days produces 95-100% males (Guerrero 1982). Development of interspecific hybrids is another means of producing all male progeny and limiting uncontrolled spawning, but this method suffers from problems of low fertility and difficulties maintaining pure brood stocks. (Hickling 1960). Additional methods for restricting overpopulation include utilizing high stocking densities, which lower reproductive rates, employing continuous harvesting, and using predatory fish species to prey on young tilapia (Popper and Lichatowich 1975). The most promising advance in the area of all male culture has been the development of reliable technology for generating greater than 97% mono-sex "YY" males, in *O. niloticus*. These YY genotypic males sire only XY male progeny, which have experimentally produced increased weight gains of 20-30% over androgen sex reversed populations (Roderick 1977).

The tilapias are tropical species whose survival, feeding responses, and reproductive activity are strongly influenced by environmental temperatures. Optimal temperatures for growth and reproduction are in the range of 23-30°C, depending on the species (Pillay 1990). Feeding responses and activity levels decline below 20°C and feeding ceases around 16°C (Avault and Shell 1968; Lim 1989). Death typically ensues at temperatures of 7-12°C (Avault and Shell 1968).

Tilapia are noted for their tolerance of poor water quality conditions and can survive dissolved oxygen concentrations as low as 0.1 mg/L. They are reported to grow over a pH range of 5-11 and tolerate an unionized ammonia level of 2.4 mg/L (Lim

1989). While considered freshwater fish, the tilapia are euryhaline and several species will thrive in full strength seawater up to 40 mg/ml (ppt) (Stickney 1986; Watanabe 1991), particularly when the transition from fresh to saltwater is made gradually. Slow acclimation allows Na^+-K^+-ATPase production to rise to compensatory levels, preventing potentially lethal ion fluxes (Dange 1985). *Tilapia mossambica* reportedly is capable of spawning at salinities up to 49 mg/ml (ppt) (Popper and Lichatowich 1975) and surviving at salinities of 60 mg/ml (ppt) (Dange 1985).

Lim (1989) has compiled an excellent review of feeding practices for tilapia. Tilapia readily digest protein from fish meals, meat, and cereal grains. The percentage of protein in the diet can be varied, depending on the amount of natural feeds present, quality of the protein, and energy level provided. Fry feeds should contain 50% protein, which should be decreased to 25-35% protein during growout. Carbohydrates are digested relatively well, with the exception of starch and highly fibrous feedstuffs. Digestible energy to protein ratios (DE/P) should range from 8.2 to 9.4 kcal DE/g protein for fry and small fingerlings. Tilapia have a dietary requirement for the linoleic (n-6) fatty acids provided by vegetable oils and they utilize these fats better than those in fish oils. Relatively little is known about vitamin requirements for tilapia, but a vitamin package must be included in the diet when natural feedstuffs are not abundant. It is assumed that tilapia have mineral requirements similar to those of other fish. The amount fed daily varies by species, size, temperature, feeding frequency, and availability of natural feedstuffs. The tilapia have small stomachs and forage continuously in the wild. Therefore, better growth occurs with multiple daily feedings, rather than with a single feeding, under artificial conditions.

Stocking densities and production yields are highly dependent upon nutrient input and degree of management intensity. Fertilized, unaerated freshwater ponds stocked with 20,000 fingerlings per hectare (ha) and fed agricultural byproducts can be expected to produce 2 tons/ha of 240g fish in five months. In contrast, intensively managed ponds with supplemental aeration, stocked at the same initial density and fed protein-rich diets, have been reported to yield as high as 25 tons/ha per annum (Pillay 1990). In intensively managed recirculating systems, stocking densities of 60-120 g/L can be achieved and 680g fish can be produced in approximately 1 year (Perera 1999).

Global Production

The potential of these fish as a cultured species has been recognized for many years and the first attempt at artificial culture took place in Kenya in 1924 (Huet 1986). Today, from a global standpoint, tilapia are second only to carp in total pounds produced, but only five species have had a significant impact under practical culture conditions: *Tilapia rendalli*, *T. zilli*, *Oreochromis mossambicus*, *O. niloticus*, and *O. aureus* (Lim 1989). Tilapia have been introduced into the wild, either accidentally or deliberately, and are cultured throughout much of the Americas, the Middle East, Asia, the Indo-Pacific, and their native Africa (Avault 1996). The market appeal of tilapia and the form in which they are sold (live, whole, or filleted) varies greatly, even within a single country, due to factors of size, coloration, physical appearance, and ethnic preferences (Avault 1996). Hybrid red tilapia are particularly appealing because they resemble other popular species like the as red snapper *Lutjanus campechanus* (Watanabe 1991).

Tilapia have been grown in almost every type of culture system imaginable, from subsistence level pond culture in Africa, to polyculture in the Philippines, and integrated culture in the rice fields of Thailand. They have been stocked into lakes and reservoirs in Israel, raised in saltwater cage culture in the Caribbean, and grown in geothermally heated raceways in Idaho. Intensive indoor culture techniques, utilizing high stocking densities with recirculating water and supplemental aeration, have become the preferred method of cultivation in temperate regions such as the U.S. and China (Pillay 1990; Watanabe 1991; Avault 1996; Martin 1997).

Production and Culture in the U.S. and Louisiana

In the United States, most tilapia are grown under intensive conditions and are fed pelleted rations. Some southern states, such as Louisiana, do not allow tilapia to be grown in ponds, for fear that fish might escape and overpopulate natural bodies of water, destroying habitat for native fish species. In cooler climates, broodfish may be stocked in earthen ponds during the summer and then returned indoors after spawning. In this system, fry and fingerlings are allowed to exploit natural feeds in the ponds during the summer and are then brought indoors, for growout, in the fall. So called "greenwater" systems, built in greenhouses, which utilize *Chlorella* algae for supplemental oxygen production and nitrogen removal, are popular in Louisiana (Martin 1997).

Five million pounds of tilapia were grown in the United States in 1991, with an increase to 16 million pounds in 1996. Despite the increased production, over 60 million pounds were imported into the U.S. in 1996 to meet the domestic consumption demand (Plante 1997). While these figures suggest great potential for continued expansion of the industry, competition with foreign markets for low priced fish and frozen fillets has had a

significant impact on profitability of the domestic product. Prices for 1.25-1.50 lb whole live fish, grown in Louisiana, fell from $1.80-$2.00/lb in February of 1998 to $0.85-$0.90/lb in July of 1999 (Perera 1999). The American Tilapia Association estimates the bulk of U.S. production is sold live to Oriental markets on the east and west coasts and in Toronto and Chicago, with only 20% of total sales as processed fish. High operating costs for labor, feed, and energy, and the inability to control diseases in intensive systems are cited as major factors affecting competition with imported products (Plante 1997).

Review of Diseases

With the exception of *Streptococcus iniae*, which is now recognized as a major global pathogen, investigations of cultured and wild tilapia have indicated that while they are more resistant to diseases than many other fish species, they are susceptible to a host of predominately opportunistic and ectocommensal agents. In the wild, a low level of external parasitism is common, generally with no clinical significance (Roberts and Somerville 1982; Lightner et al. 1988). A detailed description of these organisms and the diseases they produce is beyond the scope of this work, but a partial list of disease agents is provided below in Table 1. It is sufficient to say that the predisposing conditions, clinical signs, and lesions produced, in general, do not vary significantly from those in other fish species.

Coincidental to the emergence of *S. iniae*, additional potentially significant pathogens have been reported in tilapia, in the U.S. and abroad. In 1994, mass mortalities associated with enlarged livers, spleens, and kidneys containing multifocal to coalescing, large, white, necrotic foci occurred in several tilapia species in Taiwan. Affected fish were pale and lethargic, with a hemorrhagic and ulcerative dermatitis.

Microscopically there was systemic vasculitis, thrombosis, necrosis, and granuloma formation. Hepatocyte and macrophage cytoplasm contained rickettsia-like organisms. The diagnosis was confirmed by cell culture, electron microscopy, and infectivity trials. Outbreaks were controlled with oxytetracycline (Chen et al. 1994, Chern et al. 1994).

Diseases of fish caused by irido-like viruses were reviewed by Hedrick in 1992. Epizootic mortalities, up to 100%, associated with an irido-like virus occurred in tilapia fry in Australia in 1997. Moribund fish exhibited periods of rapid corkscrew-like swimming, and the disease was coined 'spinning tilapia (ST) syndrome'. Histopathologic changes included focal myolysis and atrophy of renal tubules, with hemorrhage and infiltration by eosinophilic granular cells. While the virus could not be isolated, the disease produced changes typical of the Bohle iridovirus (BIV) when filtrates were injected into barramundi *Lates calcarifer*. The agent is potentially transmitted by cannibalism (Ariel and Owens 1997).

In the U.S., mortalities up to 50% occurred in tilapia fingerlings transported from Florida to Idaho in 1996. Fish were dark, lethargic, and bloated with mild corneal opacification. Some gulped at the water's surface. Gills and internal organs were pale, while ascites and renomegaly were also noted. Necrosis and inflammation were present in the ocular choroid, heart, intestine, liver, renal tubules, spleen, and most severely in the hematopoietic tissues of the kidney. Large cells with finely granular cytoplasmic inclusions and eccentric nuclei were associated with necrotic foci and were identified in the circulation. The causative viral agent could not be isolated, but demonstrated features suggestive of an iridovirus on electron microscopy. A similar disease outbreak was reported at approximately the same time in Canada (Smith et al. 1997).

Table 1. Partial list of disease agents reported in tilapia.

Protozoans
**Ichthyophthirius multifiliis*[a,b]
Trichodinids (*Trichodinella spp.*, *Tripartiella spp.*, *Trichodina spp.*)[a,b]
Chilodonella spp.[a]
Ichthyobodo necatrix[a,b]
Apiosoma spp.[b]
Ambiphyra spp.[b]
Myxosporidia (Myxobolus / Myxosoma group, *Henneguya spp.*)[a]
Metazoans
Monogeneans (**Gyrodactylus spp.*[a], *Dactylogyrus spp.*[(Nguenga 1988)]
Digenean metacercariae (*Clinostomum spp.*[a], *Euclinostomum spp.*[a], *Neascus spp.*[a], *Haplorchis spp.*[a], *Diplostomum spp.*[a])
Cestodes, Nematodes (*Contracaecum spp.*), Acanthocephalans[a]
Crustaceans
Ergasilus spp.[a,b]
Lernea spp.[a,b]
Argulus spp.[a]
Gnathid isopods[(Cassua, unpublished)]
Bacterial
Flexibacter columnaris (*Flavobacterium columnare*)[a]
**Aeromonas hydrophila*[a,b], *Aeromonas spp.*[b]
Group B Streptococci (*S. difficile*)[(Eldar et al. 1994)]
S. iniae (*S. shiloi*)[(Eldar et al. 1994, Perrera et al. 1994)]
**Edwardsiella tarda*[a,b]
Mycobacterium fortuitum[a], *M. marinum*[(Noga 1990)]
Vibrio spp.[a], *V. vulnificus*[(Sakata & Hattori 1988)]
Plesiomonas shigelloides[b]
Pseudomonas putrefaciens[b], *Pseudomonas spp.*[b]
Rickettsia[(Chen et al. 1994)]
Mycotic
Saprolegnia spp.[a,b]
Branchiomyces spp.[a]
Paecilomyces spp.[b]
Viral
Lymphocystis iridovirus[a]
Bohle iridovirus (BIV)[(Ariel and Owens 1997)]
Irido-like virus[(Smith et al. 1997)]
Infectious pancreatic necrosis virus (IPNV)[(Hedrick et al. 1983)]

a=Roberts and Sommerville (1982), b=Lightner et al. (1988)

Family Streptococcaceae

Taxonomy, Morphology and Biochemical Characteristics

In the past 25 years, the taxonomy of the streptococci and allied lactic acid bacteria has undergone significant expansion and revision. This diverse group of bacteria are defined by the production of lactic acid as the sole or major end product of carbohydrate metabolism (Kilian 1989). In traditional taxonomic schemes, based on biochemical properties, morphologic characteristics and serologic grouping, members of the 'family Streptococcaceae' are classified as being non-spore forming gram-positive cocci, negative for cytochrome enzymes utilizing the catalase test, which are arranged in pairs and chains, and are predominantly facultative anaerobes (Hardie 1984).

With the advent of modern molecular techniques, the validity of the 'family Streptococcaceae' has been challenged and the lists of both streptococcal and the new streptococcus-like species have increased (Koneman et al. 1997). Several new species, including *Streptococcus iniae* (Pier and Madin 1976) have gained prominence and have been added to previously described genera, and several new streptococcus-like genera have been established to accommodate previously recognized organisms. Most notably the original genus *Streptococcus* has been divided into three genera: *Streptococcus sensu stricto*, *Enterococcus* (formerly group D fecal streptococci), and *Lactococcus* (formerly group N streptococci) (Schleifer et al. 1985; Schleifer and Kilpper-Balz 1987; Ludwig et al. 1985). Comparative analysis of 16s rRNA segments and nucleic acid hybridization studies have shown that streptococci, enterococci, and lactococci form a loosely related group within the clostridium branch of the gram-positive bacteria (Ludwig et al. 1985). Additional streptococcus-like genera now described include

Leuconostoc, *Pediococcus*, *Alloiococcus*, *Vagococcus*, *Globicatella*, *Tetragenococcus*, *Helococcus*, *Aerococcus*, and *Gemella* (Koneman et al. 1997). Members of the genera *Streptococcus*, *Lactococcus*, and *Vagococcus* are known to be pathogens of fish (Austin and Austin 1993).

Streptococcus iniae

Introduction

The gram-positive lactic acid producing bacteria are receiving increased attention from fish health specialists due to the increasing incidence of diseases caused by fish pathogenic members of the group in both wild and cultured fish (Austin and Austin 1993). Selected representatives include *Carnobacterium pisicola*, formerly *Lactobacillus pisicola* (Hiu et al. 1984; Baya et al. 1991), *Lactococcus piscium*, formerly group N Streptococcus (Williams et al.1990), *Lactococcus garvieae*, formerly *Enterococcus seriolicida* (Kusuda et al. 1976; Teixeira et al. 1996), *Enterococcus sp.*, formerly group D Streptococcus (Boomker et al. 1979), and *Vagococcus salmoninarum* (Wallbanks et al. 1990).

Streptococcosis was originally described in rainbow trout farmed in Japan (Hoshina et al. 1958), but the causative organism was incompletely characterized. Non-hemolytic group B, type Ib streptococci were first reported in the U.S. in 1966 and have since been implicated in large multiple species fish kills in natural marine environments (Robinson and Meyer 1966; Plumb et al. 1974; Baya et al. 1990). The group B streptococci, including *S. difficile*, have now been shown to possess whole-cell protein patterns and phenotypic characteristics similar to those of other type Ib variants of *Streptococcus agalactiae* (Elliott et al. 1990; VanDamme et al. 1997). Most recently,

Streptococcus parauberis has been described as a pathogen of diseased turbot (Romalde et al. 1999).

Initial Isolation and Characterization

In 1972 Stewart Madin isolated a novel *Streptococcus sp.* from purulent exudate contained within subcutaneous abscesses of a captive Amazon freshwater dolphin *Inia geoffrensis* housed at Steinhart aquarium in San Francisco. As a β-hemolytic organism, it was unusual in that it failed to react with any grouping antisera directed against recognized Lancefield serogroups utilizing antigen prepared against the hot HCl method (Fuller 1938), the hot autoclave method (Rantz and Randall 1955), the *Streptomyces albus* lytic enzyme method (Maxted 1948), or extraction with Pronase B (Ederer et al. 1972). Based on this finding and additional cultural, morphological, and biochemical features, the bacteria was recognized as a new species and was named *Streptococcus iniae* (Pier and Madin 1976). The type strain was cataloged by the American Type Culture Collection (ATCC) using accession number 29178.

Pier and Madin originally described *S. iniae* as a gram-positive encapsulated coccus, up to 1.5 μm in diameter, usually occurring in long chains in broth culture. On solid blood agar, small (up to 1 mm) colonies are white and umbonate, with an entire opaque border, an opaque center spot, and a translucent ring separating the border and center. Colonies are surrounded by a small to moderate zone of β-hemolysis, with a diffuse outer zone of α-hemolysis. Growth in broth is characteristic, producing a coarse, white, granular material growing predominately at the bottom of the tube. As with other streptococci, this organism was determined to be facultatively anaerobic.

Fermentation studies revealed acid production from dextran, fructose, galactose, glucose, maltose, mannitol, mannose, salicin, sucrose, and trehalose. Acid was not produced from reaction with arabinose, dulcitol, glycerol, inositol, inulin, lactose, melibiose, raffinose, rhamnose, sorbitol, or xylose. Biochemical test reactions are summarized in Table 2 (Pier and Madin 1976).

The DNA base composition was calculated at 32.9 mol % G+C, which is close to values reported for other streptococci. The isolate was submitted to R.R. Facklam at the Centers for Disease Control (CDC), who prepared and tested a Lancefield extract against antisera for groups A through U, plus four additional proposed groups. The *S. iniae* antigen extract failed to react with any of the grouping sera (Pier and Madin 1976).

A second *S. iniae* isolate was later cultured from an Amazon dolphin housed at the Niagra Falls Aquarium in New York. This isolate differed from the first in its ability to produce acid from lactose, but not salicin, and in its ability to hydrolyze esculin. The two isolates shared a common cell wall antigen believed to represent the C polysaccharide grouping antigen of this species. This isolate is maintained by the ATCC as number 29177 (Pier et al. 1978).

Phylogenetic Relationship to Other Streptococci

Analysis of 16S rRNA sequences was used to examine the phylogenetic relationships between 34 species of streptococci. *Streptococcus iniae* clustered with nine other organisms designated as the pyogenic group, including β-hemolytic representatives of Lancefield groups A (*S. pyogenes*), group B (*S. agalactiae*), group C (*S. equi*), group G, groups L and M (*S. canis*), groups E, P, U, and V (*S. porcinus*), and several other nongroupable species including *S. uberis*, *S. parauberis*, *S. intestinalis*, and

Table 2. Biochemical reactions of *S. iniae* (ATCC 29178)

Test	Reaction
Catalase	-
Nitrate reduction	-
Growth in/at	
Bile esculin media	-
Methylene blue milk, 0.005%	+
Methylene blue milk, 0.1%	-
6.5% NaCl	-
4.0% NaCl	+
2.0% NaCl	+
10% Bile broth	-
40% Bile broth	-
0.04% Tellurite	-
0.1% Tetrazolium	+
10°C	+
45°C	-
Hydrolysis of	
Starch	+
Sodium hippurate	-
Gelatin	-
Esculin	+

S. phocae (Kawamura et al. 1995). With the exception of *S. agalactiae*, a PCR based analysis using degenerate primers to characterize an internal fragment of the manganese-dependent superoxide dismutase gene, placed *S. iniae* in a similar group (Poyart et al. 1998). Results of restriction fragment length polymorphism (RFLP) analysis of 16S rDNA following *RsaI* digestion, support the findings of Bentley et al. (1991), who demonstrated 98.4% homology between *S. iniae* and *S. parauberis* (Eldar et al. 1997).

Chronological History of the Disease

Emergence as a Fish Pathogen: The Japanese Epizootics

The origins and evolution of *S. iniae* as a fish pathogen have been somewhat obscured by time, lack of standardization in testing procedures used by diagnostic

laboratories, and misinterpretation of certain test results, particularly hemolytic patterns on blood agar. Although it was not recognized at the time, in 1976, a β-hemolytic streptococcus with a phenotypic profile similar to that of *S. iniae* was reported from diseased yellowtail *Seriola quinqueradiata* in Japan (Minami et al.1976). From 1977 to 1980, large-scale epizootics of streptococcal disease occurred among tilapia *T. nilotica*, rainbow trout *Oncorhynchus mykiss*, ayu *Pleyoglossus altivelis*, and amago *Oncorhynchus rhodurus var. macrostomus*, on Japanese freshwater farms (Kitao et al. 1981; Ohnishi and Jo 1981; Ugajin 1981). Infections were considered chronic with affected fish described as anorectic and lethargic. Lesions included exophthalmos, petechiation, and congestion of the mouth, anus, and caudal fins. Ascites, intestinal hemorrhage, and petechiation of the liver were also noted. The causative agent was isolated from kidney, heart, and spleen, and appeared to have a high affinity for the brain. The organism failed to react with grouping sera to Lancefield groups A, B, C, D, E, F, G, H, K, L, M, N, O, or V. Antiserum to this bacterium weakly agglutinated the previously mentioned coccus isolated from yellowtail by Minami et al. (1976) (Kitao et al. 1981; Ugajin 1981).

In 1981 and 1982, a bacterial disease occurred among young cultured flounder *Paralichthys olivaceus*, in Kunda Bay, Japan. Signs of disease included hemorrhage of the opercle, corneal opacity, exophthalmos, and hemorrhage of the eyes. The organism isolated was determined to be a β -hemolytic streptococcus, but it could not be identified with any species, including *S. iniae*, recognized at the time (Nakatsugawa 1983). The first specific reference made to *S. iniae* as the β -hemolytic streptococcus responsible for these Japanese epizootics was made by Kaige et al. in 1984. *Streptococcus iniae* was

isolated predominately from the brains of yellowtail suffering from a variety of vertebral deformities. When these isolates were compared biochemically to those previously discussed, they were found to be identical (Kaige et al. 1984). The first chapter in the emergence of *S. iniae* as a fish pathogen concludes with mass mortalities of rabbitfish *Siganus canaliculatus* in floating fish farms along the coast of Singapore in 1984. While an α-hemolytic streptococcus was reported, the phenotypic profile is similar to that of *S. iniae* and it is likely that the pattern of hemolysis was misinterpreted (Foo et al. 1985).

Global Spread: Epizootics in Israel and the U.S.

The second chapter in the history of *S. iniae* begins in Israel in 1984, when outbreaks of bacterial encephalitis in tilapia *Tilapia sp.*, rainbow trout, and Coho salmon *Oncorhynchus kisutch*, spread rapidly throughout the country. Despite the considerable economic losses incurred, findings were not reported until 1994 (Eldar et al. 1994; Eldar et al. 1995b). Recurring mortalities began in tilapia hybrids *T. nilotica* x *T. aurea* at Simaron Freshwater Fish, Inc. near Houston, Texas in 1992 after fish were moved from outdoor ponds to indoor wintering tanks. A biotype of *S. iniae* was isolated and identified (Perera et al. 1994; Perera et al. 1998).

In the Israeli outbreaks, two gram-positive cocci, believed to represent previously unknown species of streptococci, were isolated and new species names proposed. The γ-hemolytic isolates were named *S. difficile*, while the α-hemolytic isolates from Israel and one previously unclassified isolate from Taiwan were designated *S. shiloi*. *Streptococcus shiloi* was differentiated from *S. iniae* based on the ability of the latter to ferment galactose, its G & C content of 32.9 mol% (versus 37.1 mol% for *S. shiloi*) and its β-hemolytic phenotype (Eldar et al. 1994).

Streptococcus difficile is now recognized as a variant of the group B capsular type Ib *S. agalactiae* (VanDamme et al. 1997). While the name *S. shiloi* was validated in 1995 (Anonymous 1995), it was later reduced to a junior synonym of *S. iniae*. The change in nomenclature was based on DNA-DNA hybridization studies with U.S. isolates from Texas and the *S. iniae* ATCC type strain 29178, phenotypic profiling using API 50 CH test kits (bioMerieux Vitek), and by demonstrating that the Israeli isolates were in reality β-hemolytic (Eldar et al. 1995a). Differences in the G & C contents were believed to have resulted from technical improvements in the methods used. Denaturation was used originally by Pier and Madin (1976) compared to the more accurate high-performance liquid chromatography used by Eldar et al. (1994).

It was suggested at the time that *S. iniae* may have come to Israel in imported fish eggs from the U.S. in the early 1980's, since the original dolphin strain was isolated in this country (Eldar et al. 1995b). This scenario now seems unlikely, however, as the dolphin strain has been shown to be non-pathogenic to fish (Perera et al. 1997). As the U.S. imports tilapia from Israel, it was also possible that fish pathogenic strains of *S. iniae* were unknowingly introduced onto the Simaron farm near Houston in shipments of fry fish originating in Israel.

An attempt was made to resolve this question using restriction fragment length polymorphisms (RFLP) of 16S rDNA and of whole rRNA genes (ribotyping) of 14 U.S. and 10 Israeli *S. iniae* isolates. The RFLP method differentiated *S. iniae* from other fish pathogens, but not U.S. from Israeli isolates. With the exception of one isolate (Dan-1) from a rainbow trout, eight other Israeli trout and tilapia strains were differentiated from nine U.S. tilapia strains by ribotyping based on *Eco*RI digests. Ribotyping of *Hin*dIII

digests produced a single ribotype for all U.S. isolates. The Israeli strains yielded three ribotypes, two were distinct from the U.S. strains, but Dan-1 again resembled the U.S. ribotype pattern (Eldar et al. 1997).

The authors concluded that since most of the Israeli isolates differed from the U.S. ribotypes, the possibility that the disease was spreading from one country to the other was unlikely (Eldar et al. 1997). Since all of the U.S. isolates that were ribotyped were from a single farm, the study probably examined only a small cross section of U.S. strains, and because *Eco*RI and *Hin*dIII ribotypes were identical for Dan-1 and the U.S. strains, this argument is not entirely convincing. The ATCC 29178 dolphin strain differed in its ribotype pattern from all the U.S. and Israeli fish isolates (Eldar et al. 1997).

In a later study following a *S. iniae* outbreak in red drum *Sciaenops ocellatus*, raised on the Israeli Mediterranean coast, five isolates fell into two different ribotype patterns. One isolate was identical to Israeli *Eco*RI strains, while the remainder possessed patterns consistent with U.S. tilapia strains. The authors admitted that isolation of the typical Israeli clone was expected, and were surprised by the presence of the U.S. ribotype and suggested it probably arrived in Israel in shipments of fry imported from this country (Eldar et al. 1999).

Additional Case Reports

Additional published reports of *S. iniae* infections in fish have involved hybrid striped (sunshine) bass *Morone saxatilis* male x *M. chrysops* female, in the U.S. (Stoffregen et al. 1996), dusky spinefoot *Siganus fuscescens*, in Japan (Sugita 1996), hybrid tilapia *T. nilotica* x *T. aurea*, in Saudi Arabia (Al-Harbi 1994), gilthead sea bream

Sparus aurata, and European sea bass *Dicentrarchus labrax*, in a mariculture facility along the Israeli Mediterranean coast, and in wild spinefoot *Siganus rivulatus*, living in proximity to the aforementioned mariculture operation (Zlotkin et al. 1998). More recently, *S. iniae* was isolated from red drum, grown in cage culture along the Israeli coast (Eldar et al. 1999). In 1999 the first isolation of *S. iniae* in Australia was made from diseased barramundi *Lates calcarifer* (Bromage et al. 1999). Infections were also reported that year in whitespotted rabbitfish *Siganus canaliculatus* in Bahrain (Yuasa et al. 1999).

Isolation of *S. iniae* at the AADDL

A review of previously unclassified streptococcal isolates archived by the Aquatic Animal Disease Diagnostic Laboratory (AADDL), School of Veterinary Medicine, Louisiana State University, revealed the first *S. iniae* isolation made by this lab was cultured from hybrid striped (Palmetto) bass, *Morone saxatilis* female x *M. chrysops* male, in November 1993, followed by the first tilapia isolate in February of 1994. In both instances, the fish were being cultured in indoor recirculating systems. Prior to October of 2000 there had been no known outbreaks involving commercial fish culture operations in the state of Louisiana. That month *S. iniae* was isolated from diseased tilapia at a commercial grow-out operation and high school aquaculture project in Abbeville, Louisiana. All of the fish involved had originated from a Mississippi facility with a known history of *S. iniae* infections. A list of the *S. iniae* isolates archived by the AADDL are presented in Table 3.

The American Tilapia Association now considers *S. iniae* to be the most important pathogen affecting the growth of the tilapia industry in this country (Bowser et

al. 1998). In a 1997-98 survey of 970 tilapia, 415 hybrid striped bass, and 158 channel catfish *Ictalurus punctatus* from 24 farms throughout the U.S., the prevalence of *S. iniae* was found to be 3.81% and 7.23 % for tilapia and hybrid striped bass, respectively. The prevalence on 12 tilapia farms ranged from 0-27.4% and on 6 hybrid striped bass farms from 0-21.6%. The organism was not isolated from channel catfish (Shoemaker et al. 2001).

Isolation, Culture and Identification

Selection of Culture Media and Primary Isolation

Streptococcus iniae grows well on a variety of solid media, particularly when enriched with 5% animal blood. Growth in broth is enhanced by the addition of 3-5% heat inactivated fetal bovine serum (unpublished). Suitable media for primary isolation and growth include heart infusion agar, brain heart infusion agar (BHI), tryptic soy agar (TSA), TSA with 5% blood, Columbia agar with 5% sheep or beef blood, Todd Hewitt agar (TH), chocolate agar, BHI broth, TH broth, TH with 0.3% yeast extract, and Columbia broth (Pier and Madin 1976; Kitao et al. 1981; Nakasugawa 1983; Foo et al. 1985; Eldar et al. 1994; Perera et al. 1994; Eldar et al. 1995b; Facklam 1997; unpublished). For selective isolation from contaminated sites, Columbia colistin-naxidixic acid agar (CNA) is particularly useful. Growth on CNA is far more luxuriant than on sodium azide blood agar (unpublished). *S. iniae* does not grow on phenylethyl alcohol agar (PEA) (unpublished) or MacConkey agar (Al-Harbi 1994).

In cases of fulminating septicemia, particularly when abdominal exudate is present, *S. iniae* can be readily isolated from livers, spleens, kidneys, hearts, and especially the brains of diseased fish (Kitao et al. 1981; Eldar et al. 1994; Perera et al.

Table 3. *S. iniae* isolates archived and partially characterized by the Louisiana Aquatic Animal Disease Diagnostic Laboratory, Louisiana State University

LSU ID	Original ID	State or Country of Origin	Species of Origin	Hemolysis	Special Features	API Strep 20 Code
93-331A		MA	HSB	α/β		4563117
94-034		MA	HSB			
94-036		IL	Tilapia	α/β		4563117
94-093A		ND	Tilapia	α/β		4563117
94-142		MS	Tilapia			
94-426		IL	Tilapia			
94-449		LA	Tilapia	β	Clumps in broth	4563117
95-066		LA	Tilapia	β	Clumps in broth	4563117
95-290		CA	HSB			
96-262		IA	Tilapia	α/β		4563117
96-290B		VA	Tilapia	α/β		4563117
96-325		IA	Tilapia	α/β		4563117
96-327		IA	Tilapia	α/β		4563117
97-003		IA	Tilapia	α/β		4563117
97-039		IA	Tilapia	α/β		4563117
97-041		IA	Tilapia	α/β		4563117
97-045		VA	Tilapia	α/β		4563117
97-103		IA	Tilapia	α/β		4563117
98-052		IA	Tilapia	α/β		4563117
98-061		MN	Tilapia			
98-071		S. Arabia	Tilapia	β	Clumps in broth	4563117
98-083	ATCC 29178	CA	Dolphin	α/β	Clumps in broth	4563117
98-113	M43B	TX	Tilapia	β		4563117
98-114	ND5C	Israel	Tilapia	α/β		4563117
98-115	Dan 12	Israel	RBT			4563117
98-116	M34	TX	Tilapia	β		4563117
98-117	M32B	TX	Tilapia	β		4563117
98-118	Dan 1	Israel	RBT	α/β		4563117
98-177		CA	HSB			4563117
98-178		CA	HSB			4563117
98-179		CA	HSB			4561115
98-240	KS S98-1	MS	Tilapia	α/β		4563117
99-091	2031-96	Canada	Human	α/β	Clumps in broth	4563117
99-092	2030-96	Canada	Human	α/β		4563117
99-093	2032-96	Canada	Human	α/β		4563117
99-094	2378-91	TX	Human	β		4563117
99-299A		FL	R. Shark			4563117
99-299B		FL	R. Shark	β		4563117

(table cont'd)

LSU ID	Original ID	State or Country of Origin	Species of Origin	Hemolysis	Special Features	API Strep 20 Code
99-301D		MN	Tilapia	β	Clumps, Man neg	4563117
99-301G		MN	Tilapia	α/β		4563117
99-456D		IL	Tilapia	β	Clumps in broth	4563117
00-296		LA	Tilapia	α/β		4563117
00-300		LA	Tilapia	α/β		4563117
00-318		MN	Tilapia	β		4563117

1994; Al-Harbi 1994). Using Columbia CNA, it can also be isolated in large numbers from the lower intestinal tract and often from skin and gill surfaces (unpublished). In chronic cases, usually displaying neurologic signs, the bacteria can often be cultured only from the brain, which appears to be the single most reliable site for isolation (Eldar et al.1995b; Bowser et al. 1998; unpublished).

In a survey of 1385 tilapia and hybrid striped bass, Shoemaker et al. (2001) isolated *S. iniae* from brain, kidney and skin with an incidence of 3.25%, 3.63%, and 2.91%, respectively. These authors concluded that the skin represented an appropriate site for non-invasive sampling. This conclusion is disputed, however, by a recent publication, which found that many *S. iniae* skin isolates have a non-virulent genetic profile on pulse field gel electrophoresis (PFGE) and may only represent commensal organisms (Fuller et al. 2001). Following experimental exposure, Bromage et al. (1999) were unable to isolate *S. iniae* from the spleen or kidney of barramundi 10 days post infection, but were able to infrequently isolate it from the brains of survivors.

Growth Characteristics

When incubated at 35-37°C, growth is usually sufficient to begin an identification procedure at 24 hours. At 22-30°C, 48 hours of incubation or longer may be required to produce adequate growth (Nakatsugawa 1983; Foo et al. 1985; Perera et al. 1994; Eldar

et al. 1995b). Colonies are milky white, semi-mucoid, and up to 2mm in diameter (Pier and Madin 1976; Eldar et al. 1995b). Additional colony morphology described by Pier and Madin (1976) is presented in the 'Initial isolation' subheading to this section. Pier and Madin (1976) also described the growth of *S. iniae* in broth as coarse and granular with a tendency to grow at the bottom of tubes. At the AADDL, it has been found that while a few strains produce this type of growth, the majority are buoyant and disperse evenly throughout the media in a more conventional fashion. In group B streptococci, buoyancy in broth culture is directly related to the level of capsular polysaccharide expression. More heavily encapsulated isolates are more buoyant and typically more virulent (Hakansson and Holm 1986, Hakansson et al. 1988).

Biochemical Characteristics

While the majority of *S. iniae* strains encountered at the AADDL are homogenous in their phenotypic profiles, some variations have been reported, which have confused identification of the organism, particularly in the early literature. Dr. Richard Facklam at the CDC in Atlanta has compiled a list of diagnostic criteria essential to the identification of *S. iniae* along with detailed instructions for performing the individual biochemical tests (Facklam 1997). As a member of the genus *Streptococcus*, *S. iniae* is a gram-positive, catalase-negative, non-motile coccus that produces long chains, especially when grown in broth. It is susceptible to 30 mg vancomycin disks, but is resistant to bacitracin. It does not produce gas in Lactobacillus Mann, Ragosa, and Sharpe broth, and is negative in the bile esculin (BE) test. As with other streptococci, *S. iniae* gives a positive leucine arylamidase (LAP) reaction, but in contrast to most others, it is also pyrrolidonylarylamidase (PYR) positive. Growth in broth containing 6.5%

NaCl usually does not occur, although some strains may grow weakly in this media. *Streptococcus iniae* usually grows at 10°C, particularly when incubated 7-14 days, but not at 45°C, and again exceptions exist. It is CAMP test positive, negative for acetoin production on the Voges-Proskauer (VP) test, and negative for hydrolysis of hippurate (HIP). It produces acid from starch, but not from sorbitol.

Facklam considers *S. iniae* to be arginine dehydrolase (ADH) negative, but indicates some strains may be positive, although reactions may be delayed up to 14 days. This is in contrast to the literature, where most strains have been reported as ADH positive (Kitao et al. 1981; Ohnishi and Jo 1981; Ugajin 1981; Nakatsugawa 1983; Foo et al.1985; Eldar et al. 1994; Perera et al. 1994; Stoffregen et al. 1996). At the AADDL, using the API 20 Strep identification system (bioMerieux Vitek, Inc.), positive ADH reactions tend to be largely dependent on the density of the inoculum used. A similar situation exists for the production of acid from glycogen.

Streptococcus iniae does not possess a defined Lancefield group antigen. Facklam indicates that when the original type culture was submitted to the CDC, grouping antisera was also included (Pier and Madin 1976). The sera and strain were type specific. No sera remains and commercial preparations are not available, so identification relies solely on phenotypic characterization. Group antigens A, B, C, D, E, F, G, L, M, P, U, and V, plus four experimental *S. porcinus* antigens have been tested and fail to react with *S. iniae* (Pier and Madin 1976; Kitao et al. 1981; Facklam 1997). Since *S. porcinus* groups E, P, U, V, and the four new groups have not been associated with any fish pathogens, Facklam believes that it is probably sufficient to use only groups

A through G and indicates that most commercially available slide agglutination kits work well.

Three β-hemolytic streptococci are LAP and PYR positive: *S. iniae*, *S. pyogenes*, and *S. porcinus*. Group A *S. pyogenes* can be differentiated by its serologic grouping and by its sensitivity to bacitracin. *Streptococcus iniae* is differentiated from *S. porcinus* based on two tests, the hydrolysis of starch and acid production from sorbitol. *Streptococcus iniae* is starch positive and sorbitol negative, while *S. porcinus* is starch negative and sorbitol positive (Facklam 1997).

Interpretation of hemolytic patterns on blood agars has created the most confusion surrounding the identification of *S. iniae*. In the original two reports on the organism, colonies were described as having a "bull's eye" pattern of hemolysis with a narrow inner zone of β-hemolysis, surrounded by a wider zone of α-hemolysis (Pier and Madin 1976; Pier et al. 1978). Based on findings at the LAADDL, most isolates follow this pattern, with pure β-hemolysis becoming readily apparent only after 48 hours of incubation at 37°C. However, the LAADDL does maintain in its collection several strains that produce an intense zone of only pure β-hemolysis within 24 hours.

The zone of "α-hemolysis" associated with many *S. iniae* isolates may not represent true α-hemolysis in the classic sense, which results from the production of hydrogen peroxide. Most strains of group A streptococci produce complete lysis of erythrocytes (β-hemolysis), but a few strains produce a zone of greenish discoloration resembling α-hemolysis that develops into complete lysis only with prolonged incubation. The initial incomplete lysis is reported to result from inhibition of hemolysis by opacity factor (Johnson et al. 1996).

Hemolytic reactions are determined on solid media containing 5% animal blood, the most common being sheep blood. Some authors have reported only partial hemolysis when human or bovine blood is used (Eldar et al. 1995a). Plates are streaked in the usual manner to test for purity and the agar stabbed with the inoculating loop to examine for hemolysis. Incubation under 5% CO_2 is recommended and plates are examined at 24 hours, or when sufficient growth is present. All *S. iniae* strains identified by the CDC have been β-hemolytic if allowed to incubate for a sufficient period of time, especially when the stab area is examined carefully (Facklam 1997).

Identification of *S. iniae* isolates at the LAADDL is based on the presence of β-hemolysis on TSA or Columbia agars supplemented with 5% sheep blood and through use of the API 20 Strep identification system. The bioMerieux Vitek company does not currently list a code for *S. iniae* in its database, but the most common codes encountered by this laboratory are 4562117, 4563117, 4562116, and 4563116. Differences are due to variation in arginine and glycogen test results. If a heavy inoculum is used and sufficient incubation times at 37°C are allowed, most strains will give a code number of 4563117. A summary of the phenotypic characteristics of the reported and suspected *S. iniae* isolates are compared to LSU isolate 94-449 and to the diagnostic criteria outlined by Facklam in Table 4.

Direct fluorescent antibody techniques, performed on heat-fixed smears, have been used to differentiate α-hemolytic *Lactococcus garvieae* (Kusuda et al. 1976) from the β-hemolytic isolates of Minami (1979), Kitao et al. (1981), Ohnishi and Jo (1981), Ugajin (1981), and Nakatsugawa (1983), now shown to be strains of *S. iniae* (Kawahara and Kusuda 1987). Slide agglutination tests using isolates of *S. iniae* as antigens have

Table 4: Reported morphological and biochemical features of *S. iniae*

SOURCE	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Characteristic:																
Size (μm)	<1.5			0.3-0.5	0.5-0.8		0.5-0.8			0.6-0.8				0.6.0.8		
Gram stain	+			+	+		+	+	+	+		+	+	+	+	
Catalase	-	-		-	-		-		-	-		-	-	-		
Hemolysis	B		β	β	β	β	β	α	α	β		β	α	β	β	β
Motility	-			-	-		-	-		-		-	-	-	-	-
Oxidase				-	-			-					-	-	+	
Capsule	+			+											+	
Facultative anaerobe	+			+				+	+	+				+		
VP				-	-		-		-	-		-	-	-		-
H_2S							-	-					-			
OF	Fer			Fer	Fer		Fer	-	Fer				-			
Lancefield Ag	-	-	-	-	-			-	-	-						-
Nitrate reduction	-	-			-			-				-	-			
G/C % ratio	32.9	32.9							37.1							
CAMP Test																+
Growth in/at:																
MRS broth										-				-		-
Bile esculin	-	-									-		+			-
Urease broth					-				-							
pH 9.6			-	-	-	-	-	+	+	-						

(table cont'd)

SOURCE	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
2.0% NaCl	+	+														
4.0% NaCl	+	+											+			
6.5% NaCl	-	-	-	-	-	-	-	+	-	-		+	-	-		-
10°C	+	+	-	-	-	-	-	+	-	+			-	+		V
45°C	-	-	-	-	-	-	-	-	-	+		-	-	-		V
40% Bile	-	-	-	-	-	-	-	-	-	-						
10% Bile	-	-		+	+			+	-							
0.04% Telluride	-	-								-						
0.1% Methylene Blue	-	-	-	-	-	-	-	+	-							
Litmus milk	A+/-			A	-		A			-						
Hydrolysis of:																
Gelatin	-				-	-	-	-		-			-			
Starch	+		+	+	+	+	+	+	+	+			+			+
HIP	-		-	-	-	-	-	-	-	-		-	-	-		-
PYRA								+	+	+		+	+	+		+
α Gal								-	-				-	-		
β Gur								+	+				+	+		
β Gal						-		-	-				-	-		
PAL									+				+	+		
LAP									+				+	+		+
Arginine			-	+	+	+	+	+	+	+		+	+	+		+/-
Esculin	+	+	+	+	+	+	+	+	+			+	+	+		
Ornithine				-			-	-					-			
Lysine				+				-								
Glycerol	-		+	-	-	-	-	+	-	-	-	-				

(table cont'd)

SOURCE	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
D-Mannose					+		+		+		+					
D-Arabinose	-			-	-	-	-	-	-	-	-	-		-		
Ribose								+	+		+		+	+		
D-Glucose	+			+	+		+	+	+	+	+	+	-			
L-Xylose	-			-	-		-		-		-					
Rhamnose					-		-	-	-		-		-			
Inositol					-		-	-	-		-		-			
Sorbitol	-		-	-	-	-	-	-	-	-	-	-	-	-		-
Amygdalin								-	-		-					
Esculin	+	+	+	+	+	+	+	+	+		+					
Cellobiose				+		-			+		+					
Lactose	-	+	+	-	-	-	-	-	-	-	-	-	-	-		
Saccharose								-	+							
Inulin	-			-	-	-	-	-	-	-	-	-	-	-		
D-Raffinose	-			-	-		-	-	-	-	-	-	-	-		
Glycogen							+	+	+		+		+	+		
Gentibiose									+		+					
D-Fucose									-		-					
D-Arabitol									-		-					
Gluconate									-		-					
α-Keto-gluconate									-		-					
Erythritol									-		-					
L-Arabinose									-		-		-			
D-Xylose							-		-	-	-	-				
Adonitol					-		-		-		-	-				

(table cont'd)

SOURCE	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
M-D-Mannoside									-		+					
Galactose	+				+		+		-	-	-	+				
D-Fructose					+		+		+		+					
L-Sorbose									-		-					
Dulcitol					-		-		-		-					
Mannitol	+			+	+		+	+	+	+	+	+	+	+		
N-A-Glucosamine									+		+					
Arbutin							-		+		+					
Salicin	+	-		+	+	+	+	+	+	-	+	+				
Maltose	+			+	+	+		+	+	+	+					
Melibiose								-	-							
Trehalose	+			+	+	+	+	+	+	+	+	+	+	+		
Melezitose									+		+					
Starch	+		+	+	+	+	+	+	+		+			+		+
Xylitol									-		-					
Toranose									-		-					
Tagalose									-		-					
L-Fucose									-		-					
L-Arabitol									-		-					
2-Keto-Gluconate									-		-					
Sucrose	+		+	+	+	+	+	+		+	+	-	-			

Legend: A = Pier and Madin 1976; B = Pier et al. 1978; C = Minimi 1976; D = Kitao et al. 1981; E = Ohnishi and Jo 1981; F = Ugajin 1981; G = Nakatsugawa 1983; H = Foo et al. 1985; I = Eldar et al. 1994; J = Perera et al. 1994; K = Eldar et al. 1995a; L = Stoffregen et al. 1996; M = Al Harbi 1994; N = Yuasa et al. 1999; O = LSU 94-449; P = Facklam 1997.

been used to screen groups of fish for presence of antibody, indicating previous exposure (Eldar et al. 1995b). Four molecular techniques have also been used to aid in the identification of *S. iniae*. The first technique utilized was direct comparison of rDNA sequences based on PCR amplified segments of 16S rRNA from Texas field isolates with the type strain of *S. iniae* (Perera et al. 1994). DNA-DNA hybridization was later used to demonstrate the identical nature of *S. iniae* and *S. shiloi* (Eldar et al. 1995a). More recently, specific identification of *S. iniae* has been achieved following PCR amplification of the universal chaperonin 60 (*cpn* 60) gene with a pair of degenerate *cpn* 60-specific primers, followed by reverse checkerboard hybridization and chemiluminescent detection (Goh et al. 1998). Finally, a set of nested oligonucleotide PCR primers have been developed that specifically amplify a 373-bp subunit from a 524-bp consensus sequence of the *S. iniae* 16S-23S rDNA intergenic spacer (Berridge et al. 1998).

Virulence, Mortality and Epizootiological Aspects

Virulence Mechanisms

While several authors have described *S. iniae* as possessing a capsule (Pier and Madin 1976; Kitao et al. 1981), the potential role of a capsule in the pathogenesis of infections by this organism has not been investigated. More recently, Fuller et al. (2001) indicated that capsule production was not evident in virulent or commensal strains of *S. iniae*. Other pyogenic streptococci produce capsules or surface protein molecules that inhibit phagocytosis or allow the bacteria to evade other non-specific immune system components. Of particular interest is the 'M-family' of surface proteins found in group A, C, and G streptococci, including the antiphagocytic Emm protein. Using a pair of primers specific to the C-terminal conserved anchor region of the 'M-family' of

molecules (Podbielski et al.1991; unpublished), PCR analysis failed to detect the presence of any related surface proteins in *S. iniae*. To date, no proven virulence factors have been identified in *S. iniae*. Hyaluronidase and DNase, purported virulence factors in other streptococci, are produced by some strains of *S. iniae* and there is some evidence to suggest the presence of a plasminogen activator (unpublished).

Mortality Rates, Patterns of Mortality and Species Susceptibility

Mortalities attributed to *S. iniae* are often described as large scale (Kitao et al. 1981) or massive (Al-Harbi 1994). Eldar et al. (1995b) determined a mortality rate of 30% in tilapia and 50% in rainbow trout. Epizootic outbreaks in barramundi have produced mortalities as high as 70% during warm summer months (Bromage et al. 1999). In the Israeli epizootics, common carp *Cyprinus carpio* in community with diseased tilapia were not affected, but other ornamental cyprinids and cichlids were involved (Eldar et al. 1995b). Hybrid striped bass were found to be susceptible while red drum and channel catfish were resistant to artificial infection (Perera et al. 1997). Natural outbreaks have since been reported in cage-reared red drum along the Israeli Mediterranean coast (Eldar et al. 1999). *Streptococcus iniae* was isolated at the AADDL from dermal ulcers, but not the internal organs, of a bluegill *Lepomis macrochirus* inadvertently reared in conjunction with tilapia. A complete list of species reported susceptible to *S. iniae* infection may be found under the heading of 'Chronological history of the disease'.

In a large indoor water recirculating facility, initial mortalities among tilapia were high, but assumed a chronic pattern of constant low mortalities over a period of months. Periodic spikes in death rate were usually associated with handling or episodes of low

dissolved oxygen or high water nitrite levels (unpublished). Similar patterns have been observed in Israel and Australia. Eldar et al. (1995b) reported that fish at various stages of the disease, along with apparently healthy fish, may be present in a pond at any one time. The presence of healthy fish may account for the observation that a tank may often continue to feed vigorously during an outbreak. Low level prolonged mortalities result with additional fish constantly becoming infected. In seawater cultured barramundi, the disease usually persists as a chronic infection with low daily mortalities interspersed with episodes of acute deaths in which few clinical signs are seen (Bromage et al. 1999).

True Pathogen or Opportunist

Compelling evidence as to the role of *S. iniae* as an obligate or opportunistic pathogen is also lacking in the literature. Eldar et al. (1995b), conclude that *S. iniae* represents a true fish pathogen, as the disease is characterized by, "a distinct pathological picture linked to the presence of the agent." Supporting their conclusion was the finding that enrichment methods were required to isolate the organism from pond water, indicating low environmental numbers. This is in contrast to common opportunists, like *Aeromonas hydrophila*, which can be isolated in large numbers from most freshwater environments (Hazen et al. 1978).

Eldar's group dismisses the possibility that *S. iniae* was acting merely as an opportunist in a stressed population of fish, despite the observation that disease outbreaks in Israel were associated with deteriorated water quality. Increased water temperatures resulted in increased feeding responses, decreased dissolved oxygen levels, and an increase in ammonia and nitrite levels that favored spread of the epizootics (Eldar et al. 1995b). Mortalities in the original Texas outbreak began only after tilapia were

moved from ponds to indoor wintering facilities, implying physical trauma, handling stress, or more intensive culture conditions may have been necessary to precipitate the outbreak (Perera et al. 1994). While the organism was not cultured from pond water or mud using BHI broth containing 150-200 mg/ml of sodium azide, it was shown that *S. iniae* could survive at least 5 days in distilled water or 9 days in 0.85% saline at 5°C. The authors suggest that survival in nutritive rich pond muds at lower winter temperatures might represent a potential environmental reservoir (Perera et al. 1997). In yellowtail, *Streptococcus sp.* are present year round in the vicinity of rearing pens. Bacterial counts are highest in seawater during summer and highest in muds during the autumn and winter (Kitao et al. 1979). The possibility of a subclinical carrier state or some other animate reservoir was not investigated. It has been observed that *S. iniae* can be isolated from the brains of asymptomatic barramundi and it has been suggested that these fish may act as carriers (Bromage et al. 1999). A similar situation has been observed in tilapia (unpublished).

Findings by Bowser et al. (1998) also suggest *S. iniae* may behave as a stress mediated opportunist. Mortalities in a water recirculation facility rearing tilapia began in one tank 7 days following an episode of elevated suspended solids and low dissolved oxygen and in a second system 7 days after a mechanical failure again resulted in low dissolved oxygen levels. Additional tanks plumbed to the same biological filter did not suffer mortalities, but it was not stated whether these tanks also experienced episodic low dissolved oxygen levels.

Attempts to experimentally reproduce the outbreak at 26°C were made by placing 100 healthy fingerlings into a tank from a system that had previously harbored

diseased fish. Allowing dissolved oxygen levels to fall below 1.0 mg/L and total ammonia levels to rise above 10 mg/L failed to produce mortalities and *S. iniae* was not isolated from any of the stressed fish. In a second trial, fingerlings were exposed to similar conditions while being exposed to two large moribund fish. *Streptococcus iniae* was isolated from only 1/100 of the cohabitation fingerlings. The authors concluded more time or higher water temperatures might have been required to induce a higher morbidity (Bowser et al. 1998).

Handling and transport was associated with an outbreak of *S. iniae* on a commercial freshwater recirculation facility in Massachusetts rearing hybrid striped bass at 26°C. Disease occurred in two lots of 3-4 gm fingerlings shipped from a pond-based hatchery in the southeastern U.S., despite an initial health screen, which failed to detect any bacterial pathogens. Fish were quarantined for an unspecified period of time and mortalities began shortly after transfer of the fish from quarantine to a main grow-out facility (Stoffregen et al. 1996). It has been suggested that standard culture methods may not be adequate to detect subclinical carriers of *S. iniae* and stresses the need for more sensitive diagnostic tests (Shoemaker et al. 2001).

Experimental Infection and Epizootiology

Experimental infections have been established in tilapia by intraperitoneal injection, gastric intubation, and by immersion. Injected intraperitoneally (IP), ATCC strain 29178 produces no mortalities and cannot be isolated from fish 4 days post-injection (Perera et al. 1997). This supports ribotyping findings, which show the dolphin strain to vary significantly from all the U.S. and Israeli fish isolates to which it was

compared (Eldar et al. 1997). Similarly, Fuller et al. (2001) indicate that the ATCC strain does not fit their genetic profile for virulence using PFGE.

Following IP injection into tilapia averaging 160 gm, the LD_{50} for tilapia strain ND2-16 passed in vitro was 10^7-10^8 colony forming units (cfu) 14 days after the first mortality was recorded. The experiment was conducted at 24°C or slightly higher. After three passages in vivo, the LD_{50} declined to 10^2 cfu with shortened lag and median death periods following death of the first fish. No difference in the mortality pattern was seen when fish were provided more (33 L/fish) or less (10 L/fish) tank space (Eldar et al. 1995b). In contrast, Shoemaker et al. (2000) found a strong correlation between mortality rates and stocking densities. Tilapia averaging 12.7 gm, maintained at 25°C in flow-through aquaria, stocked at 11.2 gm/L and above showed significantly higher mortality rates than at lower stocking densities.

The LD_{50} for trout strain Dan-1 at 10°C also declined following serial passage (Eldar et al. 1995b). The 96 and 168 hr LD_{50} levels in 30-90 gm tilapia injected IP were determined to be 4.9×10^5 and 3.18×10^5, respectively, after two serial passages of the original Texas isolate in vivo (Perera et al. 1997). Using IP injection at water temperatures of 28-30°C, LD_{50} values were determined for five species of tilapia, ranging from 2.8×10^5 in *O. mossambicus* to 9.2×10^7 in *O. niloticus* (Al-Harbi 1996).

Mortalities in intubated fish receiving bacteria suspended in water reached 50% after 21 days. In this study, no mortalities were produced by cohabitation with diseased fish (Perera et al. 1997). Plumb et al. (1974) observed mortalities in scavenger fish feeding on the infected carcasses of menhaden that had died from group B streptococcal infection and speculated the route of exposure was from ingestion. *Lactococcus*

garvieae has been isolated from rough fish used in the manufacture of yellowtail feeds in Japan, further suggesting ingestion as a potential route of exposure for streptococci and related organisms (Minami 1979). It is not uncommon for tilapia to consume the eyes and visceral organs of dead cohorts, all sites potentially laden with *S. iniae* (unpublished).

Mortalities have been induced experimentally in tilapia and barramundi by immersion. While infections have been initiated in tilapia following immersion for 30 minutes in a concentrated bacterial suspension, the number of colony forming units was not quantified and an LD_{50} was not obtained (Perera et al. 1997). It is presumed that a concentration higher than that likely to be encountered under natural conditions was utilized. Mortalities reached 34% after 21 days. In contrast to this, *S. iniae* has been shown to be highly pathogenic to barramundi following bath exposure for 1 minute. An LD_{50} of 2.5×10^5 and 3.2×10^4 cfu were reached after 48 hours and 10 days, respectively. The time to death of the first fingerling was usually between 16-24 hours and the mortalities usually reached 40% within 48 hours. The isolate was passed three times in vivo to increase its virulence (Bromage et al. 1999).

When *S. iniae* was administered directly onto the eyes of tilapia and hybrid striped bass no mortalities occurred, although some hybrid striped bass developed antibody titers. Following nares inoculation, hybrid striped bass were susceptible to 4.8×10^3 cfu, while tilapia required a dose 100 times higher, suggesting the nares might represent a portal of entry at least in some species. *Streptococcus iniae* was recovered from multiple tissue sites in 87.5% and 66.7% of the moribund hybrid striped bass and tilapia, respectively. The total mortality over all doses given was 88.9% for hybrid

striped bass and 18.9% for tilapia. Mortalities usually began 1-3 days post inoculation and peaked within about 3-10 days. Antibody titers were produced in both hybrid striped bass and tilapia following nares inoculation (Evans et al. 2000).

An association between skin damage and increased mortalities from *S. iniae* may also exist, but this possibility has not been fully explored. Group B streptococci are incapable of initiating infection in Gulf killifish *Fundulus grandis* by immersion unless the skin is injured prior to exposure (Chang and Plumb 1996). Mortalities in an indoor water recirculating facility were seen to spike several days after tilapia were passed through a vacuum apparatus used to transfer fish to a purging tank. Fish passing through the unit were observed to have numerous abrasions, which were later attributed to a loose metal plate within the vacuum mechanism. Typical clinical signs developed and *S. iniae* was isolated from multiple fish (unpublished). Foo et al. (1985) isolated *S. iniae* from the waters around a floating farm in which rabbitfish were cultured. They postulated that skin damage could have allowed the bacteria to enter the fish, initiating the outbreak. Healthy fish feeding on diseased carcasses may have promoted the subsequent spread of the infection. *Streptococcus pyogenes* possesses a surface expressed protein (protein F) that binds to fibronectin, a component of the extracellular matrix abundant in collagen (Sela et al. 1993). It is feasible that a related protein in *S. iniae* could aid attachment to subcutaneous connective tissues exposed by injury, while additional virulence factors, such as hyaluronidase, could promote spread into deeper tissue sites.

Skin damage from external parasitism is another mechanism by which the epidermis of a host might be breached, providing a portal of entry for *S. iniae*. Heavy

infestation of skin and gills by *Gyrodactylus spp.* monogenetic trematodes has been observed in association with *S. iniae* epizootics (Bowser et al.1998; unpublished). While the levels of gill parasitism may have contributed solely to respiratory compromise and stress (Bowser et al. 1998), other investigators have found that gyrodactylids feeding on diseased fish harbor pathogens in their digestive tracts (Snieszko and Bullock 1968). Brown and Gratzek (1980) suggest gyrodactylids may help maintain the bacteria in close association with perspective hosts, and aid in bacterial dissemination among other fish (Cusack and Cone 1985). Gyrodactylids may serve as a vector for *Aeromonas salmonicida* and the presence of monogenes has been implicated as an initiating cause of ulcer disease of goldfish (Brown and Gratzek 1980).

One possible explanation for the failure to reliably induce infection by cohabitation rests on the numbers of bacteria being shed by diseased fish at any given time. In cases of fulminating septicemia, *S. iniae* can usually be isolated from the skin, gills, and feces of affected individuals. This apparent shedding into the surrounding water may allow an infective dose to build, particularly in a heavily stocked or otherwise stressed closed system. In other instances, the organism can only be isolated from the central nervous system and the fish may be shedding few or no bacteria into their aquatic environment.

Zoonotic Potential

From December 1995 to December 1996, nine persons in the area of Toronto, Canada acquired bacteremic *S. iniae* infections. Eight developed localized cellulitis, fever, lymphangitis, and neutrophilic leukocytosis with a left shift 16-24 hours after puncturing their hands while preparing whole fresh fish. One patient, who had a history

of rheumatic heart disease and other chronic illnesses, became septic with endocarditis, meningitis, and arthritis. All of those affected were of Asian descent and six specifically recalled purchasing tilapia from different local retailers supplied by U.S. producers. All of the patients responded to antibiotic treatment with either penicillins, cephalosporins, or erythromycin (Weinstein et al. 1996; CDC 1996; Weinstein et al. 1997).

Another 12 suspected cases with similar clinical signs and histories were identified from Canadian hospital records. Although the diagnoses were unconfirmed, it was believed that *S. iniae* might have been incorrectly identified as a viridans streptococcus, again due to misinterpretation of hemolytic reactions and the lack of a recognized Lancefield grouping antigen. A review of the CDC database revealed two additional cases, one a cook in Ottawa, Canada with synovitis, and the second a Texas man with cellulitis. Interestingly, the Texas case occurred in 1991 (Weinstein et al. 1997), prior to the original isolation of *S. iniae* from tilapia (Perera et al. 1994). These revelations led investigators to question whether *S. iniae* represented an emerging human pathogen or a previously unrecognized disease (Weinstein et al. 1997). Despite this, news of the "jump" of a fish pathogen into the human population quickly found its way into the popular press and "fish fans" were warned to "beware" (Holden 1996). On a more ominous note for aquaculturists, it was rumored that Canada would place and import ban on tilapia grown in the U.S. Canadian retailers had been supplied through wholesalers from six U.S. farms located in five states. No one farm could be identified as the source of fish associated with the Toronto cases and the import ban never materialized (Weinstein et al. 1997).

Pulse field gel electrophoresis (PFGE) was performed using endonucleases *SmaI* and *ApaI* to compare the restriction patterns of human and tilapia *S. iniae* isolates. The tilapia isolates were obtained from fish in Toronto and Vancouver markets, from seven U.S. suppliers, and from samples obtained during the 1993 Texas epizootic. The PFGE patterns of the 11 human isolates fell into two closely related banding patterns, which were also present in some of the tilapia samples. Isolates examined from tilapia and other fish species in general indicated a wide range of genetic diversity, yielding 19 distinct banding patterns. Because only two clones produced invasive disease in humans, it was suggested that these strains must possess a virulence factor or factors important for pathogenicity in both humans and fish (Weinstein et al. 1997). Further evidence indicates this observation may be correct. Fuller et al. (2001) found that only the two related PFGE banding patterns, whether of human or piscine origin, were capable of causing disease in a mouse model of infection. The authors concluded that strains not fitting this specific genetic pattern associated with virulence represent only commensal organisms.

The significance of *S. iniae* as a human pathogen clearly has not yet been fully elucidated. Among streptococci, *S. iniae* is not unique, however, in its ability to produce zoonotic infections. In swine, *Streptococcus suis* produces pneumonia, arthritis, endocarditis, and meningitis, similar to *S. iniae* septicemia and meningitis in fish. Healthy pigs carry the bacteria asymptomatically in their tonsils and outbreaks are often associated with episodes of stress. The *S. suis* capsular type 2 is most invasive in pigs and produces meningitis in humans. To date, most patients have been swine breeders or abattoir workers in contact with pigs or pork and infected wounds represent the most

probable portal of entry (Arends and Zanen 1988). As with the *S. iniae* study, genomic fingerprinting found heterogeneity among serologically identical isolates and indicated that only certain clones were pathogenic to humans (Mogollon et al. 1990). On certain tilapia farms workers routinely enter large tanks known to harbor *S. iniae* infected fish to assist in their harvest. Under such close physical contact it seems unlikely that *S. iniae* is highly pathogenic for humans. Klesius et al. (2001) reached a similar conclusion following a prevalence survey on tilapia and hybrid striped bass farms, indicating *S. iniae* probably represents a limited threat for older or immunocompromised people who incur wounds while handling fish harboring the bacteria.

Treatment and Prevention

Antimicrobial Therapy

The use of antibiotics in animals intended for human consumption is a highly controversial subject regulated in the U.S. by the Food and Drug Administration (FDA), which severely limits the number of antimicrobial compounds approved for use in food fish. Antibiotic administration is also restricted according to the fish species being medicated, the pathogen being treated, and varies in the permissible daily dose and length of the treatment period. At present, there are no antibiotics approved for use in tilapia or hybrid striped bass. In rainbow trout, oxytetracycline (Terramycin®) and sulfadimethoxine-ormetoprim (Romet 30®) are approved only for the treatment of furunculosis caused by *Aeromonas salmonicida*.

Promising in vitro sensitivity tests indicating the susceptibility of *S. iniae* to a wide range of antibiotics are summarized in Table 5. Of particular interest are oxytetracycline, sulfadimethoxine-ormetoprim, ampicillin, amoxicillin, erythromycin, and

Table 5. Reported antibiotic sensitivities of *S. iniae* isolates compared to LSU 99-449

Antibiotic	A	B	C	D	E	F	G	H
Amikacin	S	-	-	R	-	-	-	-
Amoxicillin	-	-	-	-	-	S	S	I
Ampicillin	S	-	S	S	R	S	-	-
Bacitracin	S	-	-	S	-	-	-	-
Carbenicillin	S	-	S	-	-	-	-	-
Cefalothin	S	-	S	S	S	S	-	-
Cefalexin	-	-	S	-	-	-	-	-
Cefazolin	-	S	S	-	-	-	-	-
Cefoxitin	-	-	S	-	-	S	-	-
Cephaloridine	S	-	S	-	-	-	-	-
Chloramphenicol	I	S	S	S	S	S	-	-
Ciprofloxacin	-	-	-	S	-	-	-	-
Clindamycin	-	-	S	-	-	S	-	-
Colistin	-	R	-	R	-	-	-	R
Doxycycline	-	S	-	-	-	S	-	-
Enrofloxacin	-	-	-	-	-	-	S	I
Erythromycin	S	S	S	S	S	S	-	I
Fluorfenicol	-	-	-	-	-	-	-	S
Furazolidone/Nitrofurantoin	S	I	S	S	R	-	-	S
Gentamycin	S	-	S	R	S	S	-	S
Josamycin	-	S	-	-	-	-	-	-
Kanamycin	R	I	S	-	-	-	-	S
Lincomycin	S	-	-	-	-	-	-	-
Methicillin	-	S	-	S	S	-	-	-
Nafcillin	S	-	-	-	-	-	-	-
Nalidixic acid	R	R	R	R	-	-	R	R
Neomycin	R	-	S	-	S	S	-	I
Ofloxacin	-	-	-	S	-	-	-	-
Ormetoprim	-	S	-	-	-	-	-	R
Oxacillin	-	-	-	-	-	S	-	-
Oxolinic acid		-	-	-	-	-	R	-
Oxytetracycline	S	-	-	-	S	S	-	S
Penicillin	S	S	S	S	S	S	-	-
Polymyxin B	R	-	R	-	-	-	-	-
Sarafloxacin	-	-	-	-	-	-	-	S
Spiramycin	-	S	-	-	-	-	-	-
Sulfadimethoxine	R	-	-	-	-	-	-	-
Sulfadimethoxine/Ormetoprim	-	-	-	-	S	-	-	R
Sulfadimethoxine/Trimetoprim	-	-	-	S	-	-	-	-
Sulfamethoxazole	-	-	S	-	-	R	-	-
Sulfamonomethoxine	R	-	-	-	-	-	-	-
Streptomycin	R	I	S	-	-	-	-	-
Tetracycline-HCl	-	S	S	S	S	-	-	-
Trimethoprim	-	S	S	-	-	R	-	R
Vancomycin	-	-	-	S	-	-	-	-

Legend: A = Pier and Madin 1976, B = Kitao et al. 1981, C = Foo et al. 1985, D = Eldar et al. 1994, E = Perera et al. 1884, F = Al-Harbi 1994, G = Stoffregen et al. 1996, H = LSU 94-449.

enrofloxacin. These findings have prompted the FDA Center for Veterinary Medicine to permit the use of several of these compounds through Investigational New Animal Drug (INAD) provisions and compassionate or emergency INAD exemptions.

While several authors have reported the use of antibiotics as ineffective (Bercovier et al. 1997; Klesius et al. 1999), little published information is available concerning laboratory or field trials using specific drugs. Stoffregen et al. (1996) reported the unsuccessful treatment of *S. iniae* in hybrid striped bass with oxytetracycline, followed by successful treatment with enrofloxacin. Mortalities in a treated group were only 10.83% using an oral dose of 10 mg/kg, while those in untreated controls reached 55.5%. Treatment of a second group with enrofloxacin produced a less desirable outcome. Due to palatability problems and a moderate minimum inhibitory concentration (MIC) of 0.063 μg/ml against the initial isolate, the dose was decreased to 5 mg/kg when mortalities began in a second tank. Despite the apparent emergence of an enrofloxacin resistant strain (MIC >8.0 μg/ml) mortalities only reached 16.97% versus 39.8% in untreated controls.

Personal observations and reports from producers feeding oxytetracycline and amoxicillin for 10 day treatment periods at doses of 55.5-83.3 mg/kg/day, consistently indicate a decrease in mortalities to near normal levels during therapy followed by a rise to pretreatment levels shortly after the drugs are withdrawn. Treatment seldom appears to eliminate all diseased fish and moribund specimens will often remain circling near the water's surface. These fish with apparent chronic central nervous system involvement are incapable of feeding, but can survive for extended periods, and probably serve as the nidus for the next wave of deaths following the completion of an antibiotic regimen.

Thus, even in the face of a favorable response to an antibiotic treatment, cessation of mortalities should not be equated with elimination of the organism and mortalities should be expected to resume, particularly following any episode of stress.

Failure of antibiotic treatments may result from any or all of the following: 1) Inability of severely affected fish to consume therapeutic levels of medicated feed; 2) Improper use of antibiotics, such as their addition to system water instead of feed or their administration at inadequate dosing levels, or 3) Sequestration of *S. iniae* in the central nervous system where it is inaccessible to certain antibiotics, due to the drugs' inability to penetrate the blood brain barrier. It should be noted, however, that the severity of the meningitis usually seen microscopically would suggest breakdown of the blood brain barrier allowing antibiotics to enter the area. Ineffective length of antibiotic treatment may not eliminate chronically infected individuals that may serve as a source of renewed infection when therapy is completed. The disease could possibly be eliminated if antibiotics are fed for extended periods until all diseased fish have been eliminated from the system. Whether streptococci persist in intracellular locations is an area of debate and intense research (Cleary and Cue 2000). It could be possible that *S. iniae* sequestered within epithelial cells or macrophages might be inaccessible to antibiotics.

Vaccination

In Israel, the use of a conventional formalin killed *S. iniae* bacterin reportedly doubled rainbow trout production over a two year period. In large scale field trials on farms with active disease, a single 100 µl intraperitoneal (IP) injection of bacterin containing 10^{11} cfu/ml administered to 50 gm fingerlings, without adjuvant, provided 95.5% protection for up to 4 months. Mortalities in unvaccinated controls reached 53%.

Such a low mortality rate in vaccinates was achieved even though only 75% of the population was vaccinated, indicating a state of herd immunity, where the susceptibility of individuals is influenced by the immune status of the community as a whole. In addition, weight gains in vaccinates exceeded those of controls by 20% (Eldar et al. 1997).

In the laboratory, 90% protection was maintained for up to 6 months, versus 70-80% mortalities in unvaccinated controls. During this time agglutination titers had fallen from 1:20 one month after to 1:1 six months after vaccination. These findings suggest that the presence of any agglutination titer is significant and might be a useful indicator of exposure as well as protection. Use of a booster, given 2 weeks after the first injection, produced slightly higher titers, but afforded no greater protection at 1 month post-vaccination (Eldar et al. 1997).

Immunoblotting showed sera from vaccinated fish recognized only a single 48 kd protein. In challenged survivors, additional low molecular weight bands, believed to represent specific antibodies directed against capsular antigens, were also present. This would suggest that capsular polysaccharides might play a role in immunity. Complete protection was not produced, however, where hyperimmune serum was used to passively immunize fish, indicating participation of additional cell mediated and nonspecific immune mechanisms are required for full protection (Eldar et al. 1997).

Further evidence supporting the contribution of non-humoral defense systems in protection against *S. iniae* can be found in a study by Hurvitz et al. (1997). In this study, vaccinated rainbow trout were exposed to low (<7 μg/L), medium (50-80 μg/L), and high (180-230 μg/L) ammonia concentrations for 1 month, then challenged with virulent

S. iniae. Stress induced by medium ammonia levels enhanced protection and antibody production, whereas high ammonia levels decreased protection without affecting antibody titers. Because antibody levels did not correlate with protection, the difference in mortalities was attributed to impaired involvement of cell mediated and non-specific mechanisms, although, the effects of ammonia on other physiologic mechanisms, such as respiration, could not be ruled out. With passive immunization by hyperimmune serum, 40% of vaccinates died, versus 73.3% in unvaccinated controls.

Another formalin-killed bacterin, without adjuvant, has been laboratory tested in the U.S. In addition to whole cells, a concentrated cell free extract, derived from culture media, was included in the vaccine. A single IP injection of 100-200 µL of the 4.9×10^9 cfu/ml suspension, followed by IP challenge with 1×10^8 cfu of the virulent parental strain 30 days later, reduced mortalities 91.3% over a 60 day period. The average mortality in vaccinates was 7% compared to 86.7% in unvaccinated controls following challenge (Klesius et al. 1999).

Klesius et al. (2000) has also examined survival in tilapia vaccinated with a formalin-killed bacterin and challenged with heterologous strains of *S. iniae*. In this particular trial, survival was better in fish challenged with the heterologous, rather than homologous vaccine strain. These results indicate that antigenic heterogeneity exists in *S. iniae*, which might complicate development of efficacious vaccines in the future.

Streptococcal Emm Protein

Emm and the M family of Proteins

Pyogenic streptococci belonging to Lancefield group A (*S. pyogenes*) are so designated on the basis of a serologically specific carbohydrate contained in the cell wall

(Lancefield 1933). Immunity and protection are not related to this carbohydrate, but to a surface expressed protein that inhibits phagocytosis in human blood (Lancefield 1962) and may play additional roles in adhesion, invasion, and inflammation (Navarre and Schneewind 1999). Discovered over 70 years ago by Rebecca Lancefield (Lancefield 1928), the group A streptococcal Emm protein molecule is now one of the most intensely studied and best characterized bacterial virulence factors.

The term 'M protein" has been traditionally applied to members of a related group of surface proteins known to possess antiphagocytic properties. Similar proteins with unknown or no antiphagocytic properties were termed "M-like." The "M family" is now known to represent several distinct bioactive proteins sharing common features including a hypervariable N-terminal region, a conserved C-terminal region, heptad repeats with an α-helical coiled-coil structure, an N-terminal leader sequence, and a C-terminal cell wall sorting signal (Navarre and Schneewind 1999). For purposes of this discussion, the term M protein will be applied to all members of the M family, while specific proteins will be referred to by their genetic designations (*emm*, *enn*, and *mrp*).

Some strains of group C (*S. dysgalactiae*, *S. equi*) and group G streptococci also possess M proteins similar to those of *S. pyogenes*. Horizontal gene transfer occurs between these groups (Simpson et al. 1992; Sriprakash and Hartas 1996), but evidence suggests they may only be found on human isolates (Bisno et al. 1987; Bisno et al. 1996). Fischetti (1989) and Robinson and Kehoe (1992) reviewed group A streptococcal Emm proteins. Navarre and Schneewind (1999) reviewed Emm proteins in relation to other streptococcal surface proteins.

Structure

Emm proteins form dimeric 50-60 nm fibrils that appear as hair-like projections on the surface of the cell (Swanson et al. 1969). M proteins contain a seven-residue periodicity of non-polar amino acids that produce an α-helical coiled-coil secondary structure throughout much of the molecule. A short hypervariable random coil sequence at the N-terminus is responsible for the observed antigenic diversity of the protein and forms the basis for serotyping individual Emm types (Dale et al. 1983; Dale and Beachey 1986; Fischetti 1989). In contrast, central and C-terminal domains are almost completely conserved within each specific M subfamily (Navarre and Schneewind 1999). Over 100 distinct Emm serotypes have been identified to date (Kehoe 1991) and the production of protective antibodies is type specific (Lancefield 1962).

Determination of the Pep M5 sequence (Manjula et al. 1984; Manjula et al. 1985) and cloning of the entire M6 gene (*emm*6) (Hollingshead et al. 1986) provided the first complete structural analysis of an Emm molecule. Eleven distal amino acids form a non-helical N-terminus with a high net negative charge (Fischetti 1989). This N-terminal segment plays a central role in the biological activity of the molecule, as antibodies directed against this fragment opsonize the specific Emm type streptococcus for phagocytosis (Dale and Beachey 1986).

The α-helical coiled-coil region of the Emm6 protein forms a central rod comprising 80% of the molecule and is composed of four sequence repeat regions, designated A-D (Hollingshead et al. 1986). Within each repeat region the number and size of the repeating subunits varies, as well as the degree of sequence conservation. Regions A and B contain five direct tandem repeat blocks of 14 and 25 amino acids

each, respectively. Region C is composed of two and one-half tandem repeats of 42 amino acids, less conserved than in regions A and B. Region D consists of four seven-residue repeats (Fischetti 1989). Considerable size difference exists between Emm serotypes and even between strains bearing the same Emm typing antigen. Size variations of 41-80 kDa arise through insertions, deletions, replications, and recombinations within repeat regions of the α-helix (Fischetti and Fazio-Zanakis 1985).

The seven-residue periodicity of the α-helical repeats forms a structure in which a large number of amino acid substitutions are possible, providing antigenic diversity, without disrupting the molecule's basic conformation (Fischetti 1989). A thermodynamically stable ropelike dimer is formed from two helical chains coiling around each other (Manjula and Fischetti 1980). Periodic disruptions in the periodicity of hydrophobic residues, which normally occur at regular intervals within heptads provide more or less flexibility (Fischetti et al. 1988).

Pepsin digestion of whole Emm bearing streptococci at suboptimal pH cleaves the molecule into two approximately equally sized fragments (Beachey et al. 1974). The site of pepsin cleavage is usually located between the B and C repeats and forms a hinge region in the molecule (Hollingshead et al. 1986; Miller et al. 1988; Mouw et al. 1988). The distal or N-terminal PepM fragment retains much of the protein's biological properties, including its type-specific and antiphagocytic domains (Fischetti 1989). The proximal C-terminal half serves to anchor the molecule to the cell surface (Pancholi and Fischetti 1988; Piard et al. 1997).

The C-terminus is composed of two distinct regions. The more distal portion is characterized by a series of regularly spaced proline and glycine residues. The proximal

portion contains 19 hydrophobic amino acids with a positive charged six amino acid tail (Pancholi and Fischetti 1988; Fischetti 1989). The proline- and glycine-rich region introduces random coils believed to assist in intercolation of the molecule through the peptidoglycan layer, to which it corresponds, as it is exported to the cell surface (Navarre and Schneewind 1999). The hydrophobic region and charged tail are believed to anchor the protein in the cell membrane (Pancholi and Fischetti 1988).

Alternatively, Emm proteins may be covalently attached to the peptidoglycan cell wall by a mechanism similar to that of staphylococcal protein A. Protein A utilizes an LPXTG amino acid signal sorting motif, which is conserved among all cell wall anchored proteins of gram-positive bacteria. All members of the M family possess an N-terminal leader peptide, responsible for directing the molecule into the protein secretory (Sec) pathway, and a C-terminal LPXTG type signal sorting sequence located between their proline- and glycine-rich and hydrophobic amino acid regions. The LPXTG motif signals cell wall anchoring of protein A, while membrane retention is signaled by the positively charged tail, which is retained in the cell cytoplasm. It is proposed that the protein is then cleaved between the threonyl and glycyl residues of the LPXTG motif and that the newly formed C-terminus is transferred by a sortase enzyme to the cell wall pentaglycine crossbridge to which it is covalently bound (Piard et al 1997; Navarre and Schneewind 1999). In addition to protein A, the C-terminal regions of Emm proteins share strong sequence homology with immunoglobulin binding protein G of the group G streptococci and to all members of the M family of proteins (Pancholi and Fischetti 1988; Podbielski et al. 1991).

Function

The gram-positive peptidoglycan cell wall, cell membrane, and streptococcal lipoteichoic acids are all capable of activating the alternative complement pathway (Bisno 1979). Emm positive group A streptococci are resistant to phagocytosis in human blood in the absence of type-specific antibodies (Lancefield 1962), while strains lacking an Emm protein activate the alternative complement pathway, are opsonized, and cleared following deposition of C3b (Bisno 1979; Peterson et al. 1979; Jacks-Weis et al. 1982). The mechanism by which Emm positive strains escape the opsonizing effects of complement is not fully understood, but it is believed to involve plasma constituents known as regulators of complement activation (RCA). Several down regulators of complement activation have been identified as likely candidates, including the β-globulin factor H (FH), a truncated variant of FH known as factor H-like protein 1 (FHL-1), and C4 binding protein (C4BP).

Binding of FH to the C-repeat domain has been reported as the essential factor needed to accelerate the degradation of C3b, preventing opsonization (Fischetti et al. 1995). More recent findings, however, suggest FHL-1, which binds with higher affinity than FH, may be the more relevant ligand of class I Emm and that binding occurs to the hypervariable N-terminus (Johnsson et al. 1998; Kotarsky et al. 1998). Other Emm proteins, especially class II, bind C4BP in their N-terminal domain (Johnsson et al. 1996). It has also been reported that fibrinogen binding by Emm may exert an antiopsonic effect on the bacteria in non-immune blood by masking C3b binding sites (Whitnack and Beachey 1985; Poirier et al. 1989). The high net negative charge carried

by the Emm N-terminus may impair phagocytosis simply by contributing to electrostatic repulsion between the two cell types (Fischetti 1983).

Additional functions attributed to Emm proteins remain controversial or poorly understood. Some Emm types and other members of the M family are capable of binding immunoglobulins. However, the role of immunoglobulin binding in the pathogenesis of group A streptococcal infections, if any, is not clear (Bessen 1994). Certain class I Emm are reported to bind one or more IgG subtypes, while some class II Emm bind both IgG and IgA (Navarre and Schneewind 1999).

It has been speculated that M proteins are involved in the pathogenesis of group A streptococcal infections by acting as adhesins to host tissue, promoting tissue invasion, and by initiating an inflammatory response. Emm proteins have been demonstrated to bind primarily to epithelial cell surfaces, including human cheek cells (Ellen and Gibbons 1972), human laryngeal cells (Wang and Stinson 1994), and to human keratinocytes (Okada et al. 1994). Binding can be impaired by trypsinization and type-specific antisera (Ellen and Gibbons 1972; Okada et al. 1994), but the presence of other adhesins cannot always be ruled out (Wang and Stinson 1994). Some M proteins also bind F-actin, present on the surface of a number of cell types, including fibroblasts (Shearer and Gilliam 1984; Chalovich and Fischetti 1986). At least one Emm type has been reported to bind fibronectin, a ubiquitous component of basement membranes and the extracellular matrix present throughout the body, which would provide a distinct advantage in adherence (Reichardt et al 1995).

Binding of fibrinogen, kininogen, and plasminogen may allow Emm positive streptococci to invade deeper into tissues, by evading phagocytes, dissolving clots and

disrupting the extracellular matrix (Navarre and Schneewind 1999). Fibrinogen binding blocks the association of the bacterial surface with polymorphonuclear cells (Whitnack and Beachey 1982). Kininogen, the inactive precursor to the vasoactive pro-inflammatory mediator bradykinin promotes increased vascular permeability and plasma leakage. Binding kininogen could promote spread of the infection and provide nutrients to the multiplying bacteria (Ben Nasr et al. 1997). Plasminogen, precursor to fibrinolytic plasmin, dissolves clots and degrades extracellular matrix proteins also promoting tissue spread (Lottenberg et al. 1992; Boyle and Lottenberg 1997).

The *mga* Regulon

Organization and Regulation

A multiple gene regulator, similar to effector proteins of other two component bacterial regulatory systems, called Mga (formerly *vir*R or *mry*), regulates, at least in part, transcription of a virulence regulon including members of the M family and C5a peptidase (Caparon and Scott 1987; Simpson et al. 1990; Podbielski et al. 1995). Located outside the Mga regulon, but under its promotional effect is the OF/*sfb*II gene (McLandsborough and Cleary 1995). The *mga* gene, located immediately upstream of the M protein genes, encodes a DNA binding protein that activates transcription by binding a consensus site found in promoters of the regulon members (McIver et al. 1995b).

The *mga* regulon of OF$^+$ class II Emm group A streptococci are organized in the following manner: *mga*, *mrp*, class II *emm*, *enn*, and *scp*A. The regulons of OF$^-$ class I Emm are more variable in composition. They may resemble those of class II Emm, but most lack *mrp* and *enn* genes, while always containing a class I *emm*.

The mga gene is similar to the receiver proteins of two-component signal transducing systems, acting as the second, or effector, component in conjunction with a membrane spanning sensor protein responsive to environmental changes (Caparon et al. 1992). Using transposon mutagenesis, a potential sensor protein acting as a global regulator of virulence determinants in *S. pyogenes* M49 has been identified. Disruption of the pleiotropic effect locus (*pel*) effected both surface and secreted proteins and yielded bacteria with the following phenotypes: decreased β-hemolysis mediated by streptolysin S, reduced activity of a secreted cysteine protease (SpeB), streptokinase, and altered immunoglobulin and fibrinogen binding mediated by Emm49 (Li et al. 1999).

Mga regulated transcription of *emm* is enhanced by increased environmental levels of CO_2. Regulation of virulence factor expression based on levels of CO_2 could allow the bacteria to distinguish epithelial surfaces from deeper tissues where CO_2 is higher and the need to avoid non-specific immune system components, such as complement, is greater (Caparon et al. 1992). Increased osmolarity, low temperature, growth with free exchange of gasses, and restricted availability of iron decrease transcription of *emm* (McIver et al. 1995a). In vivo, Emm is expressed early in the course of infection, but may become undetectable in convalescent patients who have developed opsonic antibodies against the specific serotype (Simpson et al. 1990).

Class I and II Emm Proteins

Emm proteins can be divided into two classes based on the presence or absence of an antigenically conserved domain mapped to the C-repeat region of the molecule, referred to as MAP I (class I) and MAP II (class II), respectively. The absence of this conserved domain correlates to the activity of a lipoproteinase termed opacity factor

(OF/SbfII), which imparts opalescence to serum (Bessen et al. 1989). Class I Emm occur almost exclusively on group A streptococci negative for opacity factor (OF^-) and are capable of experimentally binding albumin, fibrinogen, kininogen, plasminogen, fibronectin, IgG, FH, and FHL-1. Class II Emm proteins are opacity factor positive (OF^+) and have been shown to bind albumin, plasminogen, IgA, IgG, and C4BP (Navarre and Schneewind 1999). Class I OF^- group A streptococci are most often associated with acute rheumatic fever (ARF), while class II OF^+ types primarily cause pyodermas and nasopharyngeal infections (Bessen et al. 1989).

Mrp, Enn, ScpA, and OF

Other components of the *mga* regulon include *mrp*, *enn*, and *scp*A. Mrp (FcrA), present in OF^+ cells, is always located immediately downstream from *mga* and binds human IgG subtypes 1, 2, and 4 (Stenberg et al. 1997). Some types bind fibrinogen (Krebs et al. 1996) and appear to be antiphagocytic (Podbielski et al. 1995). Enn, present almost exclusively in OF^+ cells, occupies the third open reading frame following *mga* (Podbielski et al. 1995) and have IgA binding activity (Bessen and Fischetti 1992). None are antiphagocytic and they are expressed in low levels, leading to speculation that they act primarily as sequence reservoirs used for recombination with *emm* and *mrp* to generate antigenic diversity (Navarre and Schneewind 1999).

Unrelated to the M family of proteins, but included in the *mga* regulon is *scp*A, which encodes a C5a peptidase. The protease ScpA inactivates C5a, a biologically active component of the complement cascade, which acts as a potent chemoattractant for polymorphonuclear cells (Wexler et al. 1993; Wexler and Cleary 1985). Insertional

inactivation of *scpA* demonstrated that the C5a peptidase plays a role in virulence in an experimental mouse model (Ji et al. 1996).

Located outside the *mga* regulon, but under the promotional effects of Mga, is the gene encoding OF/SfbII. Cleavage of the apolipoprotein component of the high-density lipoprotein fraction of serum renders it insoluble, leading to serum opacification. OF is also capable of binding fibronectin (Rakonjac et al. 1995; Kreikemeyer et al. 1995), whose potential role in tissue adhesion has been discussed previously. While OF^+ phenotypes are linked to class II Emm (Bessen et al. 1989), the gene for OF has been shown to occupy a separate locus (Rakonjac 1995).

Preparation and Identification of Emm Proteins

Numerous methods have been utilized to isolate and purify Emm proteins, although most yield a spectrum of heterogeneous polypeptides, non-representative of the native molecule (Fischetti 1989). Emm are destroyed by proteolytic enzymes, such as trypsin (Lancefield 1962). Trypsinized living streptococci are stripped of their surface Emm, but if they are placed in fresh media newly synthesized fibrils in the region of the forming cleavage section can be demonstrated with electron microscopy (Swanson et al. 1969).

The traditional method for preparing crude Emm involved extraction in acid. Streptococci were initially boiled in dilute HCl (pH 2.0) for 10 minutes and then were subjected to neutralization and purification steps (Lancefield 1928). Additional purification methods include extraction by sonication (Besdine and Pine 1968; Ofek at al. 1969), alkali (Fox and Whittner 1965; Cunningham and Beachey 1975), guanidine hydrochloride (Russell and Facklam 1975), nonionic detergent (Fischetti 1977; Fischetti

1978), cyanogen bromide (Vosh and Williams 1978), and nitrous acid (Hafez et al. 1981).

Pepsin digestion at the suboptimal pH of 5.8 (Beachey et al. 1974) has proven to be one of the better methods for obtaining Emm fragments. With this method, pepsin cleaves the molecule from the streptococcal surface at a specific site releasing fragments of 20,000-40,000 kDa, termed PepM (Fischetti 1989). Completion of the 197-residue PepM5 sequence made a complete analysis of the biologically active N-terminal half of the molecule possible for the first time (Manjula et al. 1984).

Extraction of Emm, following solubilization of the cell wall with phage-associated lysin, was particularly useful in the study of larger fragments of the molecule (Fischetti et al. 1974; Cohen et al. 1977). Use of phage lysin has lead to the current theory that Emm are cell wall rather than cell membrane anchored (Piard et al. 1997). Ultimately, the first complete amino acid sequence (Emm6) was determined by cloning and sequence analysis techniques (Hollingshead et al. 1986).

More recently, the polymerase chain reaction (PCR) has been applied to the study of Emm. A primer pair was constructed by evaluating homologous regions of six published *emm* that amplified whole gene sequences from 29 group A, one group C, and one group G streptococci. The forward primer corresponded to conserved regions overlapping the Shine-Dalgarno box and first two codons of the *emm* N-terminus. The reverse primer was directed toward the last five codons and extended three nucleotides beyond the stop codon of the *emm* C-terminus (Podbielski et al. 1991).

Streptococcal Virulence Factors Other Than Emm Protein

Introduction

Streptococci are obligate parasites of mucosal surfaces of humans and animals. Some are considered resident flora, only causing infection when introduced into normally sterile sites or immunocompromised hosts. Other species are true pathogens, which are spread between individuals and are capable of causing serious infections in normal non-immune hosts (Kilian 1998). Representatives of the pyogenic group of α-, β- and γ-hemolytic streptococci produce a broad spectrum of virulence factors, which allow them to invade host tissues and evade defensive mechanisms. Of these, the Group A streptococci (*S. pyogenes*) have been most intensely studied and their virulence factors best characterized.

The role of the M family of surface proteins in the pathogenesis of Group A, C and G streptococcal infections has been previously discussed. Proven or purported virulence factors found in the pyogenic streptococci include capsule production and the cytolytic hemolysins, streptolysins O and S, and the unrelated hemolysin of the Group B streptococci (*S. agalactiae*). The streptococcal pyrogenic exotoxins (SPEs) and superantigens of Group A streptococci are responsible for the rash of scarlet fever and believed to participate in the pathogenesis of toxic shock-like syndrome. Streptokinase (fibrinolysin), a plasminogen activator, and the enzyme hyaluronidase, are believed to act as spreading factors by dissolving fibrin clots and connective tissue ground substance, respectively. Neuraminidase (sialidase) is considered a potential mucosal colonization factor for pathogens and commensals (Koneman et al. 1997; Kilian 1998). DNases are believed to contribute to the virulence of Group A streptococci and other gram-positive

organisms, like *Staphylococcus aureus* (Podbielski et al. 1996). Selected factors are reviewed in detail below.

Capsule Production

Certain species of streptococci are known to produce antiphagocytic polysaccharide capsules that may have either linear repeating primary structure or may possess side chains that add secondary structure and create more complex immunodeterminants (Kasper 1986). The capsule of *S. pneumoniae* is one of the best documented of all bacterial virulence factors and represents one of the milestones in the understanding of bacterial pathogenesis. The antiphagocytic properties of capsules have been attributed to repulsion of phagocytes by electrostatic charge and through failure to activate the classical complement pathway in the absence of specific antibody. In contrast, the cell walls of non-encapsulated mutants directly activate the alternative complement pathway (Kasper 1986). While several authors have described *S. iniae* as possessing a capsule (Pier and Madin 1976; Kitao et al. 1981), its composition and potential role in the pathogenesis of this organism have not been investigated.

Some strains of *S. pyogenes* produce a hyaluronic acid capsule, which is not immunogenic, presumably because it is indistinguishable from the hyaluronic acid of mammalian connective tissues (Kilian 1998). Only 3% of isolates from uncomplicated cases of pharyngitis produce a capsule, in contrast to 21% from severe streptococcal infections and 42% of rheumatic fever isolates (Johnson et al. 1992). Transposon mutagenesis studies of capsule-negative strains confirm its importance as a virulence factor by negating the opsonic effects of complement C3 binding for phagocytic killing in

blood (Dale et al. 1996). Acapsular mutants also show a 100-fold reduction in virulence in mice (Wessels et al. 1991).

Clinical isolates of group B *S. agalactiae* also produce a polysaccharide capsule. A total of nine serologically distinct capsule types (Ia, Ib, and II-VIII) have been identified. Non-hemolytic group B, type Ib streptococci have been implicated in large multiple species fish kills in natural marine environments (Robinson and Meyer 1966; Plumb et al. 1974; Baya et al. 1990). The group B streptococci, including *S. difficile*, possess whole-cell protein patterns and phenotypic characteristics similar to those of other type Ib variants of *S. agalactiae* (Elliott et al. 1990; VanDamme et al. 1997). Encapsulation of these organisms inhibits phagocytosis and complement activation in the absence of specific antibody. Transposon mutagenesis studies of a type III capsular isolate resulted in loss of virulence in a neonatal mouse model (Rubens et al. 1987). Other mutagenesis studies have demonstrated the surface expression of sialic acid residues as an essential component of the capsule necessary for evasion of host defenses (Wessels et al. 1989). Failure to activate the alternative pathway resides in the terminal sialic acid residues of side chains, which bind complement regulatory factor H and ultimately results in cleavage of bound C3b (Wessels 1997).

The capsule gene region of several encapsulated streptococci consist of a group of polysaccharide-specific genes encoding glycosyltransferases and polymerases. Conserved sequences flanking these genes are believed to direct polymerization, transport, and regulation (Cieslewicz 2001). The size of capsule producing operons vary with number of monosaccharides present and complexity of the capsule's organization. The *has* operon encoding the hyaluronic acid capsule of *S. pyogenes* requires only two

essential genes, *has*A for hyaluronan synthase, which adds alternating N-acetyl-D-glucosamine and D-glucuronic acid residues to form the linear polymer, and *has*B, which forms glucuronic acid from D-glucose (Ashbaugh et al. 1998). The capsules of group B streptococci are more complex and their operons are correspondingly larger (Cieslewicz 2001).

Streptolysins

Zones of hemolysis surrounding colonies of streptococci growing on blood agar media were one the first characteristics used to recognize clinically significant isolates. Many pyogenic streptococci produce two distinct hemolysins, the oxygen labile streptolysin O and the oxygen stable streptolysin S. These hemolysins destroy erythrocytes to produce a zone of complete, or β-hemolysis. Other streptococci produce a zone of greenish discoloration, or α-hemolysis, due to the production of hydrogen peroxide, resulting in the conversion of hemoglobin to methemoglobin (Kilian 1998).

Streptolysin O (SLO) is one of a family of antigenically related, thiol-activated cytolysins produced by several genera of gram-positive and gram-negative bacteria, including *S. pneumoniae* (pneumolysin), *Clostridium tetani* (tetanolysin), and *C. perfringens* (θ-toxin), and *Bacillus cereus* (cereolysin). All compete for a common binding site on target cell membranes, believed to be cholesterol and are inhibited by small amounts of cholesterol (Cowell and Bernheimer 1977). This 50-60 kDa group of proteins all induce the formation of hydrophilic channels in cell membranes, which lead ultimately to their lysis (Kehoe 1987). With the exception of the mouse, SLO is capable of lysing erythrocytes, polymorphonuclear leukocytes, monocytes, and platelets from most domestic and laboratory species (Koneman 1997; Kilian 1998). Due to its

instability in oxygen, it is primarily responsible for the β-hemolysis seen in stabbed regions of blood agar plates (Koneman 1997). While the biological significance of SLO is not known, it is acutely cardiotoxic, producing death within seconds with injected intravenously into laboratory animals (Halbert et al. 1961).

Streptolysin S (SLS) is oxygen stable, largely cell bound, non-antigenic, and also damaging to the membranes of lymphocytes, neutrophils, platelets, and certain tissue culture and tumor cells. Despite decades of detailed investigations, the exact chemical nature of SLS remains unknown. By weight, it is one of the most potent cytotoxins known (Nizet et al. 2000). Its activity is seen in the classic surface and subsurface hemolysis of sheep erythrocytes on blood agar plates (Kilian 1998). The toxin is synthesized *de novo* in the bacteria and released into the medium only upon exposure to certain "carrier" substances, such as albumin and α-lipoprotein (Akao et al. 1992). Most of the oxygen stable hemolysins of the pyogenic streptococci have similar properties, except that produced by *S. agalactiae*, which is similar, but distinct (Marchlewicz and Duncan 1980).

A contiguous nine-gene locus (*sagA-sagI*) involved in SLS production has recently been identified in *S. pyogenes*. Targeted mutagenesis of each gene in the *sag* operon resulted in an SLS-negative phenotype. A recombinant plasmid carrying the *sag* operon conferred a β-hemolytic phenotype upon the non-hemolytic *Lactococcus lactis*. Sequence homologies of *sag* operon gene products suggest SLS is related to the bacteriocin family of microbial toxins, which are genetically encoded by an operon including a structural prepropeptide gene and genes necessary for chemical modification, processing, and export of the mature form of the hemolysin (Nizet et al. 2000). The

hemolytic activity of *S. equi* has also been attributed to an SLS-like hemolysin (Flanagen et al. 1998).

There is good evidence of a role for SLS as a virulence determinant in Group A streptococcal infections, including: 1) Its location bound to the cell surface; 2) Its continuous synthesis even in resting cells; 3) Its non-immunogenicity; 4) Its extractability by certain serum proteins; 5) Its ability to be transferred directly to target cells while being protected from inhibitory agents present in inflammatory exudates; 6) Its membrane pore forming capabilities; 7) Its ability to synergize host derived proinflammatory agonists; and 8) The reduced pathogenicity of SLS negative mutants (Ginsburg 1999). In a mouse model of subcutaneous infection, the virulence of *S. pyogenes* Tn916 mutants, of different Emm serotypes containing identical mutations, is markedly reduced. Injected subcutaneously, mutants were found to be incapable of inducing tissue damage or a neutrophilic response (Betschel et al. 1998, Nizet et al. 2000).

Streptokinase

Group A streptococci grown in the presence of human plasma generate the fibrinolytic enzyme plasmin from its inactive zymogen, the glycoprotein plasminogen, found in circulating blood and extravascular spaces (Lottenberg et al. 1992). Plasmin takes part in several physiological processes in mammals, including blood clot dissolution, cellular migration, trophoblast implantation, and cancer metastasis (Lottenberg et al. 1994; Johnsen et al. 1999). Once activated, plasmin is captured on a specific high affinity cell surface receptor, where it remains enzymatically active, escaping neutralization by α-antiplasmin, the normal regulatory molecule of the

mammalian host (Lottenberg 1992). The extracellular bacterial product responsible for plasmin activation is streptokinase, a product of the *skc* gene, which has been cloned and its nucleotide sequence determined (Malke and Ferretti 1984). Furthermore, if the bacteria are liberating a streptokinase-like protein into a medium containing 30% citrated human plasma, clot formation is inhibited when Ca is added back to the media to reconstitute clotting factors, (Donabedian and Boyle 1998).

Streptokinase is believed to act as a virulence factor by preventing the formation of fibrin barriers around foci of infection. In addition to degrading polymerized fibrin, it is also capable of degrading mammalian extracellular matrix proteins including fibronectin and laminin, and enhances collagenase activity. Degradation of these normal tissue barriers promotes penetration through mucosal and cutaneous barriers into deep tissue sites (Lottenberg 1992). Groups A, C and G streptococci contain an *skc* gene, produce streptokinase, and demonstrate surface receptors for plasmin. Lancefield Groups B, D, F, H, K, L, M, N, O, P, R, U, and *S. pneumoniae* do not. Although antigenic heterogeneity exists even among Group A streptococcal serotypes, there is 90% homology between the Group A, C and G streptokinases (Huang et al. 1989; McCoy et al. 1991).

A correlation exists between the host ranges of different streptococcal species and activation of plasminogens, reflecting the host origin of the isolate. Group A *S. pyogenes* has a host range limited essentially to humans, while Group C streptococci infect a variety of animals, including humans. *Streptococcus pyogenes* activates only human plasminogen and binds only activated human plasmin (Schroeder et al. 1999). Group C streptococci (*S. equi, S. equisimilis, S. zooepidemicus*) recovered from

humans, horses and pigs exhibit streptokinase activity only when grown in the presence of plasminogen molecules that parallel the host range of the microorganisms. The streptokinases are antigenically related and bind all three plasminogens, indicating conservation of binding sites despite the observed species-specific activation. Bovine isolates of Group C *S. dysgalactiae* are also capable of activating plasminogen in a species-specific manner by an activator that remains to be characterized (Leigh et al. 1998). The species specificity of this relationship indicates that streptokinase plays a role in establishing the host range of certain pathogenic streptococci (McCoy et al. 1991).

Plasminogen has two physiological activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator(uPA). Both are serine proteases and activate plasminogen by cleavage of a single peptide bond (Johnsen et al. 1999). Streptokinase is not a protease like most other prokaryotic and eukaryotic plasminogen activators. Instead it forms a 1:1 stoichiometric complex with plasminogen, triggering a conformational change and self-cleavage that results in formation of the serine protease plasmin (Huang et al. 1989; Lottenberg et al. 1992). Streptokinase from *S. equisimilis* is approximately 47 kDa, 414 amino acids in length and comprised of 3 domains. Plasminogen is believed to bind initially to the C-terminal domain of streptokinase to form the active enzyme complex, while additional substrate plasminogen binds to the N-terminal domain and is converted to active plasmin (Young et al. 1998).

Capturing host plasmin(ogen) is a mechanism used by other genera of pathogenic gram-positive and gram-negative bacteria, believed to contribute to invasiveness. *Streptococcus uberis*, *Staphylococcus aureus* (staphylokinase), *E. coli* and *Yersinia pestis* all share the ability to activate plasminogen through the expression of some type of

extracellular bacterial product (Lottenberg et al. 1994). The plasminogen activator of *S. uberis* shares only weak homology (23.5-28%) with streptokinase from the other streptococcal groups noted above (Rosey et al. 1999). There is no significant nucleotide or amino acid homology shared between the remaining families of biologically relevant plasminogen activators (Lottenberg et al. 1994).

Recently a novel plasminogen activator has been described in *S. uberis*, which also has surface associated activity and species-specificity (Leigh 1993; Leigh 1994; Leigh and Lincoln 1997). The bovine plasminogen activator of *S. uberis* has been sequenced and cloned, and is highly conserved within the species. The 33kDa, 286 amino acid protein shares only weak homology (26%) with the streptokinases of *S. equisimilis* and *S. pyogenes*. The plasminogen activator gene, *pauA*, was located at a site in the chromosome distinct from that encoding the *skc* genes for streptokinase in other streptococci (Rosey et al. 1999).

Hyaluronidase

Bacterial hyaluronidases have been described as a group of neglected enzymes in relation to their potential activity as virulence factors (Kriel 1995). Hyaluronidases depolymerize hyaluronic acid, a linear unsulfated glycosaminoglycan polymer, which forms the ground substance of connective tissues. For years hyaluronidase production has been considered a "spreading" factor, believed to aide in the pathogenesis of certain bacterial infections by promoting their dissemination through connective tissue barriers (Duran-Reynals 1942). Unlike some of the more well studied bacterial virulence factors, much of the information on the role of hyaluronidase as a virulence factor remains speculative, with little or no direct supporting evidence (Hynes and Walton 2000).

Hyaluronidase is a general term applied to three types of enzymes capable of degrading hyaluronate, however, some are also able to cleave chondroitin sulfate. In addition to bacteria, hyaluronidases are also produced by certain mammalian cell types (spermatozoa), and are found in the venoms and secretions of some reptiles and invertebrates. The hyaluronidases produced by bacteria form a distinct type classified as hyaluronate lyases, or more specifically endo-N-acetylhexosaminidases, based on their ability to attack β-1-4 linkages in hyaluronate to form disaccharides of N-acetylglucosamine and glucuronic acid (Kriel 1995).

A wide range of microorganisms, including both gram-negative and gram-positive bacteria and some yeasts, produce hyaluronidases. The enzymes produced by gram-negative organisms are periplasmic and appear less likely to act as spreading factors. Hyaluronidases produced by pathogenic gram-positive bacteria are excreted into the extracellular environment. Many of these organisms initiate infections at mucosal or skin surfaces in humans and animals, where the bulk of the bodies' hyaluronate is found (Hynes and Walton 2000).

Hyaluronidase activity has been detected in virtually all strains of *S. pyogenes* and in some strains of *S. agalactiae*, *S. equi*, *S. dysgalactiae*, *S. uberis*, *S. suis*, *S. intermedius*, *S. constellatus*, *S. salivarius*, *S. mitis*, and *S. pneumoniae* (Schaufuss et al. 1989; Kilian 1998; Hynes and Walton 2000). Representatives of other gram-positive genera capable of producing hyaluronidase include *Staphylococcus*, *Peptostreptococcus*, *Propionibacterium*, *Streptomyces*, *and Clostridium*. Some *S. pyogenes* isolates exhibit two hyaluronidases, one an extracellular bacterial product and the other of bacteriophage origin. Bacteriophages from *S. pyogenes* and *S. equi* encode a distinct non-secreted

hyaluronidase that probably plays no role in the pathogenesis of streptococcal infections (Hynes and Walton 2000).

Eight complete hyaluronidase genes and two bacteriophage genes have had their nucleotide sequences determined. Molecular weights of deduced amino acid sequences vary from 36-40 kDa in the bacteriophage encoded enzymes to 121 kDa in *S. agalactiae* (Hynes and Walton 2000). The purified enzymes from *S. pyogenes*, *S. equi*, and *S. uberis* have molecular weights of approximately 54 kDa (Schaufuss et al. 1989). Proteins from the gram-positive organisms, with the exception of the clostridial hyaluronidase, appear related, with global similarities between *S. agalactiae*, *S. pneumoniae*, and *S. aureus* of 65% and local similarities as high as 80%. Phylogenetic analysis, using amino acid sequences deduced from cloned and sequenced genes of gram-positive hyaluronidases, suggests relatedness among some of the enzymes (Hynes and Walton 2000).

The viscous consistency of connective tissue hyaluronate is believed to resist penetration by bacteria and the dissemination of their toxins. If true, then the production of bacterial hyaluronidases could play a critical role in the spread of pathogenic organisms into deeper tissue sites from epithelial surfaces. The production of metabolizable disaccharides from hyaluronate could also supply nutrients to an invading pathogen (Hynes and Walton 2000). Despite mounting evidence, however, the role for hyaluronidase as a virulence factor remains largely inferred. A few examples are included below.

Significantly more strains of hyaluronidase producing human streptococci belonging to the *S. milleri* group (*S. angiosus*, *S. intermedius*, *S. constellatus*) are

isolated from internal abscesses, when compared to isolates collected as part of the normal mucosal flora. Hyaluronidase production among these isolates was most common in β-hemolytic strains, suggesting hyaluronidase may also aide dissemination of the toxin (Unsworth 1989). Similarly, in *Clostridium perfringens*, hyaluronidase production is believed to facilitate spread of the tissue damaging α-toxin, potentiating its cytolytic activity (Canard et al. 1994). Only hyaluronidase positive strains of *S. pneumoniae* appear to be capable of causing meningitis, while only 15% of strains from carrier individuals produce the enzyme. Negative strains can be induced to cause meningitis if inoculated with exogenous hyaluronidase, suggesting it may be involved in breeching the blood brain barrier (Kostyukova et al. 1995). Using signature-tagged mutagenesis in a mouse model of pulmonary infection by *S. pneumoniae*, hyaluronidase production was identified as a factor in the development of pneumonia, but not in septicemia (Polissi et al. 1998).

DNase

Group A streptococci express up to four types of secreted DNases and are the products of *sda* genes A-D. Similar nucleases have also been demonstrated in Group B, C and G streptococci (Podbielski et al. 1996) and in *Staphylococcus aureus* (Chesbro and Walker 1972). Although Group A streptococcal infections are correlated with the production of anti-DNase B antibodies, a specific role for the DNases in the pathogenesis of these infections remains unclear. It has been debated that these enzymes could have only an indirect affect on virulence by providing an organism with oligonucleotides for growth (Wilson 1945). Despite this, DNases have been implicated by others as virulence factors in Group A streptococci, for several reasons: 1) They

attack a molecule essential to the activity of any potential target cell; 2) They are produced by all strains of Group A streptococci tested; 3) Production in less virulent Group B, C and G streptococci is less common and less enzyme is liberated; 4) Anti-DNase B antibodies appear after most infections with *S. pyogenes* (Podbielski et al. 1996).

Transposon Mutagenesis

Introduction

The use of transposons to induce mutations in the genetic material of pathogenic bacteria has proven to be a powerful tool in molecular genetics for dissecting the virulence mechanisms of these agents. A transposon is introduced by a process that results in the production of random insertions throughout the genome of the recipient population of bacterial cells. The mutants created typically carry a selectable marker that allows them to be screened for the loss of phenotypic traits believed to be associated with virulence. Ultimately, the virulence of the mutant created is compared to that of its wild type parental strain in an animal model (Caparon and Scott 1991).

Transposons and Transposition

Transposons are DNA elements capable of relocating, or transposing, from one site in a DNA molecule to another. First identified in plants in the 1950s, transposons are known to exist in all living organisms. This mechanism of non-homologous recombination may occur between plasmids, from plasmid to chromosome, or within the same genome, but the transposon always appears at a location different from the site at which it was originally found (Snyder and Champness 1997).

Relocation by a transposon is called transposition and the enzymes that promote its movement are transposases. Most transposons encode their own transposases and, through various means, tightly regulate their activity, making transposition a rare event. Transposition frequencies range from 1 in 10^3 to 10^8 cell divisions, making the chance of a transposon induced mutation only slightly greater than the spontaneous mutation rate. While transposons may confer benefits upon a bacterial cell, such as antibiotic resistance, an obvious consequence is the accumulation of deleterious or potentially lethal mutations within a population of cells (Freifelder 1987, Snyder and Champness 1997).

Some transposons regularly move among different genera of bacteria, either during transfer of promiscuous plasmids or via transducing phage. Some are themselves (Tn916) or can be induced to form phage (Mu). All bacterial transposons transpose by one of two mechanisms. The Tn3 family uses a replicative mechanism in which the entire transposon replicates during transposition resulting in two copies of the transposon. The transposase makes single-stranded cuts in the 3′ ends of the transposon and a staggered break in the target DNA. A cointegrate intermediate forms between donor and target DNAs, that must be resolved into separate molecules by transposon encoded site-specific recombinase enzymes (resolvases) at internal *res* sites (Freifelder 1987, Snyder and Champness 1997).

Other transposons, such as Tn10, transpose by a cut and paste mechanism in which the transposase cuts both strands of the transposon out of the donor DNA and pastes it into a target site. In both mechanisms, the transposase make staggered breaks in the target DNA that must be duplicated. In replicative transposition, DNA replication must proceed over the staggered break and entire length of the transposon. In cut and

paste transposition, replication proceeds only a short distance to fill the staggered breaks (Snyder and Champness 1997).

There are many different types of bacterial transposons. The simplest, called insertion sequence elements, encode only genes necessary for transposition. Larger transposons, composite or non-composite, typically contain additional genes, such as those for antibiotic resistance. All bacterial transposons are flanked by inverted repeats, which are nucleotide sequences on opposite strands of DNA that are identical, or nearly identical, when read in the 5′ to 3′ direction (Freifelder 1987, Snyder and Champness 1997).

As a result of insertion, short direct repeats, having the same 5′ to 3′ sequence, bracket the transposon. Prior to transposition, target DNA contains only one copy of the sequence, but during insertion the staggered breaks created by transposase enzymes must be duplicated to restore continuity of the target DNA. Most transposons exhibit little or no target specificity, so direct repeats will vary. However, even though the repeats may differ, the number of duplicated base pairs is characteristic of the transposon. For example, Tn3 always duplicates 5 base pairs at an insertion site (Freifelder 1987, Snyder and Champness 1997).

Transposon Tn917

Until recently, transposons useful for mutagenesis studies in gram-positive bacteria had not been identified. Enterococcal transposon Tn917 confers inducible resistance to the macrolide-lincosamide-streptogramin B (MLS) group of antibiotics, including erythromycin (Em). For purposes of transpositional mutagenesis, Tn917 is particularly useful when compared to other transposons, such as Tn916. Tn917 is

relatively small, has a relatively high frequency of transposition, high degree of insertional randomness, generates extremely stable mutations, and extensive information is available on its physical and genetic organization (Camilli et al. 1990).

Tn917 was first identified on the non-conjugative, multiple-drug-resistance plasmid pAD2 in *Enterococcus fecaelis* strain DS16. The determinant for MLS resistance, *erm*, encodes an RNA methylase, almost identical to that of *Streptococcus sanguis* pAM77. Tn917 also shares significant similarities with the Tn3 family of gram-negative transposons, including the duplication of a 5 base pair sequence at insertion sites, plus homology within their inverted repeats, *res* sites, resolvase and transposase enzymes (Shaw and Clewell 1985; An and Clewell 1991). Tn917 is capable of transposing into both gram-negative *E. coli* (Kuramitsu and Casadaban 1986) and gram-positive organisms, including *Bacillus subtilis* (Youngman 1983), *Listeria monocytogenes* (Camilli et al. 1990), *Clostridium acetobutylicum* (Babb et al. 1993), *Lactococcus lactis* (Israelsen et al. 1995), *Streptococcus mutans* (Gutierrez at al. 1996), and *Streptococcus pyogenes* (Li et al. 1997).

The 5, 257 base pair (bp) transposon is flanked by 38 bp, non-identical, inverted terminal repeats, which generate a 5 bp duplication upon insertion, as does Tn3. Tn917 is composed of 5 open reading frames (ORFs), of which the first four are preceded by Shine-Dalgarno ribosome binding sites. The right terminal repeat forms a stem-loop believed to act as a factor-independent transcription terminator. A second transcription terminator follows ORF3 and involves an internal repeat (Shaw and Clewell 1985).

The first three ORFs are involved in MLS resistance and share homology with *S. sanguis* pAM77, which suggests abortive transposition or a recombinational event with a

Tn917-like element. Erythromycin exerts its antimicrobial effect by binding to 23S rRNA free or within 50S ribosomal subunits, peptide bond formation is inhibited and protein synthesis ceases. ORF2, *erm*, encodes a methylase that acts to dimethylate a specific adenine residue in 23S rRNA, reducing its affinity for Em. As a result, protein synthesis proceeds unabated and Em resistance is established (Hahn et al. 1982; Shaw and Clewell 1985).

ORF1 encodes a short leader peptide believed to participate in translational attenuation control of methylase synthesis (Shaw and Clewell 1985). It has been proposed that similar *ermC* MLS resistance gene of *Staphylococcus aureus* pE194 is regulated at the post- translational level by its 19 amino acid leader peptide, whose mRNA normally assumes a hairpin configuration that blocks access of ribosomes to its Shine-Dalgarno sequence and initiation codon. Erythromycin slows ribosome movement along the leader sequence resulting in isomerization of its mRNA to a configuration that unmasks the *ermC* ribosome binding site, making its mRNA available for translation (Hahn et al. 1982). A similar system is believed to operate in pAM77, which shares significant homology with pE194 and Tn917 (Horinouchi et al. 1983). Since expression of *erm* requires ribosomes capable of binding Em, a paradox exists: however, it is probable that low levels of Em are sufficient to modify the leader peptide mRNA, while allowing a large enough pool of unbound ribosomes to initiate translation. Once a round of methylase translation has begun, synthesis becomes resistant to inhibition, since Em cannot bind assembled polysomes (Hahn et al. 1982).

The function of ORF3 remains unclear, but it is postulated to serve as a positive regulator of the ORF2 promoter. The 73 bp internal repeat following ORF3 represents a

composite of pAM77 and Tn3 (Shaw and Clewell 1985). ORFs 4 and 5 encode *TnpR* resolvase and *TnpA* transposase enzymes sharing homology consistent with a close evolutionary relationship to the Tn3 family of transposons (An and Clewell 1991). The ORF5 transposase appears dependent on ORF4 resolvase for translation as it lacks a Shine-Dalgarno sequence and promoter. Tn917 also possesses an internal *res* site, derived from Tn3, whose sequence begins at the point where homology with pAM77 ends (Shaw and Clewell 1985). The *res* site represents the location where integration cointegrate intermediates between donor and target DNA recombine during replicative transposition, catalyzed by the *TnpR* resolvase (Tomich and Clewell 1980; Shaw and Clewell 1985). A diagram of Tn917 is included in Figure 1 below.

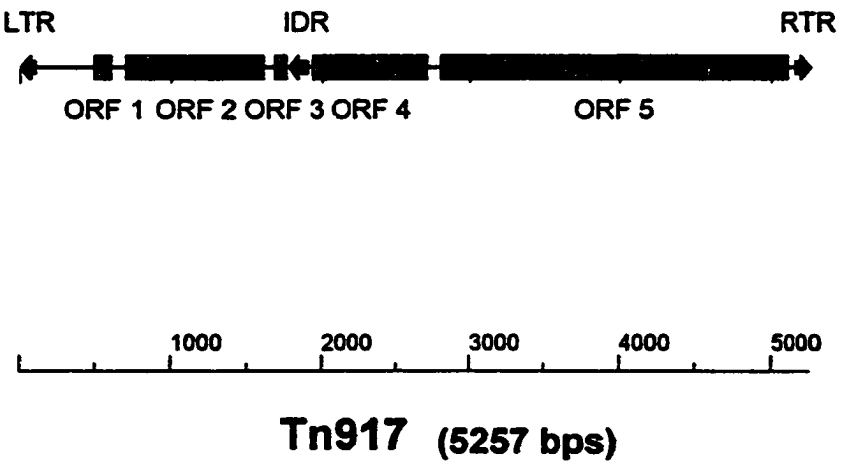


Figure 1. Diagram of transposon Tn917. ORF1 encodes a leader peptide, ORF2 the erythromycin resistance (RNA methylase) gene (*erm*), ORF3 is a positive regulator of *erm*, ORF 4 encodes *Tnp*A resolvase, and ORF 5 encodes *Tnp*R transposase.

The transposition frequency of Tn917 is inducible or enhanced in the presence of erythromycin. The structure of DNA sequences preceding ORF4 suggests drug-induced

transposition may result from a transcriptional read through from the ORF2 *erm* promoter into ORFs 4 and 5, encoding transposition functions. A stem-loop transcription terminator downstream of ORF3 allows a low level of read through that increases in the presence of Em, as a result of an increase in transcription of the *erm* promoter. Analysis of mRNAs before and after Em exposure reveals a post-exposure 4.8 kb transcript corresponding to almost the entire length of Tn917 (Shaw and Clewell 1985). In *S. fecaelis* DS16 exposed to low (0.001-0.5 μg/ml) concentrations of Em for a few hours, the frequency of transposition increased by an order of magnitude and paralleled the appearance of Em resistance (Tomich and Clewell 1980).

Mutagenesis Using Suicide Vectors

Transposons disrupt DNA sequences, causing mutations when they insert into a gene, but the mutation may not be apparent. If the transposon carries an antibiotic resistance gene, through the use of selective media, it makes its presence known. However, the location of the transposon cannot be readily determined, since the cell will be resistant, regardless of where the insertion has occurred.

One method to assay for transposition involves the use of suicide vectors, plasmid or phage, which are capable of entering a cell, but are incapable of replicating in that particular host. Once in the cell, the suicide vector will remain unreplicated and will be eventually lost. The only way that antibiotic resistance will persist is if the transposon has relocated within another DNA molecule capable of autonomous replication within the cell. Antibiotic resistant colonies growing on selective media have been mutagenized and can be screened for specific phenotypic defects (Freifelder 1987, Snyder and Champness 1997).

Plasmid pTV1-OK

Plasmid pTV1-OK is a replication-conditional (temperature-sensitive) vector based on staphylococcal replicons pE194 and pE194Ts. Plasmid pTV1-OK is analogous to pTV1, but contains the *repA*(Ts) gene from pWV01, a broad-host-range *Lactococcus lactis* plasmid capable of replication in both gram-positive and gram-negative bacteria (Gutierrez et al. 1996). The *repA*(Ts) origin of replication allows plasmid replication at permissive temperatures of 28-30°C, but not at 37-42°C. The other key feature of pTV1-OK is the *aphA3* kanamycin resistance gene, which is expressed in both *E. coli* and in gram-positive hosts (Cvitkovitch et al 1998). A diagram of pTV1-OK is included in Figure 2 below.

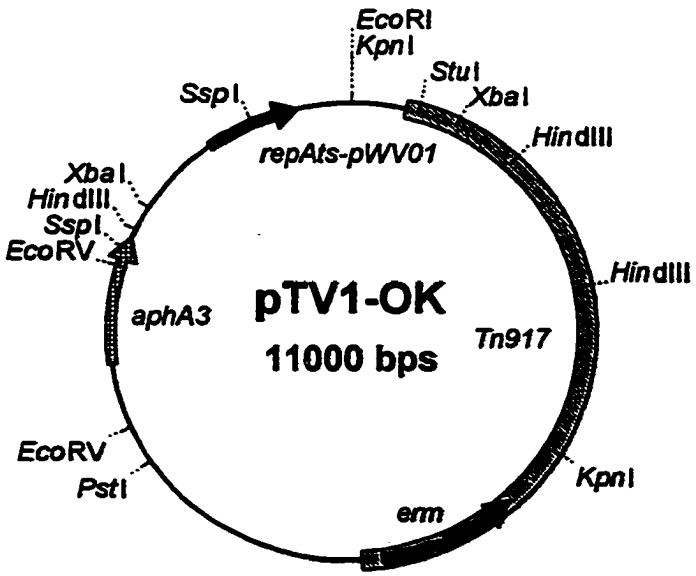


Figure 2. Diagram of pTV1-OK, a Tn917 delivery vector that uses the broad-host range replicon of *L. lactis* plasmid pWV01. Gene designations: *repA*(Ts)-pWV01, temperature sensitive replicon from pWV01, and *aphA3*, kanamycin resistance gene.

Following introduction of pTV1-OK, carrying Tn917 into a cell, the cell will exhibit resistance to both erythromycin and kanamycin. As previously discussed, transposition is induced in the presence of low levels of erythromycin. Following incubation at non-permissive temperatures, pTV1-OK is lost and only Em resistance is retained. In *S. pyogenes*, transposition occurs at a frequency of 0.01% and essentially 100% of the cells have an $erm^r kan^s$ phenotype, indicating loss of the plasmid vector (Li et al. 1997).

Electroporation

Although some gram-positive bacteria, such as *Streptococcus mutans*, are naturally competent (Gutierrez et al. 1996), the ability to introduce exogenous DNA into these organisms is complicated by their formidable cell wall and to a lesser extent their capsule (McLaughlin and Ferretti 1995). DNA is also strongly restricted by restriction-modification enzyme systems in some species of streptococci (LeFrancois et al. 1997). Despite these obstacles, certain species of streptococci and their allies can undergo conjugation, transduction, and transformation (Caparon and Scott 1991). Bacterial transformation is a process in which recipient bacterial cells acquire genetic material from free DNA molecules present in the surrounding medium. While transformation has proven most useful, the frequencies and efficiencies observed with gram-positives are lower than those for gram-negatives and seldom exceed more than 1% of the population (Chang et al. 1992).

Electroporation is a method of transformation in which foreign DNA is introduced into cells by applying a high-field strength (10^3-10^4 V/cm) electric pulse. Susceptibility to electroporation is viewed as a universal bi-layered membrane

phenomenon through which both prokaryotic and eukaryotic cells will take up DNA during permeabilization of their cell membranes. Although the precise mechanism is unknown, presumably the high voltage rearranges the membrane to produce transient pores at protein-lipid junctions and provides the driving force that carries exogenously supplied DNA traveling in the electric field into the cell (Luchansky 1988; Weaver 1995).

While numerous protocols have been advocated, most employ either distilled water or a low ionic strength buffer in conjunction with an osmotic stabilizer, such as hypertonic sucrose, to serve as a medium for electrotransformation. Bacteria are washed and resuspended in 40-200 µl of chilled buffer to which has been added 1-5 µg of purified plasmid or other DNA. Following the pulse, cells are allowed to "recover" on ice or in osmotically stabilized broth for several minutes to several hours before plating on solid agar (McLaughlin and Ferretti 1995).

Growth conditions and agents that either inhibit cell wall synthesis or enzymatically remove it, have been reported to enhance the efficiency of electrotransformation in gram-positive cells. Most authors recommend harvesting cells in early log phase (OD_{600} 0.2-0.7) for electroporation, presumably because the cell walls of rapidly dividing cells are less dense than those that have reached the stationary plateau (Powell et al. 1988; Holo and Nes 1989; Dunny et al. 1991). High concentrations of glycine have been used to inhibit cell wall synthesis in lactococci, *Enterococcus fecaelis*, and Group B streptococci to improve electrotransformation efficiencies (Holo and Nes 1989; Dunny et al. 1991; Framson et al. 1997). Similarly L-threonine (Dunny et al. 1991), and penicillin (Park and Stewart 1990) have both been used successfully to

weaken cell walls in other gram-positives. Alternatively, lysozyme or lysozyme and mutanolysin in combination have also been advocated to physically strip the cell walls of gram-positive cocci prior to electroporation (Powell et al. 1988).

CHAPTER II: GROSS AND MICROSCOPIC FEATURES OF *STREPTOCOCCUS INIAE* INFECTION IN CULTURED TILAPIA WITH COMPARISON TO THE PUBLISHED LITERATURE

Introduction

Streptococcus iniae was first isolated and characterized from purulent exudate collected from subcutaneous abscesses in a captive Amazon River dolphin *Inia geoffrensis* (Pier and Madin 1976). Since its first report as a fish pathogen in the United States (Perera et al. 1994), *S. iniae* infections in tilapia have become widely recognized throughout the United States and worldwide. The American Tilapia Association now recognizes *S. iniae* as the most significant pathogen affecting growth of the cultured tilapia industry in this country (Bowser et al. 1998). In the United States, *Streptococcus iniae* has also been identified as an important bacterial pathogen of hybrid striped bass *Morone saxatilis* male x *M. chrysops* female (Stoffregen et al. 1996).

The evolution of *S. iniae* as a fish pathogen is somewhat obscured by time, lack of standardization of testing procedures among diagnostic laboratories, and misinterpretation of certain test results, particularly hemolytic reactions on blood agar. Review of early descriptions and biochemical testing in the Japanese literature suggest that the organism may have been present in that country as early as 1976. Although it was not recognized at the time, in that year a β-hemolytic streptococcus with a phenotypic profile similar to that of *S. iniae* was reported from diseased yellowtail *Seriola quinqueradiata* (Minami et al. 1979). From 1977 to 1980, large-scale epizootics of streptococcal disease occurred among tilapia *Tilapia nilotica*, rainbow trout *Oncorhynchus mykiss*, ayu *Pleyoglossus altivelis*, and amago, *Oncorhynchus rhodurus*, on freshwater farms (Kitao et al. 1981; Ohnishi and Jo 1981; Ugajin 1981). It was not

until 1984, however, that the first specific reference was made to S. *iniae* as the β-hemolytic streptococcus responsible for the Japanese outbreaks (Kaige et al. 1984).

The disease is now prevalent throughout Asia, the Middle East and parts of the Mediterranean, affecting rainbow trout and coho salmon *Oncorhynchus kisutch*, (Eldar et al. 1994; Eldar et al. 1995b), dusky spinefoot *Siganus fuscescens*, (Sugita 1996), hybrid tilapia *T. nilotica* x *T. aurea* (Al-Harbi 1994), gilthead sea bream *Sparus aurata* and European sea bass *Dicentrarchus labrax*, spinefoot *Siganus rivulatus* (Zlotkin et al. 1998), red drum *Sciaenops ocellatus* (Eldar et al. 1999), and whitespotted rabbitfish *Siganus canaliculatus* (Yuasa et al. 1999). The disease has emerged most recently in cultured barramundi *Lates calcarifer* in Australia (Bromage et al. 1999). Additional species have been infected experimentally (Al-Harbi 1994; Yuasa et al. 1999).

Despite the large number of case reports, histopathological descriptions of the disease remain incomplete. To a large extent this may be due to the difficultly of producing an all-encompassing description of the gross and microscopic lesions associated with *S. iniae* infection, which are variable among individuals even during a single outbreak. Individual cases may present as an acute fulminating septicemia or follow a more protracted course with lesions indicative of a chronic inflammatory process. The most consistent histopathologic changes observed in this study included polyserositis, cellulitis and splenitis, with a marked predilection for the central nervous system. While severe meningoencephalitis is highly suggestive of *S. iniae* infection, a spectrum of changes may be present that are not pathognomonic for the disease and other gram-positive bacterial pathogens may have to be ruled out by ancillary diagnostic

tests. The purpose of this report is to detail the gross and histomorphological aspects of *S. iniae* infections in cultured tilapia and compare them to the published literature.

Materials and Methods

All specimens were received as clinical diagnostic cases by the Louisiana Aquatic Animal Disease Diagnostic Laboratory (LAADDL), School of Veterinary Medicine, Louisiana State University or were collected on site at commercial aquaculture facilities. Upon arrival, fish samples were assigned a case number and live fish were euthanized in 1 gm/L tricaine-methanesulfonate (MS-222). The abdomens of all fish were incised and bacterial cultures collected aseptically to confirm the presence of *S. iniae*. To promote rapid infiltration of fixative, the left abdominal walls were removed and whole specimens placed in 10% neutral buffered formalin at an approximate tissue to formalin ratio of 10:1. After 24 hours fixation, respective tissue samples were collected, trimmed to a thickness of approximately 3 mm and placed in tissue cassettes labeled with corresponding case numbers.

Prior to trimming, any tissues containing bone were decalcified in 5% formic acid for a period of 1-5 days. After trimming, the samples were neutralized in a saturated solution of sodium bicarbonate for 1-2 hours. Tissues were processed routinely, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E), Brown and Brenn tissue gram stain, Giemsa or Fite's stain for acid-fast bacteria (Prophet et al. 1992).

Cytochemical stains for oxidase and esterase activity were prepared following procedures outlined by Witten et al. (1998). For demonstration of esterase activity, smears of exudate, blood films, or cytospin preparations were air-dried, fixed for 2 min in ice-cold fixative, and gently washed in six changes of dH_2O. Fixative was composed

of 25 ml of formaldehyde, 45 ml acetone, 30 ml of dH_2O, 20 mg Na_2HPO_4, and 100 mg K_2HPO_4 (pH 6.6). After fixation, slides were incubated for 30 min at room temperature in a freshly prepared solution of 10 mg naphthol AS-D chloroacetate dissolved in 2 ml N, N-dimethylformamide (DMFA), plus 100 ml of 0.2 M phosphate buffer and 0.5 ml working paraosaniline (PRS) (pH 7.4). Paraosaniline working solution was prepared from equal volumes of 0.4 gm paraosaniline in 10 ml 2 N HCl and 0.4 gm $NaNO_2$ in 10 ml dH_2O. The incubation solution was replaced and the slides incubated for another 15 min, after which they were rinsed in six changes of dH_2O. Slides were counterstained with Mayer's hematoxylin for 10 min, rinsed in six changes of dH_2O, and allowed to stand in dH_2O for 5 min. The slides were then air-dried, dipped in xylene and coverslipped with a synthetic mounting media.

For oxidase staining, slides were air-dried, fixed 30 sec in an ice-cold solution of 60 ml acetone, 11 ml methanol, 70 µl glacial acetic acid, and 40 ml dH_2O (pH 5.4), then washed as above. Fixed slides were incubated for 30 min at room temperature in a solution of 50 mM acetate buffer pH 5 (35 ml 0.2 M sodium acetate, 15 ml 0.2 M acetic acid, 150 ml d H2O) plus 1 ml of 3-amino-9-ethyl carbazole (AEC) stock solution (13 mg AEC in 25 ml DMFA). After incubation, slides were washed, counterstained and coverslipped as described above.

Results

Gross Findings

Affected fish often exhibited diffuse external darkening or showed increased prominence of vertical banding patterns. In acute cases body condition appeared normal, while chronically infected fish tended to have tucked abdomens consistent with

prolonged periods of anorexia. In contrast, the abdomens of some fish were distended and fluctuant due to the presence of ascites (Figure 3).

Hyperemia was often present at the fin bases, particularly the paired pectoral fins, and commonly extended into the fin rays. One to 2 mm pustules were often present along the ventral aspect of the lower jaw and less frequently along the ventral midline, particularly in association with bony prominences of the pelvic girdle. These pustules typically ruptured to leave ulcers of similar size. Larger subcutaneous abscesses also occurred in the caudal peduncle at the base of the caudal fin, and less frequently at the bases of the pectoral and pelvic fins. Areas of raised scales in the peduncle, with underlying fluctuant swelling, were suggestive of abscess formation. Raised hemorrhagic bullae were sometimes present in association with abscesses (Figure 4). Rupture of pustules or incision of fluctuant areas usually initiated the flow of pale yellow-tan purulent material, which contained flecks of blood, fibrin, and tissue debris (Figure 5). Probing revealed cavities as large as 1.5-2.0 cm in diameter, sometimes containing several milliliters of exudate. Gram stains performed on exudates typically revealed large numbers of free and phagocytized gram-positive cocci.

Macroscopically, the meninges were usually congested and increased amounts of opaque, occasionally blood-tinged, fluid obscured the normally opalescent fat that surrounds the brain. Unilateral or bilateral exophthalmia was common and often accompanied by corneal opacification and ulceration (Figure 6). Periocular edema, congestion, and accumulation of inflammatory exudate within the retrobulbar space all were believed to contribute to exophthalmia. Hypopyon within the anterior chamber was sometimes grossly visible (Figure 7). In severe cases, there was rupture of the cornea

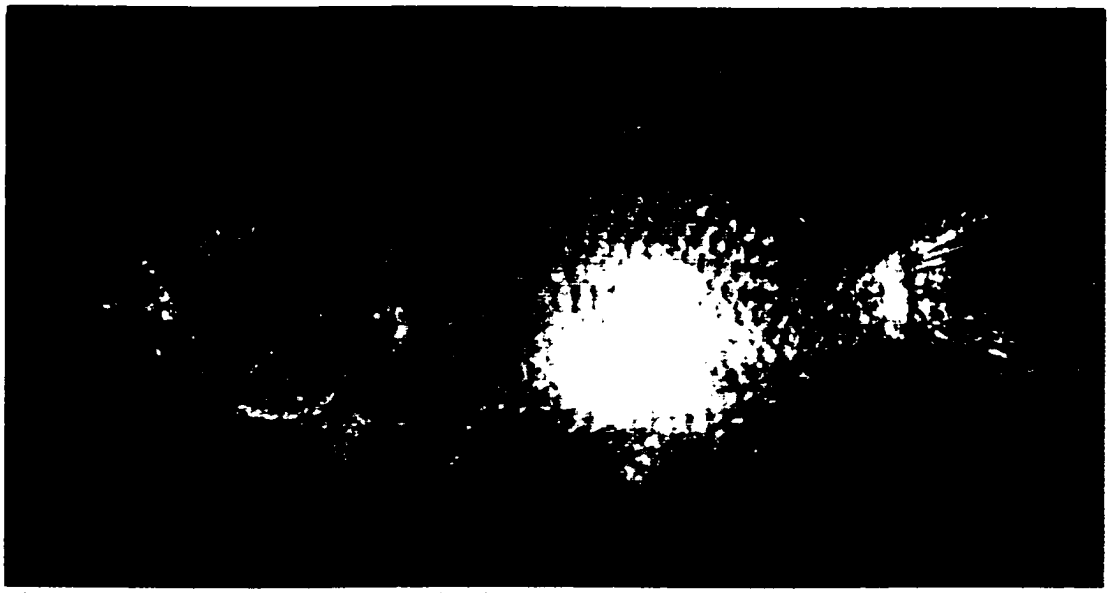


Figure 3. Gross photograph of a tilapia hybrid naturally infected with *S. iniae*. There is prominent abdominal distension due to the presence of septic ascitic fluid.

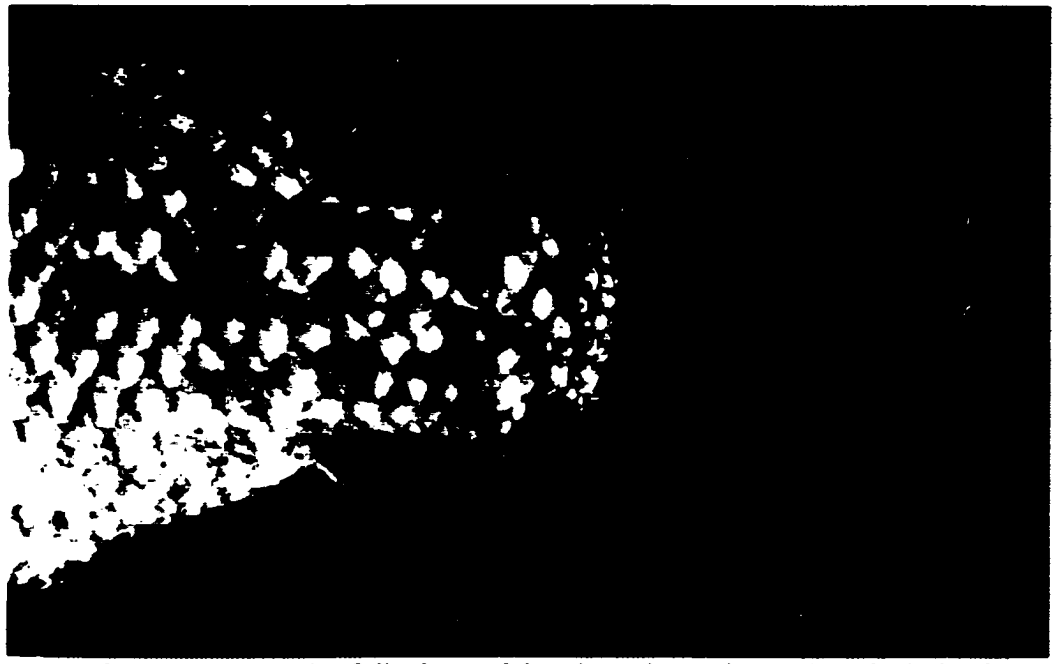


Figure 4. Gross photograph of tilapia caudal peduncular region. There is darkening of the skin with increased prominence of the vertical banding pattern. The arrow indicates a hemorrhagic bulla associated with an underlying abscess.

Figure 5. Gross photograph of tilapia caudal peduncular region. The bulla indicated in Figure 4 has been lanced to initiate the flow of purulent exudate from a large subcutaneous abscess.

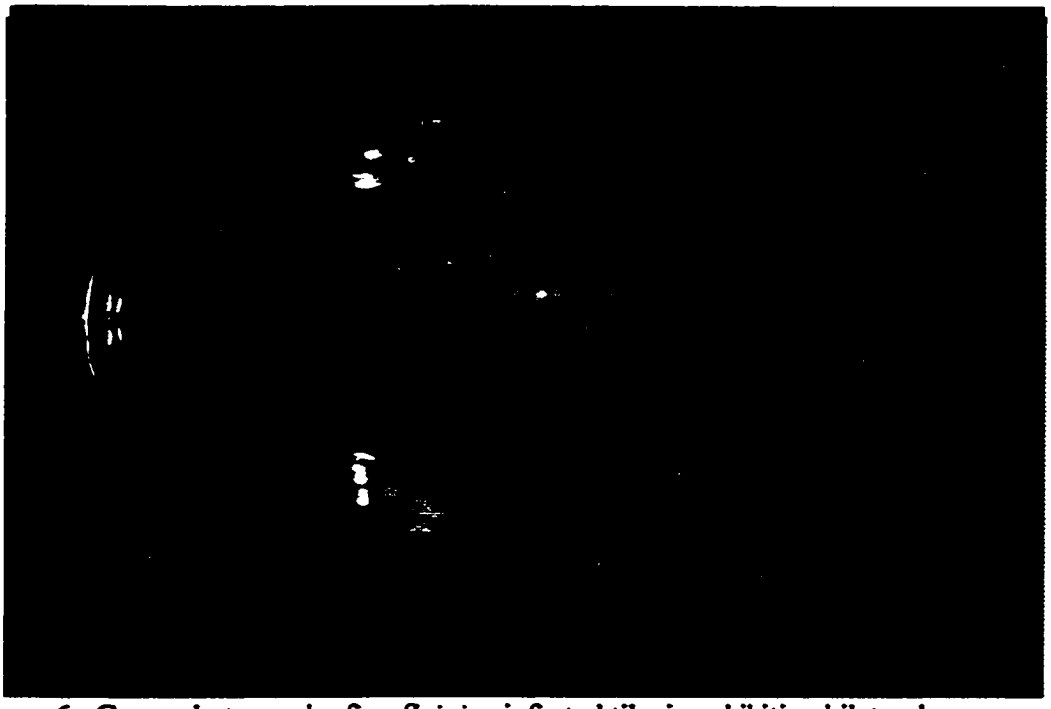


Figure 6. Gross photograph of an *S. iniae* infected tilapia exhibiting bilateral exophthalmia.

Figure 7. Gross photograph of an *S. iniae* infected tilapia demonstrating corneal ulceration and edema. Inflammatory exudate is present within the anterior chamber.

with eviceration of the retina and uveal trac. Uveal tissues sometimes sealed the corneal defect, but phthisis bulbi ultimately resulted.

Internally, the abdominal cavity often contained large amounts of slightly opaque white fluid, sometimes containing flecks of fibrin. Cytology stains performed on this fluid revealed large numbers of leukocytes and cocci, many of which had been phagocytized. If collected aseptically, this fluid typically yielded pure cultures of *S. iniae*. In contrast, the abdomens of some fish contained clear serous fluid or a mixture of clear fluid and gelatinous material. The clear fluid was usually found in fish devoid of abdominal adipose and was interpreted to represent serous atrophy of fat, while the gelatinous material suggested organ or vascular damage and protein leakage. Examination of this fluid usually revealed few leukocytes and cultures were negative or yielded only small numbers of bacterial colonies.

Consistent with anorexia, intestinal tracts were usually fluid filled and contained only scant ingesta. Gall bladders were markedly distended by bile. The amount of abdominal fat varied according to the period of inappetance. With the exception of the spleen, organomegaly was not a prominent finding in most cases. An enlarged, deeply congested, friable spleen was one of the most consistent gross findings observed in diseased tilapia. Strands of white to pale tan fibrin were occasionally loosely adhered to serosal surfaces of the abdominal viscera, particularly the spleen (Figure 8), and rarely formed a more extensive pseudomembranous covering. The epicardium was often most severely affected and in extreme cases was covered by a thick, dull, tan, granular membrane that entirely encompassed the heart.

Figure 8. Gross photograph of an *S. iniae* infected tilapia. The abdominal wall has been removed to reveal prominent splenomegaly (S) and fibrin on the splenic capsule (arrow). The gastrointestinal tract is devoid of ingesta and fluid filled.

Microscopic Findings

Inflammatory Exudates

Inflammatory changes were typically characterized by intense mixed populations of macrophages (Figure 9) and neutrophils (Figure 10), usually accompanied by the presence of small to moderate numbers of free and phagocytized gram-positive cocci (Figure 11). Inflammatory infiltrates were invariably predominated by macrophages. In severe cases there was extensive infiltration of tissues by these professional phagocytes, whose cytoplasm became distended by large numbers of cocci. Small accumulations of lymphocytes were sometimes seen, particularly in lesions involving mucous membranes. Lymphocytic infiltrates were present in the submucosa or lamina propria beneath basement membranes and could often be found migrating across epithelial surfaces, such as the olfactory mucosa. Small numbers of eosinophils were widely scattered in some lesions.

In smear preparations tilapia macrophages could be readily distinguished from neutrophils using cytochemical stains (Witten et al. 1998). In tissue sections, discrimination of macrophages and neutrophils was highly subjective, especially in areas of intense inflammation. While neutrophils are reported to be smaller, the sizes of tilapia macrophages and neutrophils are similar and may overlap. Macrophages and neutrophils average 14.6 and 12.9 μm in diameter respectively (Witten et al. 1998).

On H&E sections, macrophages were suggested by irregular cell borders, which often became indistinct when sheets of cells infiltrated limited tissue spaces. Nuclei were usually large, centrally located, and indented to irregular in outline, with vesicular to marginated chromatin patterns. A single prominent centrally located nucleolus was often

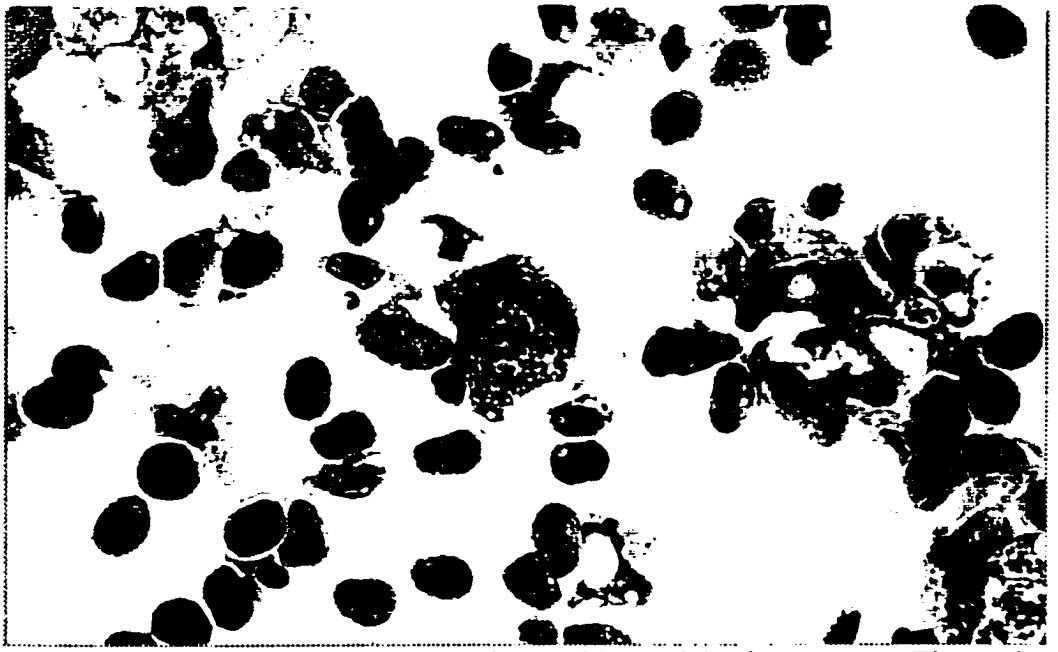


Figure 9. Tilapia macrophage demonstrating positive activity for esterase. The nucleus is displaced to the periphery of the cell and a central vacuole contains phagocytized cocci. (Esterase, X 1000).

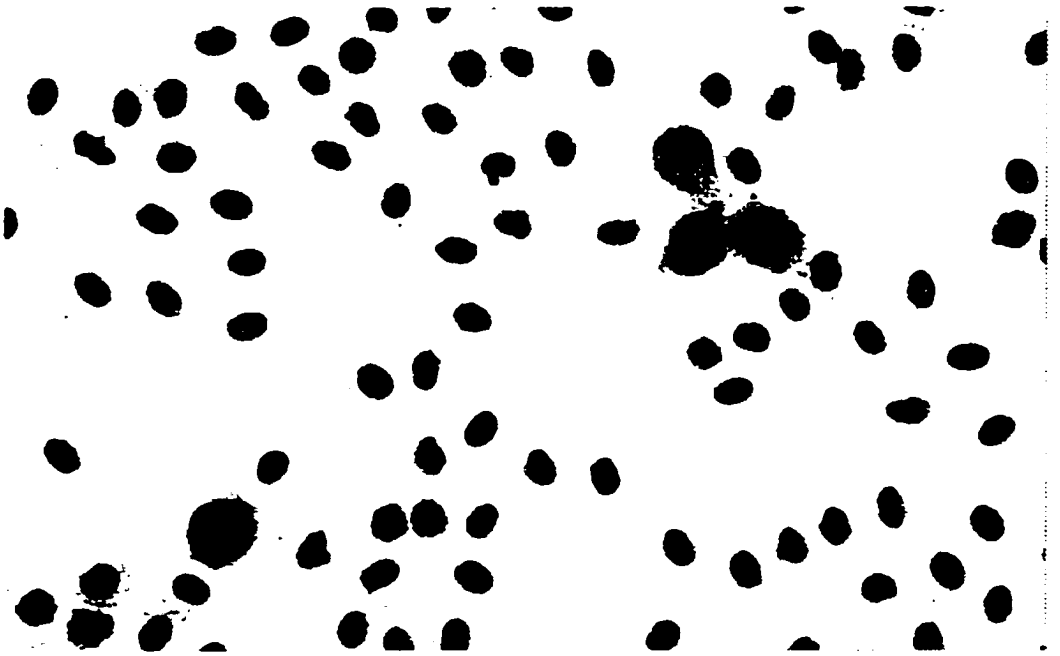


Figure 10. Tilapia neutrophils demonstrating positive activity for oxidase. The spherical cell has an oval, slightly eccentric nucleus (Oxidase, X 1000).

present. In macrophages containing abundant phagocytized material, the nucleus became highly condensed, hyperchromatic, and displaced to the periphery of the cell. Pale eosinophilic cytoplasm was abundant and finely vesicular or lacy to granular in appearance.

Neutrophils possessed a roughly spherical shape with distinct cell borders and typically had a greater nuclear to cytoplasmic ratio than mature macrophages. The round to oval, eccentrically placed nuclei of neutrophils, had dispersed to stippled chromatin patterns and exhibited deep basophilia. Nuclear banding was occasionally observed in some sections, but true segmentation was never seen (Figure 12). Cytoplasm was present in moderate amounts and more deeply eosinophilic than that of macrophages, with a finely granular appearance.

Cellulitis and deeper fasciitis were outstanding features of the disease and were often accompanied by foci of coagulative necrosis and liquefaction. Inflammatory cells resembling neutrophils were present in these lesions. In contrast, necrosis was not a prominent finding in parenchymal organs, where inflammatory cell infiltrates were composed of small numbers of macrophages.

Nervous System and Special Sensory Organs

The brains of teleosts can be divided into five embryologically determined regions. The telencephalon and diencephalon comprise the forebrain, mesencephalon the midbrain, and metencephalon and myelencephalon the hindbrain. The telencephalon forms the paired olfactory lobes and bulbs. The diencephalon or "tween brain", composed primarily of the thalamus and hypothalamus, gives rise to the pineal and pituitary glands. The diencephalon serves to connect and integrate various regions of the

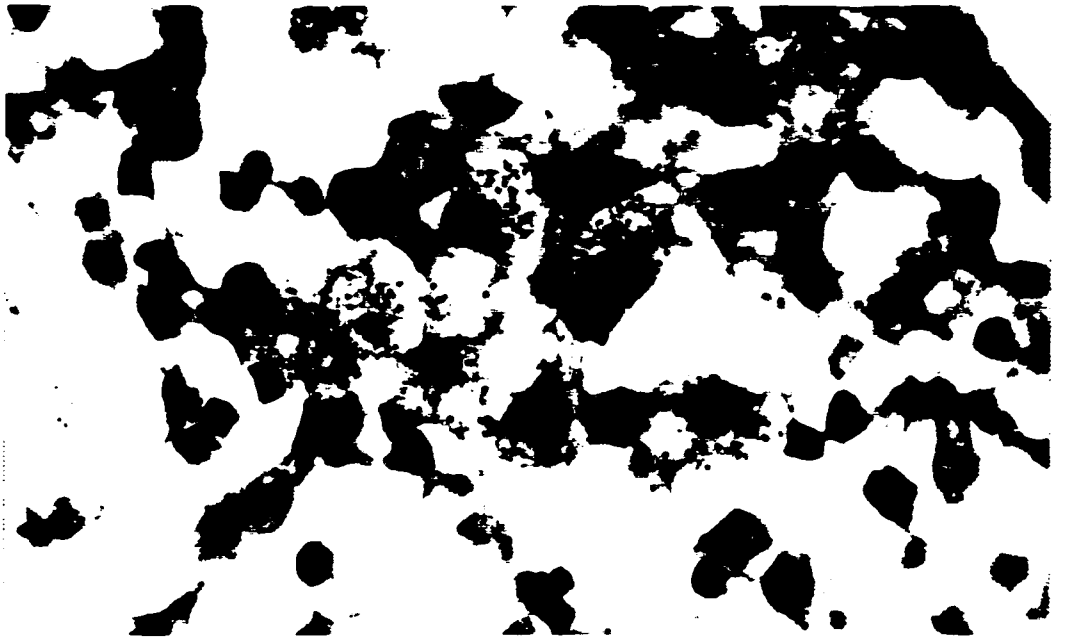


Figure 11. Inflammatory exudate surrounding the brain of a tilapia infected with *S. iniae*. There is a cluster of macrophages in the center of the field containing phagocytized gram positive cocci. (Brown-Brenn, X 1000).

Figure 12. Inflammatory cell infiltrate within an area of fasciitis. The majority of cells are neutrophils, indicated by their spherical shape and eccentric round to oval nuclei. Arrow indicates a band type nucleus. (H&E, X 1000).

brain and pituitary. Optic lobes, recognized histologically as the optic tectum, comprise the cerebral cortex, are the most prominent feature of the mesencephalon, and form the dorsal border of the third ventricle. The optic lobes are most developed in sight feeding fishes.

The anterior hindbrain or metencephalon is the cerebellum, composed of the corpus cerebelli and the valvula. The valvula extend anteriorally below the optic tectum into the third ventricle, while the corpus extends forward dorsally. The cerebellum is involved in coordination of movement, muscle tone, and postural reflexes. The posterior hindbrain or myelencephalon is composed chiefly of the medulla oblongata. The medulla gives rise to cranial nerves V-X and includes several enlarged lobes involved in somatic and visceral sensory and motor functions.

The spinal cord has a small central canal lined by ependymal cells and is divided into gray and white matter regions. Gray matter is located centrally, containing a single dorsal and two ventral horns, while myelinated nerve fiber tracts course peripherally in the spinal cord. Spinal nerves and ganglia arise segmentally along the length of the cord (Grizzle and Rogers 1976; Bond 1979; Yasutake and Wales 1983).

In tilapia a menix primitiva covers the brain, formed by a thin membranous layer of collagenous fibers through which blood vessels course. Capillaries extend from this supporting vascular layer into the brain, but do not form well-defined Virchow-Robin spaces (Grizzle and Rogers 1976). In *S. iniae* infected fish, the menix may be widened by the accumulation of edema fluid, fibrin deposition and inflammatory cell infiltrates (Figure 13). The meningitis, predominated by macrophages, may extend well beyond the menix to occupy much of the normally adipose filled extra-cranial vault (Figure 14).

Figure 13. Meningitis overlying the optic tectum. Arrow indicates the menix primitiva. Note the vascular congestion and large number of cocci laden macrophages (M). (H&E, X 400).

Figure 14. Meningitis. The cerebellum is located to the lower right. Note the large amount of exudate within the normally adipose filled extra-cranial vault. (H&E, X 100).

Meningitis may extend down the spinal cord involving nerve roots and their respective ganglia (Figure 15). Perineuritis may radiate peripherally from the spinal cord into the trunk musculature (Figure 16).

Numbers of inflammatory cells and bacteria varied dramatically between individual cases. Both free and phagocytized cocci were usually present and were best recognized using tissue gram stains, particularly when only minor inflammatory changes were present. Small granulomas with thin fibrous capsules and caseous centers were occasionally present in the menix, but bacteria were never observed within them. Gram and Fite's stains failed to reveal gram-positive cocci, gram-negative rods, or acid-fast bacteria within these granulomas.

The encephalitis produced by *S. iniae* was usually milder than the inflammation associated with the meninges and seldom extended beyond the molecular layer of the optic tectum or cerebellum. Congested meningeal capillaries extended into the neural parenchyma and macrophages were occasionally seen within their lumens. In severe cases cocci laden macrophages could be seen migrating throughout the neuropil, which assumed a vacuolated or "moth eaten" appearance, but typically exhibited remarkably little destruction of the normal architecture. It was unclear, but presumed that these phagocytes were representatives of circulating monocyte-macrophage lineage, rather than gitter cells of nervous system derivation.

Macrophages, cellular debris, and fibrillar proteinaceous material suggestive of fibrin were sometimes present in the ventricles and large numbers of macrophages infiltrated the adjacent periventricular white matter (Figure 17). Tall ciliated ependymal cells lined portions of the ventricular system of the brain and spinal canal. These

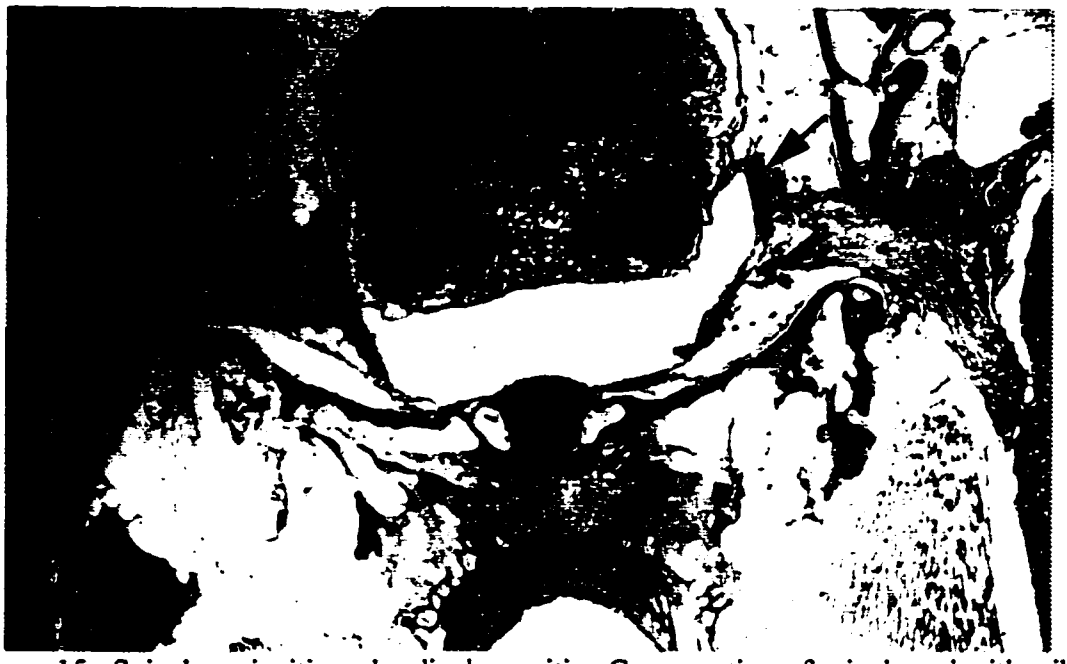


Figure 15. Spinal meningitis and radiculoneuritis. Cross section of spinal cord with mild meningitis and inflammation surrounding spinal nerve roots and ganglia (arrows). (H&E, X 20).

Figure 16. Perineuritis. Severe inflammation surrounds a spinal nerve in an *S. iniae* infected tilapia. (H&E, 200).

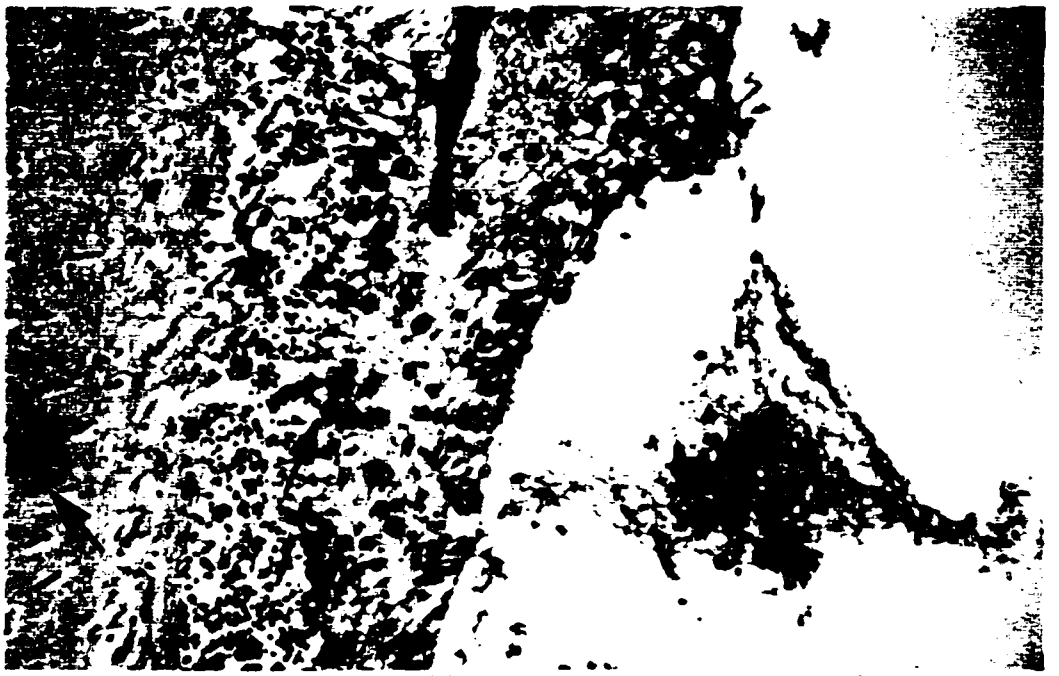


Figure 17. Encephalitis and ventriculitis. Cocci laden macrophages (M) are present within the lumen of the third ventricle and infiltrate the optic tectum (arrows). (H&E, X 200).

fusiform cells had tapering basilar portions that melded inconspicuously into the underlying nervous tissue, apparently with no or at least an inconspicuous basement membrane. In some cases, there was disruption of the ependymal cell layer due to infiltration by macrophages.

The neurocranium of tilapia is composed in part of a reticulum of cavities formed by cartilaginous and bony septa filled by adipose and to a lesser extent by areolar connective tissue, through which often course nerves and blood vessels. These cavities offered little resistance to the spread of infection and were often heavily infiltrated by bacteria and inflammatory cells. Inflammation in these sites sometimes resulted in concomitant destruction of bone and cartilage, envelopment of cranial nerves, and the involvement of additional structures of the head, such as the inner ear and olfactory epithelium (Figures 18 and 19).

The inner ear of teleosts forms an integral component of the octavolateralis system responsible for the detection of sound, vibrations, displacement of water, and postural changes. The inner ear structures are innervated by the VIII cranial, or acoustic nerve. The inner ear consists of a system of ducts and sacs, the membranous labyrinth, embedded within a corresponding set of bony cavities, the osseous labyrinth. Fish have no outer or middle ear analogs comparable to mammals and functions of the inner ear cannot be neatly divided into auditory and vestibular portions (Popper and Platt 1993). The pars superioris is composed of the semicircular canals, each with an ampulla and sensory crista, which communicate with the utriculus. The utriculus contains an otolith called the lapillus. The pars inferioris consists of two chambers, the sacculus and lagena, containing the sagitta and asteriscus, respectively. Otoliths or ear bones, sit upon a bed

Figure 18. Inflammatory exudate within the extra-cranial vault surrounding the brain and infiltrating structures of the inner ear (E). (H&E, X 20).

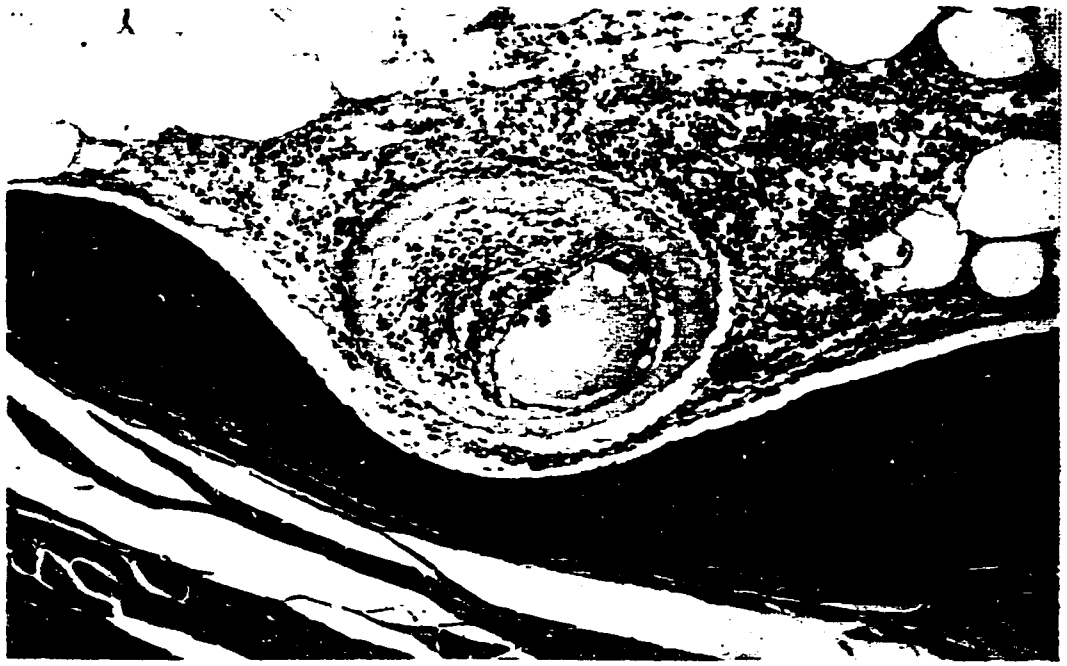


Figure 19. Otitis interna. Inflammation surrounds and partially fills endolymph spaces of an otic semicircular canal. (H&E, X 100).

of sensory hair cells or neuromasts referred to as maculae (Grizzle and Rogers 1976; Bond 1979).

Exocytosis of inflammatory cells across the collagenous tissues forming the membranous labyrinths resulted in disruption and necrosis of sensory epithelia of the cristae and maculae. Endolymph spaces became filled with exudate, ultimately resulting in obliteration of the inner ear (Figures 20 and 21). Otitis interna probably resulted from direct extension of meningitis along the acoustic nerve, although, expansion of other sites of infection with destruction of the bony labyrinth could also have occurred. In either case, it is likely that disturbance of vestibular function results in many of the abnormal swimming patterns noted in the literature (Perera et al. 1994; Eldar et al. 1994; Al-Harbi 1994; Bromage et al. 1999; Yuasa et al 2001).

The second component of the octavolateralis system is the lateral line, a mechanosensory system that detects hydrodynamic changes originating near the body. The lateral line detects obstacles and prey, is used in schooling and in predator evasion (Popper and Platt 1993). The trunk canal is located within the dermis, encased in the dermal bone of scales located along the lateral midline. The canal branches on the head where it is encased within bones of the skull. Located at regular intervals along the canals are pores that allow communication with the external environment. Neuromast organs, collections of hair cells covered by a gelatinous cupula similar to the otic cristae ampullaris, are located between pore canals (Grizzle and Rogers 1976). Changes in water pressure distort hair cell cilia generating nervous impulses carried to the medulla by the lateral line nerve (Popper and Platt 1993).

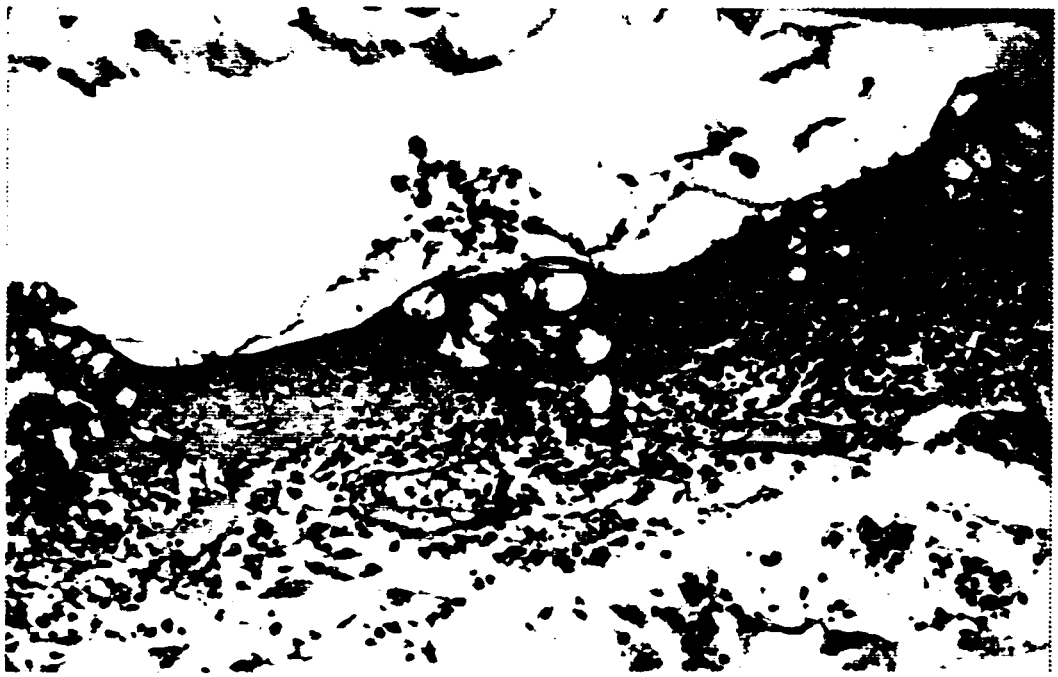


Figure 20. Otitis interna. There is vacuolization and degeneration of the membranous labyrinth associated with inflammation and the presence of cocci. (H&E, X 200).

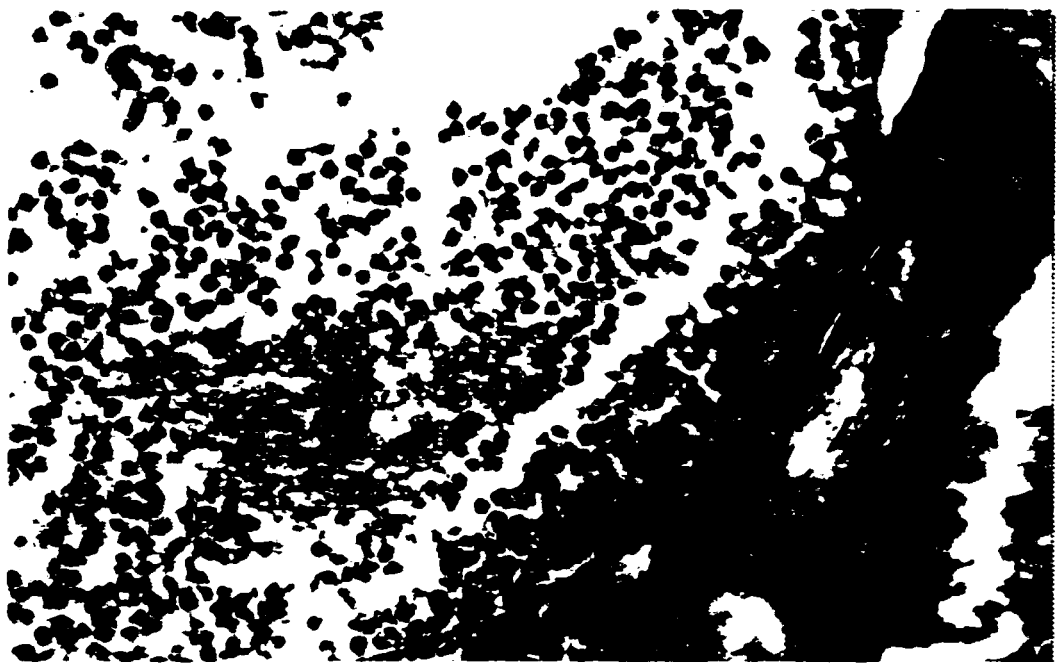


Figure 21. Otitis interna. Inflammatory exudate and cocci within the lumen of the utriculus. The basophilic structure to the right in an otolith. (H&E, X 200).

The remainder of the canal was lined by a thin stratified squamous epithelium with numerous goblet cells. The epithelium is separated from the bony canal by a zone of connective tissue adventitia, which became infiltrated by inflammatory cells. As described below for the olfactory system, cocci laden macrophages were seen traversing the epithelium and cocci were sometimes present free within the lumen of the canal.

Tilapia eyes are roughly spherical with only slight anterior flattening. The sclera is formed by a thin fibrous tunic, which contains a capsule of hyaline cartilage surrounding approximately the middle half of the globe. Scleral connective tissue is intimately associated with the abundant adipose that fills the retrobulbar space.

Corneas are covered by a 5-6 cell thick stratified squamous epithelium composed of cuboidal cells and a columnar basal layer. The epithelium sits on an inconspicuous basement membrane, with no underlying Bowman's capsule. The substantia propria is composed of two layers. The outer layer is composed of parallel bundles of collagen fibers and fibroblasts. The inner layer contains more densely packed fibers and has a more hyaline appearance. Corneas are of uniform thickness centrally, but the inner fibrous layer forms a dramatic bulge into the anterior chamber near the limbus. The outer corneal layer reflects upon itself to become part of the dermis, while the denser inner layer blends with the sclera and almost immediately envelops the ocular cartilage ring. Posteriorly, the cornea is bordered by a 1-2 cell thick endothelium. A structure analogous to Descemet's membrane could not be discerned in the sections examined. Descemet's membrane may not be readily visible or may be absent in some fish species (Tripathi 1974).

The iris is short, thin, and highly vascularized. Both surfaces are pigmented and the anterior margin contains a layer of birefringent crystals beneath the pigment cell layer that form a continuous band between the sclera and choroid coat. The iris does not appear to contain the smooth muscle sheath described in mammals (Dellman and Collier 1993), which is consistent with the immobile nature of the iris in most fish (Hargis 1991). The anterior iris endothelium is continuous with the endothelium that forms the annular "ligament," covering the trabecular network of the irido-corneal angle.

The existence of an angular aqueous drainage channel and flow of aqueous humor in the teleostean eye is controversial (Tripathi 1974). From the light microscopic level, the presence of endothelial lined vascular spaces located at the apex of the irido-corneal angle in tilapia is suggestive of the intrascleral vascular plexus present in higher vertebrates (Dellman and Collier 1993). Consistent with descriptions by other authors, a ciliary body is not present in the eyes of tilapia. It is assumed, however, that the layer of nonpigmented cuboidal cells on the posterior tilapia iris is equivalent to the cell layer suspected to secrete aqueous humor in other teleosts (Copeland 1982).

The lens is spherical and protrudes far into the small anterior chamber to almost contact the cornea. Other than its shape, the lens is conventional in most respects. The tilapia retina consists of eight layers as found in trout (Yasutake and Wales 1983) and higher vertebrates (Dellman and Collier 1983). The pigmented epithelium is thick and there is no tapetum lucidum. A well-developed choroid capillary rete mirabile is present between the retina and sclera.

Ocular changes were uni- or bilateral. It is not known whether ophthalmitis was derived as a direct extension of meningitis descending the optic nerve or established itself

hematogenously via the choroid gland. Inflammatory exudate commonly surrounded optic nerves, particularly in the area of the papillae (Figure 22). The capillary rete mirabile comprising the choroid is supplied by the ophthalmic artery originating in the pseudobranch and is believed by some to be responsible for the extremely high oxygen partial pressures in the vitreous humor (Laurent and Dunel-Erb 1984). The gland often showed extensive involvement, including inflammatory cell infiltration with both free and phagocytized cocci, intravascular fibrin thrombi, and granuloma formation (Figure 23). Perivascular edema often separated the gland from the sclera and may have been responsible for the common finding of retinal detachment (Figure 24). Retinal detachment, however, is also a common artifact of processing and must be interpreted with caution.

Both the vitreous and aqueous fluids sometimes contained inflammatory cells and bacteria, which adhered to intraocular structures such as the retina, iris and lens (Figure 25). Infrequently, the corneal endothelium was breached allowing inflammatory cells and bacteria to penetrate the substantia propria. In such cases, there was loss of tinctorial properties in the edematous cornea, accompanied by hypertrophy of proprial fibroblasts. This particular finding was encountered only infrequently.

Inflammatory exudate may also become lodged in the irido-corneal angle, and although glaucoma has not been described in fish (Hargis 1991), it is reasonable to speculate that occlusion of the filtration angle could contribute to buphthalmos resulting from increased intraocular pressure (Figures 26 and 27). In cases of retinal detachment, macrophages infiltrated the space between the retina and vascular coat of the choroid, although infiltration of the retina proper was never encountered. The iris was also

Figure 22. Optic neuritis. Severe inflammation (I) surrounds the optic nerve and there is margination of eosinophilic granulocytes at the periphery of the nerve (arrow). (H&E, X 100).

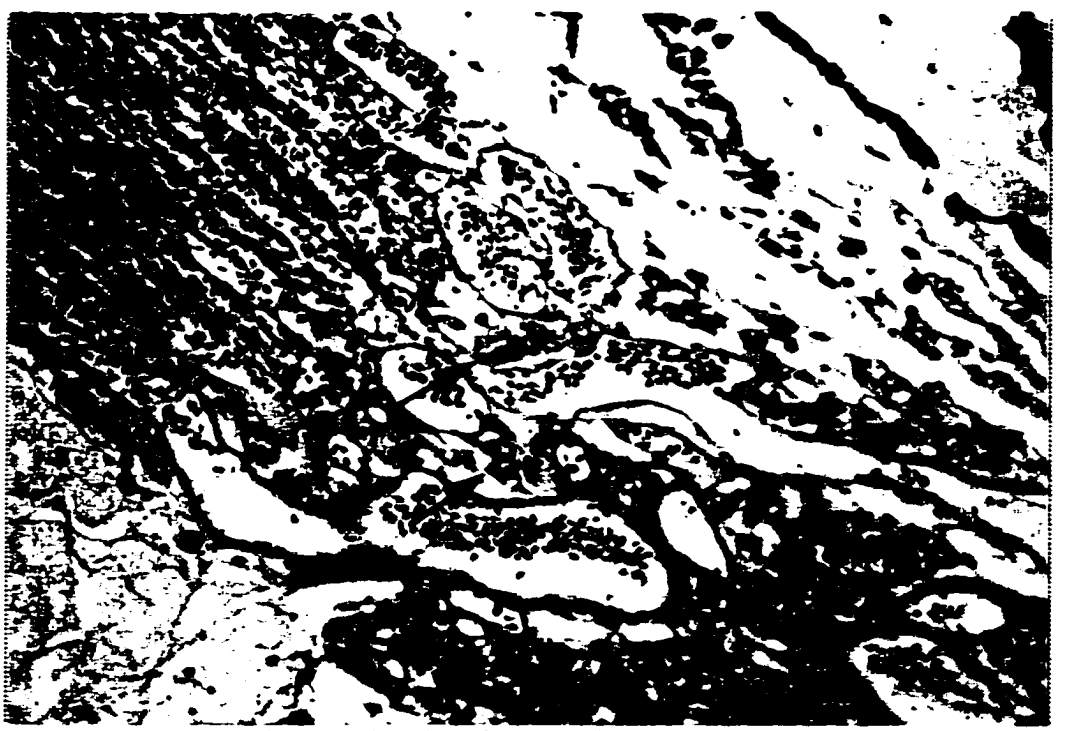


Figure 23. Vasculitis of the ocular choroid rete mirabile. There is partial occlusion of vascular lumens by macrophages (arrows) containing phagocytized cocci. (Brown-Brenn, X 200).

Figure 24. Choroiditis. There is severe inflammation and edema of the ocular choroids coat (C) and resultant retinal detachment (R). The arrow indicates scleral cartilage. (H&E, X 20).

Figure 25. Ophthalmitis. Inflammatory exudate (E) is present within the anterior chamber (hypopyon). There is erosion and infiltration of the inner layer of the cornea (C) by macrophages. (H&E, X 20).

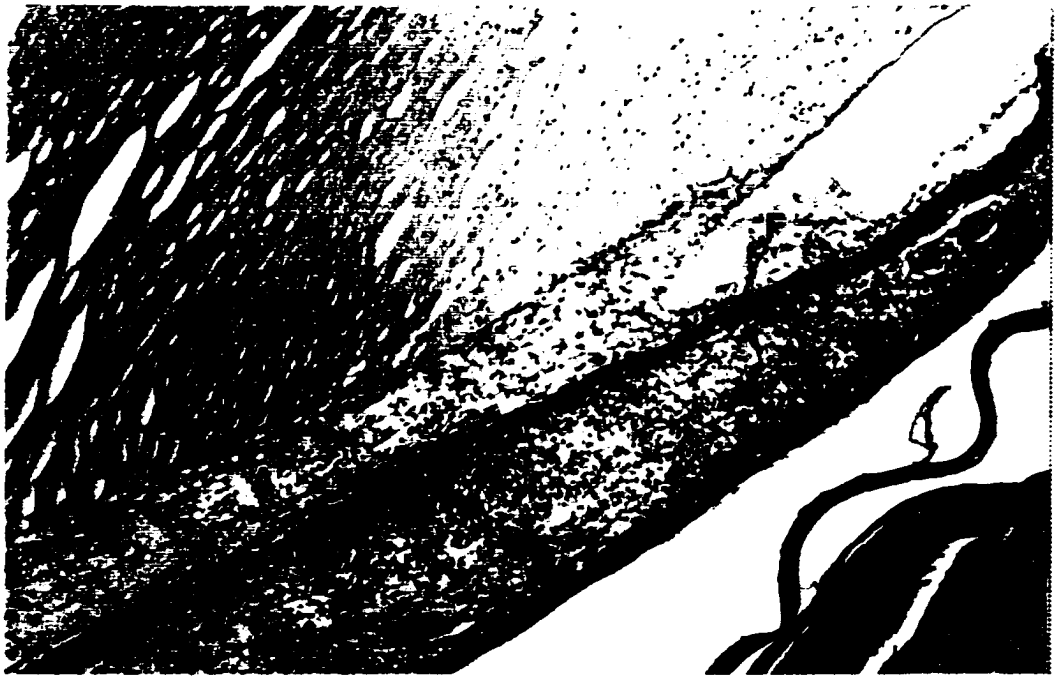


Figure 26. Iritis. There is vascular congestion and hemorrhage within the iris (I). An amorphous mass of proteinaceous material, suggestive of fibrin (F), is present in the iridocorneal angle (A). (H&E, X 100).

Figure 27. Inflammation of the iris and cornea. Inflammatory cells are present within the reticular meshwork of the annular ligament of the iridocorneal angle (A). (H&E, X 400).

heavily infiltrated in some cases by macrophages and neutrophils, possibly due to its abundant vascular supply.

The olfactory system of tilapia consists of paired olfactory sacs, each with anterior and posterior nares, lined by a stratified squamous epithelium containing abundant goblet cells. The sensory epithelium forms typical lamellae lined in part by non-sensory ciliated epithelial cells. Exiting the base of each olfactory lamellus are branches of the first cranial nerve, which tracks primarily through areolar connective tissue to the olfactory bulbs.

Severe inflammation often enveloped the olfactory nerves and infiltrated the surrounding soft tissue structures of the head. Necrotizing cellulitis surrounded the entire olfactory sac in a few cases (Figure 28). Patchy infiltration of the sensory epithelium and mucus membrane lining the remainder of the olfactory sac was present in some cases. Affected epithelial surfaces appeared spongy and vacuolated with ulceration and infiltration by macrophages and lymphocytes. Occasionally cocci laden macrophages were seen traversing the epithelium to enter the olfactory sac, which contained free cocci in some cases (Figure 29).

Cardiovascular System

The piscine heart consists of a series four chambers, only two of which contain typical striated cardiac myofibers and actively pump. The atrium is thin walled and trabecular, while the ventricular myocardium is composed of a thin compact outer layer and a wider inner layer forming a loosely organized reticulum of anastamosing myofibers. Atrioventricular valves separate the atrium and ventricle, while a set of bulboventricular valves limit blackflow from the elastic bulbus arteriosus. The heart lies

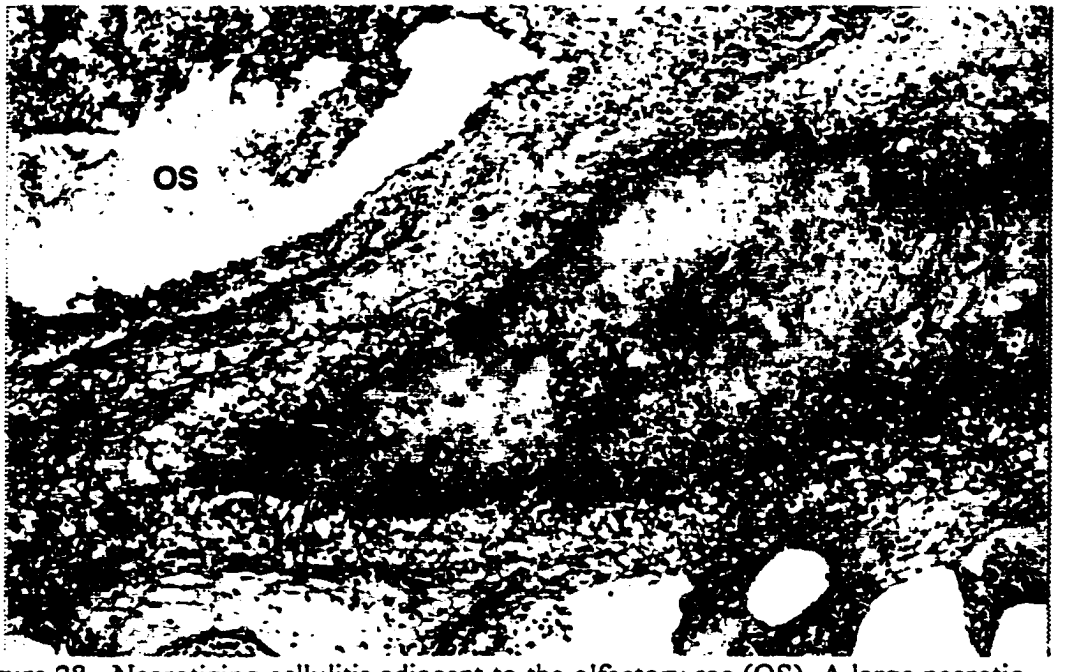


Figure 28. Necrotizing cellulitis adjacent to the olfactory sac (OS). A large necrotic focus (N) is undergoing liquifaction and is bordered by an intense zone of inflammatory cells. (H&E, X 20).

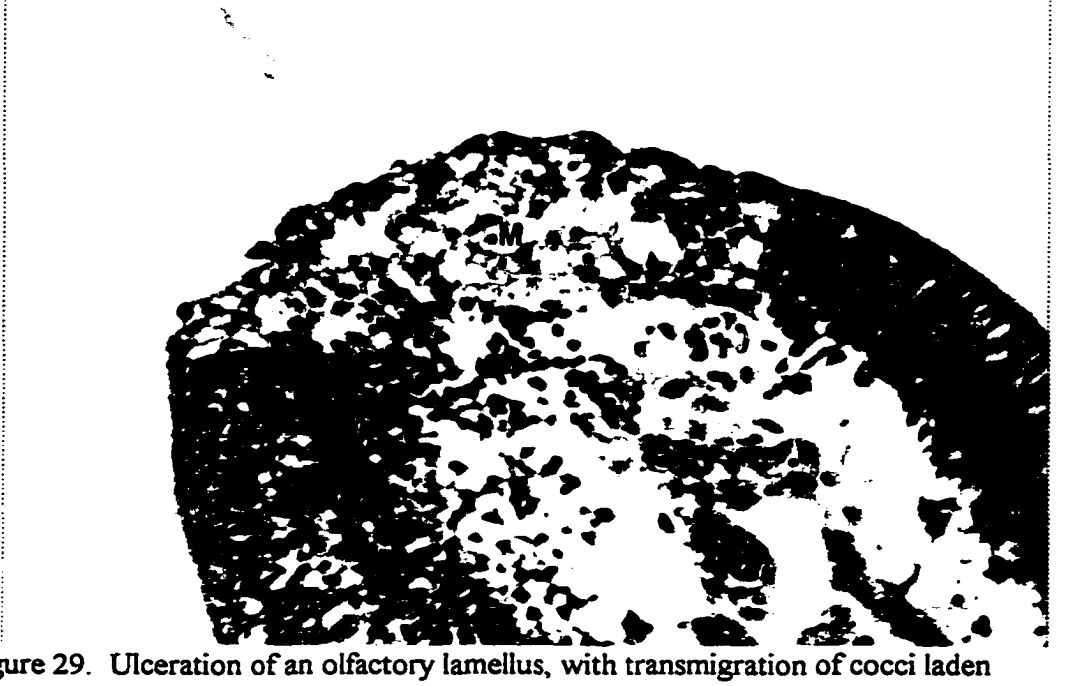


Figure 29. Ulceration of an olfactory lamellus, with transmigration of cocci laden macrophages (M) from the submucosa. Free and phagocytized cocci were sometimes present within olfactory sac lumens. (H&E, X 20).

in a pericardial cavity lined by a serous membrane and the heart itself is lined inside and out by an endo- and epicardium, respectively (Grizzle and Rogers 1976; Bond 1979, Yasutake and Wales 1983).

Lesions involving the heart varied from a mild fibrinous epicarditis to severe transmural myocarditis and endocarditis with the formation of septic thrombi. Typically there was thickening of the epicardium by a dense layer of exudate composed of pale eosinophilic fibrin, large numbers of plump macrophages, neutrophils and free and phagocytized cocci. The epicarditis penetrated both the outer compact layer and inner spongy layer of the ventricular myocardium, infiltrating between and progressively isolating small groups of cardiac fibers. There was progressive loss of myofibers with replacement by macrophages and fibroblasts resulting in obliteration of the normal tissue architecture. Neovascularization and fibrosis replaced the epicardium with a bed of granulation tissue (Figure 30). The epicarditis sometimes extended onto the bulbus arteriosis and ventral aorta.

In rare cases valvular endocarditis occurred with the development of large thrombi containing masses of cocci (Figure 31). Septic fibrin thrombi were occasionally found within the vascular system, but it was unclear whether these resulted from embolization or originated within preexisting foci of inflammation. Exocytosis of inflammatory cells across vascular walls and perivascular accumulation of inflammatory cells were common findings, however, necrotizing vasculitis was only seen in areas of intense inflammation and abscessation.

Figure 30. Transmural myocarditis. The epicardium (upper left) is being organized into a thick bed of granulation tissue, characterized by inflammation, neovascularization and fibroplasia. Arrows indicate the compact layer of the myocardium. (H&E, X 100).

Figure 31. Valvular endocarditis. A septic fibrin thrombus adhered to a bulboventricular valve leaflet. The bulbus arteriosus (BA) is present in the upper right of the field. (H&E, X 20).

Parenchymal Organs

Histology of the liver and spleen are in general similar to, but on a simpler level of organization than that of mammals, reflecting the lower position of fish on the phylogenetic tree. The piscine liver is described as tubulosinusoidal, composed of 1-2 cell thick branching and anastamosing tubules with bile canaliculi at their centers, which are surrounded by interconnecting sinusoids. There are no organized portal triads, although their components are all represented individually, and a clear pattern of lobulation does not exist. Identification of portal venules from hepatic veins can be difficult in some species, but the former are reportedly surrounded by more connective tissue and are often associated with melanomacrophage centers (Hampton et al. 1985; Hampton et al. 1988). In tilapia, exocrine pancreatic tissue is associated with portal venules and the organ is more appropriately described as a hepatopancreas.

The spleen is an encapsulated filtering meshwork perfused with blood. Its parenchyma is predominantly red pulp, a system of reticular cords and sinusoids. The white pulp consists of loose lymphoid tissue, which forms a cuff around the pulp arteries. Filtering of particulate matter from the blood occurs in the red pulp by phagocytes of the pulp cords and pale staining periarteriolar macrophage sheaths. The spleen is not a primary site of hematopoiesis in fish (Fulop and McMillan 1984).

Fish kidneys are fused bilaterally, and in tilapia form a continuous structure located retroperitoneally in the dorsal abdomen, that can be divided histologically into distinct head and trunk regions. The anterior or head kidney forms two large lobes embedded in the base of the skull and is composed entirely of hematopoietic and endocrine tissues. Hematopoietic tissues are located within a sinusoid supported by a

delicate reticular stroma. Interrenal tissue and chromaffin cells are located around ramifications of the post cardinal vein, which courses through the organ (Grizzle and Rogers et al. 1976; Yasutake and Wales 1983).

Moving posteriorly, there is a progressive decline in the amount of hematopoietic tissue, which is gradually replaced by nephrons of the trunk kidney. The nephron, or functional unit of the excretory kidney, is conventional in most respects. Compared to mammalian kidneys, glomeruli are small, hypercellular, and commonly appear to contact the visceral layer of Bowman's capsule. The renal tubular system is composed of multiple segments, which have been described in the channel catfish by Kendall and Hinton (1974). Briefly, the first proximal segment has a large lumen, is lined by a ciliated columnar epithelium with basal nuclei, and has a prominent striated luminal border. The second proximal segment is more variable with central or apical nuclei. The distal segment has a smaller diameter, more eosinophilic cytoplasm with no striated border, and basal nuclei. The tubular system of tilapia appears to follow this same basic outline.

With the exception of the spleen, involvement of the parenchymal organs in *S. iniae* infection was limited primarily to mild capsulitis. Deposition of inflammatory cells and fibrin similar to that described for the heart, but generally of less severity, was seen in cases with peritonitis (Figure 32). Involvement of the liver and kidney was usually limited to the presence of small numbers of cocci laden macrophages within the hepatic sinusoids and renal interstitium. Rarely necrosis of individual hepatocytes occurred in association with minimal numbers of macrophages and cocci. These foci were subtle and easily overlooked without the use of tissue gram stains. Occasionally sterile caseating

granulomas and little or no inflammatory cell component were noted within the hepatic parenchyma and mesentery (Figure 33).

In contrast, the spleens commonly contained phagocytized cocci, particularly in periarteriolar macrophage sheaths. In severe cases, much of the red pulp was replaced by sheets of macrophages with cytoplasm distended by phagocytized cocci (Figure 34). Reticuloendothelial hyperplasia and infiltration by circulating macrophages was probably responsible for the splenomegaly commonly seen grossly. Based on its nature as a blood filtering organ and high population of resident phagocytes, it was not surprising that the spleen was significantly involved in *S. iniae* infections.

Musculoskeletal System

The body musculature is composed of typical long striated fibers with multiple small hyperchromatic nuclei located peripherally along the length of the fiber. The bulk of the trunk musculature is composed of poorly vascularized large diameter white muscle (glycolytic) fibers organized into segmental myomeres, such that myofibers lie parallel to the long axis of the body. Connective tissue myosepta, attached to vertebrae, segregate adjacent myomeres and serve as attachment points for myofibers. The white muscle is divided into epaxial and hypaxial groups by a horizontal septum that runs roughly parallel to the lateral line.

A thin band of well-vascularized small diameter red (oxidative) muscle is located superficially along the lateral midlines of the body and is separated from the underlying white muscle by a connective tissue septum. Muscles located at the fin bases are also composed of red muscle fibers. Muscle groups of the head lack the distinct segmental organization of the trunk (Grizzle and Rogers 1976; Webb 1993).

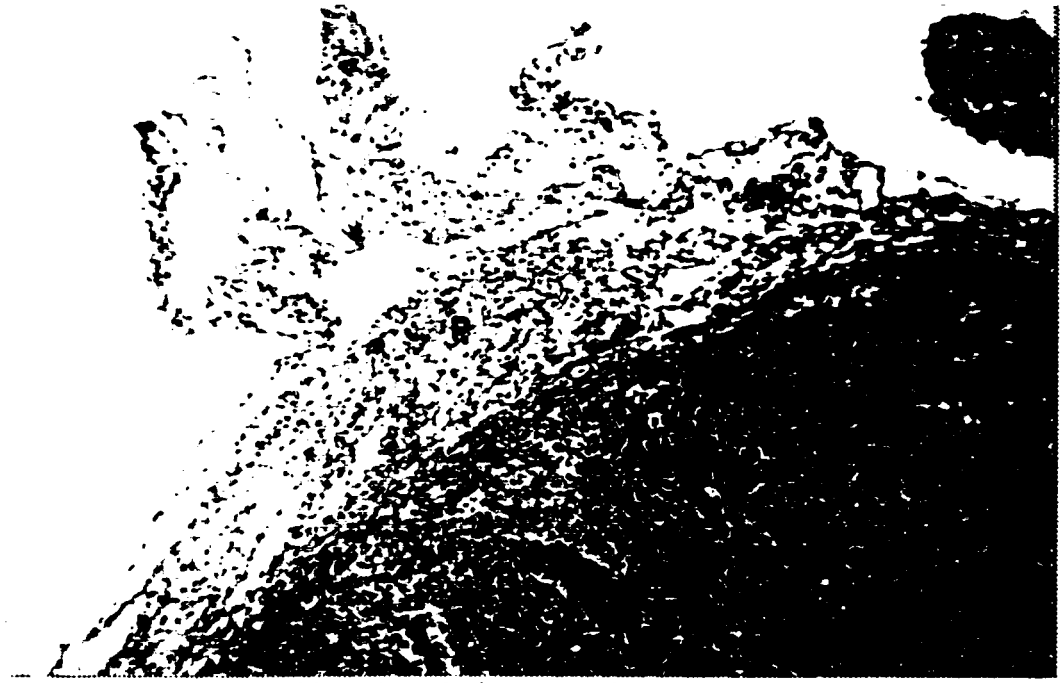


Figure 32. Splenic capsulitis. The splenic capsule is partially covered by a layer of fibrin and macrophages as part of a generalized peritonitis (P). Parenchymal changes are minimal. (H&E, X 100).

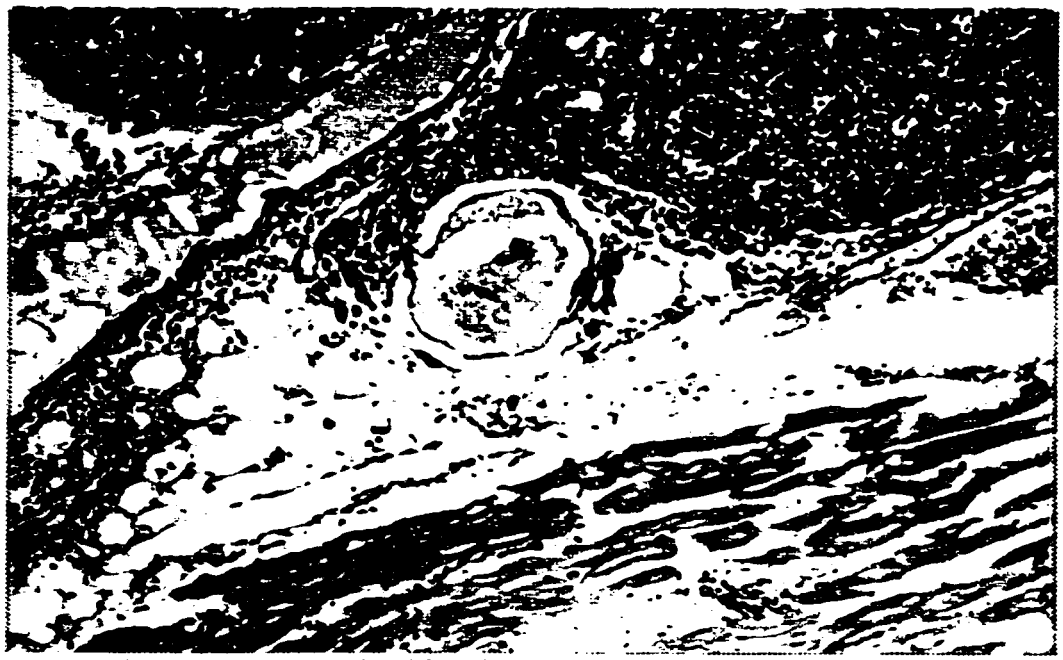


Figure 33. Granulomatous peritonitis adjacent to the head kidney. A sterile caseating granuloma is present in the center of the field. (H&E, X 100).

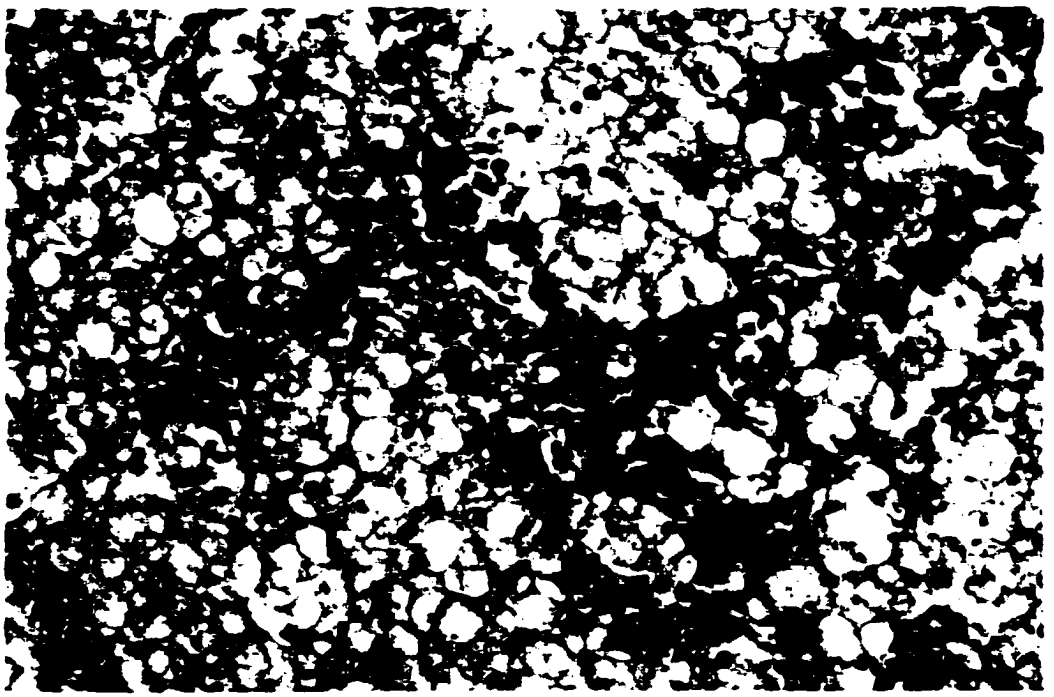


Figure 34. Granulomatous splenitis. There is distortion of the normal splenic architecture due to infiltration of white pulp areas by large numbers of plump macrophages laden with cocci. (H&E, X 400).

Muscle lesions were usually limited to mild fasciitis, originating in perivascular or perineuronal sites or as extensions of more superficial cellulitis. Less commonly, inflammation dissected progressively along interfascicular and perimysial connective tissues eventually isolating individual myofibers. Changes varied from myofiber atrophy to coagulative necrosis of entire muscle bundles characterized by increased eosinophilia, loss of striation, cytoplasmic vacuolation, and coagulation (Figure 35).

Occasionally, areas of abscessation or granulomatous inflammation and fibrosis obliterated entire muscle groups. In these areas it was often difficult to demonstrate the presence of the organism. Abscess formation appeared to have a predilection for red muscle areas, particularly at the bases of the pectoral, pelvic, and caudal fins (Figure 36).

The bones of fish are derived from two sources. Dermal bone, including the scales and some bones of the skull, arises from the dermis without a preexisting cartilaginous anlage in a manner similar to the intramembranous bone of higher vertebrates. The remainder of the fish skeleton develops from the endochondral process involving perichondral ossification of hyaline cartilage (Yasutake and Wales 1983; Sickle et al. 1993).

The histology of piscine bone, however, represents a major departure from that of higher vertebrates. As with other higher teleosts, the bone of tilapia is laminated, acellular, avascular, and lacks a system of Haversion canals. True marrow spaces are not present, although the bone may appear multilocular and contain spaces filled with areolar connective tissue and fat (Rogers and Grizzle 1976). The lack of osteocytes precludes resorption of calcium from bone such that acellular bone cannot serve as a calcium reserve (Roberts 1989). At sites of active ossification, growing bone spicules may be

Figure 35. Necrotizing myositis. The center of the field contains a focus of myolysis and inflammatory cell infiltration. Degeneration of adjacent muscle fibers is indicated by cytoplasmic coagulation and condensation. (H&E, X 200).

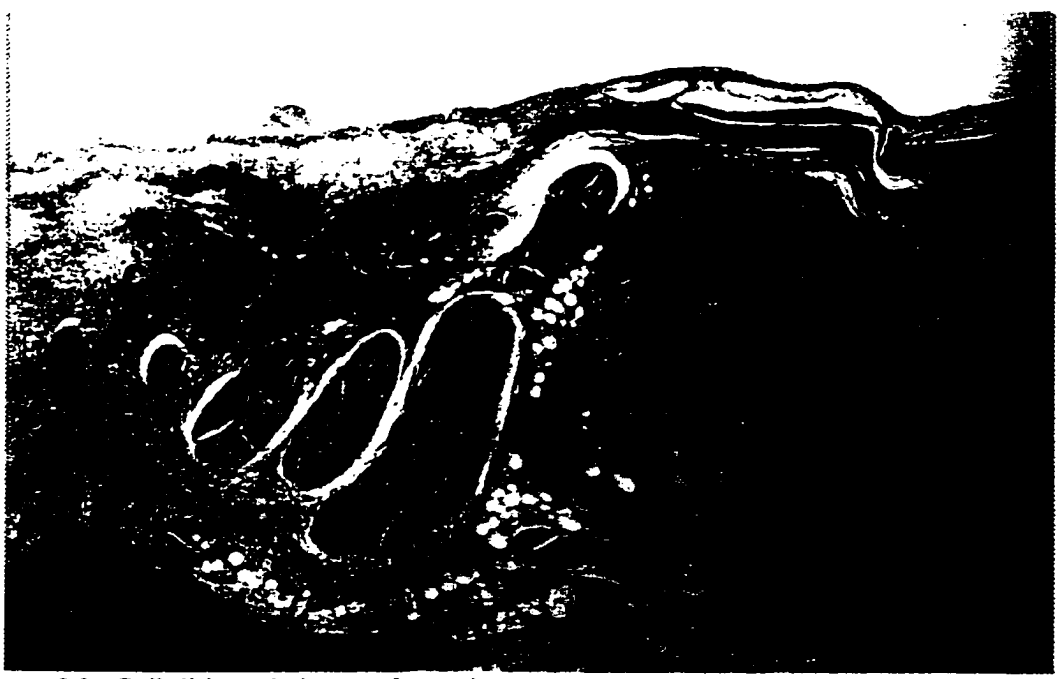


Figure 36. Cellulitis and abscess formation. An extensive area of cellulitis (C), fibroplasia and liquifaction segregates individual muscle groups (M) of the pectoral fin. (H&E, X 20).

lined by angular osteoblasts. As reported in other fish species, osteoclasts in tilapia appear to be scarce (Wendelaar Bonga 1993). As such, the use of terms such as osteitis, osteomyelitis, or even necrosis is called into question or nullified.

In *S. iniae* infections, osteolysis was seen as an extension of adjacent soft tissue inflammation. Lysis and remodeling was seen in areas of severe cellulitis or abscessation, most commonly at the fin bases and in the skull. Sequestra of necrotic bone were sometimes found within such foci. Multinucleated giant cells resembling osteoclasts were occasionally found in Howship's lacunae resorbing bone in areas of active remodeling, while simultaneous osteoblastic activity was seen along osteoid seams of preexisting bone (Figure 37).

Reproductive Organs

The ovaries of tilapia contain numerous follicles in various stages of maturation. Oogonia develop in groups associated with the germinal epithelial lining of ovigerous lamellae. The lamellae are lined by a simple squamous epithelium and supported by a scant fibrovascular stroma. Follicular development within lamellae causes them to protrude into and obscure the lumen of the hollow ovary.

Follicles are surrounded by a hyaline membrane (zona radiata), a single layer of cuboidal cells (granulosa), and a spindle cell layer (theca). The asynchronous ovaries of tilapia frequently contain atretic preovulatory follicles. Some authors have described the resorption of oocytes by granulosa cells, which hypertrophy and become phagocytic, consuming remnants of oocytes and form an irregular cellular mass containing yellow lutein pigments (Hibiya 1982; Nagahama 1983). Yolk globule fragments pass through the disrupted zona radiata to be phagocytized by large cells in the granulosa layer.

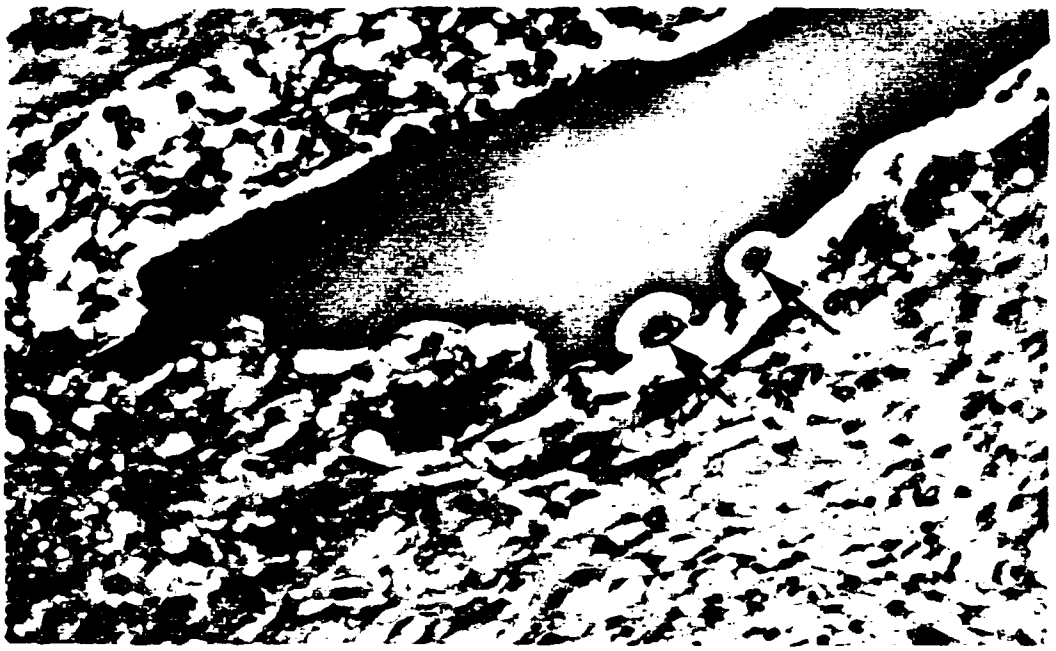


Figure 37. Bone sequestrum undergoing osteolysis within an area of liquefaction. Note the presence of osteoclasts within Howship's lacunae (arrows). (H&E, X 200).

The testicles of tilapia are of the lobular type typical of most teleosts (Grier 1981). The densely packed lobules are separated by thin fibrovascular septa containing small numbers of Leydig cells having indistinct cytoplasm and deeply basophilic nuclei with dispersed chromatin. Lobules are further subdivided into "cysts" (Henderson 1962), each containing clusters of germ cells in approximately the same stage of development formed by Sertoli cells, including spermatogonia, spermatocytes, and spermatids. Mature spermatozoa rupture from cysts and are found within the lumens of lobules (Nagahama 1983).

Associated with the basement membrane of the lobule are primordial germ cells identified by their extremely large nuclei, single prominent nucleolus, and abundant pale eosinophilic cytoplasm. Sertoli cells have been described in close association with these germ cells (Nagahama 1983), but are not readily apparent in H&E sections. Small, dense, crescent shaped nuclei can be found adjacent to germ cells, which may represent the nuclei of Sertoli cells. It was not unusual to find small numbers of large globoid cells resembling oocytes scattered among testicular lobules. It is not know if these represent vestiges of ovarian tissue from sex reversed females, or the giant spermatogonia reported in cichlid fishes (Fishelson 1996).

In diseased females, interstitial areas outside the granulosa became more prominent due to infiltration by macrophages, containing phagocytized cocci, and hyperplasia of fibroblasts. Less frequently, there was complete breakdown of follicular structure, creating lakes of fat globules infiltrated by large numbers of macrophages and cocci (Figure 38). In males, testicular interstitia also was infiltrated by variable numbers

of macrophages. Occasionally, cocci laden macrophages penetrated the lumens of lobules and intermingled with maturing spermatozoa (Figure 39).

Digestive System

The stomachs of *T. nilotica* are relatively small, but their intestinal tracts are long and possess a lengthy spiral segment consistent with their herbivorous feeding habits. The stomach has a prominent fundic region with deep rugal folds and thick mucosa formed by glands with short necks lined by mucus secreting cells. The tubular gastric glands are long, straight, densely packed, and lined by cuboidal cells with granular eosinophilic cytoplasm and central to slightly basilar round nuclei resembling chief cells.

As with other fish species, there is no morphologic differentiation of the small intestine into regions and no distinction between small and large intestinal segments (Grizzle and Rogers 1976). In the terminal portion of the intestine, villi are somewhat blunted and the number of goblet cells distributed among the tall columnar epithelium increases. No pyloric cecae are present. There is no organized gut associated lymphoid tissue, but the lamina propria does contain a resident population of lymphocytes, macrophages, and commonly, small numbers of coarse eosinophilic granulocytes, which are presumed to participate in immune surveillance.

With the aide of selective media *S. iniae* can be readily cultured from the intestines of diseased fish, suggesting enteric lesions should be expected. In the fish examined, however, it was uncommon to find the gastrointestinal tract involved to any significant degree. With mild inflammation and H&E stains, localization of *S. iniae* in the gastrointestinal tract can be missed. Gram stains revealed small numbers of

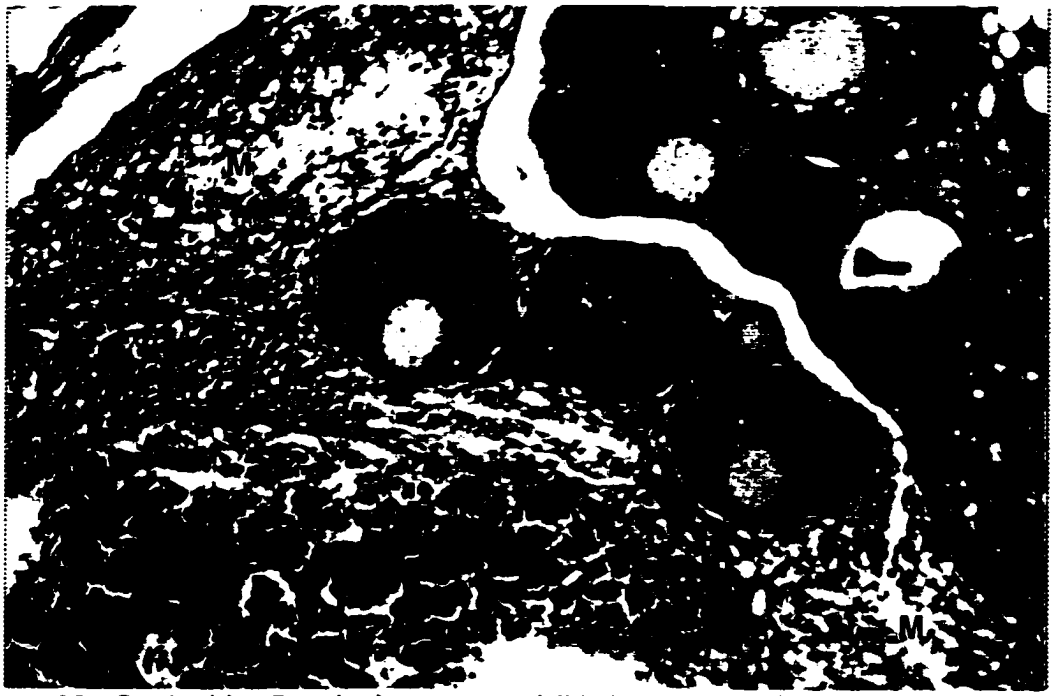


Figure 38. Oophoritis. Developing ova are visible in the upper right and a lake of degenerating yolk globules are present in the lower left. There is increased prominence of interstitial areas due to fibroplasia and infiltration by cocci containing macrophages (M). (H&E, X 100).

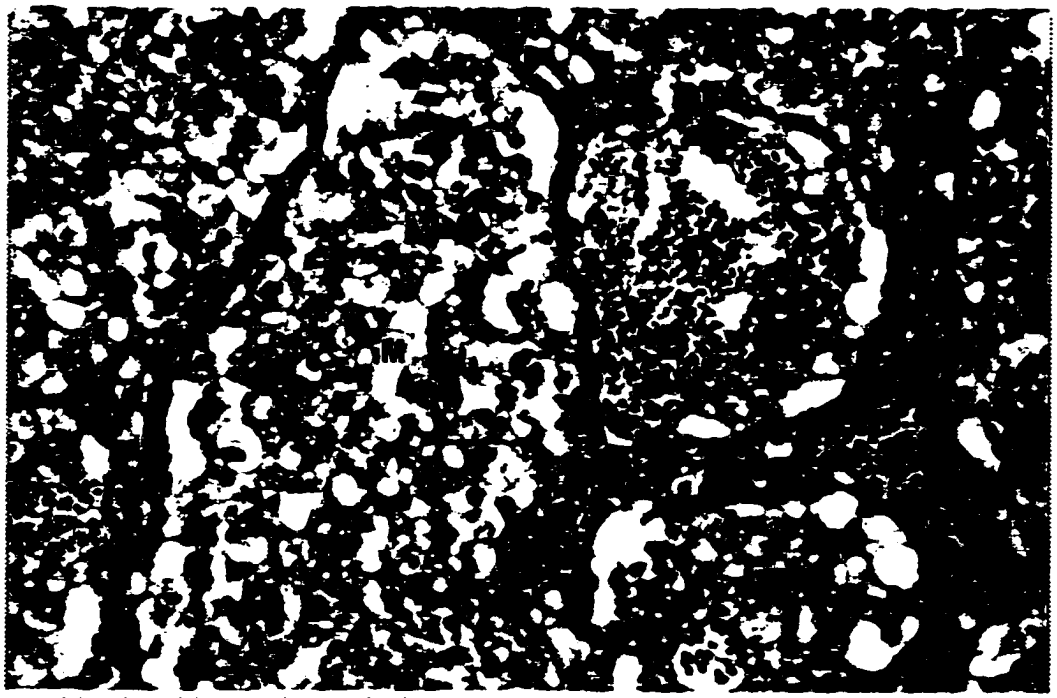


Figure 39. Orchitis. Entire testicular lobules have been replaced by invading macrophages and cocci (M). (H&E, X 100).

macrophages containing cocci in the submucosa, lamina propria, and occasionally within the mucosal epithelium. Only rarely were large foci of submucosal inflammation and necrosis seen in the stomach or intestine, but when present, exocytosis of larger numbers of cocci laden macrophages could usually be found in the mucosa (Figures 40 and 41).

In cases of peritonitis, foci of granulomatous inflammation, with small fibrin tags and cocci were associated with the gastrointestinal serosa. Similar changes, including fat necrosis, were often scattered throughout the mesentery. Extrahepatic exocrine pancreatic tissues are intimately associated with the hepatic portal vein in the area of the bile duct. Interstitial pancreatic tissues were sometimes infiltrated by macrophages and less frequently there was isolated necrosis of small numbers exocrine pancreatic cells (Figure 42). Inflammation of pancreatic islets was not seen.

The swim bladder is located retroperitoneally against the vertebral cavity and derived embryologically from an outcropping of the foregut. Tilapia are physoclistic and their swim bladder is relatively thin walled when compared to that of the channel catfish. It is bisected by a vertical septum anteriorly. The swim bladder has a dense fibrous external tunic and an internal tunic lined by a simple squamous epithelium sitting on a vascularized bed of aerolar connective tissue. The internal tunic is periodically thickened by capillary rete mirabile and overlying secretory epithelia, which collectively form portions of the gas gland (Steen 1970).

Multifocal accumulations of inflammatory cells, including neutrophils, macrophages and eosinophils were sometimes present in the submucosal layer of the tunica interna. Inflammatory cell populations were usually small but increased in association with the capillary rete mirabile and in other perivascular locations. Changes

Figure 40. Gastritis. There is focal necrosis (N) and inflammation in the submucosa. The mucosa is intact, but infiltrated by small numbers of cocci containing macrophages that occasionally traverse the epithelium. (H&E, X 20).

Figure 41. Gastritis. The submucosa is expanded by a mixture of inflammatory cells including lymphocytes, eosinophilic granulocytes, and macrophages containing cocci. (H&E, X 100).

were generally minor in the sections examined and exudate was never observed within the lumen of the swim bladder (Figure 43).

Respiratory System

With the exception of cocci laden macrophages noted within the lumens of lamellar capillaries, lesions involving the gills of tilapia were not observed.

Discussion

The early Japanese descriptions of *S. iniae* epizootics concentrate more on biochemical properties and morphology of the organism, than on morphology of the disease (Kitao et al. 1981; Ohnishi and Jo 1981; Ugajin 1981; Miyazaki 1982; Nakatsugawa 1983). Outbreaks in tilapia, hybrid striped bass and rainbow trout in the United States and Israel beginning in the early 1990s renewed interest in the disease and several recent publications have included limited descriptions of lesions resulting from *S. iniae* infection (Eldar et al. 1995b; Stoffregen et al. 1996; Perera 1998; Eldar et al. 1999; Bromage et al. 1999; Eldar and Ghittino 1999). The purpose of this paper is to supplement these narrow descriptions with one more detailed, compare findings with those published in the current literature, and to present several previously unreported findings noted in clinical cases of diseased tilapia.

In tilapia, *S. iniae* infection presents as an atypical bacterial septicemia when compared to the better known and described gram negative fish pathogens. *Streptococcus iniae* has a marked tropism for the central nervous system, and is capable of causing severe meningoencephalitis, which often extends to structures of the inner ear. In this study, multisystem involvement was seen, but with the exception of the spleen, changes in parenchymal organs were relatively mild. Polyserositis was typically present,

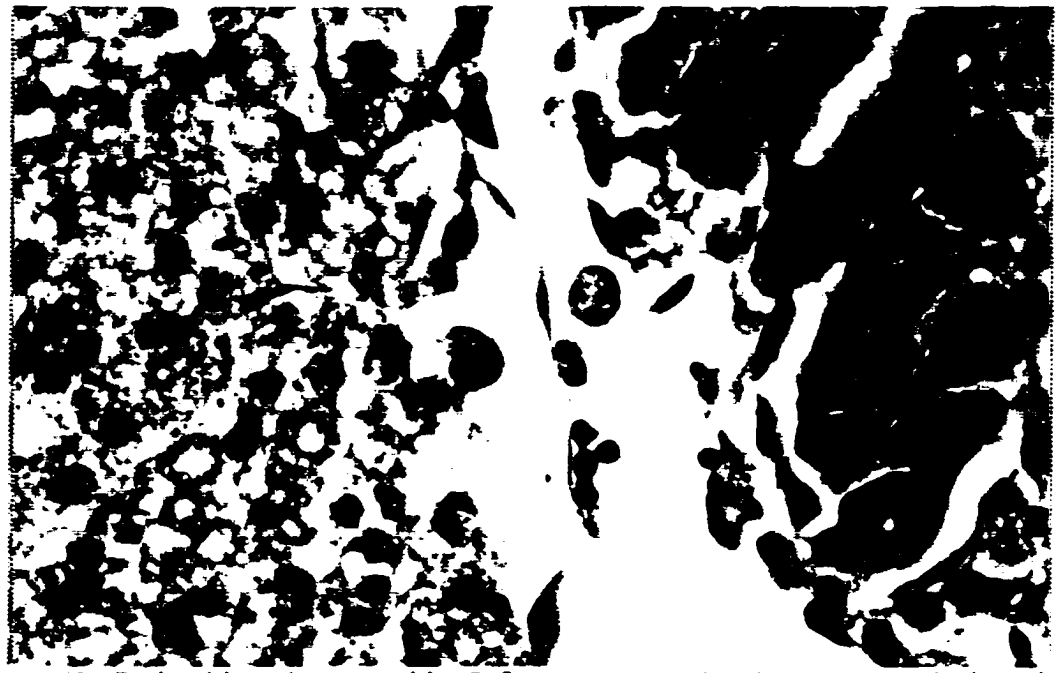


Figure 42. Peritonitis and pancreatitis. Inflammatory exudate is present on the intestinal serosa and invades interstitial areas of the exocrine pancreas. There is necrosis of fat and individual acinar cells. (H&E, X 100).

Figure 43. Inflammation of the swim bladder. The submucosa of the tunica interna is infiltrated by small numbers of inflammatory cells, including neutrophils and eosinophilic granulocytes. (H&E, X 400).

affecting serosal surfaces of the abdominal viscera and in particular the epicardium. A third notable feature of *S. iniae* infection in tilapia was cellulitis, fasciitis, and abscess formation within subcutaneous tissues and skeletal muscle. When multiple fish were examined, a spectrum of changes were usually present among individual specimens, varying in type, distribution, severity, and chronicity.

Inflammatory responses in fish have been classically interpreted as granulomatous and coagulative to caseous necrosis is the rule. While *S. iniae* belongs to the pyogenic group of β-hemolytic streptococci (Koneman et al. 1997), lesions in fish are invariably predominated by macrophages. It is interesting to note, however, that liquefaction or abscess formation does occur with *S. iniae* infections in tilapia, suggesting neutrophils and the release of hydrolytic enzymes into tissues may play a significant role in the development of cellulitis.

As stressed by Witten et al. 1998, using routine H&E stains, differentiation of tilapia macrophages from neutrophils can be difficult. Suppurative responses in fish remain incompletely characterized, but under experimental conditions neutrophils arrive at sites of inflammation within 24 hrs, peak within about 2 days, and are then gradually replaced by macrophages in a sequence similar to that in mammals (Suzuki and Iida 1992). This sequence of events is not unexpected as fish neutrophils are less phagocytic than macrophages, have a differential ability to phagocytose various bacteria, and may not kill as efficiently (Ainsworth and Dexiang 1990; Secombes and Fletcher 1992).

Using cytochemical stains on smear preparations of exudate (Witten et al. 1998) tilapia neutrophils are readily differentiated from macrophages by their oxidase or esterase positive reactions, respectively. In H&E sections, however, differentiation of

the two cell types was not always readily apparent. It is reasonable to assume that neutrophils may play a large role in early lesion development, but are outnumbered by macrophages as lesions become less acute. Peracute deaths with few clinical signs or lesions may result from *S. iniae* infection (Bromage et al. 1999), or they may follow a more protracted course with death ensuing in 1-2 weeks (Eldar et al. 1995). Often fish collected for diagnostic evaluation are exhibiting advanced clinical signs and may have passed through the acute phase of the inflammatory response. Large numbers of *S. iniae* are present in most lesions, but are best visualized by the use of tissue gram stains, such as Brown-Bren (Prophet et al. 1992), and phagocytes may become distended with engulfed cocci suggesting that intracellular multiplication might be taking place.

Early gross descriptions of *S. iniae* infected tilapia, ayu, rainbow trout, amago, and flounder report diseased fish as lethargic and anorectic, with exophthalmia, congestion of the caudal fin, and petechiation of the inner opercles, ventral abdomen, and anus. Commonly reported internal lesions include ascites, hemorrhages in the intestinal tract, liver, and pyloric cecae (Kitao et al. 1981, Onishi and Jo 1981, Ugajin 1981, Nakatsugawa 1983). In the fish examined in this study, darkening of the body with exaggeration of the vertical banding pattern, hyperemia of the fin bases, exophthalmia, hypopyon, ascites, splenomegaly, and exudate covering the meninges were the most common gross lesions. In addition, abscess formation, especially in the caudal peduncle was not unusual.

Streptococcus iniae infections share morphologic features common to other gram positive fish pathogens, particularly the group B Ib streptococci (*S. agalactiae, syn. S. difficile*), also reported to cause disease in tilapia, and *Lactococcus garviae*. A

streptococcal disease was reported to cause dermal hemorrhage, exophthalmia, panophthalmitis, meningoencephalitis, gastroenteritis, and peritonitis, with disseminated involvement of serosal surfaces and parenchymal lesions in the liver, spleen, kidney, and gonads of tilapia (Miyazaki et al. 1984). While this description could easily be applied to *S. iniae*, the organism isolated was non-hemolytic and probably represented a group B streptococcus.

Similarly, experimental infections in tilapia with a γ-hemolytic streptococcus produced neurologic signs, with granulomatous panophthalmitis, meningitis, pericarditis, myocarditis, peritonitis, splenitis, oophoritis, and submucosal inflammation in the gastrointestinal tract. Lesions were characterized by hemorrhage, macrophages, and fibroblast proliferation. The spleen, kidney and ovary sometimes contained small caseating granulomas (Chang and Plumb 1996).

Miyazaki (1982) compared the histopathological lesions of an α-hemolytic coccus, consistent with *L. garviae*, in yellowtail beak perch *Opelgnathus fasciatus*, horse mackerel *Trachurus japonica*, and striped jack *Caranx delicatissimus* to those of a β-hemolytic coccus suggestive of *S. iniae*, in flounder, ayu, and rainbow trout. The α-hemolytic organism caused granulomatous inflammation, while the β-hemolytic isolates caused septicemia with extensive suppurative inflammation of the eyes. Panophthalmitis and meningitis were reportedly identical in rainbow trout naturally infected with *S. iniae* and *L. garviae*. The two diseases were differentiated microscopically based on the lack of pseudomembranous enteritis, pericarditis, and severe peritonitis in *S. iniae* infected trout (Eldar and Ghittino 1999). These descriptions and others of group B streptococci (Rasheed et al. 1985) and *L. garviae* (Kusuda et al. 1976) illustrate enough similarities in

the pathological changes induced by the respective organisms to indicate that *S. iniae* cannot always be reliably differentiated from them at either the gross or microscopic level.

The most detailed histopathologic description of *S. iniae* infection in tilapia was published by Perera et al. (1998). Several notable differences are documented in the current study. Perera found no gross dermal or epidermal lesions and no abnormalities were present in the skeletal muscle of diseased fish. In contrast, it was common to find petechiation and pustule formation associated with bony prominences along the ventral aspect of the lower jaw and ventral midline, particularly the pelvic girdle. It was not unusual to find fluctuant swellings in the caudal peduncle, which upon closer inspection revealed large abscesses containing up to several milliliters of exudate.

Microscopically, focal cellulitis, with or without overlying epidermal necrosis, and deeper fasciitis regularly extended into muscle groups of the head and trunk. Changes varied from atrophy of isolated muscle fibers to coagulation or liquefaction of entire muscle groups. Bone remodeling with osteolysis and sequestration of bone spicules was also present within some foci of cellulitis. Severe changes appeared to have a predilection for red muscle groups located at the fin bases, possibly due to the rich vascular supply and increased oxygen tension present in these areas. Epidermal necrosis, cellulitis and necrotizing myositis have also been reported in red drum (Eldar et al. 1999).

Expansive areas of cellulitis and deeper fasciitis were often present within subcutaneous tissues of the head that sometimes surrounded lateral line canals and olfactory sacs. Inflammatory infiltrates and bacteria were present throughout much of

the bony reticulum of adipose and connective tissue filled cavities that comprise much of the skull. In the process there was envelopment of cranial nerves, blood vessels, and even tooth roots.

As described in hybrid striped bass (Stoffregen et al. 1996), tilapia (Perera et al. 1998), and red drum (Eldar et al. 1999), epicarditis and myocarditis was also a prominent feature of diseased tilapia in this study. Similar lesions were not reported in rainbow trout (Eldar and Ghittino 1999). All of the tilapia examined exhibited some degree of epicarditis and pericarditis, although changes varied dramatically in severity. In severe cases a bed of granulation tissue developed over the epicardium, sometimes forming adhesions with the pericardium, in association with transmural myocarditis. In addition, septic thrombi were occasionally found adhered to the endocardium and bulboventricular valve leaflets.

With the exception of the spleen, whose architecture could become almost unrecognizable due to massive infiltration by macrophages, lesions of the abdominal organs was usually limited to capsulitis in association with more widespread peritonitis. While small numbers of macrophages containing cocci could be identified within sinusoids or interstitial areas of various organs, necrosis or morphological disturbances were rare. This is in contrast to observations by other authors who have reported necrotizing foci and large areas of inflammatory cell infiltration in the kidney (Perera et al. 1998; Eldar et al. 1999). In the gonads, interstitial areas were sometime infiltrated by macrophages containing cocci, often accompanied by fibroplasia in the ovaries. In more severe cases, there was infiltration of both ovarian follicles and testicular lobules by the inflammatory process.

Perera et al. (1998) discussed a lack of gastrointestinal lesions in diseased tilapia in comparison to an earlier study by Miyazaki of a γ-hemolytic streptococcal disease, also in tilapia. Eldar and Ghittino (1999) considered a lack of intestinal lesions a useful criterion for discriminating between *S. iniae* and *Lactococcus garviae* infections in rainbow trout. In the current survey, lesions involving the mucosa and submucosa were less common than serosal changes associated with generalized peritonitis, but did occur. As in trout, mucosal erosions and pseudomembrane formation were not seen. Rarely there was heavy infiltration of the submucosa by mixed populations of inflammatory cells, including macrophages, neutrophils and lymphocytes, sometimes in association with foci of coagulative necrosis. More frequently, slightly increased populations of inflammatory cells, including macrophages with cocci, were encountered in the lamina propria and submucosa. Careful examination revealed exocytosis of phagocytes across the gut epithelium.

Lesions involving the swim bladder have only been reported once as a manifestation of *S. iniae* infection. In the tilapia examined here, swim bladder lesions were uncommon and typically mild in nature. Minor inflammatory changes and phagocytized cocci were occasionally found in the wall of the swim bladder, particularly in the capillary rete mirabile of the gas gland. Exudate was never found in the lumen of the bladder as reported by Perera et al. (1998).

Consistent with disease of the central nervous system, descriptions of *S. iniae* outbreaks in Israel, Texas, and Saudi Arabia all describe infected tilapia exhibiting erratic swimming, circling, or whirling near the water's surface (Eldar et al. 1994; Perera et al. 1994; Al- Harbi 1994). Kaige et al. (1984) implicated *S. iniae* as the cause of vertebral

malformation in cultured yellowtail and was first to describe infiltration of the brain and meninges by cocci laden macrophages. In addition to tilapia, central nervous system signs and lesions have also been reported in yellowtail (Kaige et al. 1984), hybrid striped bass (Stoffregen et al. 1996), red drum (Eldar et al. 1999), barramundi (Bromage et al. 1999), and rainbow trout (Eldar and Ghittino 1999).

When opening the cranium of affected tilapia, cloudy to sanguineous exudate was grossly visible, instead of the gelatinous pad of adipose that normally covers the brain, and there was congestion of the meningeal vasculature. While usually described as producing a meningoencephalitis (Kaige et al. 1984; Eldar et al. 1995; Stoffregen et al. 1996; Perera et al. 1998; Eldar et al. 1999), involvement of the brain parenchyma was usually less severe than the meningitis. Meningeal vessels were dilated and there was widening of the menix by inflammatory infiltrates, predominated by macrophages. A dense layer of exudate often filled much of the extrameningeal cranial vault. Variable, but often large numbers of free and phagocytized cocci were present in the meningeal infiltrates.

Kaige et al. (1984) reported granulomas containing cocci in the third ventricle, meninges, cerebellar cortex, hepatic capsule and peritoneum. Meningeal and hepatic granulomas have also been reported by Perera et al. (1998). Similarly, granulomas were also present in the meninges, liver, and choroid rete mirabile of some tilapia examined in this study. As noted by Perera, these granulomas had caseous centers and did not contain bacteria. The significance of granuloma formation is unclear, but they may represent foci of containment where the organism has been eliminated. An unusual

manifestation not encountered in this study was palisading of large multinucleated giant cells around foci of subarachnoid hemorrhage (Eldar et al. 1995).

In most cases, meningitis extended into the subadjacent neuropil, but seldom extended beyond the molecular layers of the optic tectum or cerebellum. Small numbers of macrophages could be seen traversing the walls of congested capillaries extending downward from the menix. In severe cases, the third ventricles contained macrophages and cocci, and there was extensive infiltration of macrophages throughout white and gray matter areas of the optic tectum and cerebral valvula.

One striking previously unreported observation made in this study was involvement of inner ear, including necrosis of the sensory epithelia of the cristae and maculae, disruption of the membranous labyrinth, and filling of endolymph spaces of the semicircular canals with exudate. Severe inflammation of the inner ear with disruption of the vestibular system probably accounts for many of the abnormal swimming patterns and postures commonly reported in the literature.

Along with structures of the inner ear, the mechanosensory lateral line forms the second component of the octavolateralis system, which is responsible for detection of short distance pressure disturbances in water (Popper and Platt 1993). In infected fish, inflammatory cells infiltrated the adventitia surrounding the canal epithelia, which was being traversed by macrophages containing cocci. Cocci were sometimes present free within canal lumens. Similar changes were also seen involving the olfactory mucosa

Ocular lesions of varying severity have been almost universally reported by authors describing *S. iniae* infections in tilapia and other fish species (Kitao et al. 1981; Miyazaki 1982; Nakatsugawa 1983; Foo et al. 1985; Perera et al. 1994; Al-Harbi 1994;

Eldar et al. 1995b; Sugita 1995; Stoffregen et al. 1996; Perera et al. 1998; Bromage et al. 1999; Eldar et al. 1999). Gross ocular changes varied from unilateral or bilateral exophthalmia with corneal opacification to more severe changes, including hypopyon. End-stage lesions were typified by panophthalmitis with perforating corneal ulcers with evisceration of the uveal tract and retina. Inexplicably, corneal ulceration and perforation was seen in tilapia suffering from *S. iniae* infection that did not possess intraocular lesions referable to the disease. Although speculative, central blindness due to inflammation in the optic tectum may have predisposed these individuals to self-induced trauma.

The existence of an irido-corneal filtration angle responsible for the drainage of aqueous fluid is debated in fish (Tripathi 1974) and glaucoma has not been reported to occur (Hargis 1991). It is unclear whether a glaucomatous condition contributes to exophthalmia, but the presence of exudate deep within the irido-corneal annular ligament, morphologically similar to the mammalian drainage angle at the light microscopic level, at least suggests this could be possible. Inflammatory changes were particularly severe in the area of the optic nerve papilla, often involving the choroid capillary rete mirabile. Choroid edema may be responsible for the common finding of retinal detachment. Inflammation and edema within the retrobulbar fat undoubtedly contributed to exophthalmia.

The goal of this study was to provide a detailed characterization of *S. iniae* infections in cultured tilapia. Several previously unreported findings may contribute to a better understanding of the pathogenesis of the disease. Abnormal swimming patterns have been commonly reported as manifestations of infection attributed to meningitis

(Eldar and Ghittino 1999). Many of the specimens examined in this study had severe inflammatory and necrotizing changes involving structure of the inner ear. Disruption of the vestibular system may be a more specific factor relating to the observed neurologic signs. Previous reports of *S. iniae* infections have also failed to include lesions involving the gastrointestinal tract. In rainbow trout, lack of gastrointestinal lesions was considered a criterion for ruling out *S. iniae* infection (Eldar and Ghittino 1999).

Little is known concerning the pathogenesis of *S. iniae* infections, in particular how the organism gains entry to fish or how it is shed into the environment. Cocci laden macrophages were observed traversing the epithelium of the olfactory, lateral line, and gastrointestinal mucosae. Evans et al. (2000) recently induced disease signs and mortalities in tilapia and hybrid striped bass following nares inoculation with a virulent *S. iniae* isolate, suggesting the nares may be a potential route of infection. The degree of submucosal inflammation and necrosis observed in diseased tilapia suggested that shedding of *S. iniae* could also have taken place across the olfactory epithelium and across mucosal surfaces in general. Infection of the gonads was also seen in many fish suggesting another potential route by which the organism might be shed. Although it is unlikely that breeding would occur in diseased fish, the possibility of a carrier state has never been addressed and the prospect of both horizontal and vertical transmission cannot be entirely dismissed.

Ocular lesions are almost universally reported in conjunction with *S. iniae* infections, but direct application of *S. iniae* to the eye fails to induce disease (Evans et al. 2000). The morphological organization of the cornea, in contrast to that of typical mucosal borders, suggests an impervious barrier and indicates that the eye would not

provide a significant portal of entry (Tripathi 1974). *Streptococcus iniae* may arrive in the eye by the hematogenous route or descend the optic nerve as a direct extension of meningitis. Other tissues with rich vascular supplies, such as red muscle, are also common sites of infection.

Variations in mortality and lesion development may reflect differences in host susceptibility or the expression of virulence factors specific to individual *S. iniae* isolates. Differences in host susceptibility have been demonstrated among various fish species (Eldar et al. 1995; Yuasa et al. 1999). Al-Harbi (1996) showed significant differences in susceptibility even among different species of tilapia experimentally challenged by intraperitoneal injection. Currently, there is little known regarding the production of virulence factors by *S. iniae*. Among 29 *S. iniae* isolates from tilapia, hybrid striped bass, rainbow trout and human sources 79 % were found to produce hyaluronidase, considered a spreading factor in other streptococcal species. In the same study 52 % were found to produce DNase, also considered a potential streptococcal virulence factor (Chapter III).

In tilapia, *S. iniae* septicemia presents with gross and microscopic lesions sharing similarities with other gram positive bacteria, most significantly a marked tropism for the central nervous system. Polyserositis and multisystem involvement occur, but lesion distribution and severity appear to vary between individual cases. As with most epizootics in closed populations of animals, a spectrum of changes may occur and the presence or lack of a specific lesion may not be a valid basis for making the diagnosis. Complete microscopic examination of multiple individuals will probably reveal a more complete picture of the disease.

CHAPTER III: INVESTIGATION OF THE PRESENCE OF POTENTIAL STREPTOCOCCAL VIRULENCE FACTORS IN *STREPTOCOCCUS INIAE*

Introduction

Streptococci are obligate parasites of cutaneous and mucosal surfaces of humans and animals. Some are considered resident flora, causing infection only when introduced into normally sterile sites or in immunocompromised hosts. Other species are true pathogens, which spread between individuals, and cause infections in normal, non-immune hosts (Kilian 1998). Representatives of the pyogenic group of hemolytic streptococci produce a broad spectrum of virulence factors, which allow them to invade host tissues and evade defensive mechanisms. Of these, the group A streptococci have been most intensely studied and their virulence factors best characterized.

Streptococcus iniae was first isolated and described from subcutaneous abscesses in a captive Amazon freshwater dolphin *Inia geoffrensis* in 1976. From 1977 to 1980, and possibly earlier, large-scale epizootics caused by β-hemolytic streptococci with biochemical profiles compatible with *S. iniae* occurred in tilapia *Tilapia nilotica*, rainbow trout *Oncorhynchus mykiss*, ayu *Pleyoglossus altivelis*, and amago *Oncorhynchus rhodurus* in Japan (Minami et al. 1976; Kitao et al. 1981; Ohnishi and Jo 1981; Ugajin 1981). Since 1994, *S. iniae* has emerged as the major bacterial pathogen of cultured tilapia (Perera et al. 1994; Eldar et al. 1994) and has steadily increased in incidence and host range worldwide (Nakatsugawa 1983; Kaige et al. 1984; Foo et al. 1985; Al-Harbi 1994; Stoffregen et al. 1996; Zlotkin et al 1998; Bromage et al. 1999; Eldar et al. 1999; Yuasa et al. 1999). There have also been cases of invasive disease in

humans, limited primarily to cellulitis of the hand, following skin injuries incurred while handling fish (Weinstein et al. 1997).

Immunity and protection against group A streptococcal (*Streptococcus pyogenes*) infections are related to a surface expressed protein that inhibits phagocytosis by blocking activation of the alternate complement pathway in human blood (Lancefield 1962; Bisno 1979) and may play additional roles in adhesion, invasion, and inflammation (Navarre and Schneewind 1999). Discovered over 70 years ago by Rebecca Lancefield (Lancefield 1928), the group A streptococcal M protein molecule is one of the best characterized bacterial virulence factors. Some strains of group C and group G streptococci also possess M or M-like proteins that share common features including a highly conserved membrane anchor region. Fischetti (1989) and Robinson and Kehoe (1992) have reviewed group A streptococcal Emm proteins. Navarre and Schneewind (1999) have reviewed M proteins in relation to other streptococcal surface proteins of the *mga* regulon.

Other proven or purported virulence factors found in the pyogenic streptococci include the production of antiphagocytic capsules, cytolytic hemolysins, streptolysins O and S, and the unrelated hemolysin of the group B streptococci (*S. agalactiae*). The streptococcal pyrogenic exotoxins (SPEs) and superantigens of group A streptococci are responsible for the rash of scarlet fever and are believed to participate in the pathogenesis of toxic shock-like syndrome. Streptokinase (fibrinolysin), a plasminogen activator, and the enzyme hyaluronidase are believed to act as spreading factors by dissolving fibrin clots and connective tissue ground substance, respectively. Neuraminidase (sialidase) is considered a potential mucosal colonization factor for both

pathogens and commensals (Koneman et al. 1997; Kilian 1998). DNases are believed to contribute to the virulence of group A streptococci and other gram-positive organisms, including *Staphylococcus aureus* (Podbielski et al. 1996).

Although virulence appears to be linked to a specific genetic profile, little is known regarding potential virulence factors associated with *S. iniae* (Fuller et al. 2001). While several authors have described *S. iniae* as possessing a capsule, there has been little evidence presented in support of these claims (Pier and Madin 1976; Kitao et al. 1981) and its composition and potential role in the pathogenesis of this organism have not been investigated. More recently, the existence of a capsule has been disputed (Fuller at al. 2001). Other pyogenic streptococci produce hyaluronic acid or other polysaccharide capsules that inhibit phagocytosis and/or complement activation (Kilian 1998). The purpose of this study was to investigate the possible presence of known or alleged streptococcal virulence factors in *S. iniae*, including M-like proteins, capsule, hyaluronidase, DNase, and streptokinase.

Materials and Methods

Bacterial Strains

Twenty-nine clinical isolates of *S. iniae*, derived from either piscine or human sources, were used. All isolates had been previously cataloged by the Louisiana Aquatic Animal Disease Diagnostic Laboratory, Louisiana State University, School of Veterinary Medicine and frozen at –70°C in broth solution containing 15 % glycerol. Bacterial isolates included in the various studies are presented in Table 6.

Table 6. *Streptococcus iniae* and control bacterial isolates used in virulence studies

LSU ID	Original ID	State or Country of Origin	Species of Origin
S. iniae 93-331		MA	Hybrid Striped Bass
S. iniae 94-036		IL	Tilapia
S. iniae 94-093A		ND	Tilapia
S. iniae 94-449		LA	Tilapia
S. iniae 95-066		LA	Tilapia
S. iniae 96-290B		VA	Tilapia
S. iniae 97-003		IA	Tilapia
S. iniae 97-045		VA	Tilapia
S. iniae 98-071		S. Arabia	Tilapia
S. iniae 98-083	ATCC 29178	CA	River Dolphin
S. iniae 98-113	M43B	TX	Tilapia
S. iniae 98-114	ND5C	Israel	Tilapia
S. iniae 98-115	Dan 12	Israel	Rainbow Trout
S. iniae 98-116	M34	TX	Tilapia
S. iniae 98-117	M32B	TX	Tilapia
S. iniae 98-118	Dan 1	Israel	Rainbow Trout
S. iniae 98-177		CA	Hybrid Striped Bass
S. iniae 98-240	KS S98-1	MS	Tilapia
S. iniae 99-091	2031-96	Canada	Human
S. iniae 99-092	2030-96	Canada	Human
S. iniae 99-093	2032-96	Canada	Human
S. iniae 99-094	2378-91	TX	Human
S. iniae 99-299B		FL	Rainbow Shark
S. iniae 99-301D		MN	Tilapia
S. iniae 99-301G		MN	Tilapia
S. iniae 99-456D		IL	Tilapia
S. iniae 00-296		LA	Tilapia
S. iniae 00-300		LA	Tilapia
S. iniae 00-318		MN	Tilapia
S. pyogenes M5			
S. zooepidemicus SzW60			
S. equi CF32			
S. dysgalactiae 98-203		LA	Tilapia
S. dysgalactiae 99-412		LA	Tilapia
S. dysgalactiae 00-351		LA	Tilapia
Group B strep 97-151			Fundulus
Staph. intermedius			
E. coli MC1061			

Investigation of M Protein Using the Polymerase Chain Reaction

Two sets of primers were used in the polymerase chain reaction (PCR) study. First was the "all M" primer pair designed by Podbielski et al. (1991), who evaluated homologous regions of published *emm* gene sequences from group A streptococci. The "all M" pair recognized entire M protein gene sequences from all 31 group A, C, and G streptococci tested. The forward primer corresponds to conserved regions overlapping the Shine-Dalgarno box and first two codons of the *emm* N-terminus. The reverse primer is directed toward the last five codons and extends three nucleotides beyond the stop codon of the *emm* C-terminus (Podbielski et al. 1991). Primer sequences are as follows: forward 5′ GGG GGG GGA TCC ATA AGG AGC ATA AAA ATG GCT 3′, and reverse 5′ GGG GGG GAA TTC AGC TTA GTT TTC TTC TTT GCG 3′.

A second primer pair was designed for this study by comparing nucleotide sequences from the National Center for Biotechnology Information (NCBI) data bank, which recognized homologous regions within conserved anchor regions of M protein and M-like genes. Included in the comparison were *S. pyogenes* emm5, enn5, emm6, emm18, emm23, emm42, emm49, enn49, emm50, enn50, emm52, and emm58; Group G emm, Group C emm, *S. zooepidemicus* SzW60, *S. equi* SeM, and *S. equi* szPSe. Almost perfect homology was found in the terminal 18 bases of all sequences. More variation existed in upstream regions, with the greatest disparity occurring in *S. zooepidemicus* SzW60. The forward primer overlaps the junction of the third C repeat and first D repeat within the cell wall associated domain of the molecule. The reverse primer was an abbreviated version of the "all M" reverse primer, terminating with the stop codon of the gene, to avoid variability within intergenic areas of the different streptococcal species.

The primer sequences are as follows: forward 5′ AAA GCT GAG CTA CAA GC 3′, and reverse 5′ CGC AAA GAA GAA AAC TAA GCT 3′.

The polymerase chain reaction was conducted for a total of 27 cycles using a Perkin Elmer model 480 Thermal Cycler. DNA preparations were denatured for 1 minute at 95°C, allowed to anneal for1 minute at 55°C, and extended for 2.5 minutes at 72°C. PCR products were subjected to gel electrophoresis in 1% agarose I (Amresco, Solon, OH) gels, for 90 min at 60V.

DNA Isolation for PCR

Crude DNA lysates were prepared following the procedure outlined by Berridge et al. (1998). Five to ten colonies grown for 24 hours on tripticase soy agar with 5% sheep blood (TSAB) agar were suspended in 250 µl of Tris-EDTA (TE) buffer (pH 8.0) and vortexed briefly. The cell suspensions were pelleted at 13,000 rpm in an Eppendorf model 5414 micro centrifuge, supernatant broth was decanted, and the pellets resuspended in 100 µl of lysis solution, containing 100 mM NaCl, 10 mM Tris-HCl (pH 8.3), 1 mM EDTA (pH 8.0), and 1% Triton X-100. Tubes were placed vertically in a floating rack and suspended in a beaker of boiling water for 10 min. After cooling to room temperature, the lysates were diluted to 1 ml with ddH_2O.

Electron Microscopy for M Protein and Capsule Production

For electron microscopy, *S. iniae* tilapia isolates 94-449, 97-003 and 98-116 were used, as was ATCC strain 29178, originally reported by Pier and Madin (1976) as encapsulated. For comparison, an M protein positive *S. pyogenes emm*5 and *S. equi* CF32 were included. Bacterial cultures used in this study were streaked for purity on TSAB agar then inoculated into Todd Hewitt broth with 0.3% yeast extract (THYE) and

incubated overnight at 36°C without agitation. The *S. iniae* and group B isolates were grown in 4.5 ml of THYE supplemented with 0.5 ml of filter sterilized tilapia serum. The *S. pyogenes* and *S. equi* broth cultures were supplemented with 0.5 ml of human serum.

For examination of M protein, 1 ml of overnight culture was gently pelleted in microfuge tubes at 2000 rpm for 10 min. Media was removed and cells fixed by resuspension in 0.8% glutaraldehyde with 2% formaldehyde, in 1 M sucrose and 0.01 M NaH_2PO_4 buffer (pH 7.2) for 1.5 hrs. After fixation cells were pelleted, rinsed in 1 ml sucrose-phosphate buffer, pelleted again, and resuspended in 0.1 ml of buffer. Post-fixation was performed in 0.5 ml of sucrose-phosphate buffer containing 1% OsO_4 for 1 hr. Following post-fixation, cells were pelleted and resuspended in 1 ml sucrose-phosphate buffer overnight.

Cells were pre-embedded by pelleting and resuspending them in 1 ml of 2% agarose II (Amresco) held in a 37°C water bath. Cell suspensions were then pipetted into individual wells of six well cell culture plates to a depth of approximately 1 mm, allowed to harden at room temperature, cut into 1-2 mm blocks, and the blocks placed in 1 ml sucrose-phosphate buffer in 2 ml glass vials. Blocks were dehydrated by passage through a graded series of ice-cold ethanol (EtOH) solutions of 30%, 50%, 70%, 80%, 90%, and twice in 100% for 10 min each. The EtOH was replaced with 1 ml of 1 part LR White (London Resin Co. Ltd.):2 parts 100% EtOH, followed by 1 hr of mixing on a horizontal rocker at room temperature, then 2 parts LR White:1 part ETOH, and 4 hrs in 100% LR White. The LR White was changed for a final time and the cell containing agar blocks were rocked overnight. On the following day, 3-4 blocks from each

specimen were placed into the tips of beam capsules and placed in a 62°C oven overnight to cure. Embedded blocks were sectioned on a Sorval MT6000 ultra microtome and examined using a Zeiss EMTC transmission electron microscope.

Capsule staining was performed using ruthenium red dye (Polysciences, Inc., Warrington, PA). A stock solution was prepared by crushing 0.32 gm of dye granules in a mortar and pestle followed by the addition of 4 ml distilled H_2O (dH_2O). The suspension was transferred to a centrifuge tube and the volume brought to 7.5 ml with dH_2O, followed by heating at 60°C for 5 min with agitation. Tube contents were then centrifuged at 10,000 x g for 5 min and the supernatant aspirated for use.

For ruthenium red staining, bacterial samples were washed twice in physiologic buffered saline (PBS) and fixed for 1 hr in a solution containing 0.5 ml each of 6% glutaraldehyde, 0.2 M sodium cacodylate, and ruthenium red stock. After fixation, cells were washed 3 times for 5 min each in 0.1 M sodium cacodylate with 5% sucrose, followed by a second fixation for 1 hr in 0.5 ml each of 4% OsO_4 in dH_2O, 0.2 M sodium cacodylate, and ruthenium red stock. Following the second fixation, the cells were again washed 3 times for 5 min each in cacodylate sucrose buffer. Dehydration and embedding were performed as described above.

Ferritin labeling and lysine-ruthenium red staining were performed as outlined by Jacques et al. (1990). For ferritin labeling, bacterial cells were fixed in cacodylate buffer and glutaraldehyde as described above. Fixed bacteria were suspended in cacodylate buffer and allowed to react with polycationic ferritin (Sigma) at a final concentration of 1.0 mg/ml for 30 min at 20°C. The reaction mixture was diluted 10 fold and the cells washed three times with cacodylate buffer, immobilized in agar and processed as above.

For glutaraldehyde-lysine fixation with ruthenium red staining, bacterial cells were suspended in 1 volume of cacodylate buffer with glutaraldehyde and ruthenium red stock and 1 volume of cacodylate buffer with 100 mM lysine for 20 min at room temperature. After lysine stabilization, cells were pelleted and fixed for an additional 100 min in cacodylate buffer with glutaraldehyde and ruthenium red. Fixed cells were immobilized in agar and processed as previously described.

Hyaluronidase Plate Assay

Detection of the presence of hyaluronidase activity in *S. iniae* was performed using a modification of the rapid plate method of Smith and Willett (1968). In the basic medium, Todd Hewitt base (BBL, Cockeysville, MD) and Bacto agar (Difco, Detroit, IL) were substituted for Brain Heart Infusion broth and Noble agar, respectively. The basic components were prepared according to manufacturers' directions by dissolving 30 gm of Todd Hewitt base and 10 gm of agar per liter of ddH_20, heating to boiling, followed by autoclaving at 121°C for 15 min. The media was cooled to 46°C, and human umbilical potassium hyaluronic acid (Sigma) was added to a final concentration of 400 µg/ml. A 5% solution of bovine serum albumin fraction V (Sigma) was prepared in ddH_20, filter sterilized using a 0.2 µm filter, then added at a final concentration of 1%. The agar was poured to a depth of 3-4 mm, cooled, and stored at 4°C until used.

In preparation for inoculation of hyaluronidase test plates, bacteria were grown to stationary phase in 5 ml THYE overnight at 36°C in 15 ml conical centrifuge tubes. Cells were pelleted by centrifugation at 3000 x g for 10 min in a Marathon 21K/BR centrifuge, supernatant broth removed, and pellets resuspended in 100 µl THYE. Plates were inoculated by pipetting 1 µl of the concentrated bacterial suspensions onto the

surface of test plates. Following incubation for 36 hrs at 36°C, plates were flooded with 2N acetic acid, and 10 min were allowed to elapse for precipitation of non-degraded hyaluronic acid-albumin complexes. Colonies producing soluble hyaluronidase developed a clear zone after the non-degraded substrate had precipitated. Hyaluronidase activity was graded (-) for no zone of clearing, (+) zone < 5 mm, (++) zone 5-10 mm, (+++) zone > 10 mm.

DNase Plate Assay

DNase Test Agar (Difco) containing 2 gm of DNA/L was prepared according to the manufacturers instructions by dissolving 42 gm of dehydrated media in 1 liter of boiling ddH_2O, followed by autoclaving at 121°C for 15 min. Prior to sterilization, 0.05 gm of methyl green was added to facilitate detection of DNase activity (Smith et al. 1969). Methyl green normally forms a complex with intact DNA and the media remains pale green. After inoculation and incubation a zone of clearing develops around colonies where DNA has been degraded. Plates were inoculated using concentrated bacterial suspensions as described above. After 24 hrs of incubation at 36°C, DNase activity was detected by the production of a zone of clearing around positive colonies. DNase activity was graded as described above for hyaluronidase.

Casein Overlay Procedure for Plasminogen Activation

To demonstrate protease activity, THYE plates were inoculated as described above for hyaluronidase and incubated at 36°C. After 24 hrs the plates were overlaid with 9 ml of 0.8% agarose I in sterile Tris-buffered saline (TBS) containing 1 ml of a 10% solution of Bacto powdered milk. Prior to use, the milk solution was heated in a boiling water bath three times for 10 min each then added to the molten agarose. The

tube was briefly vortexed, cooled to 45°C, then carefully poured onto the surface of the THYE plates. The agarose was allowed to solidify and the plates incubated for 2-6 hrs at 36°C. Caseinolysis was indicated by the development of a zone of clearing around protease positive colonies.

In a separate assay, which takes advantage of the caseinolyitc activity of activated plasmin, human plasminogen (Sigma) was added to the casein overlay described above at a final concentration of 100 μg/ml. Bacterial colonies expressing streptokinase-like activity, resulting in plasminogen activation, produce large zones of clearing around positive colonies. Colonies producing large zones of proteolysis in both assays must be disregarded since it is impossible to distinguish caseinolysis resulting from the action of a non-specific protease from that of a plasminogen activator (Li et al. 1997).

Plasminogen Activation in Citrated Plasma

Tilapia whole blood was collected from multiple fish into Vaccutainer® tubes containing 3.8% citrate in a ratio of 9 parts blood to1 part citrate. For comparison, human blood was collected in an identical manner. The blood was then centrifuged at 1500 x g for 10 min at room temperature using a Beckman JT-6 centrifuge, the plasma removed, pooled, and filter sterilized using a 0.22 μm syringe filter.

In mammalian plasma, the addition of sufficient calcium will overcome the effects of citrate and restore normal clotting function to the plasma. To determine if exogenous Ca^{2+} could re-establish clotting ability to the citrated fish plasma, a 0.1 M $CaCl_2$ solution was prepared and filter sterilized. In 3 ml glass tubes, 700 μl of THYE broth was mixed

with 300 µl of citrated plasma, followed by the addition of $CaCl_2$ to achieve Ca^{2+} concentrations of 4 mg/dl (10µl), 6 mg/dl (15 µl), 8 mg/dl (20 µl), 10 mg/dl (25 µl).

Isolates of *S. iniae* and controls were grown to stationary phase overnight at 36°C in 5 ml volumes of THYE broth. Cells were harvested by centrifugation at 4500 rpm for 10 min, the supernatant broth removed, and resultant pellets resuspended in 200 µl THYE. One hundred µl of the concentrated bacterial suspensions were added to a series of tubes containing 600 µl of THYE broth and 300 µl of citrated plasma. The cultures were incubated overnight at 36°C. The following day 25 µl of 0.1 M $CaCl_2$ was added to each tube and they were observed at 10, 30, 60 min, and 12 hrs for the presence of clot formation. Lack of clotting suggests the liberation of a streptokinase-like plasminogen activator. Clot formation was graded on the following scale: NC indicates no clotting, (+) rim of clotting at the meniscus between the media and tube wall, (++) small clot within the media, (+++) large clot unstable to inversion, (++++) complete clot stable to inversion.

Results

Investigation of M Protein Using the Polymerase Chain Reaction

As predicted, the "all M" primer pair amplified the *S. pyogenes emm5* gene sequence, included as a positive control, visualized as a band of approximately 1.3-1.5 kb, consistent with the expected size range of M protein genes. There was no amplification of any DNA sequences in *S. zooepidemicus* SzPW60, which is also considered M protein positive (Timoney et al. 1995), the group C strains isolated from dermal lesions in tilapia, the *S. iniae* isolates, or the *E. coli* negative control (Figure 44).

When the "all M" primer pair failed to detect the presence of an M protein, *stricto senso*, in *S. iniae*, another set of primers directed against the conserved region of the M family of molecules was designed, which would theoretically identify a broader spectrum of M-like proteins. The "C-conserved" primer pair, directed against the conserved cell wall associated region of the M family of proteins, amplified sequences in *S. pyogenes* M5, *S. zooepidemicus* SzPW60, and the two piscine group C isolates in the expected size range of approximately 300-400 bp. None of the *S. iniae* isolates or the *E. coli* negative control produced visible bands (Figure 45).

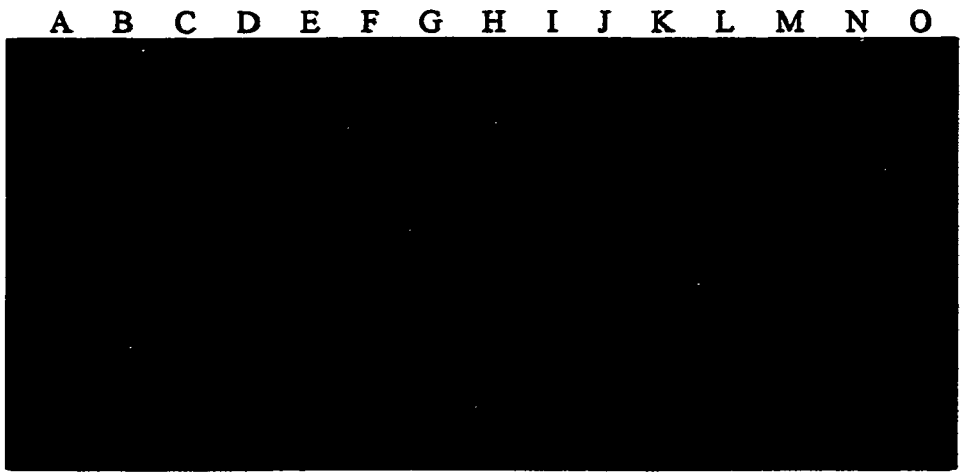
Sequence analysis of the amplified group C segment revealed 87% identity with the conserved C-terminal region of the major ligand binding protein 1 (Mlb1) of other group C streptococci, which binds to fibrinogen, albumin, and immunoglobulin G. This surface molecule shares no homology with the N-terminal half of M proteins, but shows significant homology with the C-terminal half, including the C repeats (Talay et al. 1995). Homology is also shared with C-terminal segments of over 30 M proteins and M-like proteins of streptococcal groups A, C and G, including *emm*, *enn*, *fcr*, protein G, protein H, and albumin binding proteins. The 355 bp length of the amplified mlb1 C-terminal anchor region is in agreement with band sizes seen on agarose gels and published DNA sequences (Talay et al. 1995).

Electron Microscopy for M protein and Capsule Production

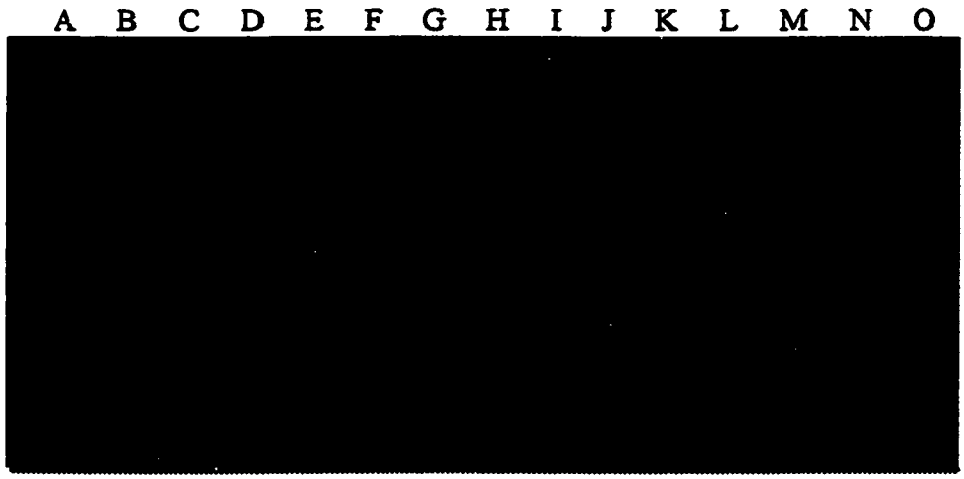
Four *S. iniae* strains isolated from tilapia were chosen at random for examination by transmission electron microscopy (TEM). For comparative purposes, one group B streptococcus isolated from tilapia, plus M protein positive *S. pyogenes emm5* and

Figure 44: Agarose gel electrophoresis of PCR products generated by the "all M" primer pair for entire streptococcal M proteins.

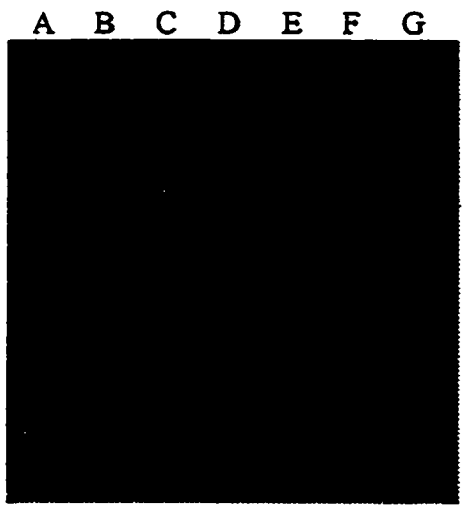
Lane	44-a	44-b	44-c
A	1 kb ladder	1 kb ladder	1 kb ladder
B	*S. pyogenes emm5*	*S. pyogenes emm5*	*S. pyogenes emm5*
C	*S. zooepidemicus*	*E. coli*	*E. coli*
D	*E. coli*	*S. iniae* 98-071	*S. iniae* 99-299B
E	Group C 98-203	*S. iniae* 98-113	*S. iniae* 99-301D
F	Group C 98-412	*S. iniae* 98-114	*S. iniae* 99-301G
G	*S. iniae* ATCC 29178	*S. iniae* 98-115	*S. iniae* 99-456D
H	*S. iniae* 93-331A	*S. iniae* 98-116	
I	*S. iniae* 94-036	*S. iniae* 98-117	
J	*S. iniae* 94-093	*S. iniae* 98-118	
K	*S. iniae* 94-449	*S. iniae* 98-177	
L	*S. iniae* 95-066	*S. iniae* 98-240	
M	*S. iniae* 96-290B	*S. iniae* 99-091	
N	*S. iniae* 97-003	*S. iniae* 99-092	
O	*S. iniae* 97-045	*S. iniae* 99-093	



44-a



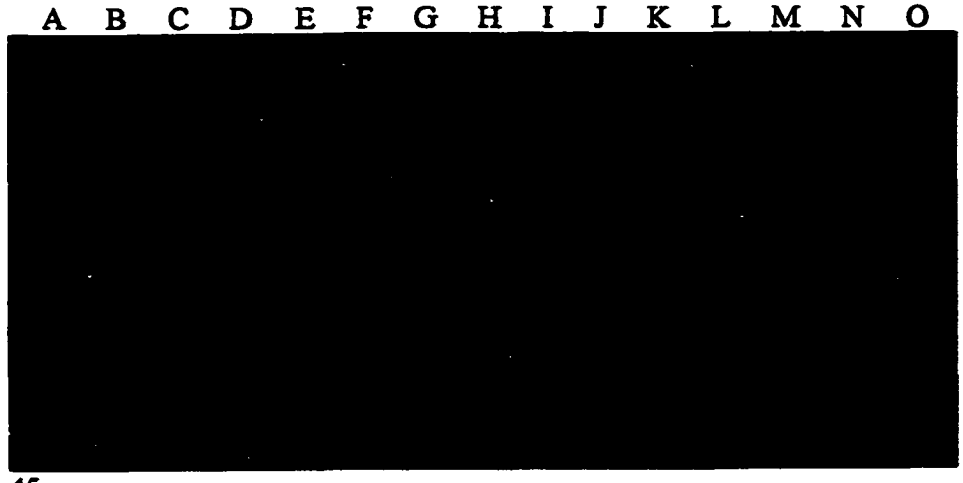
44-b



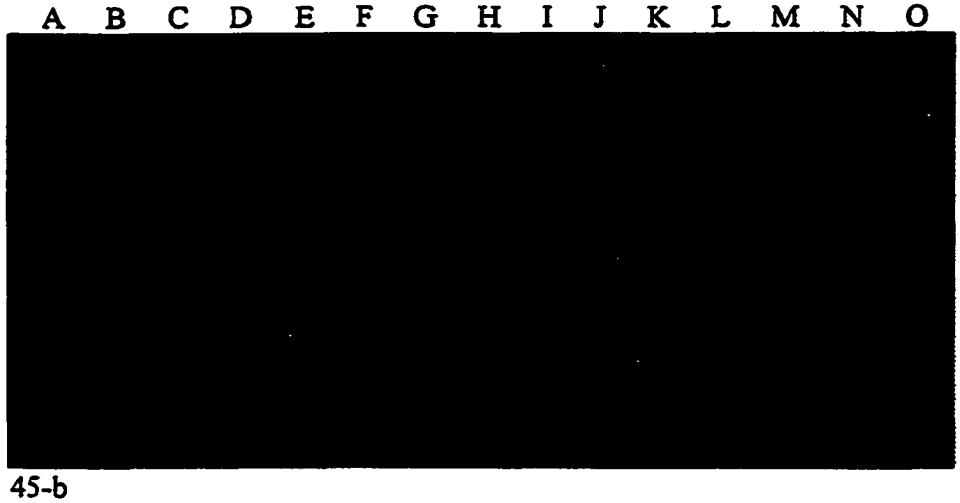
44-c

Figure 45: Agarose gel electrophoresis of PCR products generated by the "C conserved" primer pair for the conserved anchor regions of M-like proteins.

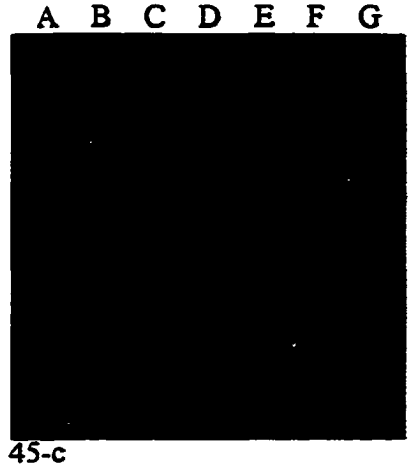
Lane	45-a	45-b	45-c
A	1 kb ladder	1 kb ladder	1 kb ladder
B	*S. pyogenes emm5*	*S. pyogenes emm5*	*S. pyogenes emm5*
C	*S. zooepidemicus*	*E. coli*	*E. coli*
D	*E. coli*	*S. iniae* 98-071	*S. iniae* 99-299B
E	Group C 98-203	*S. iniae* 98-113	*S. iniae* 99-301D
F	Group C 98-412	*S. iniae* 98-114	*S. iniae* 99-301G
G	*S. iniae* ATCC 29178	*S. iniae* 98-115	*S. iniae* 99-456D
H	*S. iniae* 93-331A	*S. iniae* 98-116	
I	*S. iniae* 94-036	*S. iniae* 98-117	
J	*S. iniae* 94-093	*S. iniae* 98-118	
K	*S. iniae* 94-449	*S. iniae* 98-177	
L	*S. iniae* 95-066	*S. iniae* 98-240	
M	*S. iniae* 96-290B	*S. iniae* 99-091	
N	*S. iniae* 97-003	*S. iniae* 99-092	
O	*S. iniae* 97-045	*S. iniae* 99-093	



45-a



45-b



45-c

S. equi CF32 were included. Visualized by TEM the *S. iniae* isolates and other group A, B, and C streptococci all possessed similar bi-layered cell wall morphology lying outside an indistinct cell membrane. The inner layer was dense and electron opaque, while the outer layer was much less dense and appeared as a nearly translucent halo. Both *S. pyogenes* and *S. equi* possessed a corona of short hair like fibrils of uniform length and density radiating from the cell surface, consistent with M protein fibers (Figures 46 and 47).

Processed conventionally using glutaraldehyde fixation, isolates of *S. iniae* possessed either a smooth surface or an irregular patchy layer of globular material with variable electron density that sometimes appeared to collapse onto the cell wall or condense into thick strands (Figures 48 and 49). Similar results were noted when compared to the encapsulated group B isolate. The presence of a capsule was confirmed by positive staining with ruthenium red dye. A more extensive capsular structure could be seen by first stabilizing the capsule with either polycationic ferritin or lysine, which prevented dehydration and collapse of the capsule during processing. Pretreatment with ferritin or lysine, in conjunction with ruthenium red staining, revealed a thick ragged layer of uniformly electron dense material consistent with the presence of a capsule (Figure 50). Figure 51 is a photomicrograph of an encapsulated group B streptococcus included for comparative purposes.

Hyaluronidase and DNase Production

Using a modification of the rapid plate method of Smith and Willett (1968), hyaluronidase production was identified in 23 of 29 *S. iniae* isolates tested. Using a commercially available test agar 15 of 29 *S. iniae* isolates tested positive for the

Figure 46. Transmission electron micrograph of *S. pyogenes emm*5 demonstrating an outer corona of hair-like M protein fibrils. Note the bi-layered cell wall (arrow) with its inner dense and outer translucent zones. The cell membrane is inconspicuous.

Figure 47. Transmission electron micrograph of *S. pyogenes emm*5. The fibrillar nature of the M protein fibers is indicated by their interdigitation with adjacent cells.

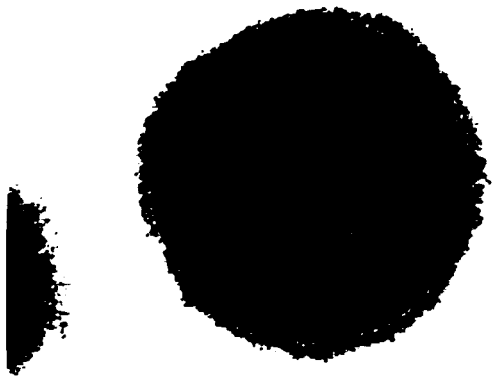


Figure 46

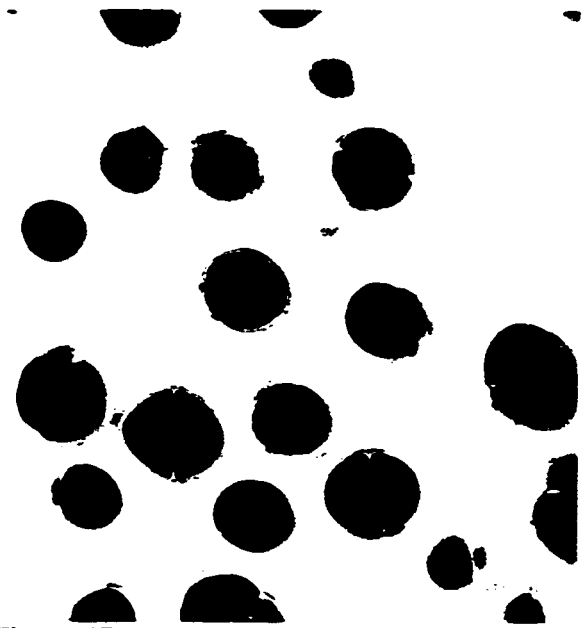


Figure 47

Figure 48. Transmission electron micrograph of *S. iniae* isolate 94-449 grown in the presence of tilapia serum and processed with conventional glutaraldehyde fixation. The cell wall is partially covered by a ragged layer of capsular material that has collapsed onto the cell surface.

Figure 49. Transmission electron micrograph of *S. iniae* 97-003 stained with ruthenium red capsule stain, which reveals a thick ragged layer of capsular material.

Figure 48

Figure 49

Figure 50. Transmission electron micrograph of *S. iniae* isolate 94-449. The capsule has been stabilized with lysine and stained with ruthenium red to reveal a more uniform layer of granular appearing capsular material.

Figure 51. Transmission electron micrograph of an encapsulated group B streptococcus stained with ruthenium red capsule stain for comparison to *S. iniae*.

Figure 50

Figure 51

production of DNase. In both assays, zones of clearing developed around positive colonies (Figures 52 and 53). Zone sizes varied greatly between individual *S. iniae* isolates in the two assays. Results are summarized in Table 7.

Caseinolytic Activity and Plasminogen Activation

None of the *S. iniae* isolates, including four of human origin, were able to activate human plasminogen using the casein overlay procedure. Proteolytic activity against casein alone was very weak and was not enhanced by the addition of human plasminogen to the overlay. In the absence of a source of purified tilapia plasminogen to use in a casein overlay test, it was decided to attempt a modification of a procedure developed by Donabedian and Boyle (1998) to detect clot formation by *S. pyogenes* in nutrient (glucose) poor media containing citrated human plasma.

As in human plasma, adding calcium was found to overcome the chelating effects of citrate and restore normal clotting ability to citrated tilapia plasma. The addition of 10 mg/dl of Ca^{2+} to the broth and plasma mixture induced a clot stable to inversion within 10 min at room temperature in both human and tilapia plasma. Adding 8 mg/dl Ca^{2+} produced a weak clot in 10 min, no clotting was observed at lower concentrations.

Liberation of a plasminogen activating substance by streptococci into broth media should negate the ability of calcium to reactivate clotting factors in citrated plasma. While the majority of *S. iniae* strains delayed clot formation in tilapia plasma, complete inhibition was seen in only 10 % of the isolates 12 hrs after the addition of calcium (Table 8). However, at 12 hrs only 52% of the clots were complete and stable to inversion, whereas, 100% of the clots were stable to inversion in human plasma by 30 min. Control plasma, with no bacteria added, formed a complete clot stable to inversion

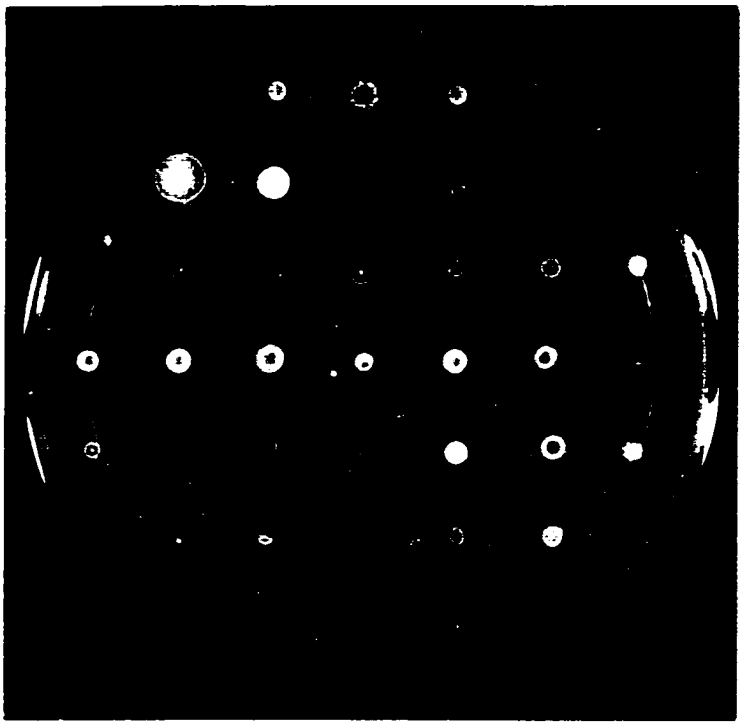


Figure 52. Hyaluronidase test plate showing variably sized zones of clearing surrounding colonies liberating hyaluronidase.

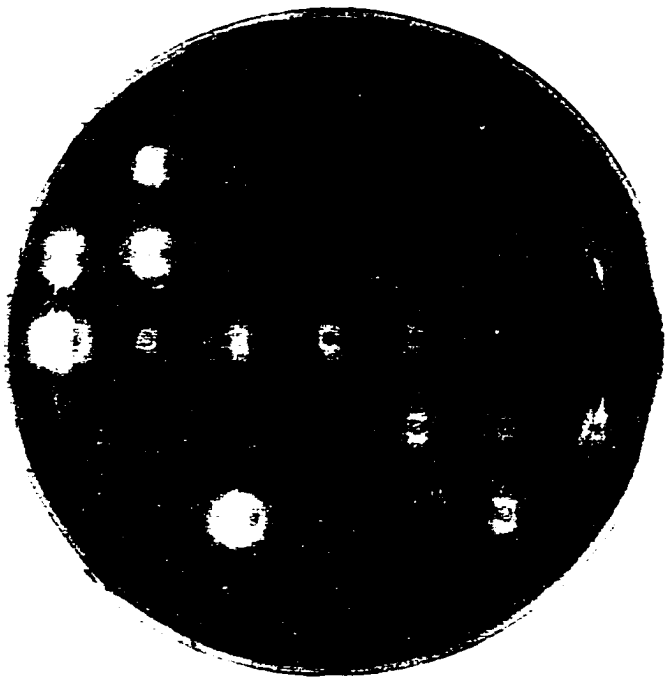


Figure 53. DNase test plate showing variably sized zones of clearing surrounding colonies liberating DNase.

Table 7. Hyaluronidase and DNase activity in representative isolates of *S. iniae*

LSU ID	Hemolysis	Hyaluronidase Activity	DNase Activity
S. iniae 93-331	α/β	++	-
S. iniae 94-036	α/β	++	-
S. iniae 94-093A	α/β	++	-
S. iniae 94-449	β	-	+++
S. iniae 95-066	β	-	+++
S. iniae 96-290B	α/β	+	-
S. iniae 97-003	α/β	+	-
S. iniae 97-045	α/β	++	-
S. iniae 98-071	β	-	+++
S. iniae 98-083	α/β	++	+++
S. iniae 98-113	β	+++	+++
S. iniae 98-114	α/β	+++	+
S. iniae 98-115	γ	-	++
S. iniae 98-116	β	++	++
S. iniae 98-117	β	+++	++
S. iniae 98-118	α/β	+++	+
S. iniae 98-177	β	+	-
S. iniae 98-240	α/β	++	-
S. iniae 99-091	α/β	++	-
S. iniae 99-092	α/β	++	-
S. iniae 99-093	α/β	++	-
S. iniae 99-094	β	+++	+++
S. iniae 99-299B	β	+++	+++
S. iniae 99-301D	β	-	+++
S. iniae 99-301G	α/β	++	-
S. iniae 99-456D	β	-	+++
S. iniae 00-296	α/β	++	-
S. iniae 00-300	α/β	++	-
S. iniae 00-318	β	++	+++
S. pyogenes	β	-	++
S. zooepidemicus	β	+++	+
S. dysgalactiae 00-351	β	++	-
Staph. intermedius	β	-	+
E. coli	γ	-	+

within 10 min following the addition of calcium. The *S. iniae* isolates showed no evidence of clotting at 10 min, but 20% showed some clot development by 20 min. By 30 min 45% of the isolates showed some degree of clot formation and by 1 hr 72 % had formed partial or complete clots.

Unexpectedly, an *S. pyogenes emm5* isolate totally inhibited clot formation in tilapia plasma after 12 hrs incubation in the presence of added calcium, as did a tilapia isolate of group C *S. dysgalactiae*. Consistent with findings in the casein-plasminogen overlay, there was no delay in clot formation in human plasma following calcium addition, indicating no interaction between *S. iniae* and human plasminogen. Following the addition of calcium, viable *S. pyogenes* cells completely inhibited clot formation in human plasma out to 12 hrs, whereas clotting occurred normally within 10 min when heat killed *S. pyogenes* cells were used in the assay. Results are summarized in Table 8.

Discussion

The antiphagocytic group A *S. pyogenes* M protein molecule is one of the most intensely studied and best characterized bacterial virulence factors (Lancefield 1928; Fischetti 1989; Navarre and Schneewind 1999). Isolates of group C and G streptococci also possess M proteins, but evidence suggests that they may only be found on human isolates (Bisno et al. 1987; Bisno et al. 1996). Similar proteins with unknown or no antiphagocytic properties were termed "M-like." The "M family" of surface proteins is now known to represent several distinct bioactive proteins sharing common features such as a hypervariable N-terminal region, conserved C-terminal region, heptad repeats with an α-helical coiled-coil structure, an N-terminal leader sequence, and a C-terminal cell wall sorting signal (Navarre and Schneewind 1999).

Table 8. Delay of clot formation in representative isolates of *S. iniae*

	Clot formation in tilapia plasma				Clot formation in human plasma			
LSU ID	10 min	30 min	60 min	12 hrs	10 min	30 min	60 min	12 hrs
S. iniae 93-331	NC	+++	+++	+++	++++	++++	++++	++++
S. iniae 94-036	NC	NC	NC	+++	NC	++++	++++	++++
S. iniae 94-093A	NC	NC	++	++++	NC	++++	++++	++++
S. iniae 94-449	NC	+++	++++	++++	NC	++++	++++	++++
S. iniae 95-066	NC	NC	++	+++	NC	++++	++++	++++
S. iniae 96-290B	NC	NC	++	+++	NC	++++	++++	++++
S. iniae 97-003	NC	NC	++	+++	++++	++++	++++	++++
S. iniae 97-045	NC	NC	NC	++	++++	++++	++++	++++
S. iniae 98-071	NC	+++	++++	++++	NC	++++	++++	++++
S. iniae 98-083	NC	+	++++	++++	NC	++++	++++	++++
S. iniae 98-113	NC	NC	++	++++	NC	++++	++++	++++
S. iniae 98-114	NC	NC	++	++++	NC	++++	++++	++++
S. iniae 98-115	NC	NC	NC	NC	NC	++++	++++	++++
S. iniae 98-116	NC	NC	NC	+++	NC	++++	++++	++++
S. iniae 98-117	NC	+++	+++	+++	++++	++++	++++	++++
S. iniae 98-118	NC	NC	+++	+++	NC	++++	++++	++++
S. iniae 98-177	NC	++	++++	++++	++++	++++	++++	++++
S. iniae 98-240	NC	NC	NC	NC	NC	++++	++++	++++
S. iniae 99-091	NC	NC	NC	+++	NC	++++	++++	++++
S. iniae 99-092	NC	NC	NC	++	NC	++++	++++	++++
S. iniae 99-093	NC	++	+++	++++	NC	++++	++++	++++
S. iniae 99-094	NC	++	++++	++++	NC	++++	++++	++++
S. iniae 99-299B	NC	+	+++	++++	++++	++++	++++	++++
S. iniae 99-301D	NC	NC	++	++++	NC	++++	++++	++++
S. iniae 99-301G	NC	NC	NC	NC	NC	++++	++++	++++
S. iniae 99-456D	NC	++	++++	++++	NC	++++	++++	++++
S. iniae 00-296	NC	++	++++	++++	++	++++	++++	++++
S. iniae 00-300	NC	++	++++	++++	++++	++++	++++	++++
S. iniae 00-318	NC	+++	++++	++++	NC	++++	++++	++++
Group C 00-351	NC	NC	NC	NC	NC	NC	++++	++++
Group C 00-412	NC	NC	NC	NC	NC	NC	++++	++++
Group C 00-421	NC	NC	NC	NC	NC	NC	++++	++++
S. pyog. (live)	NC	NC	NC	NC	NC	NC	NC	NC
S. pyog. (dead)	++++	++++	++++	++++	++++	++++	++++	++++
S. zooepidemicus	NC	++	++++	++++	+++	++++	++++	++++
E. coli	NC	NC	+++	++++	NC	NC	NC	++++
No bacteria	++++	++++	++++	++++	++++	++++	++++	++++

Symbols: NC = no clot; (+) = narrow rim of clotting at meniscus; (++) = small clot in media; (+++) = large unstable clot; (++++) complete clot stable to inversion.

Pepsin digestion of whole Emm cleaves the molecule into two approximately equally sized fragments (Beachey et al. 1974). The variable distal N-terminal fragment retains much of the protein's biological properties and forms the basis of antigenic diversity and serotyping among the 100 plus recognized M types (Fischetti 1989; Robinson and Kehoe 1992). The proximal C-terminal half anchors the molecule to the cell surface (Pancholi and Fischetti 1988, Piard et al. 1997) and is highly conserved among the various M-subfamilies (Navarre and Schneewind 1999).

The ability of *S. pyogenes* to survive in whole blood of non-immune individuals has been attributed to the presence of M protein on the bacterial surface. Virulent strains of *S. iniae* have also been reported to survive in whole blood, suggesting a similar surface expressed antiphagocytic molecule may be present (Fuller et al. 2001). The traditional method for preparing crude M protein involved extraction in boiling hydrochloric acid (Lancefield 1928). More recently, the polymerase chain reaction has been applied to the study of the M protein gene family (Podbielski et al. 1991). Results of PCR based studies using primer pairs directed against entire M protein gene sequences and conserved regions of M-like family did not amplify any sequences from *S. iniae* isolates. These findings indicate that survival in blood must be due to another surface component, such as a capsule, that imparts resistance to non-opsonized phagocytosis.

The M protein of group C *S. zooepidemicus* SzPW60 is truncated, lacking the A, B, and C repeats of group A streptococcal M protein molecules, but shows significant homology with the carboxy termini of both group A and G streptococci (Timoney et al. 1995). Both SzPW60 and Mlb1 have shorter N-terminal signal peptides than M proteins

and both have less homology with the region immediately upstream of the M protein start codon, including the Shine-Dalgarno ribosome binding site, which corresponds to the "all M" forward primer.

Visualized by transmission electron microscopy, the typical surface morphology for glutaraldehyde-fixed M protein positive streptococci consists of the bi-layered cell wall, lying outside an inconspicuous cell membrane. The inner layer is electron opaque, while the outer layer has little electron opacity and appears as a nearly transparent band beneath the corona of 50-60 nm hair-like M protein fibrils (Swanson et al. 1969). Typically M protein fibers can also be seen to interdigitate with like fibers on adjacent cells (Fischetti 1989). The surface features described above were readily apparent in the *S. pyogenes* and *S. equi* isolates examined. In contrast, isolates of *S. iniae* possessed either a smooth surface or a poorly visualized irregular layer of material outside the cell wall of variable electron density more suggestive of a capsule than M protein.

Bacterial capsules are composed of highly hydrated polymers, usually polysaccharide, subject to collapse during dehydration steps in preparation for electron microscopy. Ruthenium red is a polycationic dye with specificity for polyanionic polymers such as the acidic polysaccharides found in bacterial capsules, but does not protect against collapse during processing. Capsules can be stabilized, however, by pretreatment with polycationic ferritin or a primary amine, such as lysine (Jacques et al. 1990). Pretreatment with ferritin or lysine in conjunction with ruthenium red staining proved superior techniques for demonstrating the presence of a capsule surrounding isolates of *S iniae*. Both methods revealed an outer layer of thick uneven to ragged material of moderate electron opacity consistent with the presence of a capsule. This

conclusion was supported by comparison to a heavily encapsulated group B streptococcus.

Certain streptococcal species produce antiphagocytic polysaccharide capsules that may have only linear repeating primary structure or may possess side chains that add secondary structure and create more complex immunodeterminants (Kasper 1986). The polysaccharide capsule of *S. pneumoniae* is one of the best documented of all bacterial virulence factors and represents one of the milestones in the understanding of bacterial pathogenesis. The antiphagocytic properties of capsules have been attributed to repulsion of phagocytes by electrostatic charge and through failure to activate the classical complement pathway in the absence of specific antibody. In contrast, the cell wall peptidoglycan of non-encapsulated mutants directly activates the alternative complement pathway (Kasper 1986). Many capsular polysaccharides are immunogenic, however, and induce formation of type-specific antibodies (Rubens et al. 1987). While several authors have described *S. iniae* as possessing a capsule (Pier and Madin 1976; Kitao et al. 1981), its composition and potential role in the pathogenesis of this organism have not been investigated.

Some *S. pyogenes* strains produce a hyaluronic acid capsule, which is not immunogenic, presumably because it is indistinguishable from the hyaluronic acid of mammalian connective tissues (Kilian 1998). Only 3% of isolates from uncomplicated cases of pharyngitis produce a capsule, in contrast to 21% from severe streptococcal infections and 42% of rheumatic fever isolates (Johnson et al. 1992). Transposon mutagenesis studies of *S. pyogenes* reveal the importance of the capsule as a virulence factor in capsule-negative mutants, which are unable to resist the opsonic effects of

complement C3b binding for phagocytic killing in blood (Dale et al. 1996). Acapsular mutants also show a 100-fold reduction in virulence in mice (Wessels et al. 1991)

Like *S. pneumoniae*, clinical isolates of group B *S. agalactiae* produce a polysaccharide capsule. A total of nine serologically distinct capsule types (Ia, Ib, and II-VIII) have been identified. Encapsulation inhibits phagocytosis and complement activation in the absence of specific antibody. Transposon mutagenesis studies of a type III capsular isolate resulted in loss of virulence in a neonatal mouse model (Rubens et al. 1987). Other mutagenesis studies have demonstrated the surface expression of sialic acid residues as an essential component of the capsule necessary for evasion of host defenses (Wessels et al. 1989). Failure to activate the alternative pathway resides in the terminal sialic acid residues of side chains, which bind complement regulatory factor H, which ultimately results in cleavage of bound C3b (Wessels 1997).

The capsule gene regions of several streptococci consist of a group of polysaccharide-specific genes encoding glycosyltransferases and polymerases. Conserved sequences flanking these genes are believed to direct polymerization, transport, and regulation (Cieslewicz 2001). The size of capsule producing operons varies with the number of monosaccharides present and complexity of the capsule's organization. The *has* operon encoding the hyaluronic acid capsule of *S. pyogenes* requires only two essential genes, *has*A for hyaluronan synthase, which adds alternating N-acetyl-D-glucosamine and D-glucuronic acid residues to form the linear polymer, and *has*B, which forms glucuronic acid from D-glucose (Ashbaugh et al. 1998). The capsules of group B streptococci are more complex and their operons are correspondingly larger (Cieslewicz 2001).

Twenty-nine clinical isolates of *S. iniae* from piscine and human origin were tested for the production of other known or purported virulence factors identified in pyogenic streptococci, including hyaluronidase, DNase and streptokinase. Seventy-nine percent were found to liberate hyaluronidase. Bacterial hyaluronidases have been described as a group of neglected enzymes in relation to their potential activity as virulence factors (Kriel 1995). Hyaluronidases depolymerize hyaluronic acid, a linear unsulfated glycosaminoglycan polymer, which forms the ground substance of connective tissues. Hyaluronidase is considered a "spreading" factor, believed to aide in the pathogenesis of certain infections by promoting bacterial dissemination through connective tissue barriers (Duran-Reynals 1942).

Hyaluronidase is a general term applied to three types of enzymes capable of degrading hyaluronate and some are also able to cleave chondroitin sulfate. In addition to bacteria, hyaluronidases are also produced by certain mammalian cell types (spermatozoa), and are found in the venoms and secretions of some reptiles and invertebrates. The hyaluronidases produced by bacteria form a distinct type classified as hyaluronate lyases, or more specifically endo-N-acetylhexosaminidases, based on their ability to attack β-1-4 linkages in hyaluronate to form disaccharides of N-acetylglucosamine and glucuronic acid (Kriel 1995).

A wide range of microorganisms, including both gram-negative and gram-positive bacteria, and some yeasts, produce hyaluronidases. The enzymes produced by gram-negative organisms are periplasmic and less likely to act as spreading factors. Pathogenic gram-positive bacteria excrete hyaluronidase into the extracellular environment. Many of these organisms initiate infections at mucosal or skin surfaces in

humans and animals, where the bulk of the bodies' hyaluronate is found (Hynes and Walton 2000).

Hyaluronidase activity has been detected in virtually all strains of *S. pyogenes* and in some strains of *S. agalactiae*, *S. equi*, *S. dysgalactiae*, *S. uberis*, *S. suis*, *S. intermedius*, *S. constellatus*, *S. salivarius*, *S. mitis*, and *S. pneumoniae*. Representatives of other gram-positive genera capable of producing hyaluronidase include *Staphylococcus, Peptostreptococcus, Propionibacterium, Streptomyces, and Clostridium* (Schaufuss et al. 1989; Kilian 1998; Hynes and Walton 2000).

Eight complete hyaluronidase genes from *S. agalactiae*, *S. pneumoniae*, *Streptomyces griseus*, *Streptomyces coelicolor*, *Staphylococcus aureus*, *Clostridium perfringens*, *Propionibacterium acnes*, and *Proteus vulgaris* and two streptococcal bacteriophage genes have had their nucleotide sequences determined. Molecular weights of deduced amino acid sequences vary widely from 36–40 kDa in the bacteriophage encoded enzymes to 121 kDa in *S. agalactiae* (Hynes and Walton 2000). The purified enzymes from *S. pyogenes*, *S. equi*, and *S. uberis* have molecular weights of approximately 54 kDa (Schaufuss et al. 1989). Proteins from the gram-positive organisms, with the exception of the clostridial hyaluronidase, appear related, with global similarities between *S. agalactiae*, *S. pneumoniae*, and *S. aureus* of 65% and local similarities as high as 80% (Hynes and Walton 2000).

The viscous consistency of connective tissue hyaluronate is believed to resist penetration by bacteria and the dissemination of their toxins. If true, the production of bacterial hyaluronidases could play a critical role in the spread of pathogenic organisms into deeper tissue sites from epithelial surfaces. The production of metabolizable

disaccharides from hyaluronate could also supply nutrients to an invading pathogen. Unlike some of the better studied bacterial virulence factors, much of the information on the role of hyaluronidase as a virulence factor remains speculative or inferred (Hynes and Walton 2000).

Significantly more strains of hyaluronidase producing human streptococci belonging to the *S. milleri* group are isolated from internal abscesses, when compared to isolates collected as part of the normal mucosal flora. Hyaluronidase production among these isolates was most common in β-hemolytic strains, suggesting hyaluronidase may also aide dissemination of the toxin (Unsworth 1989). Similarly, in *Clostridium perfringens*, hyaluronidase production is believed to facilitate spread of the tissue damaging α-toxin, potentiating its cytolytic activity (Canard et al. 1994). Only hyaluronidase positive strains of *S. pneumoniae* appear to be capable of causing meningitis, while only 15% from carrier individuals produce the enzyme. Negative strains can be induced to cause meningitis if inoculated with exogenous hyaluronidase, suggesting it may be involved in breeching the blood brain barrier (Kostyukova et al. 1995). Using signature-tagged mutagenesis in a mouse model of pulmonary infection by *S. pneumoniae*, hyaluronidase production was identified as a factor in the development of pneumonia, but not in septicemia (Polissi et al. 1998).

Using a commercial test agar, 52 % of the *S. iniae* isolates examined were positive for the production of DNase. Group A streptococci express up to four types of secreted DNases and are the products of *sda* genes A-D. Similar nucleases have also been demonstrated in groups B, C, and G streptococci (Podbielski et al. 1996) and in *Staphylococcus aureus* (Chesbro and Walker 1972). Although group A streptococcal

infections are correlated with the production of anti-DNase B antibodies, a specific role for the DNases in the pathogenesis of these infections remains unclear. It has been debated that these enzymes could have only an indirect affect on virulence by providing an organism with oligonucleotides for energy (Wilson 1945). Despite this, DNases have been implicated by others as virulence factors in group A streptococci, for several reasons: 1) They attack a molecule essential to the activity of any potential target cell; 2) They are produced by all strains of Group A streptococci tested; 3) Production in less virulent group B, C and G streptococci is less common and less enzyme is liberated; 4) Anti-DNase B antibodies appear after most infections with *S. pyogenes* (Podbielski et al. 1996).

Plasmin is normally found in the blood and extravascular spaces of mammals, where it takes part in several physiological processes, including blood clot dissolution, cellular migration, trophoblast implantation, and cancer metastasis (Lottenberg et al. 1994; Johnsen et al. 1999). Group A streptococci grown in the presence of human plasma also generate the fibrinolytic enzyme plasmin from its inactive zymogen, the glycoprotein plasminogen (Lottenberg et al. 1992). Once activated, plasmin is captured on a specific high affinity cell surface receptor, where it remains enzymatically active, escaping neutralization by α-antiplasmin, the normal regulatory molecule of the mammalian host (Lottenberg 1992).

The extracellular bacterial product responsible for plasmin activation is streptokinase, product of the *skc* gene, which has been cloned and its nucleotide sequence determined (Malke and Ferretti 1984). Under normal conditions, the addition of sufficient calcium to broth media containing bacteria and 30% citrated human plasma

will allow clotting to proceed unabated. However, if the bacteria are liberating a streptokinase-like plasminogen activator, the addition of calcium will not reconstitute clotting factor activity and the broth will remain in a fluid state (Donabedian and Boyle 1998). It has been reported that only 62% of *S. pyogenes* isolates exhibit streptokinase activity in human plasma, although all strains tested carry the *skc* gene (Huang et al. 1989).

Plasminogen has two physiological activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Both activate plasminogen by cleavage of a single peptide bond (Johnsen et al. 1999). Streptokinase is not a protease like most other plasminogen activators. Instead it forms a 1:1 stoichiometric complex with plasminogen, triggering a conformational change and self-cleavage that results in formation of the serine protease plasmin (Huang et al. 1989; Lottenberg et al. 1992). Streptokinase from *S. equisimilis* is approximately 47 kDa, 414 amino acids in length and comprised of 3 domains. Plasminogen is believed to bind initially to the C-terminal domain of streptokinase to form the active enzyme complex, while additional substrate plasminogen binds to the N-terminal domain and is converted to active plasmin (Young et al. 1998).

Streptokinase is believed to act as a virulence factor by preventing the formation of fibrin barriers around foci of infection. In addition to degrading polymerized fibrin, it is also capable of degrading mammalian extracellular matrix proteins including fibronectin and laminin, and it enhances collagenase activity. Degradation of these normal tissue barriers promotes penetration through mucosal and cutaneous barriers into deep tissue sites (Lottenberg 1992). Groups A, C and G streptococci contain an *skc*

gene, produce streptokinase, and demonstrate surface receptors for plasmin. Lancefield groups B, D, F, H, K, L, M, N, O, P, R, U, and *S. pneumoniae* do not. Although antigenic heterogeneity exists even among group A streptococcal serotypes, there is 90% homology between the group A, C and G streptokinases (Huang et al. 1989; McCoy et al. 1991).

Recently a novel plasminogen activator has been described in *S. uberis* (Leigh 1993, Leigh 1994, Leigh and Lincoln 1997). The bovine plasminogen activator of *S. uberis* has been sequenced and cloned, and is highly conserved within the species. The 33kD, 286 amino acid protein shares only weak homology (26%) with the streptokinases of *S. equisimilis* and *S. pyogenes*. The plasminogen activator gene, *pau*A, was located at a site in the chromosome distinct from that encoding the *skc* genes for streptokinase in other streptococci (Rosey et al. 1999).

Capturing host plasmin(ogen) is a mechanism used by other genera of pathogenic gram-positive and gram-negative bacteria, believed to contribute to invasiveness. *Staphylococcus aureus* (staphylokinase), *E. coli* and *Yersinia pestis* all share the ability to activate plasminogen through the expression of plasminogen activating extracellular bacterial products. There is no significant nucleotide or amino acid homology shared between these families of biologically relevant plasminogen activators (Lottenberg et al. 1994).

Streptococcus iniae, including several human isolates, did not activate human plasminogen. Results of plasminogen activation studies for streptokinase-like activity by *S. iniae* in tilapia plasma were equivocal. The majority of *S. iniae* strains examined only delayed clot formation in tilapia plasma, but did not completely prevent it.

Group A *S. pyogenes* have a host range limited essentially to humans, while group C streptococci infect a variety of animals, including humans. *Streptococcus pyogenes* activates only human plasminogen and binds only activated human plasmin (Schroeder et al. 1999). Group C streptococci (*S. equi, S. equisimilis, S. zooepidemicus*) recovered from humans, horses and pigs exhibit streptokinase activity only when grown with plasminogen that parallels the host range of the microorganism. The streptokinases are antigenically related and bind all three plasminogens, indicating conservation of binding sites despite the observed species-specific activation. Bovine isolates of group C *S. dysgalactiae* are also capable of activating plasminogen in a species-specific manner by an activator that remains to be characterized (Leigh et al. 1998). The species specificity of this relationship indicates that streptokinase plays a role in establishing the host range of certain pathogenic streptococci (McCoy et al. 1991). Bearing this in mind, it was less surprising that a group C *S. dysgalactiae* isolate of tilapia origin also totally inhibited clot formation in tilapia plasma.

Although it was hoped that tilapia and human isolates would behave in a similar fashion, results using this crude assay do not entirely rule out the possibility of a plasminogen activator in *S. iniae*. They may only be a reflection of the evolutionary distance between fish and higher vertebrates and further investigation of this possibility is indicated. Only limited information is available regarding hemostatic mechanisms of fish. While platelet aggregation and fibrin deposition are well demonstrated, the existence of a clotting cascade leading up to the generation of fibrin remains to be fully elucidated (Rowley et al. 1997).

Plasminogen has been isolated from the lamprey (Order Petromyzontiformes) and the partially sequenced protein showed 54% homology with human plasminogen. The lamprey plasminogen failed to react with human urokinase or tissue plasminogen activator, streptokinase, or a 1:1 streptokinase/human plasminogen complex (Affolter et al. 1993). A protein of similar molecular weight and 50% identity with human and bovine plasminogen has also been isolated from Atlantic salmon *Salmo salar* (Lund and Olafsen 1998). Both the lamprey and salmon proteins exhibited cross-reactivity to antibodies against human plasminogen (Affolter et al. 1993; Lund and Olafsen 1998).

Proteins have also been purified from goldfish *Carassius auratus* and rainbow trout *Oncorhynchus mykiss* with molecular weights similar to human plasminogen, but they were not further characterized. The purported goldfish plasminogen was converted to an active protease by a plasminogen activator isolated from goldfish aorta. Human plasminogen activators did not significantly activate either of the piscine plasminogens (Salles et al. 1990). Proteins with molecular weights of 50-55 kDa have been purified from the skin of *Xiphophorus sp.* sharing immunoreactivity with human urokinase and the ability to convert human plasminogen to plasmin (Takahashi et al. 1987). It is probable that if fish are capable of generating fibrin, a means of fibrinolysis would exist concurrently, as it does in higher vertebrates. It would also seem possible that if *S. iniae* is truly adapted as a fish pathogen, it may produce a novel plasminogen activator that interacts specifically with fish plasminogen such as the one associated with *S. uberis*.

This study was undertaken to investigate the presence of potential virulence mechanisms in *Streptococcus iniae*, an important fish pathogen and potential zoonotic agent. Several known or purported virulence factors, identified in other species

belonging to the pyogenic group of streptococci, were examined including M protein, capsule production, hyaluronidase, DNase, and a streptokinase-like plasminogen activator. Using a PCR based analysis, there was no evidence to indicate the presence of an M protein molecule on *S. iniae*, using a primer pair reported to amplify entire *emm* protein genes. Similarly, a primer pair designed to amplify conserved regions of M-like molecules failed to detect any related surface proteins. In retrospect, this study would have been strengthened by using PCR products derived from M protein positive *S. pyogenes* controls, particularly the conserved anchor region, as probes in Southern Blot analyses of *S. iniae* DNA.

Electron microscopic examination of *S. iniae* isolates revealed surface features more suggestive of capsular material than M protein. Pretreatment of samples with ferritin or lysine prior to dehydration steps used in processing for TEM proved to be superior methods for demonstrating the presence of a capsule. Positive staining with ruthenium red dye indicates a capsule composed of acidic polysaccharides. These findings support earlier observations concerning the presence of a capsule surrounding *S. iniae*, a known streptococcal virulence factor whose association with this microorganism has recently come under scrutiny.

Seventy-nine percent of the *S. iniae* isolates were positive for hyaluronidase production, 52% were positive for DNase, and 31% were positive for both. Hyaluronidase is a spreading factor that promotes dissemination through connective tissue barriers and is used particularly by bacteria that initiate infections at skin or mucosal surfaces. Hyaluronidase is reported to be an essential virulence factor in *S. pneumoniae* strains capable of causing meningitis. In a limited study, an *S. iniae* isolate

negative for hyaluronidase production, was incapable of initiating septicemic infection when injected intraperitoneally, but produced typical signs of meningitis when injected intracranially (unpublished). The role of DNase as a virulence factor is less well established and may simply provide streptococci with a source of oligonucleotides.

Various species of gram-positive and gram-negative bacteria liberate plasminogen activators that prevent the formation of fibrin barriers around foci of infection allowing invasion of deeper tissue sites. Activity is often highly host specific and may help determine the host range of the pathogen. One of the best characterized of these bacterial plasminogen activators is streptokinase, produced by groups A, C and G streptococci. Other streptococci, like *S. uberis*, liberate novel plasminogen activators. *Streptococcus iniae* isolates showed no interaction with human plasminogen. Results in tilapia plasma were equivocal and warrant further study, delaying but not completely inhibiting the formation of fibrin clots.

CHAPTER IV: PRODUCTION OF NON-HEMOLYTIC MUTANTS OF *STREPTOCOCCUS INIAE* USING pTV1-OK, A TEMPERATURE CONDITIONAL PLASMID DELIVERY VECTOR, AND INSERTIONAL MUTAGENESIS WITH Tn917

Introduction

Streptococci are obligate parasites of cutaneous and mucosal surfaces of humans and animals. Some are considered resident flora, causing infection only when introduced into normally sterile sites or in immunocompromised hosts. Other species are true pathogens, which spread between individuals, and cause infections in normal non-immune hosts (Kilian 1998). Representatives of the pyogenic group of streptococci produce a broad spectrum of virulence factors, which allow them to invade host tissues and evade defensive mechanisms. One example is streptolysin S, a potent cytolytic agent and β-hemolysin, identified as an important virulence factor in the arsenal of *S. pyogenes* (Betschel 1998; Ginsburg 1999; Nizet et al. 2000).

Streptococcus iniae is a β-hemolytic pyogenic streptococcus first isolated and described from subcutaneous abscesses in a captive Amazon freshwater dolphin *Inia geoffrensis* in 1976. From 1977 to 1980 large-scale epizootics caused by β-hemolytic streptococci with biochemical profiles compatible with *S. iniae* occurred in Japan in a variety of fish species (Minami et al. 1976; Kitao et al. 1981; Ohnishi and Jo 1981; Ugajin 1981). Since 1994, *S. iniae* has emerged as a major bacterial pathogen of cultured tilapia (Perera et al. 1994; Eldar et al. 1994) and has steadily increased in incidence and host range worldwide (Nakatsugawa 1983; Kaige et al. 1984; Foo et al. 1985; Al-Harbi 1994; Stoffregen et al. 1996; Zlotkin et al 1998; Bromage et al. 1999; Eldar et al. 1999; Yuasa et al. 1999). There have also been reports of invasive disease in

humans, limited primarily to cellulitis of the hand, following skin injuries incurred while handling fish (Weinstein et al. 1997).

Identifying and understanding genes encoding or regulating bacterial virulence factors is essential to the study of pathogenic mechanisms in these microorganisms. Transposon mutagenesis is a powerful tool in molecular genetics for dissecting the virulence mechanisms of pathogenic bacteria. Transposons are DNA elements capable of relocating, or transposing, from one site in a DNA molecule to another. Transposons are introduced by a process that results in the production of random insertions throughout the genome of the recipient population of bacterial cells. The mutants created typically carry a selectable marker and can then be screened for loss of phenotypic traits believed to be associated with virulence. Ultimately, the virulence of the mutants created is compared to that of its wild type parental strain, usually in an animal model (Caparon and Scott 1991).

Until recently, transposons useful for mutagenesis studies in gram-positive bacteria had not been identified. *Enterococcus faecalis* transposon Tn917 confers inducible resistance to the macrolide-lincosamide-streptogramin B (MLS) group of antibiotics, including erythromycin (erm). For mutagenesis studies, Tn917 is particularly useful when compared to other transposons, such as Tn916, as it is relatively small, has a relatively high frequency of transposition, high degree of insertional randomness, generates extremely stable mutations, and extensive information is available on its physical and genetic organization (Camilli et al. 1990).

Tn917 is capable of transposing in both gram-negative *E. coli* (Kuramitsu and Casadaban 1986) and several gram-positive organisms, including *Bacillus subtilis*

(Youngman 1983), *Listeria monocytogenes* (Camilli et al. 1990), *Clostridium acetobuttylicum* (Babb et al. 1993), *Lactococcus lactis* (Israelsen et al. 1995), *Streptococcus mutans* (Gutierrez et al. 1991), and *Streptococcus pyogenes* (Li et al. 1997). One method to assay for transposition involves the use of suicide or replication-conditional vectors, plasmid or phage, which are capable of entering a cell, but are incapable of replicating in that particular host or under particular culture conditions. Plasmid pTV1-OK is a replication-conditional (temperature-sensitive) vector capable of replication in both gram-positive and gram-negative bacteria (Gutierrez et al. 1996).

Little is known concerning virulence factors associated with *Streptococcus iniae*. The purpose of this project was to generate non-hemolytic mutants of *S. iniae* using the pTV1-OK::Tn917 mutagenesis system, under the hypothesis that its β-hemolysin is similar in design and function to that of *S. pyogenes* streptolysin S and that it also contributes to virulence of the microorganism. Non-hemolytic mutants were screened in a tilapia model of infection for changes in virulence following the loss of this phenotypic trait.

Materials and Methods

Streptococcus iniae, pTV1-OK and Tn917

Streptococcus iniae isolate LSU-99-301D was chosen for mutagenesis experiments due to its strong, purely β-hemolytic phenotype and ability to grow well at 38°C. This particular isolate is negative for mannitol fermentation, an unusual property among *S. iniae* biovars, but otherwise has a typical biochemical profile. It is negative for hyaluronidase and positive for DNase production. The strain was originally isolated at the Louisiana Aquatic Animal Disease Diagnostic Laboratory, Louisiana State

University, School of Veterinary Medicine, from a natural outbreak of disease in cultured tilapia.

Plasmid pTV1-OK is an 11 kb replication-conditional (temperature-sensitive) vector based on staphylococcal replicons pE194 and pE194Ts, which contains the *repA*(Ts) gene from pWV01, a broad-host-range *Lactococcus lactis* plasmid capable of replication in both gram-positive and gram-negative bacteria (Gutierrez et al. 1996). The *repA*(Ts) origin of replication allows plasmid replication at the permissive temperature of 28-30°C, but not at 35-42°C (Maguin et al. 1992). The other key feature of pTV1-OK is the *aphA3* kanamycin resistance gene, which serves as a phenotypic marker for the presence of the plasmid backbone (Cvitkovitch et al. 1998).

Plasmid pTV1-OK carries transposon Tn917. The 5257 bp transposon shares significant homology with the Tn3 family of gram-negative transposons (Shaw and Clewell 1985; An and Clewell 1991) and is capable of transposing in both gram-negative *E. coli* (Kuramitsu and Casadaban 1986) and several gram-positive organisms (Youngman 1983; Camilli et al. 1990; Babb et al. 1993; Israelsen et al. 1995; Gutierrez et al. 1991; Li et al. 1997). Tn917 is composed of five open reading frames (ORFs), the first three of which are involved in MLS resistance, while ORFs four and five encode resolvase and transposase functions, respectively, of this replicative transposon. Coding sequences are flanked by 38 bp terminal inverted repeats, which generate a 5 bp duplication upon insertion (Tomich and Clewell 1980; Shaw and Clewell 1985). Transposition of Tn917 is inducible or enhanced in the presence of low levels of erythromycin. In *S. faecalis* exposed to 0.001-0.5 μg/ml concentrations of erythromycin

for a few hours, the frequency of transposition increased by an order of magnitude and paralleled the appearance of erythromycin resistance (Tomich and Clewell 1980).

Preparation of Plasmid and Chromosomal DNA

Plasmid isolations were performed using a QIAprep Spin kit® (Qiagen Inc., Valencia, CA), as per the manufacturers instructions. To isolate pTV1-OK from *S. iniae*, however, it was necessary to add 500 U of egg white lysozyme (Sigma, St. Louis, MO) to 250 µl of buffer P1 containing RNaseA. The suspension was briefly vortexed and incubated for 30 min in a 37°C water bath.

Crude DNA preparations were made following the procedure of Berridge et al. (1998). In microfuge tubes, 500 µl of stationary phase *S. iniae* cells were pelleted at 3000 x g for 10 min using an Eppendorf model 5414 micro centrifuge and resuspended in 250 µl Tris-EDTA buffer (TE), pH 8.0. Alternatively, 10 colonies growing on agar plates were suspended in 250 µl TE. The cells were centrifuged again, the supernatant TE removed and replaced by 100 µl of lysis buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.3), 1 mM EDTA, 1% Triton X-100]. The microfuge tubes were placed vertically in a raft and floated in a beaker of boiling water for 10 min, cooled to room temperature, and diluted to 1 ml with ddH_2O. Five µl of boiled cells were used in the polymerase chain reaction (PCR) to amplify *aph*A3 and *erm* gene sequences for pTV1-OK and Tn917, respectively.

Isolation of purified chromosomal DNA was performed using modifications of procedures used by Berridge et al. (1998) and Proft et al. (2000). A small quantity of the respective bacterial strains and mutants were removed from storage at –70°C, streaked onto blood agar plates, and checked for purity by overnight culture. A single

colony was inoculated into 5 ml of Todd Hewitt broth with 0.3% yeast extract (THYE) and incubated overnight at 36°C without aeration. When working with Tn917 mutants, solid and broth media were supplemented 5 μg/ml erm. The following day, overnight starter cultures were inoculated into 40 ml of THYE broth and incubated an additional 8 hrs under the same conditions. The expanded cultures were transferred to conical centrifuge tubes and centrifuged in a Marathon 21K/BR centrifuge at 4500 rpm for 10 min at 4°C.

Supernatant THYE was carefully decanted and pellets resuspended by vortexing in 5 ml of lysis buffer [25 mM Tris (pH 8.0), 10mM EDTA (pH 8.0), 150 mM NaCl, 50 mM glucose]. The bacterial suspensions were centrifuged a second time and the pellets resuspended in 2 ml of lysis buffer. To the suspensions were added 10 μl of 50 mg/ml RNase A, 25 μl of 5000 U/ml mutanolysin (Sigma), and 25 mg of egg white lysozyme (Sigma) dissolved in 0.5 ml of lysis buffer. Following 1 hr of incubation in a 37°C water bath, 10 μl of 20 μg/ml proteinase K was added and tubes gently inverted to mix. The suspensions were incubated for an additional 30 min at 37°C followed by the addition of 200 μl of 20% Sarkosyl (Sigma). The tubes were gently rocked to mix and became mucoid almost immediately, indicating bacterial lysis. The mixtures were then incubated for 30 min at 37°C, at which time they were almost completely clear and extremely viscous.

To the lysed bacteria were added 325 μl of 5M NaCl and 250 μl of Na CTAB, prewarmed to 65°C. The tubes were gently mixed, then incubated for 10 min in a 65°C water bath. Contents were transferred to 50 ml round bottom centrifuge tubes and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Amresco, Solon, OH)

added. The tubes were gently inverted five times to mix, then centrifuged for 15 min at 6000 x g in a Beckman J-25 centrifuge at 4°C. The upper aqueous layers were removed by pipette and transferred to fresh 50 ml conical centrifuge tubes. One-tenth the volume of 3 M sodium acetate (pH 5.2), was added and the tubes gently inverted to thoroughly mix.

The DNA was precipitated by the addition of 2 ½ times the volume of ice-cold 100% ethanol, removed with a small hooked Pasteur pipette tip and transferred immediately to a clean microfuge tube containing 300 µl of ice-cold 70% ethanol to rinse. The condensed DNA was gently blotted on the tube wall and immediately transferred to a fresh microfuge tube containing 200-400 µl of ddH_2O. The precipitated DNA was allowed to dissolve for 24-48 hrs at 4°C. On subsequent days additional ddH_2O was added as needed to dissolve any remaining DNA. All plasmid and chromosomal DNA preparations were quantified using a Beckman DU® 640 spectrophotometer.

Electrotransformation of pTV1-OK

Electroporation was conducted using a modification of the procedure of Powell et al. (1988). Archived streptococcal isolate 99-301D, previously frozen at –70°C, was streaked onto a TSA blood agar plate and incubated overnight at 28°C. The following day a single colony was inoculated into 5 ml THYE broth containing 5% fetal bovine serum and incubated overnight at 28°C without aeration. On the third day, the culture was diluted 1:25 by inoculating 50 ml fresh THYE with 2 ml of the starter culture. An initial OD_{600} reading was taken, and flasks were incubated at 28°C without aeration. At 2 hour intervals OD_{600} measurements were taken until an OD of 0.15-0.3 was reached.

Cells were transferred to a conical centrifuge tube and harvested by centrifugation at 4,500 rpm for 10 min at 4°C in a Marathon 21K/BR centrifuge, then washed in 25 ml ice-cold electroporation buffer (EPB). The EPB was composed of 0.5 M sucrose, 1 mM $MgCl_2$, and 7 mM K_2HPO_4-KH_2PO_4 (pH 7.4). The cells were centrifuged a second time and resuspended in 5 ml of EPB pre-warmed to 37°C. To the cell suspension was added 0.05 gm of egg white lysozyme (Sigma) and 20 µl of 5000 U/ml mutanolysin (Sigma), followed by 30 min of incubation in a 37°C water bath. Cells were then centrifuged at 3000 x g for 15 min at 4°C and washed in 10 ml ice-cold EPB to remove residual enzymes. Following a final centrifugation step under the same parameters, cells were resuspended in 1 ml of ice-cold EPB. From this suspension, 150 µl was removed and a plate count performed.

To each of two 200 µl aliquots of washed and concentrated bacteria was added 1 µg (21 µl) of previously prepared and frozen pTV1-OK. The suspensions were gently mixed in a microfuge tube, transferred to chilled 2 mm gap electroporation cuvettes (BTX®), and placed on ice. To two additional cuvettes bacterial suspension only was added without plasmid. Bacteria were electroporated using a Bio Rad Gene Pulser® and Controller using 200 Ω and 25 µF. Voltages of 1.25 kV and 2.5 kV were applied to one cuvette each with and without plasmid, generating field strengths of 6.25 kV/cm and 12.5 kV/cm, respectively.

The electroporated cells were rested for 25 min on ice. Cell suspensions electroporated in the presence of plasmid were added to 10 ml of THYE broth containing 0.5 M sucrose for osmotic stabilization. Plate counts were performed on control suspensions subjected to electroporation without added pTV1-OK. After

incubating without aeration for 5 hrs at 28°C, broth cultures were transferred to 15 ml conical tubes and centrifuged at 3000 x g for 10 min at room temperature. The supernatant broth was removed and cells resuspended in 1.5 ml of fresh THYE with 0.5 M sucrose.

To the surface of six THYE agar plates, containing 0.5 M sucrose and 300 µg of kanamycin, 200 µl each of the concentrated bacterial suspensions was spread. Plates were placed in a 28°C incubator and observed daily for growth. All colonies growing on kanamycin at the permissive temperature of 28°C were patched onto THYE plates containing 300 µg kanamycin and 5 µg/ml erythromycin and incubated for 24 hrs at 28°C. Successful transformation of 99-301D with pTV1-OK was indicated by the acquisition of a $kan^r erm^r$ phenotype at the permissive temperature of 28°C. Three individual transformant colonies were grown to log phase in 4.5 ml THYE with 300 µg/ml kanamycin. To these cultures were added 750 µl of sterile glycerol. The cultures were dispensed in 1 ml aliquots and frozen at –70°C.

Insertional Mutagenesis, Transposition Frequency and Mutant Stability

A single transformant colony of *S. iniae* 99-301D, designated 99-301-22, growing on THYE agar with 300 µg/ml kanamycin at 28°C was inoculated into 5 ml of THYE broth with 300 µg/ml kanamycin and incubated overnight at 28°C without aeration. An initial 1/50 dilution was prepared, followed by series of two-fold serial dilutions from 1/100 to 1/1600 made in triplicate, by adding 150 µl of the overnight broth culture to 15 ml of fresh THYE broth containing 0.5 µg/ml erythromycin. The remaining dilutions were made by serially transferring 5 ml of the previous dilution into 5

ml of THYE containing the same antibiotic concentration. The five dilutions were incubated without aeration for 18 hours at the non-permissive temperature of 36°C.

To measure the transposition frequency, cell counts were performed in triplicate on the above five cultures by performing a series of 1/10 (20µl/180µl) dilutions in THYE broth in microtiter plate wells and plating 20 µl drops onto tripticase soy agar with 5% sheep blood (TSAB) from 10^{-1} to 10^{-6}. Following overnight incubation, the total number of cfu/ml was calculated and the transposition frequency determined by dividing the number of erm^r cfu/ml by the total number of viable cfu/ml at that dilution. From each of the five dilutions, 10, 50 and 100 µl aliquots were also spread onto the surface of THYE agar plates containing 5 µg/ml erythromycin and incubated overnight at 36°C. This was necessary to determine a volume that would provide 100-200 cfu/plate. Typically 2000-4000 mutants must be screened to produce one colony with the desired mutant phenotype (Li et al. 1997).

To measure the efficiency of concomitant plasmid loss, 100 suspected Tn917 mutant colonies growing on erm plates were patched in duplicate onto THYE plates containing 300 µg/ml kanamycin. After overnight incubation of one plate at 36°C and the other at 28°C, the percentage of mutants that exhibited Tn917 insertion with attendant plasmid loss (erm^r kan^s) was calculated for the two temperatures by dividing the number of erm^r kan^s by the number of erm^r colonies times 100%.

Polymerase Chain Reaction

For PCR analysis two sets of primers were utilized. Primers for the erythromycin methylase gene of Tn917 were designed from published DNA sequences (Shaw and Clewell 1985). Presence of the pTV1-OK backbone was detected using the *aph*A3

primer pair directed towards its kanamycin resistance gene. The primer pair sequences had been published previously by VanHoof et al. (1994). Sequences for the two primer pairs are as follows: erm forward: 5'-CATGCGTCTG ACATCTATCTG-3', erm reverse: 5'-CGATTGACCC ATTTTGAAAC-3', *aph*A3 forward: 5'-CTGATCGAAA AATACCGCTG C-3', *aph*A3 reverse: 5'-TCATACTC TTCCGAGCAAAGG-3'. The erm pair amplified a 248 bp DNA segment, while the *aph*A3 segment was 269bp in length.

Gene amplification was conducted by PCR using a Perkin Elmer model 480 Thermal Cycler. All PCR reactions were performed in 50 µl volumes containing 5 µl Perkin Elmer 10 X PCR Buffer with 15 mM $MgCl_2$, 5 µl dNTP mix (New England Biolabs, Inc., Beverly, MA) providing a 200 µM final concentration of each dNTP, 0.5 µM final concentration of each primer, 0.5 µg of template DNA, and 2.5 U Perkin Elmer AmpliTaq DNA polymerase. Thirty-five cycles of denaturation at 94°F for 30 sec, annealing at 55°F for 60 sec, and extension at 72°F for 2 min were followed by a 5 min extension at 75°C. At the completion of the reaction, the amplified products were held at 4°C until analyzed by gel electrophoresis.

To examine the stability of Tn917 insertions, 301-22-16 and 301-22-17 were subcultured on blood agar plates with and without erythromycin for a total of 20 passes. The two mutants were also passed in vivo in tilapia and reisolated for a total of five passes.

Southern Blot Analysis

Chromosomal DNA was isolated from eight *S. iniae* colonies selected at random, which demonstrated temperature conditional resistance to kanamycin, the two non-

hemolytic mutants, and two colonies erm^r kan^r at both permissive and non-permissive temperatures. The 99-301 erm^s kan^s wild type strain was used as a negative control. Chromosomal DNA was enzymatically digested using 20 U of 10,000 U/ml *Stu* I (New England Biolabs) in 50 µl reactions containing 10 µg of chromosomal DNA. *Stu* I was chosen because it cuts only once in pTV1-OK, 34bp upstream from the right terminal repeat of Tn917 outside the erm resistance gene (Shaw and Clewell 1985). Digestions were conducted for 4 hrs in a 37°C water bath. From each preparation, 2 µg digested of DNA were loaded into the wells of two 0.6% agarose gels. As positive controls, 1 µl of previously amplified erm and aphA3 PCR products were used. The gels were then electrophoresed at 45 V for 3 hrs.

Southern blot analysis was performed with minor variations following the ECL™ Direct Nucleic Acid Labelling and Detection System (Amersham Pharmacia, Piscataway, NJ). Gels were processed by covering with 500 ml of 0.2 M HCl and gently agitating for 10 min, followed by two washes in ddH_20. Denaturation of DNA was accomplished by rocking gels twice for 15 min in 500 ml of a solution of 1.5 M NaCl and 0.5 M NaOH. Gels were neutralized for 30 min in a solution of 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5), again with gentle agitation.

Neutralized gels were placed on transfer "pyramids" composed of a glass bread mold on top of which was placed a 15 x 15 cm glass plate. On top of the glass was placed an approximately 35 x 10 cm Whatman 3MM paper "wick," the free ends of which were draped into the bread mold. The wick was then thoroughly wetted and the mold half filled with 20X SSC [0.3 M Na_3Citrate, 3.0 M NaCl (pH 7.0)]. Individual gels were placed on top of the wicks and covered with a Hi-Bond nylon transfer membrane

cut approximately 3 mm smaller than the gel. The membranes had been wetted with ddH_2O for 1 min and then soaked in 20X SSC for 10 min. Centered on top of the membranes was a sheet of Whatman paper cut 5 mm smaller than the membrane that had been previously wetted with 20X SSC and slightly blotted. Care was taken to assure no air bubbles were present between the glass, wick, membrane, and Whatman paper. Two additional sheets of Whatman paper and a 3 cm stack of absorbent paper towels were then placed on top of the growing pyramid, followed by a second glass plate and a 400 gm weight. The entire pyramid was then loosely wrapped in plastic wrap to limit evaporation and the transfer was allowed to proceed overnight.

On the following day, the pyramid was disassembled and the transfer membranes covered in 2X SSC for 5 min. Membranes were gently blotted on Whatman paper, placed DNA side up in a pre-warmed Stratagene UV Stratolinker and the DNA auto-crosslinked. The membranes were wetted with 5X SSC, placed DNA side up on sheets of fine plastic mesh, and inserted into hybridization tubes of a Hybaid Micro-4 hybridization rotisserie oven. The mesh surfaces were placed against the glass tubes with no overlapping of the membranes and a small volume of 5X SSC was added, ensuring no air bubbles between the membranes and tube walls. The SSC was then poured off and 12 ml of hybridization buffer (ECL™) pre-warmed to 42°C was added to the tubes. The blots were then pre-hybridized for 1 hr at 42°C.

Labeled probes were prepared using ECL™ reagents. In microfuge tubes, to 3 ul of PCR product (same products used as positive controls) was added 7 µl of water supplied by the manufacturer. The probe DNAs were denatured by heating for 5 min in a boiling water bath and cooled on ice for 5 min. Contents were collected at the bottom

of the tubes by brief centrifugation, followed by the addition of 10 µl of labeling reagent. After gently mixing, 10 µl of glutaraldehyde were added to each, the tubes were mixed, and briefly centrifuged. The labeled probes were incubated at 37°C for 10 min and added immediately to the pre-hybridized blots. Hybridization was then allowed to proceed at 42°C for 4 hrs.

When hybridization was complete, the buffer was poured off, replaced with 100 ml of pre-warmed 5X SSC, and the blots returned to the rotisserie for 5 min. The SSC was then removed and replaced with 100 ml of pre-warmed primary wash buffer composed of 6.0 M urea, 0.4% SDS, 0.5X SSC. One 20 min wash was followed by two 10 min washes in fresh buffer on the rotisserie, all at 42°C. The blots were then removed from the tubes, placed in trays, and washed twice for 5 min each in 2X SSC.

Detection was accomplished by mixing 5 ml each of ECL™ detection reagents 1 and 2. The reagents were applied to the entire surface of the two membranes, which had been placed DNA side up on a sheet of plastic wrap, and allowed to incubate for 1 min at room temperature. Excess reagents were poured off the blots, which were wrapped in fresh plastic wrap, and placed DNA side up in an 8 x 10 in Fisher Scientific autoradiograph cassette. A sheet of Kodak X-OMAT™ scientific imaging film was placed on top of the blots, exposed for 5 min, and processed routinely in an automatic film processor.

Single-Primer PCR for Identification of Transposon Insertion Sites

Single-primer PCR was used to identify Tn917 insertion sites and to generate PCR products, up and downstream from the transposon, to be used for sequencing. As implied, the procedure uses only a single primer, specific for one end of the transposon

to amplify genomic sequences adjacent to the insertion site, and a single PCR reaction, consisting of three rounds of amplification. The first round amplifies a single-stranded transposon-specific template at conventional annealing temperatures. In the second round, a low annealing temperature allows non-specific binding of the single primer on the opposite DNA strand in the flanking *S. iniae* chromosomal DNA. Finally, round three allows further amplification of the double stranded product from round two, again at conventional annealing temperatures. The final products consist of a mixture of transposon-specific and unrelated fragments, however, round one enriches for and favors amplification of the transposon related insertion in the later rounds. A second transposon-specific primer, external to the first, is used for sequencing purposes (Karlyshev et al. 2000).

The PCR began with an initial denaturation at 94°C for 1 min. Round one consisted of 20 cycles of 94°C for 20 sec, 57°C for 30 sec, and 72°C for 3 min; round two, 30 cycles of 94°C for 20 sec, 45°C for 30 sec, and 72°C for 2 min; round three, 30 cycles of 94°C for 20 sec, 57°C for 30 sec, and 72°C for 2 min. Upon completion, final extension at 72°C was conduction for 7 min. Primers used for amplification and sequencing are included below: upstream amplification (UTP-1): 5' - AGAGAGATGT CACCGTCAAG - 3', upstream sequencing (UTP-2): 5' - AATGTACAAAATAACAG CGAA - 3', downstream amplification (DTP-1): 5' - CTAAACACTTAAGAGAATTG - 3', and downstream sequencing (DTP-2): 5' - TAGGCCTTGAAACATTGGTT - 3'.

The PCR products were visualized on a 2% agarose gel, and purified on a QIAquick™ Spin PCR purification kit (Qiagen) following the manufacturers directions

to remove residual primer, nucleotides, enzymes, and salts. Sequencing was carried out using a Perkin Elmer ABI Prism 377 DNA Sequencer.

Competitive Elimination Trial

The *S. iniae* 99-301-WT and 301-22-17 non-hemolytic mutant were passed through and reisolated from tilapia a total of three times. A single colony of each was inoculated into 5 ml of Todd Hewitt broth and grown to stationary phase at 28°C. The mutant colony was grown in the presence of 5 μg/ml of erythromycin. Starter cultures were diluted 1:50 into 125 ml of Todd Hewitt broth and incubated at 28°C until mid-log phase growth was achieved (OD_{600} 0.7-0.8). The two cultures were pelleted, washed in sterile physiologic saline (PBS), and resuspended in 1.5 ml of PBS. Prior to combining the concentrated wild-type and mutant bacterial suspensions, plate counts were performed on each.

A total of 60 tilapia *T. niloticus*, with an average weight of 49.7 gm, were lightly anaesthetized with tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) and injected intracranially with 20 μl of the combined bacterial suspension. The fish were placed in a single 100 gallon tank receiving a constant flow of 27 +/- 1°C water. Five fish were collected at random daily and euthanized in 1000 μg/ml MS-222. Following this, brains were aseptically removed, suspended in 500 μl of sterile PBS, and weighed. The brains were homogenized and plate counts performed to determine the total number of cfu/gm of tissue for both the wild-type and mutant strains on TSAB blood agar and THYE with 5% sheep blood and 5μg/ml erythromycin, respectively.

Results

Electrotransformation of *S. iniae* 99-301D

Streptococcus iniae 99-301D ($erm^s kan^s$) wild type cells were harvested in log phase growth at an OD_{600} of 0.32, then incubated in a combination of lysozyme and mutanolysin to remove the cell wall. To an aliquot of prepared bacterial suspension, containing 1.75×10^9 cfu/ml, was added 1 µg of pTV1-OK. Following plasmid delivery, cells were allowed time to osmotically stabilize in broth with 0.5 M sucrose in the absence of antibiotics. Successful transformation was determined by demonstrating growth on agar containing 300 µg/ml kanamycin. After approximately 60 hrs of incubation at 28°C, 1-1.5 mm colonies were visible. A total of 93 colonies were counted from six plates inoculated with 200 µl each of cells electroporated at 2.5 kV. Electroporation at 2.5 V resulted in a 9.5% decrease in viable cell numbers. There was no growth on plates inoculated with cells electroporated at 1.25 kV.

Eighty of the 93 colonies growing on kanamycin at 28°C were selected at random and patched onto THYE agar plates containing 300 µg/ml kanamycin and 5 µg/ml erythromycin. After incubation for 24 hrs at 28°C, only two colonies failed to grow on erythromycin. Successful transformation was thus indicated by acquisition of an $erm^r kan^r$ phenotype under the permissive temperature of 28°C. The presence of pTV1-OK was further confirmed in three transformant colonies by PCR amplification using primer pairs directed towards the erm methylase gene of Tn917 and aphA3 kanamycin resistance gene of the pTV1-OK backbone (Figure 54).

Insertional Mutagenesis, Transposition Frequency and Mutant Stability

As reported in other bacterial species, pTV1-OK exhibited temperature conditional replication in *S. iniae* 99-301-22. Transposition of Tn917 into the 99-301-22 chromosome occurred with concomitant loss of the pTV1-OK backbone at the non-permissive temperature of 36°C. Following 18 hrs of incubation only two dilutions (1:50 and 1:100) showed visible growth, regardless of erythromycin level. Plate counts were performed on these two dilutions on THYE agar with and without 5 μg/ml erythromycin and incubated at 36°C. Calculated transposition frequencies ranged from 0.01-0.02%. The efficiency of plasmid loss following incubation at non-permissive temperatures was found to be 100%.

A total of 2180 colonies grew on THYE agar plates supplemented with 5μg/ml erythromycin and 5% sheep blood. Two non-hemolytic mutants were detected after 24 hrs of incubation at 36°C (Figures 54 and 55). No spontaneous non-hemolytic mutants were detected among a total of 1971 wild type colonies of *S. iniae* 99-301 spread on TSA blood agar plates. The two mutants were designated 301-22-16 and 301-22-17. PCR analysis was performed on the two mutants using the erm and *aph*A3 primer pairs, which confirmed the presence of Tn917 in the *S. iniae* chromosome and loss of the pTV1-OK backbone (Figure 56). There was no reversion by the two mutants to a hemolytic wild type phenotype following five *in vivo* passes, or in 20 *in vitro* passes, both in the presence and absence of erythromycin.

Southern Blot Analysis

Examination of Southern blots indicated a high degree of random insertion by Tn917 into the *S. iniae* chromosome. The presence of a kanamycin band, in one of the

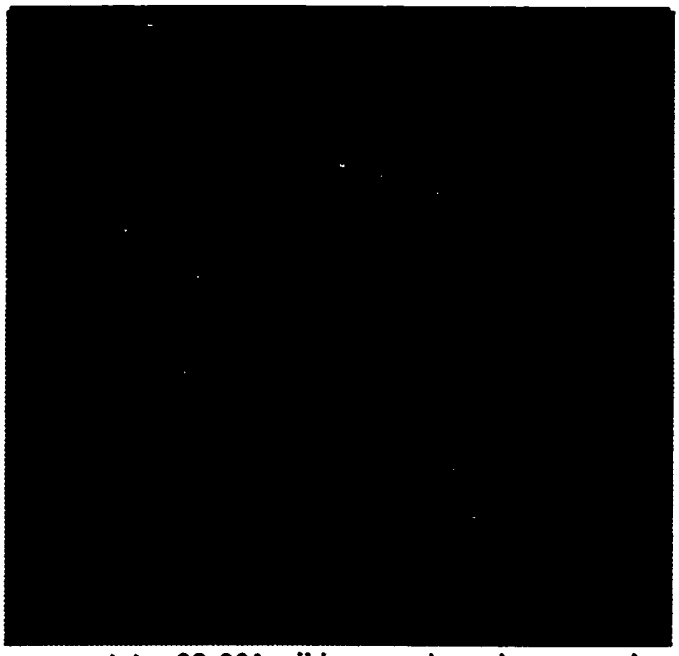


Figure 54. *Streptococcus iniae* 99-301 wild type colony demonstrating typical pattern of β-hemolysis.

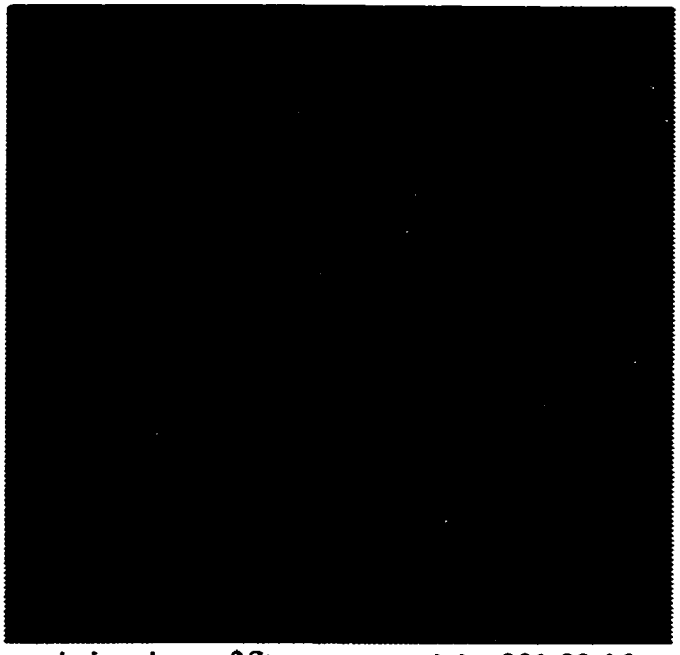


Figure 55. Non-hemolytic colony of *Streptococcus iniae* 301-22-16 resulting from transpositional mutagenesis with Tn917.

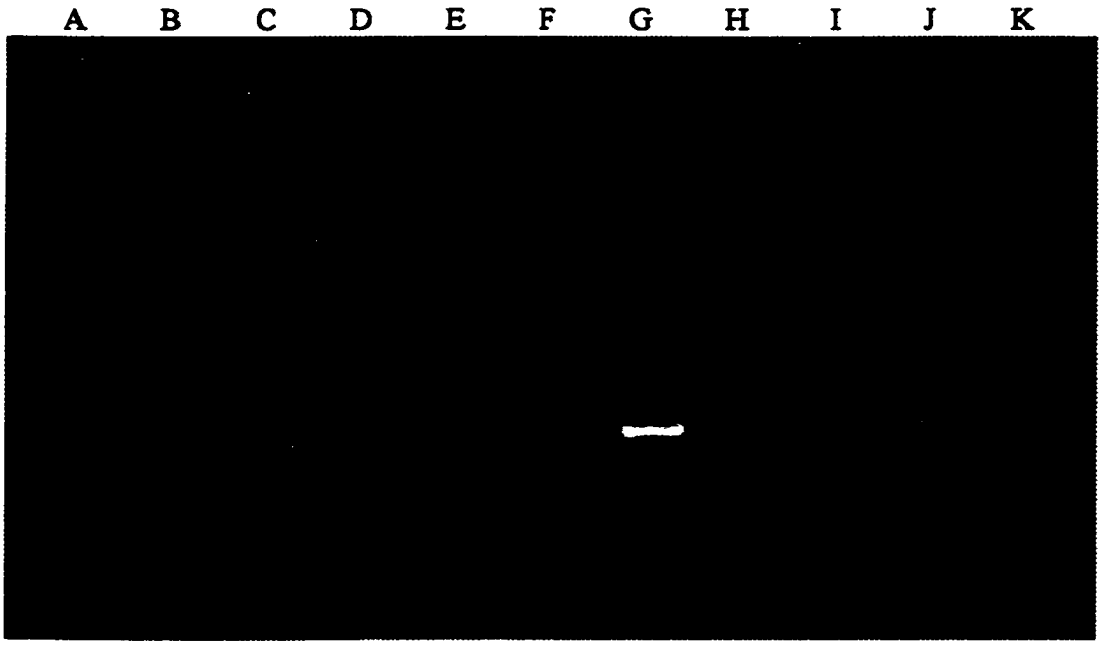


Figure 56. Agarose gel electrophoresis of PCR products generated by the aphA3 and erm primer pairs indicating loss of the pTV1-OK backbone and transposition of Tn917 into the S. iniae chromosome following incubation at non-permissive temperatures.

Lane		Primers
A	Φ174 ladder	
B	pTV1-OK	*aph*A3
C	pTV1-OK	erm
D	99-301-WT	*aph*A3
E	99-301-WT	erm
F	99-301-22	*aph*A3
G	99-301-22	erm
H	99-301-22-16	*aph*A3
I	99-301-22-16	erm
J	99-301-22-17	*aph*A3
K	99-301-22-17	erm

erm^{r}kan^r insertional mutants suggested integration of the entire pTV1-OK backbone. The two non-hemolytic mutants produced different bands indicating insertion at two different sites, both resulting in phenotypically identical mutants (Figure 57).

Single Primer PCR for Identification of Transposon Insertion Sites

For unknown reasons, repeated attempts to clone *Eco*RI, *Xho*I, and *Hin*dIII digested chromosomal fragments from the two non-hemolytic mutants failed. On agarose gels, the single primer PCR procedure yielded one predominant band of approximately 320 bp upstream and 1100 bp downstream and 320 bp upstream and 1400 bp downstream from Tn917 non-hemolytic mutants 301-22-16 and 301-22-17, respectively (Figure 58). Comparison of the *S. iniae* DNA sequences indicated distinct Tn917 insertions in the two mutants. Sequence analysis revealed 74-85% identity with recently published segments of *sag*A, *sag*B, and *sag*C, components of the *S. pyogenes* *sag* operon responsible for production of streptolysin S (Nizet et al. 2000).

Competitive Elimination Trial

Streptococcus iniae wild-type isolate 99-301 was harvested in mid-log phase of growth at an OD_{600} of 0.75. A plate count performed on the concentrated suspension equaled 6.0×10^{10} cfu/ml. Similarly, the OD_{600} for mutant 99-301-17 was 0.74 and corresponded to a plate count of 2.5×10^{10} cfu/ml. The 20 µl volume injected intracranially contained 4.0×10^8 cfu and 1.3×10^8 cfu of the wild-type and mutant bacteria, respectively. Numbers of wild-type and non-hemolytic mutant bacteria per gram of brain tissue are presented in Figure 59.

Figure 57. Southern blot analysis of *S. iniae* mutants demonstrating random insertion of Tn917 into the bacterial chromosome. Figure 55a demonstrates the presence of the *erm* erythromycin resistance gene of Tn917. Figure 55b demonstrates the presence of the *aphA3* kan^r gene of pTV1-OK. Lanes K and L represent the two non-hemolytic mutants, 99-301-22-16 and 99-301-22-17.

Lane	57-a	57-b
A	99-301-22-2	99-301-22-2
B	99-301-22-3	99-301-22-3
C	99-301-22-4	99-301-22-4
D	99-301-22-5	99-301-22-5
E	99-301-22-6	99-301-22-6
F	99-301-22-7	99-301-22-7
G	99-301-22-8	99-301-22-8
H	99-301-22-9	99-301-22-9
I	99-301-22-13	99-301-22-13
J	99-301-22-14	99-301-22-14
K	99-301-22-16	99-301-22-16
L	99-301-22-17	99-301-22-17
M	99-301-22-WT	99-301-22-WT
N		
O	erm^r	kan^r

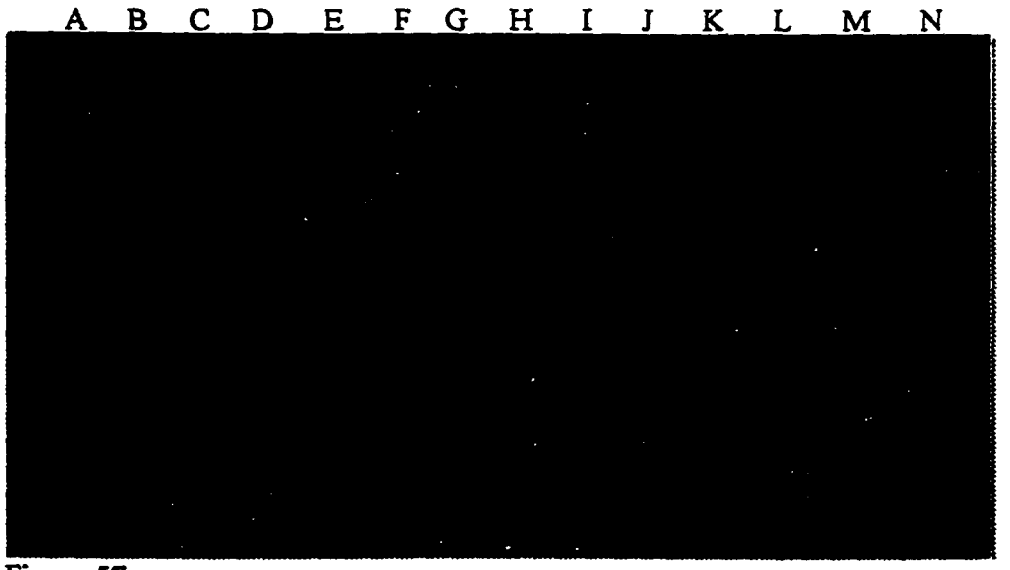


Figure 57-a

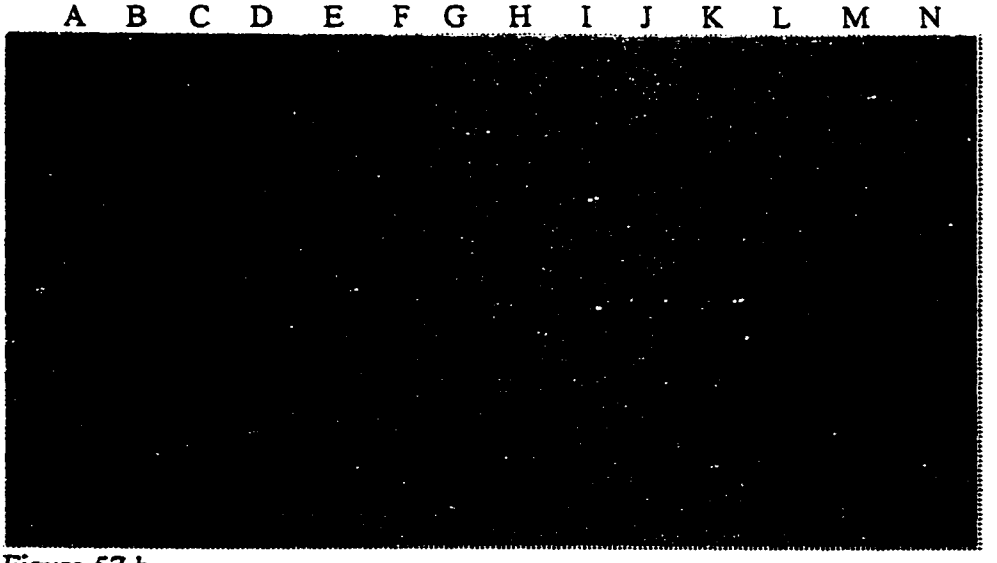


Figure 57-b

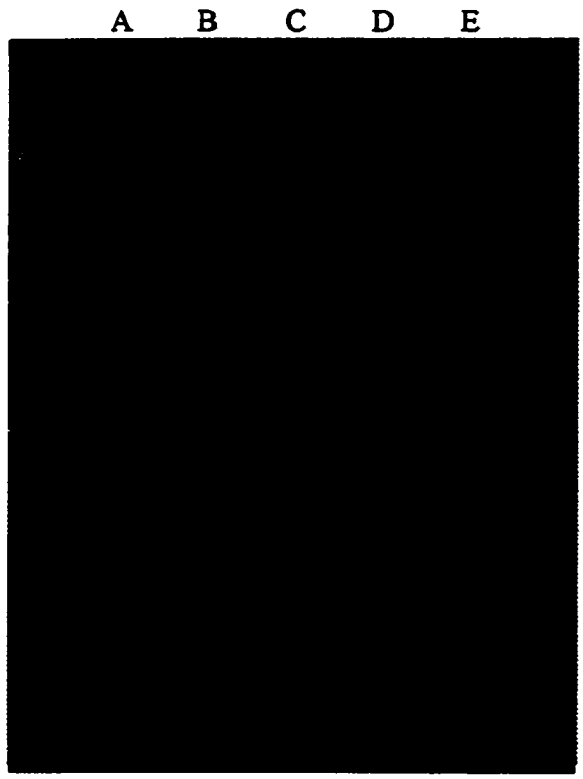


Figure 58. Agarose gel electrophoresis of PCR products generated by single primer PCR, indicating a single prominent band amplified from up and downstream Tn917 insertion sites

Lane		Primer
A	Φ174 ladder	
B	301-22-16	UTP-1
C	301-22-16	DTP-1
D	301-22-17	UTP-1
E	301-22-17	DTP-1

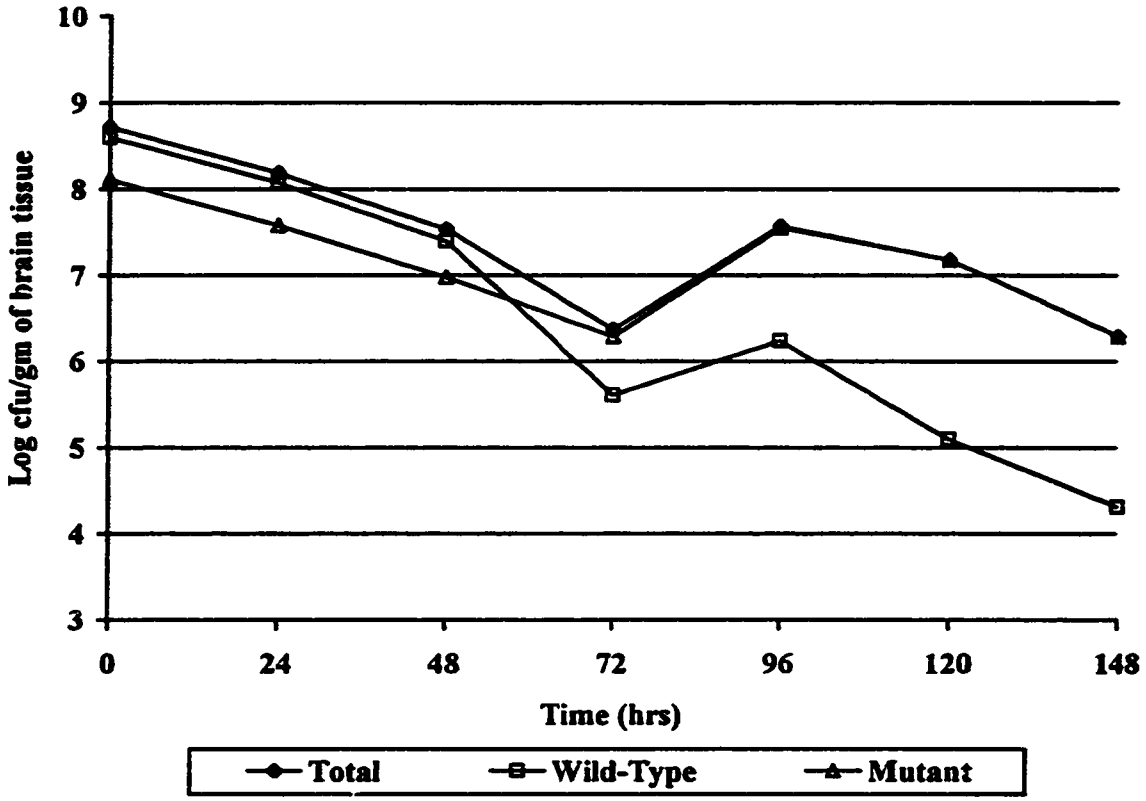


Figure 59. Competitive elimination trial. Daily bacterial counts (cfu/gm) from the brains of tilapia injected intracranially with 4 x 10^8 cfu of *Streptococcus iniae* 99-301 wild-type and 1.3 x 10^8 cfu of non-hemolytic mutant 310-22-17.

Discussion

The purpose of this research was to examine the utility of Tn917 mutagenesis in *S. iniae*, an important fish pathogen and potential zoonotic agent (Perera et al. 1994; Weinstein et al. 1997). Transposon mutagenesis has proven to be a powerful tool for elucidating virulence mechanisms in gram-negative bacteria, but has only recently been successfully applied to gram-positive pathogens. Using plasmid pTV1-OK, a replication-conditional (temperature-sensitive) delivery vector, which carries transposon Tn917, two distinct non-hemolytic mutants of *S. iniae* were ultimately produced.

S. iniae strain 99-301D was isolated originally from a natural outbreak of disease in cultured tilapia. This particular isolate was chosen because of its strong β-hemolytic phenotype and ability to grow well at 38°C. The majority of *S. iniae* isolates produce a small inner zone of β-hemolysis surrounded by a larger outer zone of α-hemolysis. Growth of other *S. iniae* isolates examined was rapidly inhibited above 36°C, near the reported minimum temperature of 35°C for efficient inhibition pTV1-OK replication (Maguin et al. 1992). To investigate the utility of the pTV1-OK-Tn917 system in *S. iniae*, the β-hemolytic characteristic was targeted for mutagenesis studies because of the ease of direct screening on blood agar plates. It has also been recently demonstrated that the β-hemolysin of *S. pyogenes* (streptolysin S) contributes to the virulence of this pathogen (Betschel et al. 1998; Ginsburg 1999; Nizet et al. 2000).

The cell wall of *S. iniae* proved to be a formidable barrier to the introduction of pTV1-OK by electroporation. Plasmid pTV1-OK was introduced into three different strains of *S. iniae* using variations of two different methods (data not shown). In the first, cells were made competent for transformation by growth in broth media containing

0.5% glycine, which results in a weaker cell wall, as described for *S. agalactiae* (Framson et al. 1997). The second method used enzymatic removal of the cell wall with lysozyme (Powell et al. 1988) or a combination of lysozyme and the muralytic agent mutanolysin.

The addition of mutanolysin presumably resulted in protoplast formation and was found to yield the greatest number of transformants, although, numbers of transformants obtained may reflect differences between strains rather than the methodologies used. Protoplast formation is considered a disadvantage because more time is required for regeneration of cell walls and a greater number of cells are lysed during the electrical pulse (Powell et al. 1988). Regardless of the method used only a single transformant colony is needed to proceed with mutagenesis experiments.

For successful electroporation it was necessary to harvest cells in early log phase, OD_{600} of approximately 0.3. It was also found that following electroporation 4-5 hrs of incubation was needed to allow expression of antibiotic resistance before plating on selective media, as reported for *S. pyogenes* (Li et al. 1997). Expression of erythromycin resistance appeared to lag behind that for kanamycin and it was generally necessary to plate first on kanamycin and if necessary transfer over to erythromycin at a later time.

For unknown reasons, attempts to clone fragments of chromosomal DNA from the two non-hemolytic *S. iniae* mutants using pBluescript and *rpsL* mutant *E. coli* strain MC1061 failed using multiple restriction enzymes. Similar results were obtained in another laboratory working with *S. iniae* using pUC19 and *rpsL* mutant *E. coli* DH10B (De Azavedo 2001). It has been reported that the "shot gun" approach for cloning of Tn917 insertions in *E. coli* cannot be used because the Tn917 erm gene could not be

expressed at selectable levels in the host or that the erm gene or sequences proximal to it were toxic. These difficulties reportedly can be overcome by using *E. coli* MC1061 or another *rpsL* negative strain (Gutierrez et al. 1996; Cvitkovitch et al. 1998).

After numerous unsuccessful attempts using traditional cloning techniques, a sequencing of DNA segments flanking the Tn917 insertions was achieved after a single primer PCR technique was adopted (Karlyshev et al. 2000). This novel method for the identification of transposon insertion sites eliminates the need for cloning by using a single transposon primer, specific for each end of a transposon. Amplification and sequencing primer sequences were graciously provided by Dr. Joyce De Azavedo, Mt. Sinai Hospital, Toronto, Canada.

Zones of hemolysis surrounding colonies of streptococci growing on blood containing agar were one the first characteristics used to recognize clinically significant isolates. Many pyogenic streptococci produce two distinct hemolysins, the oxygen labile streptolysin O and the oxygen stable streptolysin S. These hemolysins destroy erythrocytes to produce a zone of complete, or β-hemolysis. Other streptococci produce a zone of greenish discoloration, or α-hemolysis, due to the production of hydrogen peroxide, resulting in the conversion of hemoglobin to methemoglobin (Kilian 1998).

Sequence data collected from the two mutants shared a high degree of homology with the first three open reading frames of the *S. pyogenes sag* operon. These findings are in agreement with unpublished data that indicate extensive similarities between the entire *S. iniae* hemolysin and *S. pyogenes sag* operons (De Azavedo 2001). Sequence analysis of the nine-gene *sag* locus (*sag*A-*sag*I) indicates that SLS is related to the bacteriocin family of microbial toxins, which are encoded by an operon including the

structural prepropeptide gene and genes required for posttranslational modification, processing, and export of the mature toxin using an ABC type transporter system (Nizet et al. 2000). Transposon insertions for the two mutants occurred in the suspected promoter region upstream from *sag*A, the gene encoding the streptolysin S pre-propeptide, and in *sag*B, whose gene product in believed to be involved in the posttranslational modification of the streptolysin propeptide.

Streptolysin S is oxygen stable, largely cell bound, non-antigenic, and also damaging to the membranes of erythrocytes, lymphocytes, neutrophils, platelets, and certain tissue culture and tumor cells. Despite decades of detailed investigations, the exact chemical nature of SLS remains unknown. By weight, it is one of the most potent cytotoxins known (Nizet et al 2000). Its activity is seen in the classic surface and subsurface hemolysis of sheep erythrocytes on blood agar plates (Kilian 1998). The hemolytic activity of *S. equi* has also been attributed to an SLS-like hemolysin (Flanagen et al. 1998).

There is good evidence of a role for SLS as a virulence determinant in group A streptococcal infections, including: 1) Its location bound to the cell surface; 2) Its continuous synthesis even in resting cells; 3) Its non-immunogenicity; 4) Its extractability by certain serum proteins; 5) Its ability to be transferred directly to target cells while being protected from inhibitory agents present in inflammatory exudates; 6) Its membrane pore forming capabilities; 7) Its ability to synergize host derived proinflammatory agonists; and 8) The reduced pathogenicity of SLS negative mutants (Ginsburg 1999). In a mouse model of subcutaneous infection, the virulence of *S. pyogenes* Tn916 mutants, of different Emm serotypes containing identical mutations, is

markedly reduced. Mutants were found to be incapable of inducing necrotizing skin lesions (Betschel et al. 1998; Nizet et al. 2000).

Unexpectedly, when injected intracranially, in tilapia, in a competitive elimination trial, the non-hemolytic mutant showed greater persistence than the hemolytic wild-type strain. These findings suggest greater virulence in the non-hemolytic mutant and are in direct contrast to findings in the mammalian models discussed above. At this time it is unclear whether these findings are accurate or represent loss of virulence of the *S. iniae* 99-301 wild-type following *in vitro* passage in the laboratory and prolonged storage at –70°C. Currently, mutagenesis studies using another virulent β-hemolytic *S. iniae* strain are under way.

In conclusion, this study was undertaken to investigate the utility of the pTV1-OK::Tn917 mutagenesis system in *S. iniae* and ultimately resulted in the production of two non-hemolytic mutants. Using single primer PCR, approximately 1600 bp of DNA sequence was obtained from up and downstream transposon insertion sites from the two mutants. Analysis of sequence data revealed a high degree of identity with extensive segments of the recently published *S. pyogenes sag* operon, responsible for the production of streptolysin S (Nizet et al. 2000). Transposition frequencies, the efficiency of plasmid loss, and frequency of the mutant phenotypes were all consistent with findings in S. mutans (Gutierrez et al. 1996) and S. pyogenes (Li et al. 1997). Additionally, Tn917 insertion occurred with a high degree of randomness and produced extremely stable mutations. The findings indicate that Tn917 should prove to be a useful tool in the study of additional potential *S. iniae* virulence mechanisms.

SUMMARY

Outbreaks of *Streptococcus iniae* infection in tilapia, hybrid striped bass and rainbow trout in the United States and Israel in the1990s renewed interest in the disease. Despite this, the literature contains only limited descriptions of histopathologic lesions. To provide a more complete picture, a composite of histopathologic changes was compiled from clinical cases of diseased tilapia submitted to the Aquatic Animal Disease Diagnostic Laboratory, Louisiana State University, School of Veterinary Medicine. *Streptococcus iniae* infections share enough morphologic features common to other gram-positive fish pathogens to indicate that it cannot always be reliably differentiated from them at either the gross or microscopic level.

The most detailed histopathologic description of *S. iniae* infection in tilapia was published by Perera et al. (1998). Several notable differences are documented in the current study. In common with certain other streptococci, *S. iniae* has a marked tropism for the central nervous system, causing severe meningoencephalitis. Previously unreported is extension of the meningitis to involve structures of the inner ear, a likely cause of commonly observed vestibular signs.

Ocular lesions have been widely reported and were common in this study. Typical changes included panophthalmitis with inflammation of the choroid rete mirabile, retinal detachment, and optic neuritis. Inflammatory cells and fibrin were sometimes present in the irido-corneal angle. While glaucoma has not been reported to occur in fish, the presence of this material suggests this condition could be possible.

Perera found no gross skin changes or abnormalities in skeletal muscle. In contrast, pustules were often associated with bony prominences and large abscesses

regularly occurred. Cellulitis and fasciitis extended into muscles of the head and trunk. Bone remodeling with osteolysis and sequestration was also seen. There was a predilection for red muscle groups of the fin bases and within bony cavities of the skull.

Polyserositis was typically present affecting serosal surfaces of the abdominal viscera. Multisystem involvement was seen, but with the exception of the spleen and to a lesser extent the head kidney, changes in parenchymal organs were mild. All tilapia exhibited some degree of epicarditis and myocarditis, often severe. Septic thrombi were occasionally found adhered to the endocardium and bulboventricular valve leaflets.

Perera discussed a lack of gastrointestinal lesions in diseased tilapia and other authors have considered a lack of intestinal lesions a useful criterion for discriminating between *S. iniae* from other gram-positive pathogens. While uncommon, necrotic lesions were found in the submucosa of the stomach and intestine. Swim bladder lesions have only been reported once as a manifestation of *S. iniae* infection. In the tilapia examined here, they were uncommon and typically mild in nature.

Little is known concerning the pathogenesis of *S. iniae* infections, in particular how the organism gains entry to fish or how it is shed into the environment. Cocci laden macrophages traverse the epithelium of the olfactory, lateral line, and gastrointestinal mucosae, suggesting these may all represent sites of bacterial shedding. Infection of the gonads also suggests another potential route by which the organism might be shed. While the possibility of a carrier state has never been addressed, this study indicates both horizontal and vertical transmission via the gonads should not be dismissed.

Inflammatory changes were invariably predominated by macrophages, although differentiation from neutrophils in tissue sections was sometime difficult. Using

cytochemical stains neutrophils were readily differentiated from macrophages. It is reasonable to assume that neutrophils play a role in early lesion development, but are outnumbered by macrophages over time. These findings indicate that inflammatory responses in tilapia may more closely parallel those of higher vertebrates than previously thought. The participation of neutrophils is not surprising when one considers *S. iniae* belongs to a group organisms classically described as pyogenic. Large numbers of *S. iniae* are present in most lesions, but are best visualized by the use of tissue gram stains. Distension of phagocytes distended with engulfed cocci suggests that intracellular multiplication might be taking place.

Using other pyogenic streptococci, particularly *S. pyogenes*, as models, 29 S. iniae isolates, of fish and human origin, were examined for the presence of known or purported virulence factors found in these microorganisms. Potential virulence factors evaluated were M protein, capsule production, hyaluronidase, DNase, and streptokinase. There was no indication of an antiphagocytic M-like protein molecule using PCR primers designed to amplify entire M protein genes or conserved anchor regions of related M-like proteins. Findings were supported by electron microscopy, which revealed features more consistent with a capsule than M protein. The use of lysine, in combination with ruthenium red, to stabilize and stain the capsule proved superior to glutaraldehyde fixation alone. Positive ruthenium red staining indicates a capsule composed of acid-polysaccharides. Using a hyaluronidase plate assay 79% of the isolates demonstrated activity for this enzyme, which is considered a spreading factor in other bacterial pathogens. Fifty-two percent of the isolates showed positive activity using DNase test

agar, also considered a potential streptococcal virulence factor. Thirty-one percent were positive for both enzymes.

Similar to hyaluronidase, streptokinase also acts as a spreading factor. Streptokinase-like activity was evaluated using a casein overlay containing human plasminogen and a broth assay using human and tilapia plasma. *Streptococcus iniae* showed no activity with human plasminogen. In tilapia plasma, results were equivocal, delaying but not completely inhibiting clot formation.

A final study was undertaken to evaluate the utility of the temperature conditional delivery vector, pTV1-OK, carrying the transposon Tn917 as a mutagenesis system applicable to use in *S. iniae*. Insertional mutagenesis using Tn917 ultimately resulted in the production of two non-hemolytic *S. iniae* mutants. Using single primer PCR, approximately 1600 bp of DNA sequence was obtained from up and downstream transposon insertion sites from the two mutants. Analysis of sequence data revealed a high degree of identity with extensive segments of the recently published *S. pyogenes sag* operon. The *sag* operon is responsible for the production, modification and export of streptolysin S, a potent cytolytic agent considered to be an important virulence factor in that organism. In contrast, a competitive elimination trial revealed greater persistence of the non-hemolytic *S. iniae* mutant than the wild-type strain.

Transposition frequencies, the efficiency of plasmid loss, and frequency of the mutant phenotypes were all consistent with findings in other streptococci. Additionally, Tn917 insertion occurred with a high degree or randomness and produced extremely stable mutations. The findings indicate that Tn917 should prove to be a useful tool in the study of additional potential *S. iniae* virulence mechanisms.

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APPENDIX: BESTFIT ANAYSIS OF *STREPTOCOCCUS INIAE* NON-HEMOLYTIC MUTANT DNA SEQUENCES TO THE *STREPTOCOCCUS PYOGENES SAG* OPERON

301-22-16 (Primer UTP-1)

```
16up2.txt x sagabc.txt    April 5, 19101 13:57  ..

                .         .         .         .         .
  3 TGATAAGAGTTAGGTAGTTGTTTCGTTACAATAATACAAATTTGCTAGCC 52
    ||||||||  ||| ||||||||  |||||||  | |||||  |  |||||||
159 tgataagaactagatagttgttgtgttacaacagtacaattgagctagcc 208
                .         .         .         .         .
 53 TTG...TTTTATGGATAAC...GCTTATAGATTAAGTTCTAAAAT..ACT 94
    |||   || |   | ||||     || || | |||| |  |||||  ||
209 ttgtccttgttgtgttaactttatttttaaaataaggttaaaaataaacg 258
                .         .         .         .         .
 95 ATTTGAATTCTTATCAGTTGTTTTACTTATTTGTATAAGGAGGTAAGCGT 144
    | | |  ||||||||||     |||||||||  | ||||||||||||  | |
259 actcgtgttcttatcag.....ttacttattag.ataaggaggtaaacct 302
                .         .         .         .         .
145 TATGTTACAATTTACTTCAAATATCTTAGCTACTANTGTAGCTGAAACAA 194
    |||||||  ||||||||||||||||  ||||||||||:|||||||||||||
303 tatgttaaaatttacttcaaatattttagctactagtgtagctgaaacaa 352
                .         .         .         .         .
195 CTCAAGTTGCTCCTGGTGGCTGTTGCTGCTGCTGCTGCACATGTTGTGTG 244
    ||||||||||||||||  |||||  ||  ||||||||     ||  |||||  |
353 ctcaagttgctcctggaggctgctgttgctgctgtactacttgttgcttc 402
                .         .         .         .         .
245 GCGGTAAATGTTGGAAGTGGTTCTGCTCAAGGTGGTAGTGGTACTCCANC 294
     |  |    |  ||||||||||  | ||||||||||||| ||  | |   :|
403 tcaattgctactggaagtggtaattctcaaggtggtagcggaagttatac 452
                .         .         .         .         .
295 ACCAGCTCCAAAGTAATTCACTCTTCTTAGCATCTCTATTTAATGGTTAA 344
     ||||        |||| |  | |||  |||||||||||||  | ||  ||  |
453 gccag.......gtaaat.aatctatttagcatctctatgtgatagtgat 494
                .         .         .         .         .
345 GTGTTGAAGATTAgTTTGCTAGCGGCTAACTATACTGCATCTAACTTGCG 394
     |    |   || ||||||||||||  |||| | ||        ||||||  |
495 attaaggtaatgagtttgctagcaactaagt.tatctttggtaacttctg 543
                .         .         .         .         .
395 ATGtCAAGCTCATTNTCGAATAGGGATGTTACATGTCACGAGACTGNTGT 444
    || |  || | |  :| | |||| | ||||| |   || |||||  : ||
544 at.tatagatga..atggcatagaggtgttagaaaacatgagacaaaagt 590

                .         .         .         .         .
445 AATCATCTTGGTTCTCATGTCTCAAGGTAATTAGCAGGTaCTAGNCAGTG 494
    ||||  |||||  | ||  | |||||  ||||||||||||||||||||: |||
591 aatcttcttgatgctaacgtctc.aggtaattagcaggtactagatagta 639
                .         .         .         .         .
495 CCTGCTAATTACTATATGTATACTANAATAATGNCGAAAATATA..TATG 542
    ||||||||||||||||||||  ||  ||:||| |  : | ||| |||  ||||
640 cctgctaattactatatgtttagtaaaatgagataggaaagatagttatg 689
                .         .         .
543 TCTTTTTTTTTCTAAGGAAAAACCATTTCANAAAA 577
    ||  ||||||  | ||||||  |||  |  | |: | |
690 tc.atttttacaaaggaacaacaacctaaagaga 723
```

301-22-16 (Primer DTP-1)

```
16dwn.txt x sagabc.txt    April 5, 19101 13:32  ..

                .         .         .         .         .
   107 TCTTTTTTAATAA.ATTACAAAGAAAGGGTTTACATATCTATTATTTTTT 59
       || ||||  |||| |||| ||||||||||||||||||  || |||||||
    96 tcattttcgataatattaaaaagaaagggtttacatattaatcatttttt 145

    58 AAt 56
       | |
   146 act 148



                .         .         .         .
   267 TCAAAAGAGAATGCAAAAAAAGCAGTCTCATTCTTTGAAA 306
       | ||| ||||  | ||| | ||   | ||||| |||  ||
   663 taaaatgagataggaaagatagttatgtcatttttacaa 702
```

301-22-17 (Primer UTP-1)

```
17up.txt x sagabc.txt     April 5, 19101 13:23  ..

          .         .         .         .         .
  495 AGCAGTTGGTGGCTCAACATTTAATGCCTAATGAAACAGACAATCTTAGT 446
      ||||| | || ||||||||||||||||||||||| ||| || |||||||||
  829 agcagctagttgctcaacatttaatgcctaatgcaaccgataatcttagt 878
          .         .         .         .         .
  445 CAACGTTTTTTGATGAATTATAAGTCAAATANNAATAAATGNNGCNGTCA 396
      |||| |||||||||||||| |||||  | ||||::||| | | ::||:  ||
  879 caacatttttgatgaactataaagctaataataattatttaggcttcca 928
          .         .         .         .         .
  395 AGCCAGTGTCGTTGATTTTTTTACGGATTCTGCGGTGACCACTTTTTCTA 346
      ||| ||| | || || |||||||| |||||||| ||  | | |||||| |
  929 agctagtattgtcgacttttttacagattctgccgttgctaatttttcaa 978
          .         .         .         .         .
  345 ACAGTGATTTTTTTGAAAGTCAGGATGAGACGATTTCTTTACCAAAGGCC 296
        ||| |  ||| ||||||||||||  |||  |||  |||||||||  |
  979 gtagttacgtttatgaaagtcaggaaaagataattcgtttaccaaaacct 1028
          .         .         .         .         .
  295 A.GAAAATTGTGACTTCTCTGTCTACTTGTATTACAAAAAGGAGGAGTCA 247
      |  || ||    ||| ||||||| || ||||||| |||| | || |||||
 1029 accaagatatcaactgctctgtcgacatgtattataaaacgaagaagtca 1078
          .         .         .         .         .
  246 TAGGCAGTTTTCAGGTTTGGAAATGCCTCTTCAAGACTTAGCAAATCTTT 197
      | | || |||||||  |    ||||||||||||||| ||| ||||  ||
 1079 tcgtcaattttcagatagacaaatgcctcttcaagatttatcaaacattc 1128
          .         .         .         .         .
  196 TGTATTATGCTTGTGGTGTTAGCTCTGAAGCAAGTATTAAAGAAGGCATG 147
      | |||||||| |||||||||| ||  |||||   |||| ||| ||
 1129 tttattatgcatgtggtgttagttcacaagcatcaattagagatggagca 1178
          .         .         .         .         .
  146 ACAAAAACAGTTAGTCTAAGAAATTGCGCTTCTGGTGGAGGTTTATACCC 97
       || | |   |||  || ||||| || ||||| ||||||||||||||||
 1179 tcagataagattacactcagaaactgtgcttcaggtggaggtttatаccc 1228
          .         .         .         .         .
   96 AATTACCTTATTCTTTTATGCTCGCAATATTACAAAACTTAAAGACGGGG 47
       |||   ||| | |||||||||| | || || |  ||| | | ||| ||
 1229 tattcatttagtttttatgctagaaacatcagtaaattaatagatggtt 1278
```

301-22-17 (Primer DTP-1)

```
17down.txt x sagabc.txt   April 5, 19101 11:35  ..

     2 AGACGGTTTTTATGAATACCTTCCTTATCAGCATGCTTTAAGATGTCATC 51
       ||| ||||| |||||||| || || |||||||||||  |||| ||| |||
  1271 agatggtttctatgaatatctaccctatcagcatgcactaaggtgttatc 1320

    52 GTGTCAGTGAAGAAGAAGATGTCAGAGCTTTTGCTGAGTATGGTAGCATA 101
       |    ||    || ||| | || |||| |||||| || || |||   ||
  1321 ggcatagctctgaggaaaacgttagagattttgcggaatacggtgctatt 1370

   102 AATGCCGAGAACTGTAACGTGATTATGATATACGTCTACAATTACATTAA 151
       ||||| || || |||||  | ||||| || || |||||| ||||||| ||
  1371 aatgctgaaaattgtaatattattattatttatgtctaccattacatgaa 1420

   152 AAACACACGCAAATATGGCAATCAAGCGACTGCTTACGCTTTTATTGAGT 201
       ||| ||||| |||||||| ||||| |||||||| || ||||||||||| |
  1421 aaatacacgtaaatatgggaatcaggcgactgcctatgcttttattgaat 1470

   202 CTGGTGAAATAGCACAGAACATTCAATTGGTTTCGACTGCTTTAGCATAT 251
       | || |||||||| ||||| |||||||||  | | ||||| ||| | |||
  1471 caggagaaatagcccagaatattcaattgactgcaactgccttaacttat 1520

   252 GGTAGTGTTGATATTGGAGGTTATAGTAAGGAATATCTCCAAGAAAAGTT 301
       || ||| |||||||||| ||||||| ||||||||||||||||||   ||
  1521 ggaagtattgatattggtggttataataaggaatatctccaagaattatt 1570

   302 AGCTTTAGATGGATTAAACCAGCATGTCATTCACATGACATTAGTAGGAA 351
       || |||||||||  ||    ||||||| |||||||||||| | |||||||
  1571 agatttagatgggctaggagagcatgtgattcacatgacactcgtaggaa 1620

   352 ACAAGGAGTCGCAATGAGATACCAATTAAATAGTAATGTTCGTATTGTGC 401
         |||||||| |||||| ||| ||| | ||||||||||||||| | |||
  1621 ctaaggagtctcaatgaaatatcaacttaatagtaatgttcgtgtcgtga 1670

   402 AATTTGATGATACCTTTTGTTTTAGAAAAGGATTATGGGATTTTAATGAA 451
        |||| | ||||| |||||||||||||||||| | ||||| |||||||||
  1671 catttcaagatacattttgttttagaaaaggaatctgggactttaatgaa 1720

   452 GCTGTATTAGATTTGTCACAAGAACCACAAATGTTAAAAGAAGTTTACCA 501
       ||  |  |||||||| |||||||||| |||| |||||   |||||| ||
  1721 gccattctagatttgacacaagaacctcaaaatttaaagatagtttatca 1770

   502 GCAAATTGTTGC.TGCCTCCTAAAAGGGCTAGTGGTCGATACAGATGATT 550
         |||| ||| |      |  | || ||  |||   | ||||| ||   ||
  1771 agaaatagtttcacagttggttaagggagtagccattgatactgaaactt 1820

   551 ATGAAAAAGAGTTAGAAGCTGA 572
       |||||||    |||||| ||||
  1821 atgaaaatactttagaacctga 1842
```

VITA

Alvin C. Camus, Jr., was born to Alvin and Cecile Camus on June 1, 1959, in New Orleans, Louisiana. Al graduated from O. Perry Walker High School in New Orleans in 1977. Following high school he enrolled in the pre-veterinary curriculum at Louisiana State University, Baton Rouge, and was accepted into the professional program in 1980. Al received the degree of Doctor of Veterinary Medicine in 1984 and worked as a private practitioner in both mixed and small animal practices in Albany, Oregon and New Orleans for four years.

Al returned to graduate studies in aquatic pathology at the University of Rhode Island, Kingston, in 1988. In 1991, he accepted a graduate assistantship and residency position in the Department of Veterinary Pathology, Louisiana State University, School of Veterinary Medicine and attained the rank of Instructor in 1992. While working toward the doctoral degree in veterinary medical sciences, Al has served as pathologist for the Aquatic Animal Disease Diagnostic Laboratory at LSU. He is married to Dr. Melinda (McWilliams) Camus and has a 15 month old daughter, Caroline Elisabeth. At the August Commencement, 2001, he will receive the degree of Doctor of Philosophy.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Alvin C. Camus

Major Field: Veterinary Medical Sciences

Title of Dissertation: Pathobiology of Streptococcus iniae Infections in Cultured Tilapia

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

May 15,2001