Title
Understanding and Controlling the Widespread Piscine Pathogen Streptococcus iniae

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Publication Date
2021

Peer reviewed|Thesis/dissertation

Understanding and Controlling the Widespread Piscine Pathogen *Streptococcus iniae*

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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Committee in Charge

2021

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ACKNOWLEDGEMENTS

This dissertation is a product of the many sources of support, guidance, and love I've been so fortunate to have in my life. If I fully articulated the extent of my gratitude, this section would be a manuscript unto itself, but to all of my friends, family, and mentors, please know how much I appreciate you. I'd like to start by thanking my advisor Dr. Esteban Soto. I am extremely lucky to have found an amazing mentor and supportive lab family in the AAHL. You have taught me so much, pushed me forward out of self-doubt, and inspired me to reach for new academic endeavors. It has been a privilege to work with you. I would also like to thank the other members of my dissertation committee, Dr. Barbara Byrne and Dr. Matt Griffin. Your insights throughout my PhD have challenged me to consider new angles and have made my writing and research considerably stronger.

I also have several incredible mentors to thank from the journey leading up to this PhD program. To Dr. Melissa Marks, getting the opportunity to be part of the MEM lab was truly the best part of undergrad, and I would not have even applied to graduate school without your encouragement. You were an indispensable resource for scientific knowledge and excellent advice. To Team Monk Seal at NOAA, I am so happy to have learned from you and played my small part in protecting those charismatic lumps of blubber. To Dr. James Anderson and Dr. Amanda Shore, thank you for giving a high schooler who dreamed of studying shark microbes the chance to make it a reality. And thank you to Dr. Lisa Privitera/Dr. P/Auntie Lisa, who encouraged me to pursue such research, beyond the limits of the classroom setting. Thank you also to Manning Taite and Dr. David Krupp, for guiding a me through the fundamentals of scientific study.

To my first teachers, my family. I am grateful for the talented, tenacious women in my life who accomplished so much and ensured I never saw any limits to my own future. Thank you to my grandmothers Jean Compton and Jean Heckman, with whom I share my middle name and love for words and reading. To my mother Terry Heckman, selfless, smart, and determined. We share the Compton nose and our laugh, but I hope I can emulate you in many more ways. To my father, Mark Heckman, watching you I learned to appreciate and question the complexities of life, to approach it with all the serious thoughtfulness and joyous wonder it inspires. I owe my early love of science and the ocean to you both, and immeasurably more. To my siblings, Robert and Skye, it is an absolute honor to be your older sister, and you are continuous sources of inspiration (and memes) as we grow together.

To my friends, to those who have been there since before I could form cognizant thoughts, and those I have met in the years since. I have cherished the cake, commiserations, hikes, beach days, puppy play dates and other adventures. A massive and heartfelt thank you, to all of you, for everything. And finally, to my partner, Joshua Bumgarner. We took a chance and ended up navigating graduate school together, stronger and happier than we ever would have been apart. It is difficult to concisely articulate all the ways you have influenced my progress as a scientist and person, but you have been an unwavering source of support, laughter, intellectual inspiration, and incredible food. Spending extra time with you and our dog Willow was the silver lining of this pandemic, and I cannot wait to marry you.

PREFACE

The chapters comprising this dissertation are collective works in various stages of the publication process. All chapters have been modified from published form to fulfill formatting requirements.

Chapter 1 is being adapted for a literature review under the title "Recent advances in understanding and controlling *Streptococcus iniae*" with coauthor Dr. Esteban Soto.

Chapter 2 is a full reprint of the publication: Heckman, T. I., Griffin, M. J., Camus, A. C., LaFrentz, B. R., Morick, D., Smirnov, R., Ofek, T., & Soto, E. (2020). Multilocus sequence analysis of diverse *Streptococcus iniae* isolates indicates an underlying genetic basis for phenotypic heterogeneity. *Diseases of Aquatic Organisms*, *141*, 53–69. https://doi.org/10.3354/dao03521, with permission from the journal and coauthors.

Chapter 3 is a full reprint of the publication: Heckman, T. I. & Soto, E. (2021). *Streptococcus iniae* biofilm formation enhances environmental persistence and resistance to antimicrobials and disinfectants. *Aquaculture*, 540: 736739. https://doi.org/10.1016/j.aquaculture.2021.736739, published under a Creative Commons CC-BY license with coauthor Dr. Esteban Soto.

Chapter 4 is a modified version of a manuscript submitted for publication: Heckman, T.I, Shahin, K., Henderson, E.E., Griffin, M.J., & Soto, E. Development and efficacy of *Streptococcus iniae* live-attenuated vaccines in Nile tilapia, *Oreochromis niloticus*, with permission from all coauthors.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance

BHI(A): brain heart infusion (agar)

CAMHB: cation-adjusted Mueller-Hinton broth with lysed horse blood

CFU: colony forming unit

CPS: capsular polysaccharides

CNS: central nervous system

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

FW: freshwater

gDNA: genomic deoxyribonucleic acid

GMO: genetically modified organism

H&E: hematoxylin and eosin

HSWB: high-salt wash buffer

IC (IP): intra-coelomic (intra-peritoneal)

IgM/G: immunoglobulin M/G

LAV: live-attenuated vaccine

LDH: lactate dehydrogenase

LSWB: low-salt wash buffer

MBC: minimum bactericidal concentration

MBEC: minimum biofilm eradication concentration

MIC: minimum inhibitory concentration

MEM: minimal essential media

mL: milliliter

µL: microliter

MLSA/T: multilocus sequence analysis/typing

MOI: multiplicity of infection

MW: marine water

OD: Optical density

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFGE: pulsed field gel electrophoresis

Rep-PCR: repetitive element palindromic polymerase chain reaction

RIF: rifampin

RPS: relative percent survival

rRNA: ribosomal ribonucleic acid

RT: room temperature

SBA: sheep's blood (5%) agar

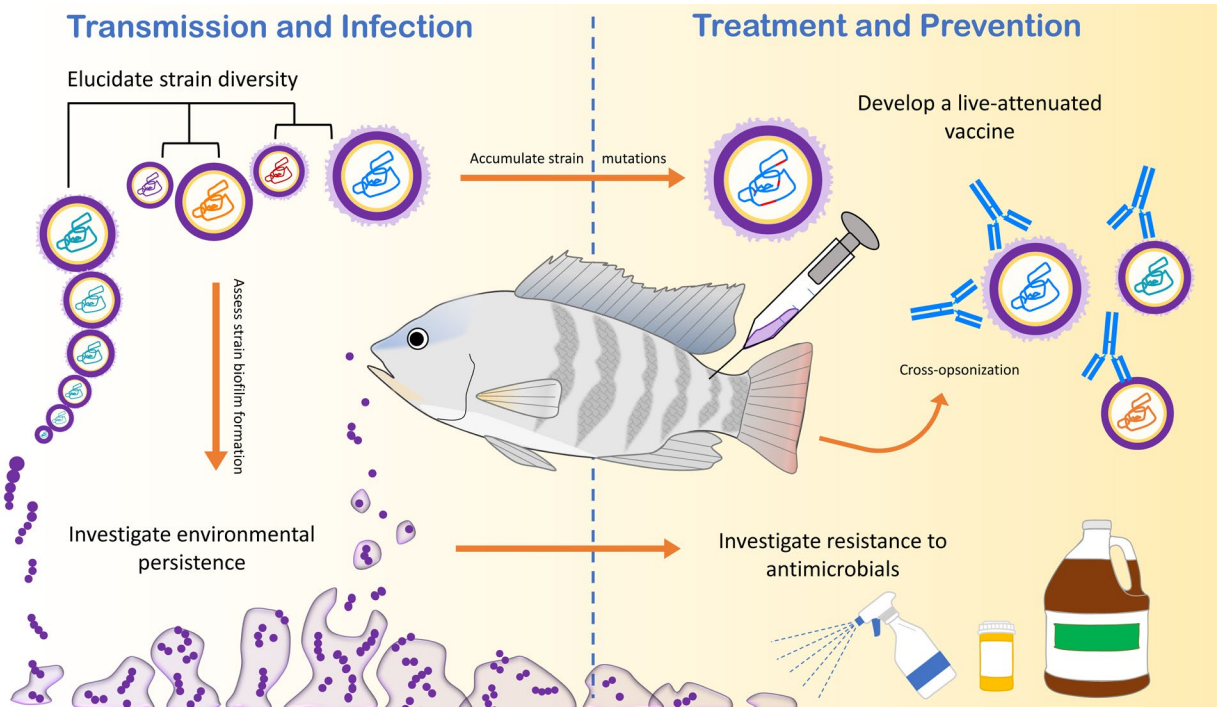
SNP: single nucleotide polymorphism

TmBs: *Oreochromis mossambicus* bulbus arteriosus cell line

US(A): United States (of America)

WT: Wild-type

ABSTRACT



Streptococcus iniae is a gram-positive bacterium and one of the primary etiologic agents of piscine streptococcosis, a pervasive disease that costs the global aquaculture industry billions of dollars in annual losses. Treatment by antimicrobial administration and prevention by vaccination have had limited success in controlling *S. iniae.* The primary obstacle for developing effective and sustainable control strategies is a lack of understanding regarding the genetic and antigenic diversity of *S. iniae* in relation to its pathogenesis. As a re-emerging pathogen, our understanding of the geographic and host range of *S. iniae* is still expanding. Since the first isolation of *S. iniae* from skin lesions of a captive Amazon river dolphin (*Inia geoffrensis*) in 1976, it has been reported from almost 100 wild, farmed, and ornamental fish species inhabiting fresh, euryhaline and marine environments across every continent except Antarctica. Additionally, *S. iniae* is an opportunistic zoonotic pathogen, capable of causing systemic disease in humans and other mammals. The work presented in this dissertation aims to elucidate the

genetics, ecology, and infection dynamics of strains from varied isolation sources with translatable results for aquaculture. A multilocus sequence analysis (MLSA) scheme was developed to understand the genetic relationships between diverse isolates and uncover shared phenotypic and virulence characteristics. The MLSA phylogenies were comparable to established genotyping methods and placed the *S. iniae* isolates into 5 major clades relating to phenotype and host species. Characterized strains from different genetic backgrounds were assessed for their ability to form biofilms, and for the role of biofilms in environmental persistence and resistance to treatment and disinfection. All strains formed biofilms within 72 hours using the minimum biofilm eradication concentration (MBEC) assay® system, and biofilms drastically increased the aquatic persistence of *S. iniae* and resistance to antimicrobials commonly used in aquaculture. Finally, live-attenuated vaccine candidates were developed from representative strains from North American clades by rifampin passaging. Candidate strains were attenuated in virulence by *in vitro* and *in vivo* assays and elicited a protective immune response in tilapia following intra-coelomic immunization.

CHAPTER 1. An introduction to *Streptococcus iniae*

1. Identification and diversity

1.1 *Streptococcus iniae*

Streptococcus iniae is a gram-positive, chain-forming coccoid bacterium belonging to the order Lactobacialles. It is a non-Lancefield, β-hemolytic streptococci, although the extent of hemolysis may vary with oxygen availability and blood type [1,2]. *Streptococcus iniae* is facultatively anaerobic, with hemolytic activity most consistently observed under anaerobic conditions on 5% sheep's blood agar (SBA) [3,4]. On SBA, *S. iniae* forms slightly translucent to white, round, and umbonate colonies, generally surrounded with a zone of β-hemolysis and a diffuse outer ring of α-hemolysis (Figure 1.1) [1,2]. Colony size may range from 1-3 mm depending on strain, with some strains reported to be more mucoid [5]. The bacterium is catalase and oxidase negative, and chains of up to 10 cocci can be observed by Gram staining and light microscopy [1,6,7]. Many strains produce some degree of polysaccharide capsule detectable by electron microscopy, the extent of which impacts morphology, cell surface charge, chain length, buoyancy, and serotype [8–12]. While two distinct serotypes were originally identified based on capsulation and arginine dihydrolase and ribose reactions [8,13], there is no established serotyping system for *S. iniae,* and the number of serologically distinct strains is unclear. The genome of *S. iniae* is ~2 million bases, with an average G+C content around 36.7% [14–16].

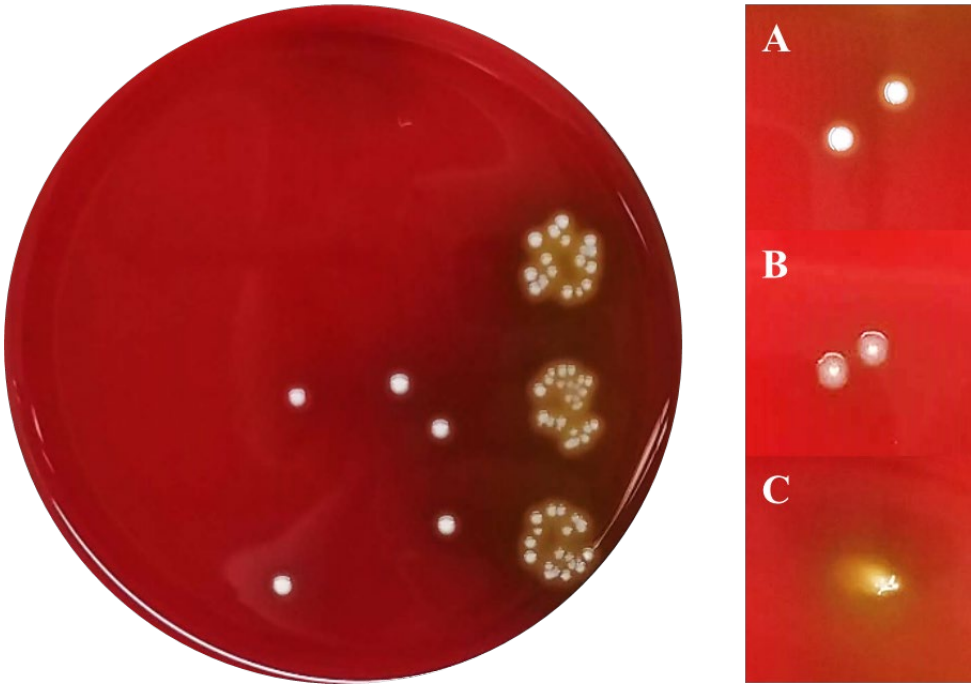


Figure 1.1: Morphology and hemolysis of *Streptococcus iniae* isolates spot plated on 5% sheep's blood agar. (A) Classical presentation of round white colonies with a tight ring of β-hemolysis and more diffuse α-hemolysis. (B) Alternative translucent colony morphology with reduced hemolysis under aerobic conditions. (C) Agar stab demonstrating increased hemolysis under more anaerobic conditions.

1.2 Diagnosis and typing methods

Streptococcus iniae can be readily isolated from infected tissues, particularly the brain, kidney and spleen, and grown on SBA, trypticase soy agar (TSA), brain heart infusion agar (BHIA) or selective agars at 25-37°C [2,6,17,18]. An agar stab in SBA facilitates confirmation of β-hemolysis (Fig. 1.1C) [3]. Biochemical profiles can be compared using API® 20 Strep, API® Rapid ID 32 Strep and Vitek® 2 systems (bioMérieux, France), but identification of suspected *Streptococcus* spp. beyond genus level by these methods is not recommended. Biochemical tests preclude precise identification and classification of streptococcal isolates due to differences in strain metabolism and growth rates, and pathogen databases can be incomplete or incorrect [19–23].

To definitively identify *S. iniae* versus the other agents of piscine streptococcosis, molecular methods are required. Sequencing of the 16S rRNA gene is a standard method for diagnosis of bacterial pathogens and is generally applicable for *S. iniae* [17,24]. Species-specific primers for the 16S rRNA (Sin-1/Sin-2) [25], 16S–23S rDNA intergenic spacer (ITS; SP-1/SP-2) [26] and lactate oxidase (*lctO*; Lox-1/Lox-2) [27] genes are also available. Loop-mediated isothermal amplification (LAMP) assays targeting *lctO* [28] and phosphoglucomutase (*pgmA*) [29] genes have enhanced sensitivity to conventional PCR methods and can be used for rapid and specific diagnosis. Recently developed quantitative PCR (qPCR) methods are similarly sensitive and can be useful for diagnosis of subclinical infections by amplification of DNA from fish tissues [30].

These single gene systems, however, cannot provide the resolution needed for intraspecies strain comparisons. Pulsed field gel electrophoresis (PFGE) [31], repetitive sequence mediated PCR (rep-PCR) [32] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [33,34] methodologies have varying degrees of discriminatory power to identify and type *S. iniae*. Pulsed field gel electrophoresis was essential in early studies of strain diversity, revealing greater homogeneity among virulent isolates from fish and humans, suggesting chromosomally encoded virulence factors [31,35]. Later work using the same method determined more than one genetic profile associated with invasive disease [19,36]. Multiple genotypes have been found within and between fish farms, and in some cases rep-PCR or PFGE have shown connections between genotype and geography, host type, virulence, or phenotype [32,35,36]. This supports the need for further phylogenetic studies on the more intricate relationships between genetic variability and virulence. More comprehensive sequencing-based techniques such as multilocus sequence typing or analysis (MLST/A) alone, or in combination with whole genome analysis, can provide a greater depth of information and are more replicable

and more easily disseminated than rep-PCR and PFGE [20,37,38]. As new genomes become publicly available, these approaches will become increasingly valuable for determining the genetic and phenotypic diversity of *S. iniae.*

2. Host species and geographic range

Streptococcus iniae has few restrictions in its geographic and teleost host range. Outbreaks have been reported on almost every continent – Asia, the Americas, Australia/Oceania, Europe and Africa [1,18,20]. They have occurred in fresh, brackish and marine water environments at warm, temperate and cool temperatures. Host species include fish of high economic importance for aquaculture such as tilapia (*Oreochromis* spp.) [6], sturgeon (*Acipenser* spp.) [39] and rainbow trout (*Oncorhynchus mykiss*) [40], as well as ornamental varieties [41] and wild fish [25,42,43]. The bacterium is also capable of infecting mammalian species, where it sporadically causes disease [44]. By 2007, Agnew and Barnes reported *S. iniae* from 27 fish species in addition to dolphins and humans [1]. A comprehensive literature search revealed an additional 58 fish and 6 non-fish species susceptible to infection by *S. iniae*, expanding its reach to nearly 100 different hosts (Table 1.1).

Streptococcus iniae has also been isolated from asymptomatic animals, or those where the disease status was not reported. These include shrimp (*Penaeus indicus* and *Neomysis awatschensis*) as well as additional species of wild and farmed fish [45–47]. The ability of *S. iniae* to colonize carrier animals that may interact with more susceptible species is an important factor for transmission. There have been a few species that have demonstrated higher resistance to streptococcal infection. Common carp (*Cyprinus carpio*) were not susceptible to infection when reared with infected tilapia [48], and to date, *S. iniae* has only been isolated from apparently healthy carp [47]. Channel catfish (*Ictalurus punctatus*) were initially considered

similarly resistant [49,50], but outbreaks of high mortality were later reported in China [51,52]. In an outbreak in wild reef fish, zooplanktivores were notably underrepresented in the collected carcasses, and most mortalities were in large adult carnivores and benthic feeders [42]. Juvenile fish are somewhat less susceptible to infection, and the consumption of infected prey may explain the higher representation of omnivores [1,42]. In the inaugural descriptions of *S. iniae,* mice, guinea pigs and rabbits were found to be resistant to infection [2,53], but this may be strain dependent and a murine model of subcutaneous infection has been established [35]. While humans and a few other mammals have been infected naturally, these instances are infrequent, and *S. iniae* can be considered opportunistically zoonotic to a susceptible demographic. Cases of human disease are primarily in older (> 60 yrs) individuals with one or more underlying conditions and a history of preparing fresh fish [3,5,19,31,54,55].

Table 1.1: Species reported to be susceptible to *Streptococcus iniae* infection in addition to the 29 first reported by Agnew and Barnes (2007).

Common name	Scientific name	Location first reported	References
Amazon catfish	*Leiarius marmoratus x Pseudoplatystoma corruscans*	Brazil	[56]
Angelfish	*Pomacanthus* sp.	Australia	[57]
Black saddled grouper	*Epinephelus bleekeri*	China	[58]
Blacktip grouper	*Epinephelus fasciatus*	Israel	[59]
Borneo mullet*	*Liza macrolepis*	Bahrain	[60]
Cardinalfish	*Cheilodipterus* sp.	Israel	[42]
Channel catfish	*Ictalurus punctatus*	China	[61]
Chinese sturgeon	*Acipenser sinensis*	China	[62]
Clown loach	*Chromobotia macracanthus*	USA	[44]
Cobia	*Rachycentron canadum*	Taiwan	[63]
Common mackerel	*Scomber scombrus*	Japan	[64]
Crimson snapper	*Lutjanus erythropterus*	China	[58]

Damselfish	*Pomacentridae* sp.	Australia	[57]
Eeltail catfish	*Plotosus japonicus*	Israel	[42]
Filefish	*Stephanolepis* sp.	Israel	[36]
Flat bream	*Rhabdosargus sarba*	China	[58]
Flying fox[a]	*Epalzeorhynchos kalopterus*	Australia	[44]
Golden pompano	*Trachinotus ovatus*	China	[58,65,66]
Gold-saddle goatfish	*Parupeneus cyclostomus*	Israel	[42]
Guppy*	*Poecilia reticulata*	Iran	[67]
Hussar	*Lutjanus adetti*	Australia	[57]
Hybrid sturgeon	*Huso dauricus x Acipenser schrencki*	China	[68]
Jade perch	*Scortum barcoo*	Australia	[44]
Kelp grouper	*Epinephelus bruneus*	China	[58]
Korean rockfish	*Sebastes schlegeli*	Korea	[69]
Linear blenny	*Ecsenius lineatus*	Israel	[42]
Lionfish	*Pterois volitans*	Australia	[57]
Longfin yellowtail	*Seriola rivoliana*	USA	[70]
Northern red snapper	*Lutjanus campechanus*	St. Kitts and Nevis	[71]
Orange-spotted grouper	*Epinephelus coioides*	Vietnam	[72,73]
Parrotfish	*Scarus* sp.	Israel	[42]
Princess parrotfish	*Scarus taeniopterus*	St Kitts and Nevis	[71]
Rainbow shark	*Epalzeorhynchos erythrurus*	USA	[41]
Randall's threadfin bream	*Nemipterus randalli*	Israel	[74]
Red hind	*Epinephelus guttatus*	St. Kitts and Nevis	[71]
Red porgy	*Pagrus pagrus*	Spain	[75]
Red sea bream	*Pargus major*	Japan	[76]
Red-tail black shark	*Epalzeorhynchos bicolor*	USA	[35]
Rosy barb*	*Barbus conchonius*	USA	[77]
Russian sturgeon	*Acipenser gueldenstaedtii*	Uruguay	[78]
Sand bass	*Psammoperca waigiensis*	Australia	[57]
Siberian sturgeon	*Acipenser baerii*	China	[79]
Silver perch	*Bidyanus bidyanus*	Australia	[80]
Silver shark*	*Balantiocheilos melanopterus*	Iran	[81]

Snubnose pompano	*Trachinotus blochii*	Vietnam	[73]
Spotted rose snapper	*Lutjanus guttatus*	Central America	[82]
Spotted silver scat	*Scatophagus argus*	China	[58]
Striped snakehead	*Channa striata*	Thailand	[44]
Stripey snapper	*Lutjanus carponotatus*	Australia	[57]
Thread-sail filefish	*Stephanolepis cirrhifer*	Japan	[64]
Threeband sweetlips	*Plectorhynchus cinctus*	China	[58]
Tiger oscar	*Astronotus ocellatus*	India	[83]
Turbot	*Scophthalmus maximus*	China	[84,85]
White sturgeon	*Acipenser transmontanus*	USA	[39]
Yellow catfish	*Pelteobagrus fulvidraco*	China	[86,87]
Yellow sea bream	*Acanthopagrus latus*	China	[58]
Yellowtail grunter	*Amniataba caudavittata*	Australia	[57]
Zebrafish*	*Danio rerio*	USA	[88]
American bullfrog	*Rana castesbeiana*	USA	[89]
Bottlenose dolphin	*Tursiops truncatus*	China	[90]
Flatback sea turtle	*Natator depressus*	Australia	[57]
House mouse*	*Mus musculus*	Canada	[91]
Pygmy hippopotamus	*Choeropsis liberiensis*	USA	[82]
White leg shrimp*	*Litopenaeus vannamei*	China	[92]

[a] A case of mistaken identity based on shared common name previously attributed infection to the flying fox bat species (*Pteropus alecto*) [1,44]

* indicates experimental infection without published report of natural outbreaks.

3. Epizootiology

3.1 Environmental parameters

Environmental conditions have substantial impact on the growth, persistence, transmission, and infectivity of *S. iniae. Streptococcus iniae* grows best at temperatures between 25-35°C [2,6,17,18], but some strains can proliferate between 10-45°C [6,7,49]. Optimal growth occurs at neutral pH and 0% salinity, but pH 5.5-8.5 and salinity of 30ppt (equivalent to marine water) are within the suitable ranges for viability and replication [6,49]. There is limited information on the

extra-host persistence of *S. iniae.* Detection of the bacteria in the water and sediments has relied on bacterial culture, which has low sensitivity and may miss bacteria present in low concentrations. Still, the year-round presence of *Streptococcus* spp. in the environment near fish farms has been reported [93,94], and moderate concentrations (10^4-10^5 CFU/mL) of *S. iniae* have been isolated from tanks and adjacent water systems amidst outbreaks [95,96]. In an early study, Perera et al. [49] found the bacterium persisted longer in cold and saline conditions, but viable cocci were only recovered up to 9 days after inoculation in saline water at 5°C. At 25°C, *S. iniae* levels dropped below their level of detection within 24 hours. The ubiquitous and recurrent nature of streptococcal infections despite this limited persistence suggests alternative factors, such as biofilms or carrier fish, contribute to the debated reservoir of *S. iniae.* There is no previous information on the role of biofilms in persistence, but the potential contributions of carrier fish in *S. iniae* transmission is discussed further in section 3.2.

The infectivity of *S. iniae* and susceptibility of its hosts are greatly influenced by the aquatic environment. Environmental stressors elevate the probability and severity of streptococcosis. Warm and alkaline (pH > 8) water conditions, low dissolved oxygen concentrations and high ammonia or unbalanced toxin levels all enhance disease. High stocking densities and the presence of parasites similarly contribute to unfavorable conditions and increased risk of infection [1,18,49,97,98]. Changes in temperature may also initiate outbreaks. Transfer of fish to higher or lower temperatures, especially without adequate acclimation, can lead to immunosuppression and decreased resistance to *S. iniae* [99]. Both the rate and extent of temperature change influence its effect on infection. Two recent mass mortality events in wild reef fish were attributed to extreme temperature changes, and increased mortalities in farmed fish

have also been associated with abrupt rises in temperature [42,43]. It has been suggested that these mortality events might intensify with global climate change [42].

3.2 Transmission

Fish have been experimentally infected by multiple routes – oral (including intragastric gavage and coated pellets), immersion, cohabitation, intraceolomic or intramuscular injection – and exposure route impacts the course of the disease [1,95]. Challenge by injection bypasses the biological defenses of the mucosal compartments, generally resulting in higher mortality rates than natural infections [18,49]. Comparatively, cohabitation and immersion challenges have shown inconsistent results. Immersion typically requires high concentrations of bacteria and/or abrasions and a compromised state [7,18,95,100]. Oral exposure results in a slight lag in onset and a more subacute form of disease than injection or immersion challenge [95].

Ingestion of infected material is thought to be the most likely mode of transmission in natural outbreaks. Cannibalism of the eyes and viscera of dead and dying fish by cohorts is frequently observed, subsequently infecting the cannibalizing fish, and amplifying the outbreak [95,100]. Similar consumption of pathogen-laden carcasses by uninfected reef fish is thought to prolong mortality events in wild species [42]. *Streptococcus iniae* has additionally been found in "trash fish", undesirable bycatch that may be used as fish meal. Contaminated food is a potential source of infection for farms that prefer this cheap alternative to commercial pellets [46]. Ingestion of carrier fish or bacteria-laden feces has also been suggested, as *S. iniae* has been isolated from the skin and internal organs of apparently healthy farmed and wild fish [7,74,101]. The brain and intestine are potential reservoirs for the bacteria in carrier fish, the later providing an avenue for release of bacteria into the water [1,7,95]. Subacute infection of a susceptible subset of a population by this oral-fecal route could result in elevated bacterial concentrations in the water,

sufficient to exacerbate disease outbreaks through exposure by the skin, gills, or nares [18,95,96].

4. Disease and Pathogenesis

4.1 Presentation of disease

Acute outbreaks of piscine streptococcosis caused by *S. iniae* are associated with heavy economic losses and high mortality rates in cultured fish. Although difficult to quantify, outbreaks in wild fish also result in economic losses stemming from disruption of important ecosystems and ecotourism [42]. Mortality rates in cultured fish commonly fall between 30-50%, but may be as high as 75% [6,48,95,102,103]. Clinical signs of affected fish are generally nonspecific [18]. Afflicted fish may demonstrate behavioral changes such as lethargy, disoriented or erratic swimming, and anorexia. These are accompanied by observations of melanosis, dorsal rigidity, and uni- or bi-lateral exophthalmia, often with corneal opacity (Figure 1.2A). Coelomic distention, congestion of the skin, and other surface lesions may also be observed [1,17]. There are often no outwardly visible clinical signs preceding mortality in the septic form of the disease [95]. Internally, gross lesions may include darkened and enlarged spleens, pale livers, and bloody ascites (Figure 1.2B) [17].

Histopathological findings in piscine streptococcosis largely involve the central nervous system (CNS). Meningitis, accompanied by ophthalmitis, and inflammation of regions of the heart are most commonly reported, but the spleen, muscle, serous membranes, kidneys and diverse other organs can also be affected [18,96,104,105]. Inflammation may be granulomatous and/or necrotic, with observations of intra- and extracellular gram-positive cocci distributed throughout the lesions (Figure 1.2C) [18,104–106]. Severity and extent of the lesions is generally

reflected in the mortality patterns displayed in an outbreak – slow chronic, daily losses to sub-acute or acute episodes of heavy loss [1,7,95].

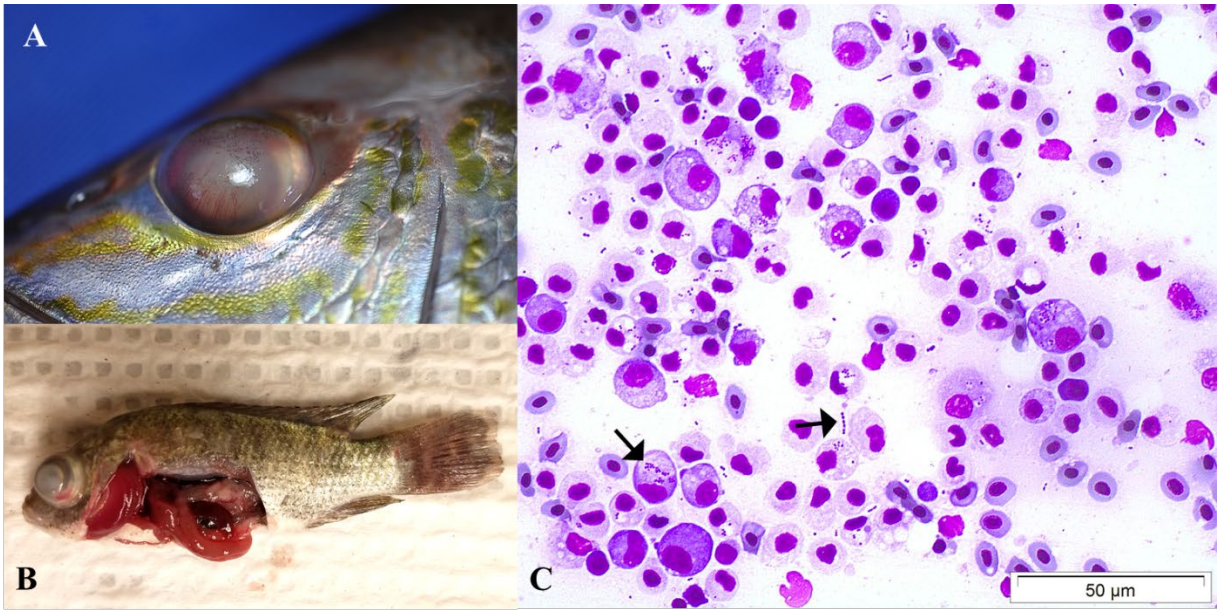


Figure 1.2: Gross and histopathological changes in clinically affected fish associated with *Streptococcus iniae* infection. (A) Exophthalmia with corneal opacity in a rose spotted snapper (*Lutjanus guttatus)* and (B) a Nile tilapia (*Oreochromis niloticus*) also exhibiting splenomegaly. (C) Intracellular and extracellular chains of cocci (arrows) visible in a heart-imprint smear with Giemsa staining, scale bar is 50µm.

4.2 Pathological mechanisms

These different presentations of streptococcosis are outcomes of multistep pathogenesis pathways. The basic pathway proposed in the early 2000s [1] of colonization followed by dissemination in the bloodstream and invasion of the CNS can be further elucidated with details on strain-specific interactions with the relevant host cells. *Streptococcus iniae* is likely transmitted to fish through the gut, skin, gills or nares [18]. Adherence to the epithelial cells lining these mucosal surfaces is an essential step for establishing host-pathogen interaction. Both virulent and avirulent strains are proficient at adhering to epithelial cells, with so called "commensal" strains often demonstrating higher efficiency [91,107,108]. This may be a result of

the reduced capsulation observed in less virulent strains, as capsulation can conceal surface proteins involved in adhesion [12,91]. Internalization of *S. iniae* is facilitated by pseudopodia, involving actin mobilization of the host cytoskeleton and cell surface receptors of the bacteria [91,107,109]. Bacteria first exist intracellularly in pseudopodia-derived membrane bound vacuoles, which may degrade and release the microbes directly into the cytoplasm [107]. There may be a strain-dependent period of limited intracellular replication, but transcytosis proceeds swiftly and without damage to the epithelial cells or structure [107]. This lack of damage can be advantageous for the bacteria, as the early innate immune response to *S. iniae* has a significant impact on disease progression [108,110]. By avoiding activation of cellular alarms, the bacteria prolong their opportunity to establish infection.

The ability to balance both evasion of the immune response with exploitation may be a key driver to the observed diversity of disease presentation among different strains. Certain isolates of *S. iniae* have been found to trigger enhanced inflammatory responses, either through reducing apoptosis of nonspecific cytotoxic cells (NCC) with simultaneous increases in necrosis [111], or through production of extracellular polysaccharide (EPS) – a potent inducer of the inflammatory cascade akin to gram-negative lipopolysaccharide [112]. Strong induction of the inflammatory response may be responsible for the septic shock-like presentation of the disease observed in some outbreaks [95,112]. Most strains appear more adept at modulating or resisting host immune defenses, and resistance to phagocytic killing is a primary strategy for invasive bacteria. Initial evasion of neutrophils and macrophages is largely dependent on the presence of the polysaccharide capsule (CPS) [113]. Similarly to other streptococcal pathogens, CPS is the primary virulence factor for *S. iniae* and is thought to limit phagocytosis by masking cell surface components and preventing opsonization [10,91]. Capsulated strains show significantly higher

survival in blood from relevant hosts, allowing establishment of bacteremia and their rapid dissemination in the vasculature [12,110,114]. A number of other virulence factors may contribute to preventing phagocytic uptake, including surface-associated M-like protein of *S. iniae* SiM [115,116], sortase A [117], nuclease SpnAi [118,119] and interleukin-8 protease [120], highlighting the importance of this step (Table 1.2).

Even the subset of bacteria that are phagocytosed during bacteremia have methods for surviving the killing mechanisms of macrophages or neutrophils, allowing persistence and proliferation within the phagocytes [108]. This intracellular survival is not dependent on the capsule [11,110], but is instead associated with other virulence factors such as phosphoglucomutase (PgmA) [114], nucleotidase S5nAi [118,119] and CpsY regulation of polysaccharide aceytlases (OatA), deactylases (PgdA and Pdi) [121], and putative autolysins [122,123]. Strain-dependent resistance has been reported [108,113], but further elucidation of the mechanisms for survival in both neutrophils and macrophages is warranted. Indeed, intracellular survival appears to be a significant part of pathogenesis for this microbe that was initially considered exclusively extracellular. *Streptococcus iniae* has been observed to survive and proliferate in multiple cell types *in vitro* and *in vivo,* including neutrophils, mammalian and teleost macrophages, fibroblasts, and epithelial and endothelial cells from skin, brain, liver, and other tissues [35,124–127].

Residence in macrophages has been suggested as a mechanism for systematic spread, as bacteria migrating within macrophages can be released in dispersed tissues through induction of apoptosis [113]. Bacteria can pass the blood brain barrier through this “trojan horse” mechanism, or as free bacteria, by cytotoxic effect on brain endothelial cells [91,113]. Both free and macrophage associated cells have been observed with brain tissue cells [125]. Within the brain,

S. iniae can cause damage to host cells by expression of toxins like streptolysin S (SLS) [128,129]. This establishes the most common disease presentation of meningoencephalitis. Strains with reduced virulence causing atypical infection appear deficient in their ability to avoid phagocytosis or intracellular killing, limiting their dissemination [12,110,114]. However, they are not just avirulent commensals as once suggested, and may cause more localized disease – such as the original skin lesions observed in freshwater dolphins, bone lesions in previously vaccinated barramundi, or co-infections with other microbes [2,39,130]. The impact of other established (Table 1.2) and putative [11,85,131] virulence factors on pathogenesis must be further resolved for a more complete understanding of strain-dependent disease variation. Connecting strain phylogenetics with virulence factor expression and clinical presentations will resolve the pathological mechanisms of *S. iniae* for improving outbreak response and control strategies.

Table 1.2: Established virulence factors of *S. iniae* and their proposed roles in pathogenesis.

Virulence factor	Role	Description	References
Streptolysin S (SLS)	Host cell damage	A surface associated toxin encoded by 9 genes in the *sag* operon, responsible for erythrocyte hemolysis and host epithelial cell damage. SLS was required for necrosis but not bacteremia or resistance to phagocytosis in a murine model. Despite its cytotoxic properties, variable effects of SLS on virulence have been observed in mice and fish.	[108,128,129]
Exopolysaccharide (EPS)	Inflammation	A loosely attached to secreted polysaccharide complex distinct in composition from CPS. EPS triggers intense inflammatory cascade akin to that of septic shock in mammals.	[112,132]

α-Enolase	Adherence & invasion	A plasmin/plasminogen binding enzyme facilitating tissue invasion.	[133]
Polysaccharide deacetylase (Pdi)	Immune evasion Adherence & invasion	A paralogous and nonredundant protein to putative virulence factor PgdA, Pdi deacetylation of cell wall residues promotes resistance to lysozyme killing and adherence and invasion of epithelial cells. Δ*pdi* mutants were attenuated in whole blood survival and host cell binding.	[134]
M-like protein (SiM)	Immune evasion Adherence & Invasion	Binds immunoglobulin by the Fc region and contributes to macrophage resistance and adherence to epithelial cells through fibrinogen binding. SiM expression is regulated by Mgx which responds to iron availability and NaCL levels. SiM expression has a significant contribution to virulence.	[115,135–137]
Capsule (CPS)	Immune evasion Adherence & Invasion	An outermost layer of polysaccharides encoded by a 21-kb operon. CPS reduces opsonophagocytosis and is essential for systematic disease. Isogenic mutants and isolates with naturally reduced CPS are significantly attenuated in virulence, but generally more efficient at binding host cells.	[10,11,110,138]
Phosphoglucomutase (PgmA)	Immune evasion	PgmA interconverts G6P and G1P and is involved in capsular biosynthesis, cell wall structure, and general metabolism. Insertional mutants were attenuated in hybrid striped bass and more sensitive to AMPs and whole blood killing	[139]
Sortase A (SrtA)	Immune evasion	A cell wall anchoring enzyme impacting surface protein positioning. Isogenic mutants were attenuated in tilapia and whole blood survival.	[117]
C5a peptidase	Immune evasion	Prevents neutrophil recruitment by cleaving complement factor C5a.	[137]

		Role appears minor in *S. iniae* virulence.	
Interleukin-8 protease	Immune evasion	Cell envelope protease that cleaves chemokine IL-8, increasing resistance to neutrophil phagocytosis	[120]
Bacteriocin Sil	Immune evasion	A secreted bacteriocin with activity against *B. subtilis* and an additional role in virulence. Sil interacts directly with monocytes to inhibit respiratory burst and acid phosphatase activity.	[140]
Streptococcal 5'-nucleotidase A *iniae* (S5nAi)	Immune evasion	A secreted nucleotidase that hydrolyzes AMP and ADP to produce immunomodulatory adenosine. Adenosine decreases phagocytic activity by suppressing generation of nitric oxide, superoxide, proinflammatory cytokines and inhibits neutrophil degranulation.	[118,119]
Streptococcus pyogenes nuclease A *iniae* (SpnAi)	Immune evasion	An extracellular nuclease that degrades neutrophil extracellular traps (NETs).	[118,119]
Glutamate racemase (MurI)	Immune evasion Metabolism	A cofactor-independent enzyme that catalyzes the interconversion of *L*-glutamate and *D*-glutamate and is crucial for peptidoglycan synthesis and cell wall integrity maintenance. MurI-deficient mutants were attenuated in sturgeon whole blood survival.	[141]
Phosphotransferase system (PTS)	Metabolism	Involved in uptake of carbohydrates by sequential phosphoryl group transfer. Strains without a fructose specific IIABC component sequence or transposon insertion in a PTS sequence homologous to *S. agalactiae* IIC component were attenuated.	[11,142]
ABC metal transport system (MtsABC)	Metabolism	An ABC transporter system involved in heme utilization as a source of essential iron in physiological settings. Likely regulated by MtsR in response to iron availability.	[143]
ABC metal transport system (FtsABCD)	Metabolism	An ABC transport system responsible for heme utilization in *S. iniae.*	[144]

CpsY	Metabolism Regulation	A transcriptional regulator involved in metabolism and defense against neutrophil-mediated killing Regulates stabilization of the cell wall by proposed activation of peptidoglycan acetylase OatA, and repression of cellular autolysin MurA.	[123]
SivS/R	Regulation	A two-component system responsible for regulating virulence factors SLS and CPS, and potentially additional putative virulence factors.	[145,146]
Multigene regulator protein Mgx	Regulation	Mgx is homologous to GAS Mga and regulates SiM and potentially other virulence factors. Responds to environmental cues such as iron and salt levels.	[115,135,137]
Metal-dependent transcriptional regulator MtsR	Regulation	A transcriptional regulator homologous to the DxtR family. Position and co-expression data suggest it represses *mtsABC*	[147]

5. Prevention and Control

5.1 Antimicrobials

Control of existing outbreaks relies mainly on antimicrobial treatment to reduce mortality. However, several practical and biological factors limit the effect of this approach. In most countries, the use of antimicrobials in aquaculture is tightly regulated and there are few efficacious options available [1,148]. In the United States, for example, the only approved antibiotic for controlling mortality from *S. iniae* is florfenicol (AQUAFLOR®) [18]. Even with regulation, there is evidence of increasing antimicrobial resistance in *S. iniae,* lending to concerns for human and animal health [20,41,56]. Medicated feed may also be ineffective due to disease-induced anorexia and the ability of the bacteria to survive intracellularly, necessitating multiple costly treatments and favoring development of a carrier state in subclinically infected

fish [1,148]. Recurrent infections following antimicrobial treatment and disinfection are usually the result of failure to eradicate the pathogen from the population or environment [41,130,149]. Complete eradication of bacterial pathogens, especially those that form biofilms, is logistically challenging in flow-through or net-cage systems, and even closed recirculation systems are difficult to completely disinfect [130]. Prevention of outbreaks through alternate methods is therefore advantageous.

5.2 Vaccines

In salmonids, vaccination programs enacted against bacterial diseases such as yersiniosis and furunculosis have largely been effective [150,151], resulting in dramatic and permanent reductions in mortalities, antibiotic usage, and associated treatment and mitigation costs [152,153]. In contrast to these success stories, vaccination efforts against piscine streptococcosis have seen mixed results. The traditional formalin-killed whole-cell preparations (bacterins) most frequently employed in aquaculture can provide high levels of protection against homologous strains [9,130]. However, the significant circulating strain diversity coupled with the genetic and immunogenic plasticity of *S. iniae* have limited the extent of that protection, leading to vaccine escape and reinfection of vaccinated stocks with new serotypes [13,36,130,154].

Variation in the coverage and composition of the antigenic capsular polysaccharides is largely responsible for serotype switching, and a direct correlation has been reported for deviant *cps* genes and vaccination failure on Australian barramundi farms [13,130]. Production of secreted extracellular polysaccharides has also led to vaccine escape in Israel [154]. There is still some evidence of cross-reactive antibodies raised against different serotypes, particularly against less encapsulated strains, where other antigenic surface components are more exposed [139,155,156]. In some cases, though, this cross-reactivity does not translate into cross-

opsonization [9,115]. Protection against *S. iniae* involves both humoral and cellular immunity [78,103,115], and lack of cross-opsonization is thought to contribute to vaccine escape. A more detailed understanding of serotypical diversity and host response will be necessary to develop a more comprehensive vaccine.

There has been considerable effort to improve vaccines available for *S. iniae.* Implementation of a vaccine in the aquaculture setting requires consideration of the cost and ease of administration. Most vaccines are delivered by intracelomic or intramuscular injection, which is labor intensive and stressful for the fish [153,157]. Mucosal vaccines are preferred from both practical and immunological perspectives, as they are delivered needle-free through more natural routes and stimulate both systematic and mucosal responses [157,158]. Mucosal tissues are the primary points of entry for *S. iniae* and many other pathogens and are important sites for generating immunity [157]. Currently, commercial mucosal vaccines for *S. iniae* are limited to AQUAVAC® Strep Si (MSD Animal Health) and ME-VAC Aqua Strept® (MEVAC), a polyvalent vaccine for *S. iniae, S. agalactiae, L. garvieae* and *E. faecalis,* which are both labeled for injection or immersion delivery. The availability of these bacterins, however, is restricted to certain countries and host species. There are no mucosal or injectable commercial vaccines for piscine streptococcosis approved in the US [18].

A range of experimental vaccines seeking to improve upon the conventional killed whole-cell preparations have been developed, including modified bacterins [159,160], DNA vaccines [161-163], recombinant subunit vaccines [115,164], live-attenuated vaccines (LAV) [78,117,137–139,165,166], and bacterial ghosts [167]. A modified bacterin consisting of two strains of formalin killed *S. iniae* with extracellular products (ECP) was demonstrated to have improved efficacy against heterologous strains by intramuscular injection [160], while coating bacterins

with chitosan/alginate had increased their protection by oral delivery [159]. Improved bacterins however, may still be limited in their ability to stimulate cellular immunity, a relevant concern for this facultatively intracellular pathogen [139,149,150]. Subunit vaccines for *S. iniae* are similarly more limited in their initiation of a strong immune response, though the ease of including multiple antigenic proteins offers an advantage for an extended range of protection [158,168,169]. Delivery with adjuvants or a bacterial carrier [170] may augment immunity from subunit vaccines, but the former increases undesirable side effects, and the latter is complicated by public perception of genetically modified organisms (GMOs) [158,171]. DNA vaccines, such as those developed based on the Sia10 [163], SLS [162], or α-enolase [161] genes stimulate both arms of immunity, but are delivered by injection [168]. The bacterial ghost vaccine of *S. iniae* is essentially an empty cell wall with antigenic surface components and has also been restricted to injection [167]. Live attenuated vaccines have been developed by targeted [117,137–139,166] or random [78,165] removal of virulence genes including *pgmA, srtA, simA, cpsD* and a fructose specific IIABC component sequence of the PTS system (Table 1.2). LAV strongly stimulate robust humoral and cellular immunity by mimicking natural infection, and some LAV have promise for mucosal delivery and/or protection against multiple strains [152,158,168]. Those developed by targeted mutagenesis, however, face dual concerns with safety regarding live vaccines and GMOs [168,171]. Continued enhancement of these vaccine strategies in response to new pathogenesis and immunologic data remains an ongoing process.

5.3 Management practices

In addition to chemical therapeutics and prophylactics, management practices to optimize fish health can be extremely beneficial for prevention of streptococcosis and other diseases. Keeping water quality high, balancing stocking rates, strengthening biosecurity measures, and

promptly responding to signs of infection can help prevent outbreaks [1,18]. Proper nutrition is also important, and dietary supplementation with probiotics and immunostimulants is a growing area of research. There is some evidence that rosemary (*Rosmarinus officinalis*) can reduce mortality rates in tilapia similarly to oxytetracycline [172], and garlic has also been shown to enhance resistance to *S. iniae* in several species of fish [63,173,174]. Including probiotic bacteria such as *Bacillus* spp., *Lactobacillus acidophilus*, and *Lactococcus lactis* in feed may also boost immune responses in fish, though there may be a period of delay before significant resistance to infection is conferred [18]. A large-scale study by LaFrentz et al. [175] found that selective breeding to propagate host varieties with reduced susceptibility to *S. iniae* may also be possible.

6. Conclusions

Piscine streptococcosis caused by *S. iniae* is a widespread and multifactorial disease. The patterns and outcomes of infection are determined by a complex interplay of host, bacterial, and environmental factors. Host species, age, and immune status can all influence their susceptibility, while environmental conditions impact both host immune response, bacterial transmission, and virulence. It has been clearly established that the pathogenesis of *S. iniae* is strain dependent, but the nuances of different pathologies in relation to strain genetic background and associated virulence factor profiles are still being parsed apart. Understanding the molecular and cellular processes contributing to transmission, infection, and immunogenicity will be crucial for development of a sustainable, cross-protective vaccine for widespread control of this important global fish pathogen.

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CHAPTER 2. Multilocus sequence analysis of diverse *Streptococcus iniae* isolates indicates an underlying genetic basis for phenotypic heterogeneity

Abstract

Streptococcus iniae is a gram-positive, opportunistically zoonotic bacterium infective to a wide variety of farmed and wild fish species worldwide. Outbreaks in wild fish can have detrimental environmental and cultural impacts, and mortality events in aquaculture can result in significant economic losses. As an emerging or re-emerging pathogen of global significance, understanding the coalescing factors contributing to piscine streptococcosis is crucial for developing strategies to control infections. Intraspecific antigenic and genetic variability of *S. iniae* has made development of autogenous vaccines a challenge, particularly where the diversity of locally endemic *S. iniae* strains is unknown. This study genetically and phenotypically characterized 11 *S. iniae* isolates from diseased wild and farmed fish from North America, Central America and the Caribbean. A multilocus sequence analysis (MLSA) scheme was developed to phylogenetically compare these isolates to 84 other strains of *Streptococcus* spp. relevant to aquaculture. MLSA generated phylogenies comparable to established genotyping methods, and isolates formed distinct clades related to phenotype and host species. The endothelial *Oreochromis mossambicus* bulbus arteriosus cell line and whole blood from rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), and white sturgeon (*Acipenser transmontanus*) were used to investigate the persistence and virulence of the 11 isolates using *in vitro* assays. *In vivo* challenges using an *O. niloticus* model were used to evaluate virulence by the intragastric route of infection. Isolates showed significant differences ($p < 0.05$) in virulence and persistence, with some correlation to genogroup, establishing a basis for further work uncovering genetic factors leading to increased pathogenicity.

1. Introduction

Aquaculture is the world's fastest growing food production sector, playing a critical role in providing livelihoods and protein sources for a population expected to approach 10 billion by 2050 [1]. Infectious diseases such as streptococcosis have substantial economic impacts on the industry through diseases outbreaks, treatment expenditures, and other production losses [2,3]. A major etiologic agent of piscine streptococcosis, *Streptococcus iniae*, is estimated to cost the global aquaculture industry over 100 million dollars annually [4], and the number of hosts, habitats, and countries impacted continues to expand. Infection by *S. iniae* is multisystemic, but is commonly associated with meningitis, panophthalmitis, and septicemia. Pathogenesis varies depending on the bacterial strain, host species, and environmental conditions [5]. The host range of *S. iniae* includes over 30 species of fresh, euryhaline, and saltwater fish [5,6]. Outbreaks with high mortalities occur in commercially valuable fish, including tilapia (*Oreochromis* spp.) [7,8], rainbow trout (*Oncorhynchus mykiss*) [9,10], white sturgeon (*Acipenser transmontanus*) [11], and others. Ornamental species [12], wild marine fish [13–16], and mammalian species, including humans [17–22], can also be affected. As such, *S. iniae* is a pathogen of concern in the fields of aquaculture, conservation, and animal health. Disease transmission between farmed and wild fish has been implicated in several outbreaks [14,23–25], illustrating the need for a better appreciation of the disease and its transmission directly between hosts and indirectly in the environment. Despite this, the pathogenic mechanisms used by *S. iniae* and its epidemiology remain incompletely understood, and vaccination efforts have been met with variable success [5,26–28]. A contributing factor to these difficulties is our incomplete knowledge of the antigenic and genetic diversity of *S. iniae*, especially related to relevant differences in pathogenesis between strains.

To this end, *S. iniae* collected from wild and cultured fish species in marine and freshwater environments across North America, Central America, and the Caribbean were genetically and phenotypically characterized. Genotyping schemes, such as repetitive sequence mediated fingerprinting (Rep-PCR) and pulsed field gel electrophoresis (PFGE), have been used for *S. iniae* typing [19,29], but the portability of data from these techniques is cumbersome, results are often poorly reproducible, and reliable comparisons require isolates to be processed simultaneously. In contrast to these image-based approaches, multi-locus sequence analysis (MLSA) identifies variation in housekeeping genes in a reproducible and disseminable manner. MLSA has been used successfully to genetically characterize a number of bacterial species [30–34] and publicly accessible databases facilitate inclusion of MLSA and whole-genome data from isolates across the globe [34–36]. However, no MLSA scheme has been established for *S. iniae*. Herein, isolates previously typed by Rep-PCR [11] were used to evaluate an MLSA method for *S. iniae*. The MLSA was designed to be inclusive of other piscine streptococcal pathogens, giving it broader relevance to fish health research and diagnostics. To further characterize these isolates and improve laboratory techniques used to assess *S. iniae* pathogenicity, *in vitro* and *in vivo* assays were employed to assess isolate virulence in relevant fish hosts. Through the *in vivo* assay, this study also validates a biologically relevant intragastric gavage challenge model for *S. iniae* infections.

2. Materials and Methods

2.1 Bacteria

Eleven clinical isolates of *S. iniae* from five wild and farmed fish species from North America, Central America, and the Caribbean were used in all aspects of this study. Thirty-five additional *S. iniae* isolates from piscine and mammalian sources, as well as representative *S.*

agalactiae, S. dysgalactiae and *S. ictaluri* isolates, were included for the MLSA (Table 2.1). Isolates were stored in 1 mL aliquots in brain heart infusion broth (BHI, MP Biomedicals, LLC, USA) with 20% glycerol at -80°C. Before each assay, isolates revived from frozen stocks were grown at 30°C for 24 h in BHI, with shaking, or for 48 h on trypticase soy agar supplemented with 5% sheep's blood (SBA; University of California, Biological Media Services) unless otherwise noted. The 0.5 McFarland standard (~$1.5x10^8$ CFU mL^{-1}) used for the *in vivo* and *in vitro* virulence assays corresponded to a bacterial suspension in phosphate buffered saline (PBS) with an optical density measurement of 0.14-0.155 at 600 nm, read on a UV/Vis photometer (BioPhotometer Plus, Eppendorf AG, Hamburg, Germany). Isolates were phenotypically characterized using the API 20 STREP system following the manufacturer's instructions and read at 48h (bioMérieux Inc., Durham, NC, USA).

Table 2.1: *Streptococcus* isolates used in this study. Isolates in bold were used for both MLSA typing and further phenotypic characterization. NECB and SECB stand for Northern and Southern Caribbean basin respectively.

Isolate	Origin	Geography	Reference
***S. iniae* ECO86-17**	Spotted Rose snapper (*Lutjanus guttatus*)	Central America	(Heckman et al. unpubl. data)
***S. iniae* B8**	Wild reef fish	SECB	[11]
***S. iniae* K08-409H**	Wild reef fish	NECB	As above
***S. iniae* F15-4-3**	Tilapia (*Oreochromis spp.*)	California	As above
***S. iniae* WS-6B**	White sturgeon (*Acipenser transmontanus*)	California	As above
***S. iniae* ARK PB03-62B**	Albino rainbow shark (*Epalzeorhynchos frenatum*)	Florida	As above
***S. iniae* LSU 01-105**	Tilapia (*Oreochromis spp.*)	Minnesota	As above
***S. iniae* LSU 10-070**	Tilapia (*Oreochromis spp.*)	Florida	As above
***S. iniae* LSU 94-034**	Tilapia (*Oreochromis spp.*)	Massachusetts	As above
***S. iniae* LSU 96-525**	Tilapia (*Oreochromis spp.*)	Iowa	As above
***S. iniae* LSU 94-036**	Tilapia (*Oreochromis spp.*)	Illinois	As above
S. iniae WS-6H	White sturgeon (*A. transmontanus*)	California	As above

S. iniae 831	Pygmy hippo (*Choeropsis liberiensis*)	Texas	This study
S. iniae 832	Pygmy hippo (*C. liberiensis*)	Texas	This study
S. iniae 837	Pygmy hippo (*C. liberiensis*)	Texas	This study
S. iniae 105-04	Human (*Homo sapiens*)	California	[20]
S. iniae 4780-01	Human (*H. sapiens*)	California	As above
S. iniae 4787-01	Human (*H. sapiens*)	California	As above
S. iniae 4989-04	Human (*H. sapiens*)	California	As above
S. iniae 143-01	Human (*H. sapiens*)	California	As above
S. iniae 2388-02	Human (*H. sapiens*)	California	As above
S. iniae 1056-03	Human (*H. sapiens*)	Pennsylvania	As above
S. iniae SS1440	Human (*H. sapiens*)	Canada	As above
S. iniae SS1543	Human (*H. sapiens*)	Canada	As above
S. iniae 8278	Tilapia (*Oreochromis spp.*)	Israel	This study
S. iniae 8679	Hybrid striped bass (*Morone saxatilis x Morone chrysops*)	Israel	This study
S. iniae 11042	Tilapia (*Oreochromis spp.*)	Israel	This study
S. iniae 11979	Tilapia (*Oreochromis spp.*)	Israel	This study
S. iniae 12302	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae 12424	Hybrid tilapia (*O. aureus x O. niloticus)*	Israel	This study
S. iniae 14957	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae 15091	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae 15414	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae 15843	Barramundi (*Lates calcarifer)*	Israel	This study
S. iniae 15957	Barramundi (*L. calcarifer)*	Israel	This study
S. iniae 16029	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae 16616	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae 17105	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae 17737	Barramundi (*L. calcarifer)*	Israel	This study
S. iniae 19735	Barramundi (*L. calcarifer)*	Israel	This study
S. iniae 20130	Barramundi (*L. calcarifer)*	Israel	This study
S. iniae 20960	Hybrid striped bass (*M. saxatilis x M. chrysops*)	Israel	This study
S. iniae WS-10A	White sturgeon (*A. transmontanus*)	California	This study
S. iniae WS-10B	White sturgeon (*A. transmontanus*)	California	This study
S. iniae WS-10C	White sturgeon (*A. transmontanus*)	California	This study
S. iniae WS-10D	White sturgeon (*A. transmontanus*)	California	This study
S. ictaluri CNA2848	Catfish (*Ictalurus* spp.)	Unknown	This study

S. agalactiae RUSVM-CR	Nile tilapia (*O. niloticus*)	North America	This study
S. dysgalactiae subsp. *equisimilis* STC3	White sturgeon (*A. transmontanus*)	Uruguay	This study

2.2 Buoyant density assays

Buoyant density assays can be used to estimate the quantity of capsular polysaccharide (CPS), as the buoyant density is inversely related to the amount of CPS [37]. Assays were performed as described previously [38] with some modifications. Briefly, a standard isotonic Percoll solution was prepared by mixing 9 parts Percoll with 1 part 1.5 M NaCl. Each isolate was grown to mid-exponential phase in BHI and 1 mL pelleted by centrifugation, washed in PBS and resuspended in 500 µL PBS. The suspension was layered onto the Percoll solution and centrifuged at 4,000 x g for 90 min. The experiment was repeated with new cultures three times.

2.3 Genomic DNA extraction

Swabs of each isolate from freezer stocks were used to inoculate 5 mL aliquots of BHI and were expanded at 30°C overnight with shaking at 150 rpm. One milliliter of the expanded bacterial suspension was centrifuged for 10 min at 5000 x g (7500 rpm). Genomic DNA (gDNA) was isolated from the concentrated pellet using the DNeasy® Blood and Tissue kit (Qiagen, Germantown, MD, USA) following manufacturer recommendations for gram-positive bacteria. The quality and quantity of recovered DNA was assessed using a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific™, Waltham, MA USA) and samples with 260/280 ratios of 1.8-2.0 were cryogenically stored (-20°C) until further analysis.

2.4 Multilocus sequence analysis

The purified gDNA from each isolate was used for polymerase chain reaction (PCR) amplification and sequencing of 9 housekeeping genes: *arcC, glnA, groEL, gyrB, mutS, pheT, prkC, rpoB,* and *tkt.* Degenerate primers were initially designed based on alignments of publicly

available *Streptococcus* spp. genomes available in the National Center for Biotechnology Information's Microbial Genome Database (*Streptococcus iniae* strain YSFST01-82, GenBank Acc. No. CP010783; *S. iniae* strain FP5228, GenBank Acc. No. CP024843; *S. iniae* strain QMA0248, GenBank Acc. No. CP022392; *S. agalactiae* strain WC1535, GenBank Acc. No. CP016501, *S. agalactiae* strain NGBS061, GenBank Acc. No. CP007631; *S. agalactiae* strain SGEHI2015-113, GenBank Acc. No. CP025026, *S. agalactiae* strain SG-M29, GenBank Acc. No. CP021866) and verified *in silico* by BLASTn searches of *Streptococcus* spp. in GenBank. Primer sequences are listed in Table 2.2. PCR using Phusion high-fidelity DNA polymerase (Thermo Scientific™, USA) was carried out following manufacturer recommendations with 3% added DMSO, and annealing temperatures 6°C below the suggested melting temperature for each primer set (Table 2.2). Aliquots of amplification reactions, along with concurrently run molecular weight standards (Quick-Load® Purple 100 bp DNA Ladder, New England BioLabs, Ipswich, MA, USA), were electrophoresed through 1% agarose gels supplemented with SYBR® Safe DNA gel stain (Invitrogen, Waltham, MA, USA) (1 μL mL^{-1}) and visualized under ultraviolet light to confirm the presence of appropriately sized bands.

Table 2.2: Primers used in the MLSA scheme for genotyping *S. iniae*.

Name	**Forward Primer**	**Reverse Primer**
Carbamate kinase (*arcC*)	GCWAAAGCACAACAAGAAGC	CGCCADCCACGRCCWGCATC
Glutamine synthetase (*glnA*)	MAAATGGGYTTTGAAGTDGAAGC	RTCAATTTCCCATTGWGAMAY
Chaperonin (*groEL*)	TAAATTTTCAGCAGATGCSCGY	ACTTCAAGYTCTGTYTCCATACC
Gyrase B (*gyrB*)	GGWGARGATGTTCGTGAAGG	TCCATTGTTGTTTCCCAAAG
DNA mismatch repair protein (*mutS*)	WAAAAATTCTGARCGYTATGG	AAGGTTGATTGCCCAGAAAT

Phenylalanine-tRNA ligase subunit beta (*pheT*)	GGTCAACCWATGCATGCTTT	WCATYGGCCACATVAGTTC
Serine/threonine-protein kinase (*prkC*)	TATTTGCTGGTCGTTATCGS	YCCCATSGCATAAATATCAC
RNA polymerase B (*rpoB*)	TGTTGGTACTGGTATGGA	AAACGTTGTCCACCAAAT
Transketolase (*tkt*)	CAGAAGATGTKAAAGGACGTT	GCCATKGCAAATTCACGWAC

PCR products were purified using the AccuPrep® PCR Purification Kit (Bioneer, Oakland, CA, USA) or QIAquick PCR Purification Kit (Qiagen) and their concentration and purity assessed by Nanodrop. Purified products and corresponding forward primers were diluted and submitted for Sanger sequencing at the University of California, Davis Sequencing facility (UC Davis, CA, USA) or through GENEWIZ (South San Francisco, CA, USA). Sequences for each housekeeping gene were aligned by MUSCLE with default settings in Geneious Prime (2019.0.4). Sequence ends were annotated with a 0.01 error probability limit and trimmed to the region of quality bases shared by all isolate sequences. A representative trimmed sequence of each housekeeping gene was used in BLAST searches of a localized database populated by *S. iniae, S. agalactiae, S. dysgalactiae, S. ictaluri,* and *S. pyogenes* genomes downloaded from GenBank. The database represented 46 fish and mammalian isolates derived worldwide (Table 2.3). Trimmed sequences for each housekeeping gene were concatenated alphabetically for each isolate. Concatenates were exported to MEGA-X [39] and aligned by MUSCLE using the default settings. A maximum likelihood tree was generated using the Tamura 3-parameter model with a Gamma distribution [40], selected based on Bayesian and Akaike Information Criterion in MEGA-X. The percentage bootstrap confidence levels calculated from 1000 re-samplings of the original data.

Table 2.3: *Streptotoccus* strains with full genome assemblies available on GenBank used in MLSA analysis.

Isolate	Origin	Accession number	Assembly level
S. agalactiae 2603V/R	Human (*Homo sapiens*), Italy	NC_004116	Complete
S. agalactiae QMA0271	Giant catfish (*Netuma thalassina*), Australia	CP029632	Complete
S. agalactiae S13	Nile tilapia (*Oreochromis nioliticus*), Brazil	CP018623	Complete
S. dysgalactiae subsp. *dysgalactiae* ATCC 27957	Cow (*Bos taurus*), UK	NZ_CM001076	Complete
S. dysgalactiae subsp. *equisimilis* 167	Human (*H. sapiens*), Japan	AP012976	Complete
S. dysgalactiae subsp. *equisimilis* AC-2713	Human (*H. sapiens*), Germany	NC_019042	Complete
S. ictaluri 707-05	Channel catfish (*Ictalurus punctatus*), USA	ASM18801v3	Contigs
S. iniae 89353	Nile tilapia (*O. niloticus*), Taiwan	NZ_CP017952	Complete
S. iniae FP5228	Olive flounder (*Paralichthys olivaceus*), South Korea	NZ_CP024843	Complete
S. iniae ISET0901	Nile tilapia (*O. niloticus*), Israel	CP007586	Complete
S. iniae ISNO	Attenuated strain from ISET0901, Nile tilapia (*O. niloticus*), Israel	CP007587	Complete
S. iniae QMA0071	Barramundi (*Lates calcarifer*), Australia	GCA_003675245.1	Scaffold
S. iniae QMA0074	Barramundi (*L. calcarifer*), Australia	GCA_003675285.1	Scaffold
S. iniae QMA0080	Barramundi (*L. calcarifer*), Australia	GCA_003675195.1	Scaffold
S. iniae QMA0084	Black flying fox (*Pteropus alecto*), Australia	GCA_003675145.1	Scaffold
S. iniae QMA0140	Amazon river dolphin (*Inia geoffrensis*), USA	GCA_003697605.1	Contig
S. iniae QMA0141	Amazon river dolphin (*I. geoffrensis*), USA	GCA_003675085.1	Contig
S. iniae QMA0142	Barramundi (*L. calcarifer*), Australia	GCA_003697565.1	Contig
S. iniae QMA0155	Barramundi (*L. calcarifer*), Australia	GCA_003697585.1	Scaffold
S. iniae QMA0165	Barramundi (*L. calcarifer*), Australia	GCA_003674995.1	Contig
S. iniae QMA0177	Barramundi (*L. calcarifer*), Australia	GCA_003674985.1	Contig
S. iniae QMA0186	Rainbow trout (*Oncorhynchus mykiss*), Israel	GCA_003674945.1	Scaffold
S. iniae QMA0187	Striped snakehead (*Channa striata*), Thailand	GCA_003674935.1	Contig
S. iniae QMA0188	Rainbow trout (*O. mykiss*), Israel	GCA_003674925.1	Scaffold
S. iniae QMA0189	Rainbow trout (*O. mykiss*), Reunion	GCA_003674875.1	Scaffold
S. iniae QMA0190	Striped snakehead (*C. striata*), Thailand	GCA_003674865.1	Scaffold
S. iniae QMA0233	Bone, barramundi (*L. calcarifer*), Australia	GCA_003674715.1	Contig
S. iniae QMA0248	Barramundi (*L. calcarifer*), Australia	NZ_CP022392	Complete

S. iniae QMA0249	Bone, barramundi (*L. calcarifer*), Australia	GCA_003674565.1	Scaffold
S. iniae QMA0371	Jade perch (*Scortum barcoo*), Australia	GCA_003674385.1	Scaffold
S. iniae QMA0373	Barramundi (*L. calcarifer*), Australia	GCA_003674425.1	Contig
S. iniae QMA0445	Tilapia (*Oreochromis sp.*), USA	GCA_003674345.1	Contig
S. iniae QMA0446	Tilapia (*Oreochromis sp.*), USA	GCA_003674395.1	Contig
S. iniae QMA0447	Hybrid striped bass (*Morone saxatilis x Morone chrysops*), USA	GCA_003674285.1	Scaffold
S. iniae QMA0448	Hybrid striped bass (*M. saxatilis x M. chrysops*), USA	GCA_003674305.1	Scaffold
S. iniae QMA0458	Red-tailed Black Shark (*Epalzeorhynchos bicolor*), USA	GCA_003674315.1	Contig
S. iniae QMA0462	Clown loach (*Chromobotia macracanthus*), USA	GCA_003674265.1	Contig
S. iniae QMA0463	Clown loach (*C. macracanthus*), USA	GCA_003674215.1	Scaffold
S. iniae QMA0466	Tilapia (*Oreochromis sp.*), USA	GCA_003674205.1	Contig
S. iniae QMA0468	Tilapia (*Oreochromis sp.*), USA	GCA_003674235.1	Scaffold
S. iniae QMA0490	Tilapia (*Oreochromis sp.*), Honduras	GCA_003674165.1	Scaffold
S. iniae SF1	Olive flounder (*P. olivaceus*), China	CP005941	Complete
S. iniae YM011	Attenuated strain from GX005, Nile tilapia (*O. niloticus),* China	NZ_CP032400	Complete
S. iniae YSFST01-82	Olive flounder (*P. olivaceus*), South Korea	NZ_CP010783	Complete
S. pyogenes M1 GAS SF370	Human (*H. sapiens*), USA	NC_002737.2	Complete
S. pyogenes NS53	Human (*H. sapiens*), Australia	NZ_CP015238.2	Complete

2.5 Whole blood survival assays

Isolate survival in whole blood was assessed using methods adapted from Locke et al. [41,42]. Whole, lithium-heparinized blood was collected from healthy, anesthetized rainbow trout, Nile tilapia, and white sturgeon by caudal venipuncture. Aliquots of 300 µL were transferred to sterile microcentrifuge tubes. A 0.5 McFarland suspension was generated for each bacterial isolate from 48 h cultures on SBA and suspensions diluted to ~$1.5x10^5$ CFU mL^{-1} in sterile PBS. Two microliters (~300 CFU) of bacterial suspension were added to each tube of blood, in triplicate, and incubated for 1 h with shaking at 20°C for the trout and sturgeon blood and 30°C for the tilapia blood. Two microliters of bacterial suspension were added to 300 µL

sterile PBS to establish baseline CFU. Six, 25 µL aliquots of blood or PBS were spot plated onto SBA and colonies counted after 72 h. Percent survival was calculated by dividing colony counts from blood by respective colony counts from PBS. Spots with >100 colonies were excluded from analysis due to increased probability of counting error. Experiments were repeated three times using blood from the same group of fish. Statistical significance was determined in GraphPad Prism (version 8.3.0, GraphPad Software, La Jolla, CA USA) using Brown-Forsythe and Welch ANOVA (Analysis of Variance) tests with Dunnett's T3 multiple comparisons test, with individual variances computed for each comparison.

2.6 Cytotoxicity assays

To test the ability of *S. iniae* to kill endothelial cells, the *Oreochromis mossambicus* bulbus arteriosus cell line (TmBs) [43,44] was challenged with the different bacterial isolates at 20 and 30°C. This cell line was chosen as the heart is commonly affected in *S. iniae* infection [15,45]. TmBs were plated in 24-well dishes ($2.5x10^5$ cells/well) in 500 µL of minimal essential media-2 + HEPES + 10% fetal bovine serum (MEM) and grown to confluence at 25°C. A 0.5 McFarland standard was generated for each isolate and 100 µL added to their respective cell wells (MOI 1:100). Sterile PBS (100 µL) was added to uninfected cells as a negative control. Cells were incubated for 3 h at 20 or 30°C, washed 3 times with 1 mL of MEM to remove non-adherent bacteria and placed back in the same incubator for 24 h.

The Cytotox96© Non-Radioactive Assay (Promega, Durham, NC, USA) was used according to the manufacturer's instructions to quantitate release of the stable cytosolic enzyme lactate dehydrogenase (LDH). An enzymatic assay is used to measure LDH release into culture supernatant, converting a tetrazolium salt (INT) into a red formazan product. The amount of color produced is proportional to the number of lysed cells. Adsorption at 490 nm was measured

using a Cytation™ 5 Imaging Reader (BioTek, Winooski, VT, USA). Adsorption values were standardized against negative controls and percent toxicity calculated by dividing experimental treatment values by the positive control value. Statistical significance was determined using Kruskal-Wallis with Dunn's multiple comparisons test in GraphPad Prism (version 8.3.0, GraphPad Software, La Jolla, CA USA).

2.7 Intragastric challenge

Challenges were conducted under the University of California, Davis School of Veterinary Medicine Institutional Animal Care and Use Committee approved protocol #19645. To investigate *in vivo* pathogenesis, Nile tilapia fingerlings were challenged with each of the 11 North American *S. iniae* isolates. Fresh 0.5 McFarland solutions (~$1.5x10^8$ CFU mL^{-1}) were generated for each isolate. Sixteen fish were gavage challenged with 0.1 mL of the McFarland solutions (~$1.5x10^7$ CFU) or with a sterile PBS control using a 20G X 1.5", 1.9 mm flexible plastic feeding needle (Cadence Science®, Cranston, RI, USA). Fish were kept in aerated flow through tanks at 28-30°C and morbidity and mortality recorded daily for 21 d. Moribund fish or those exhibiting abnormal swimming, lethargy, or exophthalmia were euthanized with buffered MS-222 (500 mg L^{-1}) in sodium bicarbonate and necropsied. Posterior kidney and brain swabs were plated on SBA and incubated at 30°C. Whole fingerlings were fixed in 10% neutral buffered formalin, decalcified, processed routinely, and stained with hematoxylin and eosin (H&E) for light microscopic examination. Select sections were stained with a Brown and Hopps Gram stain. At the end of the 21 d challenge, all surviving fish were euthanized. Three fish per treatment were fixed and processed for histologic evaluation. Three fish per treatment were necropsied and posterior kidney and brain swabs plated on SBA then incubated at 30°C for 48 h. Survival curve analysis and statistics were performed using GraphPad Prism (version 8.3.0,

GraphPad Software, La Jolla, CA, USA). Statistical significance was determined by Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.

3. Results

3.1 Genetic Characterization

The MLSA primers successfully amplified target regions of the selected housekeeping genes in *S. iniae, S. agalactiae, S. dysgalactiae* and *S. ictaluri.* MLSA of *S. iniae* isolates generated a maximum likelihood tree consisting of 5 major clades A-E (Figure 2.1), with several lineages consisting of one or two isolates. There was an overall high level of similarity between isolates among the chosen housekeeping genes (99.9% pairwise identity). Isolates assigned to the same vertical line had no base pair differences. Clusters were largely determined by only a few single nucleotide polymorphisms (SNPs) in the *gyrB, pheT, prkC*, and *rpoB* genes, with exception of lineages from pygmy hippopotamuses (*Choeropsis liberiensis;* 831, 832, and 837) and the second Amazon River dolphin (*Inia geoffrensis;* QMA0141), which had multiple SNPs in every gene fragment.

Isolates from North America fell into four of the five clades (A, B, D and E), three of which consisted exclusively of isolates from this region (A, B and D). Clade E, the largest, contained isolates from diverse species and countries. Clade A contained isolates from tilapia in the United States and humans from Canada, Clade B, isolates from fish in California, and Clade D, isolates from outbreaks in wild and farmed marine fish in Central America and the Caribbean. Clade C, the second largest clade, consisted of isolates from marine and freshwater fish from Israel and Australia. Several smaller lineages of one or two isolates did not fit into the other major clades, likely a result of undersampling. Isolates WS-6H, QMA0188, QMA0187 from a white sturgeon, rainbow trout, and striped snakehead (*Channa striata*), respectively, branched separately from

the main cluster. Tilapia isolate ISET0901 and its attenuated derivative ISNO grouped together, as did the isolates from clown loaches (*Chromobotia macracanthus*). Non-human mammalian isolates were generally divergent from the other isolates, except for QMA0140, the original Amazon River dolphin type strain isolate (ATCC29178), which fell into clade E. QMA0084 from a black flying fox (*Pteropus alecto*) formed its own lineage but shared a high degree of homology with the fish isolates. The second dolphin isolate (QMA0141) and isolates from pygmy hippos (831, 832, 837) were the most divergent lineages. Including isolates of different streptococcal species slightly reduced intraspecies resolution but grouped isolates according to their respective species and subspecies, consistent with phylogenies derived from whole genome comparisons [46] (Figure 2.2). The concatenated sequences of *S. iniae, S. agalactiae, S. dysgalactiae, S. ictaluri* and *S. pyogenes* shared 87.3% pairwise identity.

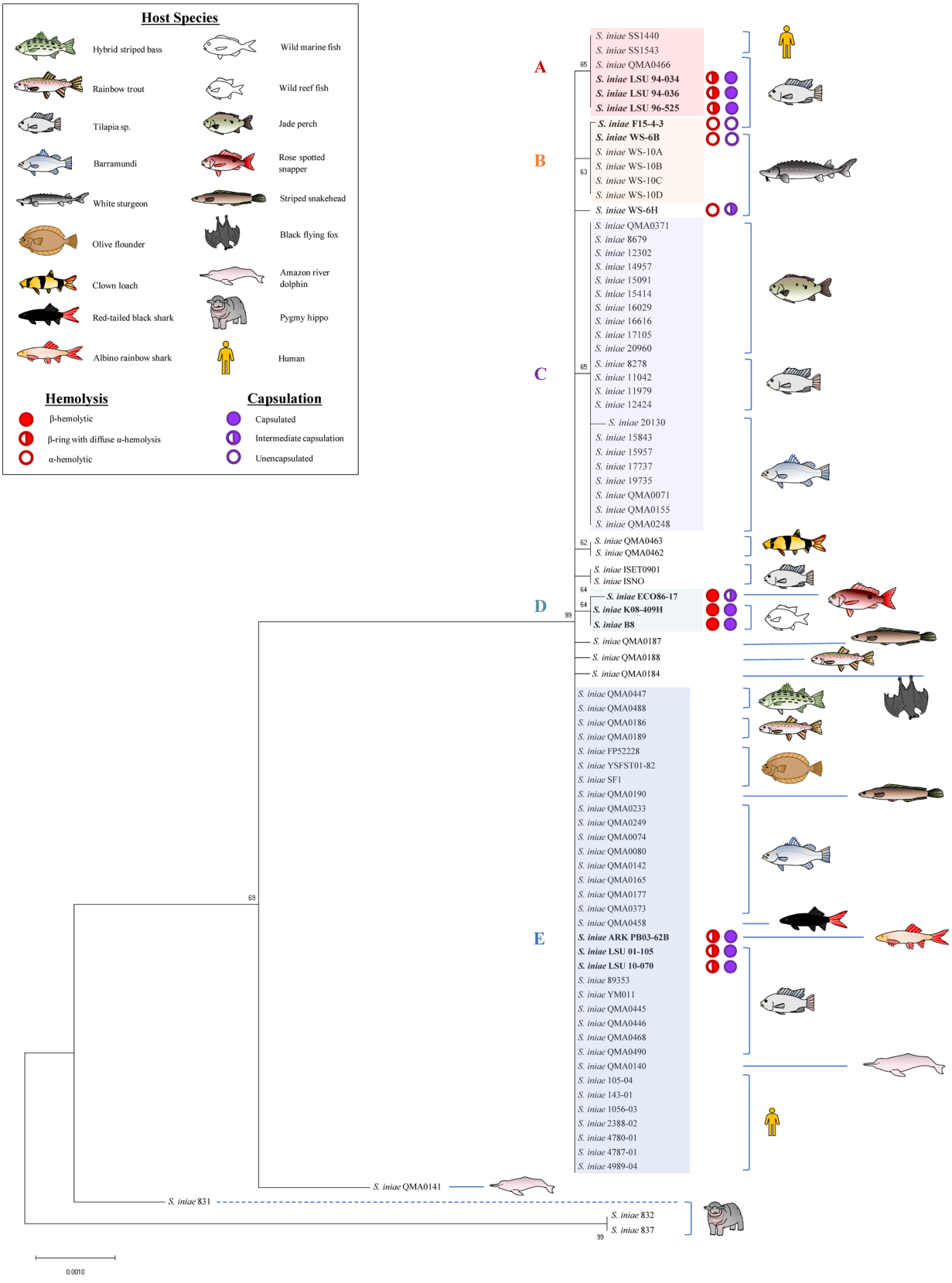
Host Species
Hybrid striped bass
Rainbow trout
Tilapia sp.
Barramundi
White sturgeon
Olive flounder
Clown loach
Red-tailed black shark
Albino rainbow shark
Wild marine fish
Wild reef fish
Jade perch
Rose spotted snapper
Striped snakehead
Black flying fox
Amazon river dolphin
Pygmy hippo
Human
Hemolysis
β-hemolytic
β-ring with diffuse α-hemolysis
α-hemolytic
Capsulation
Capsulated
Intermediate capsulation
Unencapsulated
A
B
C
D
E
S. iniae SS1440
S. iniae SS1543
S. iniae QMA0466
S. iniae LSU 94-034
S. iniae LSU 94-036
S. iniae LSU 96-525
S. iniae F15-4-3
S. iniae WS-6B
S. iniae WS-10A
S. iniae WS-10B
S. iniae WS-10C
S. iniae WS-10D
S. iniae WS-6H
S. iniae QMA0371
S. iniae 8679
S. iniae 12302
S. iniae 14957
S. iniae 15091
S. iniae 15414
S. iniae 16029
S. iniae 16616
S. iniae 17105
S. iniae 20960
S. iniae 8278
S. iniae 11042
S. iniae 11979
S. iniae 12424
S. iniae 20130
S. iniae 15843
S. iniae 15957
S. iniae 17737
S. iniae 19735
S. iniae QMA0071
S. iniae QMA0155
S. iniae QMA0248
S. iniae QMA0463
S. iniae QMA0462
S. iniae ISET0901
S. iniae ISNO
S. iniae ECO86-17
S. iniae K08-409H
S. iniae B8
S. iniae QMA0187
S. iniae QMA0188
S. iniae QMA0184
S. iniae QMA0447
S. iniae QMA0488
S. iniae QMA0186
S. iniae QMA0189
S. iniae FP52228
S. iniae YSFST01-82
S. iniae SF1
S. iniae QMA0190
S. iniae QMA0233
S. iniae QMA0249
S. iniae QMA0074
S. iniae QMA0080
S. iniae QMA0142
S. iniae QMA0165
S. iniae QMA0177
S. iniae QMA0373
S. iniae QMA0458
S. iniae ARK PB03-62B
S. iniae LSU 01-105
S. iniae LSU 10-070
S. iniae 89353
S. iniae YM011
S. iniae QMA0445
S. iniae QMA0446
S. iniae QMA0468
S. iniae QMA0490
S. iniae QMA0140
S. iniae 105-04
S. iniae 143-01
S. iniae 1056-03
S. iniae 2388-02
S. iniae 4780-01
S. iniae 4787-01
S. iniae 4989-04
S. iniae QMA0141
S. iniae 831
S. iniae 832
S. iniae 837
65
63
65
62
64
64
99
69
99
0.0010

Figure 2.1: Maximum likelihood tree of *S. iniae* isolates generated from MLSA analysis. Isolates used in further phylogenetic analysis are in bold. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model [40]. The tree with the highest log likelihood (-7587.37) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 0.0500]). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X [39].

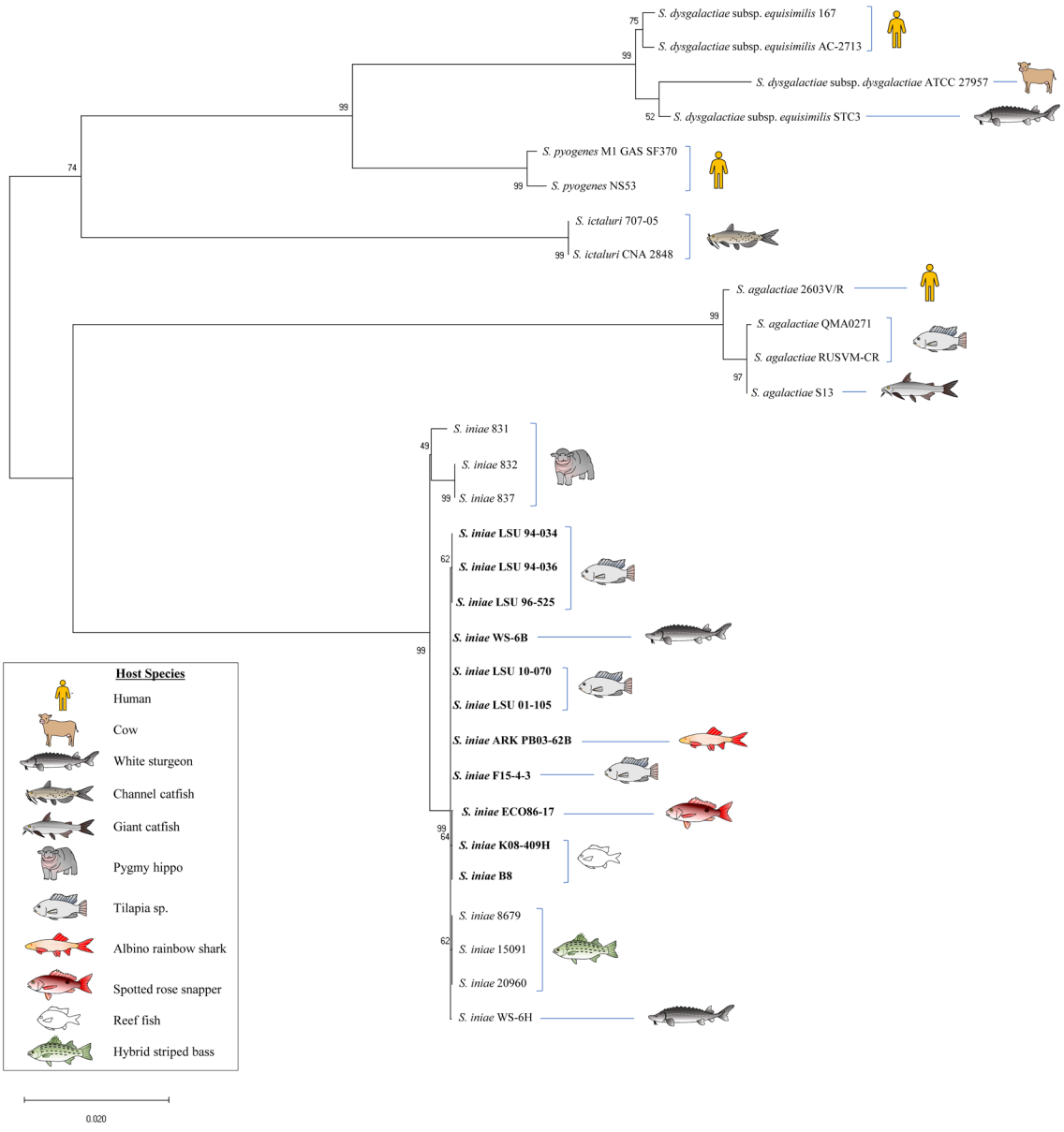

Figure 2.2: Maximum likelihood tree of *Streptococcus* sp. isolates generated from MLSA analysis. Isolates used in further phylogenetic analysis are in bold. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model [40]. The tree with the highest log likelihood (-10418.63) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0598)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X [39].

3.2 Morphology and hemolysis

The North American isolates formed small translucent colonies within 24 h on SBA that became white within 48 h. Colony size differed between phylogroups, with Clade D isolates forming larger (≤3mm) colonies than isolates in clades A, B, and E (≤1.5mm). Isolates from clade D were also clearly β-hemolytic, while isolates from A and E had a tight ring of β-hemolysis with a surrounding zone of α-hemolysis. Isolates from clade B showed the most limited hemolysis on SBA, with a diffuse ring of α-hemolysis (Figure 2.1). Hemolytic activity increased in agar stabs under more anaerobic conditions, consistent with reports [20] (Figure 1.1C). Isolate WS-6H, a Clade B isolate by Rep-PCR, formed its own lineage with MLSA and was morphologically distinct from all other isolates. All tested isolates are suspected to be serotype I based on their ability to react with arginine dihydrolase (ADH) and ribose in the API 20 STREP system [47], although caution should be exercised in concluding serotypes by this method [48]. Buoyant density assays indicated that the California isolates displayed reduced capsulation, as they traveled furthest through the Percoll gradient (Figure 2.1). Isolate Eco86-17 from Costa Rica had a higher density than the Caribbean isolates, indicating slightly reduced capsulation, while LSU 96-525 from clade A had the lowest overall density, suggesting overexpression of CPS.

3.3 Resistance to whole blood killing

Establishing bacteremia is important in streptococcal pathogenesis, and resistance to phagocytic killing is known to correlate with differences in virulence [42,49]. As such, the ability of isolates to survive exposure to whole blood killing factors was investigated in fresh blood from three relevant fish hosts. Survival differed between isolates and by host, but all isolates demonstrated some resistance to killing (Figure 2.3). Overall, survival was highest in rainbow trout blood, where in addition to persisting, most isolates were also able to replicate [20]. Survival ranged from 37%-292%, compared to 21-117% in tilapia blood and 17-133% in sturgeon blood. There were some trends related to phylogroup, as clade B generally showed lower survival than isolates in clades A, D, and E, but variability within groups and across blood types precluded statistical comparisons. Differences in survival were not due to differences in isolate growth rate or incubation temperature (data not shown).

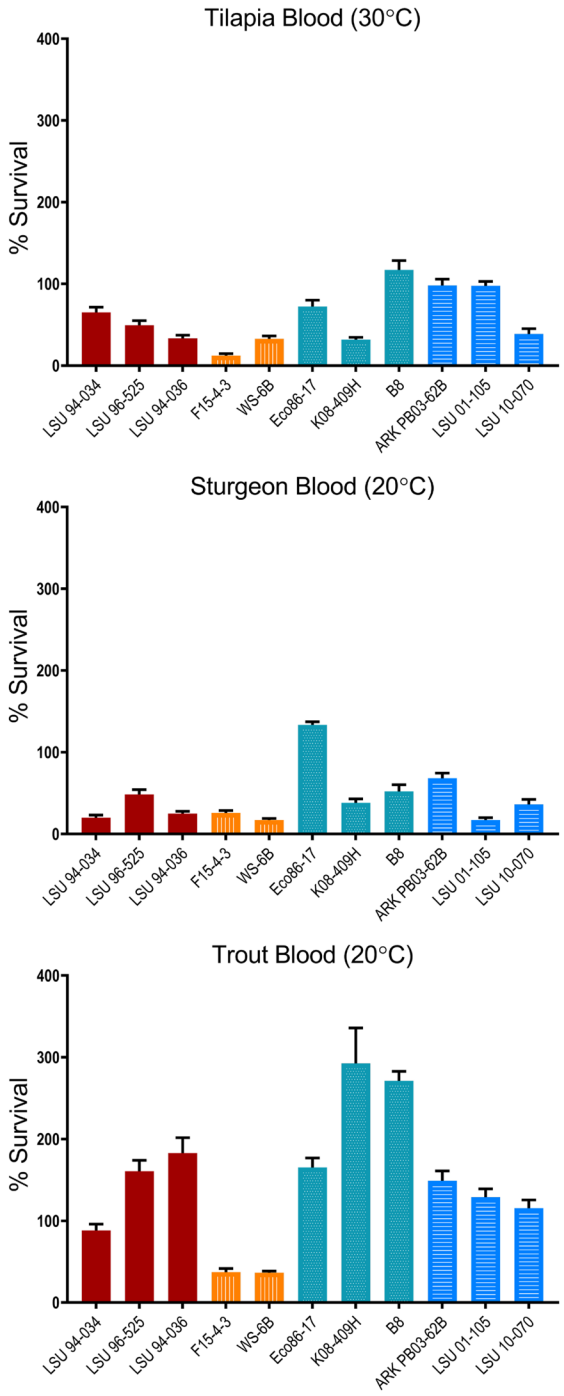

Figure 2.3: Percent isolate survival after incubation for 1hr in whole heparinized blood from three fish species incubated at biologically relevant temperatures. Species from top to bottom: Nile tilapia (*Oreochromis nioliticus*), white sturgeon (*Acipenser transmontanus*), and rainbow trout *(Oncorhynchus mykiss*). The experiment was performed twice in biological duplicate using the same groups of fish. Error bars represent standard error. Color and patterning reflect isolate phylogroup: clade A – solid red, clade B – orange with vertical stripes, clade D – teal with dots, clade E – blue with horizontal stripes**.**

3.4 Cytotoxic effects of *Streptococcus iniae* on tilapia endothelial cells

The ability of isolates to damage or kill host cells was assessed by colorimetric assay measuring release of LDH from monolayers of TmBs cells. Three main patterns of virulence were observed, and temperature was positively correlated with isolate cytotoxicity (Figure 2.4). Most isolates induced a higher percentage of LDH release at 30°C compared to 20°C. For the remaining isolates there was no statistically significant difference between temperatures ($p > 0.05$). At 30°C, the marine clade D showed significantly higher cytotoxic effects than isolates in clade A, B, or E ($p < 0.05$). Clade B isolates consistently did not cause release of LDH above background levels. Members of clades A and E induced similar trends in cytotoxicity and there was no statistically significant difference between the two groups at either temperature.

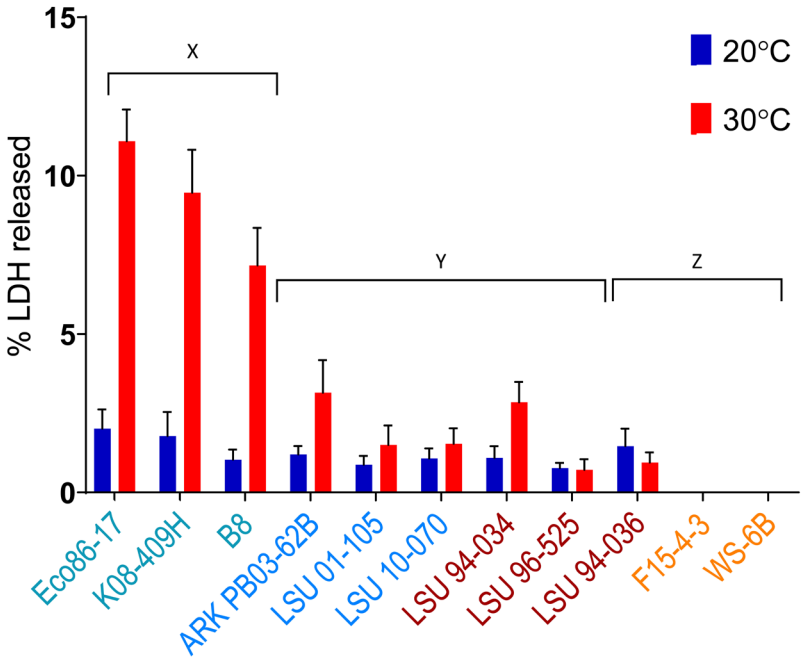

Figure 2.4: Percent cytotoxicity as measured in terms of LDH release from TmBs compared to lysed cells serving as a positive control. Cells were incubated with bacteria for 3hrs, washed, and incubated for 24 hrs at 20, 25, or 30 °C. Experiments were carried out in technical triplicate and repeated four times. Colors denote clade: clade A – red, clade B – orange, clade D – teal, clade E – blue. Letters denote statistical significance at 30°C ($p < 0.001$) Statistical significance was determined using one-way ANOVA with post-hoc Tukey HSD, error bars are standard error.

3.5 Intragastric Challenge

In-vitro assays lack the immunological complexities of a living organism, so an *in vivo* challenge using the intragastric method was used to test the virulence of representative isolates. Tilapia challenged by clade E isolate LSU 10-070 had significantly lower survival (56.25%) than the control fish challenged with sterile PBS ($p < 0.005$). There were some mortalities in tanks challenged with each of the tested isolates from clade A and one isolate from clade D, but the survival curves were not significantly different from the controls (Figure 2.5).

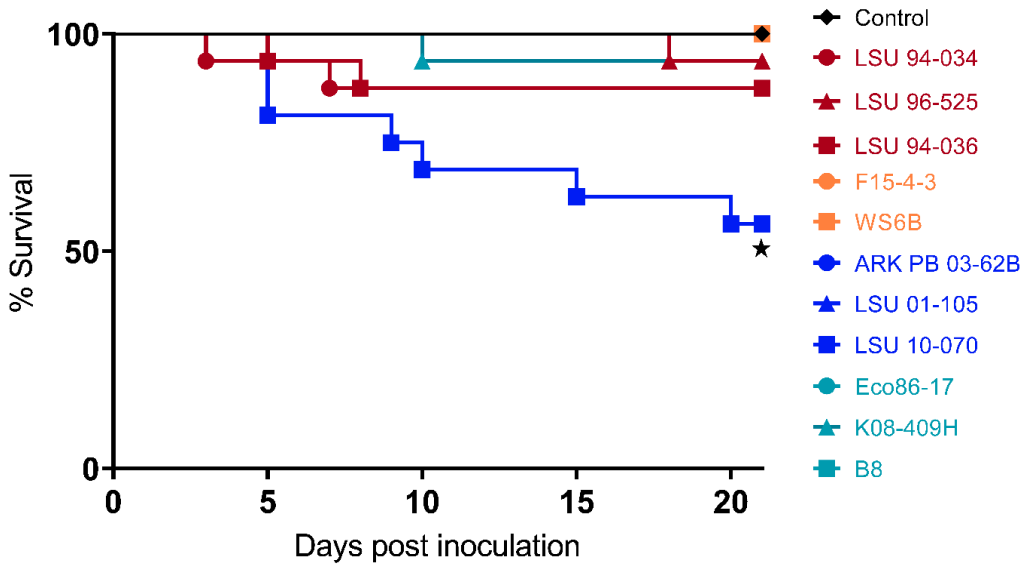

Figure 2.5: Survival curve for Nile tilapia fingerlings intragastrically challenged with North American isolates for 21 days. Sixteen fish were challenged per treatment. A star denotes statistical significance as determined by Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. Color reflects isolate phylogroup: clade A – red, clade B – orange, clade D – teal, clade E – blue.

In most cases, fish were often observed eating and swimming normally before dying rapidly, although a few fish did display disoriented swimming with or without additional clinical signs. Lack of clinical signs and swift autolysis due to high water temperatures and cannibalism limited collection of moribund or freshly dead fish. In the collected fish, the most common gross lesion

was bilateral or unilateral exophthalmia. Other gross lesions included reduced mucus and congestion of dorsal and caudal fin bases (Figure 2.6).

Figure 2.6: Tilapia collected and euthanized during the intragastric challenge with *S. iniae*. (A) Congestion along the dorsal fin. (B) Severe exophthalmia with hyperemia. (C) Gradient of exophthalmia in challenge survivors.

Isolates LSU 10-070 and LSU 94-034 were recovered from the brain tissue of freshly dead fish. The tissues from tilapia challenged with LSU 94-036 and K08-409H were heavily cannibalized before collection and were not sampled for re-isolation of bacteria. No histological changes indicating infection were noted in the controls. Figure 2.7 depicts histopathologic changes in moribund and surviving fish following intragastric challenge with isolate LSU 10-070. Examination of a moribund fish revealed extensive severe granulomatous meningoencephalitis and ventriculitis dominated by macrophages laden with gram-positive cocci, neutrophils, and scattered lymphocytes and plasma cells. Similar severe inflammatory changes affected the intraocular humors, uveal tract, choroid rete, and periocular connective tissues. Edema, foci of necrosis, and less intense inflammatory infiltrates were widespread in

cranial adipose, skeletal muscle, and associated interstitial areas. Some surviving fish were free of microscopic changes, while the meninges and cranial adipose of others contained small organizing granulomas characterized by central regions of degenerate macrophages, surrounded by mantles of lymphocytes, with scattered plasma cells and coarse eosinophilic granulocytes. Bacteria were not observed in routine H&E sections or with tissue Gram stains. Visceral organs were not affected in any of the fish examined. No histological changes indicating infection were noted in the controls.

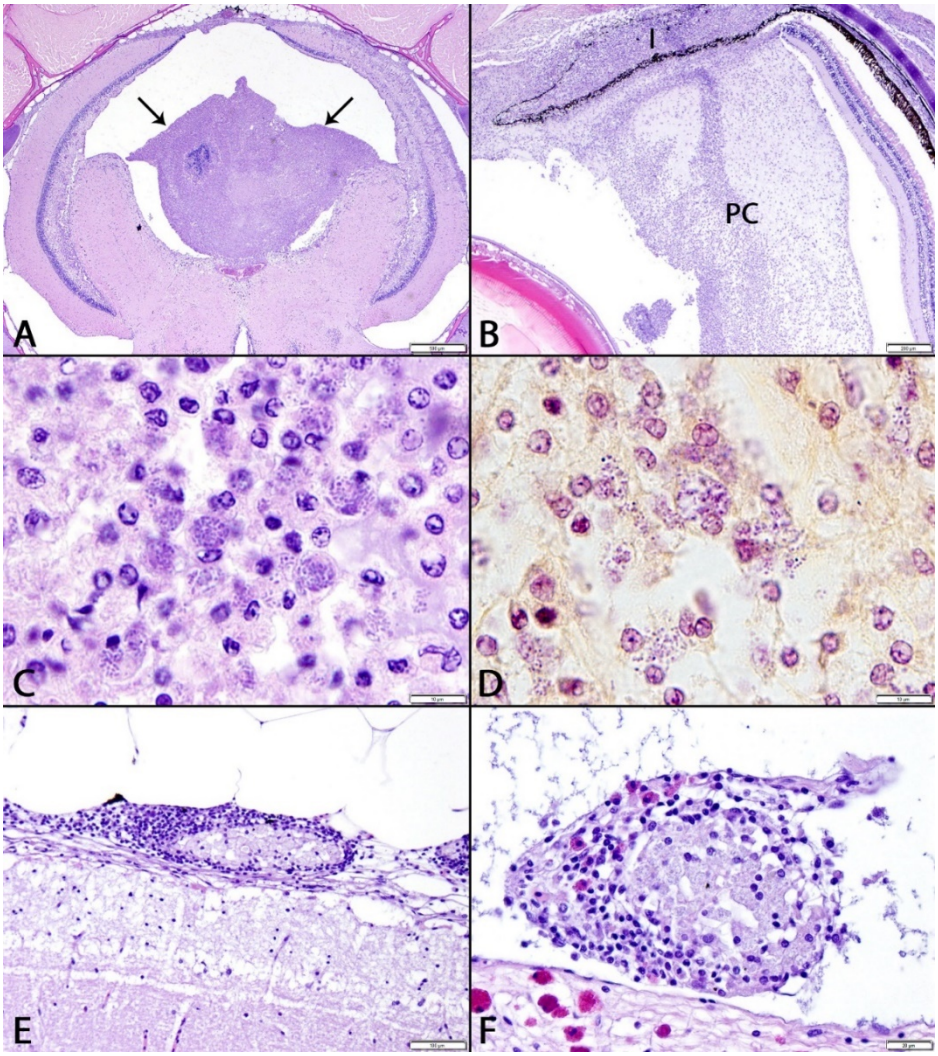

Figure 2.7: Histologic sections of *Streptoccoccus iniae* isolate LSU 10-070 (Clade E) induced lesions in Nile tilapia following intragastric challenge. A) Brain from moribund fish with granulomatous meningitis and inflammatory infiltrate within the third ventricle (arrows) below

the optic tectum (H&E; Bar = 500 μm). B) Eye with heavy infiltration of the iris (I) and posterior chamber (PC) by inflammatory cell infiltrate (H&E; Bar = 200 μm). C) High magnification image of inflammatory infiltrate within the third ventricle containing macrophages with cytoplasm distended by bacterial cocci (H&E; Bar = 10 μm). D) Optic tectum of the brain infiltrated by macrophages laden with gram-positive cocci (Brown & Hopps; Bar = 10 μm). E) Developing meningeal granuloma in a 21-day post-challenge survivor. A central focus of degenerate macrophages is surrounded by a broad mantle of lymphocytes (H&E; Bar = 100 μm). F) Higher magnification image of developing granuloma bordered by lymphocytes and eosinophilic granular cells. The absence of bacteria in these chronic lesions suggests resolution on the infection (H&E; Bar = 20 μm).

4. Discussion

During the early emergence of *Streptococcus iniae* infections in fish and humans, efforts to define an effective identification and typing workflow system were complicated by similarities of *S. iniae* to other streptococcal species, and by the lack of commercial bacterial identification systems that included *S. iniae* in their databases [20,50,51]. Genetic techniques such as 16S rRNA sequencing [52,53], PFGE [19], and Rep-PCR [29] were developed to resolve this issue, but each approach has distinct advantages and limitations in their replicability and ability to differentiate closely related species and strains [54]. An alternative to these methods is multilocus sequence analysis (MLSA). MLSA characterizes bacterial isolates by using the concatenated sequence fragments of multiple “housekeeping” genes to determine phylogenetic relationships. It generates precise, reproducible data that can be compared to sequence data from other organisms through database query and is useful for analysis of strains recovered around the globe and identification of unknown isolates. The molecular markers chosen for MLSA should have low heterogeneity within a species or genotype but provide the highest amount of separation between species or genotypes [33]. The gene fragments chosen were largely homogenous between *S. iniae* isolates but were still able to differentiate groups similarly to established methods (Figure 2.1). Furthermore, this MLSA scheme was able to incorporate and differentiate divergent *Streptococcus* spp. including *Streptococcus pyogenes,* which is an almost

exclusively human pathogen (Figure 2.2) [55]. This gives this scheme broader applicability than typical species specific MLSA formats.

The 11 North American isolates used in the study were previously genotyped by an established Rep-PCR method that yielded four major clusters (Heckman et al. unpubl. data). These clusters were largely maintained in the MLSA maximum likelihood phylogeny, where there were five major clades consisting of 3 or more isolates in the intraspecific comparison tree (Figure 2.1). Clade D, containing marine isolates from Costa Rica and the Caribbean, maintained the same isolates from the Rep-PCR, while clades A, B, and E were expanded by additional isolates. White sturgeon isolate WS-6H, co-infected with Acipenserid herpesvirus 2, had previously grouped with the other sturgeon isolates from California by Rep-PCR, but formed its own lineage in the MLSA generated maximum likelihood tree (Figure 2.1). This distinction supports the use of MLSA over Rep-PCR as WS-6H has relevant differences from the other sturgeon isolates. The colony morphology of WS-6H differed from Clade B isolates, it had a lower density indicative of more capsulation, and it better resisted killing in whole blood (data not shown). Previous work also demonstrated that WS-6H has a different biochemical profile than other sturgeon isolates [56]. The remainder of the isolates fell into two different clades but showed similar patterns of virulence and no significant differences in the *in vitro* assays. These clades both contained isolates cultured from tilapia in the United States and their similarities may be related to a closer genetic relationship and host type. Clade C contained isolates from fish cultured in Israel and Australia. Unfortunately, isolates of clade C were unavailable at the time of study for inclusion into the phenotypic and challenge experiments, and were only utilized in the phylogenetic analyses.

Many of the *S. iniae* reference genomes used were generated by Silayeva et al. [6] in a study investigating the role of mutator strains in *S. iniae* epidemiology and evolution. The authors found six major clades, one lineage with two strains, and three lineages with a single strain. Our analysis incorporated isolates from each of these lineages and produced a maximum likelihood tree with some differences from the one generated by Silayeva et al. using non-recombinant core genome SNPs. Comparably, some clades were geographically diverse (C, E), while others displayed some degree of endemism (A, B). Both trees maintained the grouping of human isolates from Canada with tilapia cases from the USA (clade A vs E2 in [6]), the singular lineage of the striped snakehead isolate QMA0187 from Thailand, and the second Amazon river dolphin isolate QMA0141 from the USA (Figure 2.1). The remaining lineages, however, were either condensed into clade A or split into smaller lineages with only one or two members. Inclusion of an additional mutation repair gene into the analysis could potentially resolve these differences, but the reduced heterogeneity in the housekeeping genes compared to the whole genome could still be beneficial. "Mutator" strains like QMA0141 can have enormous branch lengths in whole genome analysis due to deleterious changes in their mutation repair genes, potentially distorting evolutionary evaluations. QMA0141 had the highest mutation rate among the isolates investigated by Silayeva et al. [6], although the pygmy hippo isolates used in this study had an even larger number of SNPs in the *mutS* fragment, as well as the other fragments investigated. This lends support to the hypothesized role of mutators in the jump to atypical hosts. It would be interesting to further characterize these isolates using whole genome and mutation rate analyses.

The importance of host type in streptococcal infection was demonstrated in the whole blood survival assay. The 11 representative North American isolates showed varying degrees of survival in blood from three commercially valuable fish species at biologically relevant

temperatures. All isolates survived to some degree in all blood types, but survival and replication was conspicuously higher in rainbow trout blood (Figure 2.3). Additionally, although isolates did not display the exact same relative patterns across host types, there were still trends related to phylogroup. Isolates from the marine clade D largely showed higher survival, while isolates in clade B were generally more susceptible to killing. Isolates in clades A and E shared similar levels of survival. Because blood from same fish was not used across trials, intragroup variability was significant in this assay and the isolate demonstrating the highest level of survival also varied between hosts. This speaks to the complicated nature of host-pathogen interactions, where virulence depends on multiple factors related to the microbe and its target species. It would be interesting to repeat the *in vivo* challenge in trout and sturgeon to determine whether mortality patterns differed from that in tilapia. While the complete array of pathogenic mechanisms used by *S. iniae* to initiate infection and produce disease remain unclear, establishment of bacteremia is crucial for the dissemination of bacteria from local sites of infection to target organs [41,42,49]. Differences in survival between isolates may be related to proposed virulence factors affecting resistance to innate immune clearance such as expression of a cell surface Fc binding factor [47], polysaccharide deacetylase [57], M-like protein [58] or extent of capsulation [42,59–61].

In addition to virulence factors involved in resistance to the host immune response, *S. iniae* and related streptococcal species express proteins that damage tissues directly or via the host inflammatory reaction [25,41,62–65]. Cytotoxicity of the different *S. iniae* isolates was investigated in a tilapia endothelial cell line by a colorimetric assay for LDH release. Cellular damage was significantly higher at 30°C compared to 20°C (Figure 2.4), supporting previous observations linking increased temperature to outbreaks of streptococcosis [66]. The trend may

be related to higher bacterial replication rates at 30°C, or to changes in virulence factor expression, which could enhance tissue invasion and subsequent cellular injury. However, further experimentation would be necessary to determine specific causes. At 30°C, the isolates displayed cytotoxicity patterns that mirrored their survival in whole blood, but with less intragroup variability. Isolates in marine clade D exhibited the greatest cytotoxicity, isolates in clades A and E were intermediate, and those in clade B showed no cytotoxic effects. One of the isolates representing this clade was from sturgeon, a cold or temperate water host. However, decreasing the assay temperature did not result in increased LDH release. In cell cultures challenged with clade B isolates, bacterial numbers approximated those of the other clades, but produced no microscopic damage to the cell monolayer.

Results of the *in vitro* assays suggested that marine clade D isolates would be the most virulent. Despite this, isolates from the intermediate *in vitro* phenotype (clades A and E) caused the highest mortality in the tilapia challenge (Figure 2.5). This discrepancy may be due to differences in the functional capabilities of an isolated cell type versus a fully immunocompetent animal. However, it is important to note that these isolates were originally collected from tilapia, suggesting certain isolates may be better adapted to cause disease in specific hosts. Additionally, most of the isolates that caused mortality had been recently passaged through tilapia, while isolates from clades B and D are of unknown passage number. Efforts to standardize passage number were not possible due to the inability of isolates in clade B to fulfill Koch's postulates when originally cultured [11]. Regardless, the challenge did demonstrate differences in virulence, and validated effectiveness of the intragastric challenge model for *S. iniae*.

In addition to investigations of *S. iniae* associated disease pathogenesis, isolates are used to model other streptococcal species pathogenic to humans [67], test dietary supplements such as

immunostimulants, probiotics and prebiotics for aquaculture [68,69], and to study fish immune responses [70,71]. Intraperitoneal injection is the most commonly used infection route for *in vivo* *S. iniae* studies, although it bypasses host protective barriers and may cause mortalities (up to 100%) inconsistent with natural infections [42,72]. Although transmission dynamics of *S. iniae* infection remain unresolved, evidence supports transmission through the consumption of contaminated tissue, including cannibalism of moribund or dead fish by tank mates [25,73]. As a result, gavage challenge may more closely approximate natural outbreaks of disease. Direct delivery of *S. iniae* by gavage was used successfully in a limited study by Perera et al. [72] and its effectiveness further validated in this investigation. Intragastric challenge proved to be a useful alternative and straightforward method for the delivery of a standardized bacterial dose by a biologically relevant exposure route. Overall, disease progression was consistent with previous reports [25,72,74]. Mortalities began within 72 h of gavage challenge and continued over the 21-day trial, reaching a ~45% maximum (Figure 2.5). Exophthalmia was the most commonly observed gross lesion (Figure 2.6), although most fish died without clinical signs, similar to the "acute" form of disease reported by Barnes and Owens [25]. Histologic evaluation revealed typical lesions of meningoencephalitis, panophthalmitis, and cellulitis with abundant intracellular cocci in moribund specimens. Some survivors were free of microscopic changes, suggesting infection had never occurred. In others, early granuloma formation in the absence of bacteria was associated with a shift in the inflammatory reaction from predominantly macrophages to lymphocytes and indicates resolution of the infection. The presence of granulomas is consistent with descriptions of chronic *S. iniae* induced lesions and suggests elimination of the infection [74,75].

MLSA characterization of a diverse panel of *S. iniae* isolates from wild and farmed fish species demonstrated a genetic basis for strain phenotype, and further validation of the protocol as a predictor of virulence is warranted. The MLSA scheme proved robust, and readily discriminated between *Streptococcus* spp. relevant to aquaculture and human health. Increasing the number of disparate isolates included in analyses, as well as the inclusion of additional nucleotide and genome sequences in public databases as they become available will increase the applicability of this genotyping method and expand understanding of *S. iniae* genetic diversity. In addition, intragastric challenge proved to be an effective and biologically relevant alternative to other challenge methods. Results from this study will be used to inform the design for future investigations by providing a diverse group of genetically profiled isolates of differing virulence.

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CHAPTER 3. *Streptococcus iniae* biofilm formation enhances environmental persistence and resistance to antimicrobials and disinfectants

Abstract

The globally distributed bacterium *Streptococcus iniae* is responsible for outbreaks of disease resulting in high mortality in a wide range of economically important freshwater and marine fish species. Despite the significance of *S. iniae*, our understanding of its transmission and infection dynamics remains incomplete. Biofilms are important for the survival and pathogenesis of many bacteria, but there is a paucity of information on their role in the ex-host persistence of *S. iniae*. This study aimed to compare biofilm formation by isolates representing different *S. iniae* genotypes and to investigate the effect of biofilm formation on environmental persistence and resistance to common disinfectants and antimicrobials. Eleven clinical isolates of *S. iniae* representing 4 distinct genetic groups and diverse host types were assessed for their ability to form biofilms. Planktonic bacteria or mature biofilms were exposed to in vitro aquatic microcosms of different temperatures to quantify the number of culturable bacteria in each system over time. The minimum biofilm eradication concentration (MBEC) assay® system was used to determine biofilm resistance to 18 antimicrobials and 4 disinfectants commonly used in food producing animals and aquaculture, respectively. All isolates formed biofilms within 72 h. Bacteria remained culturable notably longer in the biofilm form compared to the planktonic, with a significant impact from temperature and salinity ($p < 0.05$). The MBEC was higher than the planktonic minimal inhibitory concentration (MIC) for at least one isolate in 15 out of the 18 antimicrobials tested. The MBEC was also higher than the minimum biocidal concentration (MBC) for 11 out of 18 tested, including oxytetracycline and florfenicol, the two most common antimicrobials used against *S. iniae* infections in fish. While both forms were susceptible to

disinfection by bleach, hydrogen peroxide and Virkon® Aquatic, treatment with povidone-iodine did not eliminate biofilms. The ability of *S. iniae* to form resilient biofilms provides an effective mechanism for their persistence in the environment, which must be considered and further researched to control this widespread pathogen.

1. Introduction

Streptococcosis, caused by the gram-positive bacterium *Streptococcous iniae,* is a prevalent disease worldwide in both farmed and wild fish, causing outbreaks of high mortality in a wide range of species and costing the aquaculture industry hundreds of millions in annual losses [1–3]. Streptococcosis is also considered to be a re-emerging disease, as the already extensive list of hosts and countries impacted continues to increase [4–6]. While streptococcosis has historically been associated with warmwater systems [7,8], *S. iniae* infections are not constrained to species reared in higher temperatures. *Streptococcus iniae* has been isolated from fish or the surrounding environment in warm, temperate and cold-water conditions, and in fresh, brackish and marine habitats [4–6,9–13]. Vaccination efforts against *S. iniae* have been met with mixed success, and current approaches to control the pathogen depend heavily on antimicrobials [6,14–16]. This reliance on antimicrobial administration, however, is complicated by issues of persistent or recurring outbreaks and concerns of developing antibiotic resistance [16–21]. The isolation of *S. iniae* from hosts or aquatic systems in a range of temperatures and salinities [22–25], along with issues of recurrent infection following disinfection or antimicrobial treatment [16–18], indicate the need for a more comprehensive understanding of *S. iniae* ecology.

Biofilms are well understood to be important for the persistence and pathogenesis of a range of bacteria and bacterial infections [26]. These ubiquitous communities of microbes embedded in adherent extracellular matrices, play an important and potentially dichotomous role in

aquaculture. Nutrient cycling by biofilm bacteria can reduce levels of excess nutrients and toxic compounds, improving water quality and reducing production costs [27,28]. However, biofilms may also act as a reservoir for infectious organisms, protecting and harboring them to increase the possibility of recurring diseases and development of antimicrobial resistance [28–30].

There is an extreme paucity of information on biofilm formation by *S. iniae* and its significance to the aquaculture industry. Our understanding is restricted to a small handful of studies investigating the ability of isolates to form biofilms as a predictor of virulence, with no investigation into the actual effect of the biofilm in transmission or infection [31–34]. Biofilm formation by other streptococcal species can lead to increased resistance of the bacteria to antimicrobials, disinfectants, host defenses, desiccation, and further stressors, protecting and preserving them in the host and environment [35,36]. It is reasonable to expect similar trends for *S. iniae,* but as the extent, composition, and behavior of biofilm formation differ between streptococcal species and strains, a targeted investigation is necessary to understand their role for this broad-base pathogen [37,38]. In this study we begin to elucidate the poorly understood process and function of biofilm formation in the ex-host persistence of *S. iniae* and provide guidance on improved therapeutic and disinfection guidelines with direct applications in aquaculture.

2. Materials and Methods

2.1 Bacterial strains

Eleven clinical isolates of *S. iniae* from the North American continent were used in this study, representing 4 distinct genetic groups and diverse host types (Table 3.1) [5,39]. Isolates were stored in 1 mL aliquots in brain heart infusion broth (BHI; MP biomedicals) with 20% glycerol at -80°C. Before each assay, isolates revived from frozen stock were grown at 30°C for

48 h on trypticase soy agar supplemented with 5% sheep's blood (SBA; University of California, Biological Media Services). A 0.5 McFarland standard (~1.5 x 10^8 CFU mL^{-1}) corresponded to a bacterial suspension in phosphate-buffered saline (PBS) with an optical density measurement of 0.14-0.155 at 600 nm, read on a UV/Vis photometer (BioPhotometer Plus, Eppendorf AG).

Table 3.1: *Streptococcus iniae* isolates used in this study. NECB and SECB stand for Northern and Southern Caribbean basin respectively. FW and MW stand for fresh and marine water respectively. Clade denotation was determined by MLSA analysis [5]

Strain	**Source**		**Isolation environment**	**MLSA Clade**
LSU 94-034	Tilapia (*Oreochromis* sp.)	Massachusetts	FW	A
LSU 96-525	Tilapia (*Oreochromis* sp.)	Iowa	FW	A
LSU 94-036	Tilapia (*Oreochromis* sp.)	Illinois	FW	A
F15-4-3	Tilapia (*Oreochromis* sp.)	California	FW	B
WS-6B	White sturgeon (*Acipenser transmontanus*)	California	FW	B
ECO86-17	Spotted rose snapper (*Lutjanus guttatus*)	Costa Rica	MW	D
K08-409H	Reef fish	NECB	MW	D
B8	Reef fish	SECB	MW	D
ARK PB 03-62B	Albino rainbow shark (*Epalzeorhynchos frenatum*)	Florida	FW	E
LSU 01-105	Tilapia (*Oreochromis* sp.)	Minnesota	FW	E
LSU 10-070	Tilapia (*Oreochromis* sp.)	Florida	FW	E

2.2 Biofilm formation assay

The extent and kinetics of *in vitro* biofilm formation by the *S. iniae* isolates were compared using the MBEC Assay® system, a small-volume, high-throughput assay for cultivating biofilms [40]. Biofilms were formed using an inoculator with a 96-well base and hydroxyapatite coated pegs (Innovotech Inc, Edmonton, Canada) following methods adapted from the manufacturer protocol [41]. A 0.5 McFarland solution was generated for each bacterial isolate from 48 h cultures on SBA and solutions diluted to ~1.5×10^5 CFU mL^{-1} in sterile BHI. Aliquots of 150

µL diluted inoculum or sterile media controls were added to the respective wells of the MBEC™ biofilm inoculator. Outer wells were filled with 200 µL of sterile double distilled water (DD H_2O). The plates were covered with the pegged lid and incubated at 30°C with shaking (110 rpm). Pegs were exposed to inoculated media or sterile media controls for 24, 48 or 72 h before they were collected to quantify biofilm-associated bacteria. At each time point, respective pegs were rinsed in sterile PBS for 10 s to remove non-adherent bacteria, then broken off with flame sterilized pliers for transfer to 200 µL sterile PBS in a round bottom 96-well plate. Plates were sonicated on high for 30 m to dislodge biofilms into suspension. Suspensions were serially diluted, and 10 µL spot-plated in triplicate to quantify live, biofilm associated bacteria for each strain at each time point. All subsequent sonification and quantification steps follow this method. Each isolate was tested in triplicate, repeated in two independent experiments.

2.3 Persistence assay

The persistence of *S. iniae* in the planktonic or biofilm state was investigated by comparing how long culturable organisms are recovered from *in vitro* aquatic micro-systems (microcosms). Methods were adapted from Soto and Revan [42]. Freshwater (FW) was collected from the Center for Aquatic Biology and Aquaculture at the University of California, Davis in July (2018) and February (2019). Marine water (MW) was collected from Spud Harbor near the Bodega Marine Laboratory in Bodega Bay, California in July (2018) and March (2019). Water was filter sterilized through a 0.2 µm filter (Millipore Sigma, USA).

2.3.1 Planktonic persistence

A 0.5 McFarland was generated for each isolate from 48 h culture on SBA and 2.5 µL of each suspension transferred to 3 replicate tubes of 25 mL FW or MW for a starting concentration of ~1.5×10^4 CFU mL^{-1}. Tubes were vortexed and 200 µL immediately transferred to a 96-well

plate. Suspensions were serially diluted, and spot-plated on SBA to confirm inoculum. Tubes of inoculated water were incubated at 20, 25, or 30°C and thoroughly vortexed on collection days to prevent stable biofilm formation. At days 1, 3, 5 and 7 post inoculation, 200 µL of water was collected from each tube, serially diluted and spot-plated to quantify the number of viable, culturable organisms present in each microcosm. Log survival was calculated by dividing the average CFU mL^{-1} by the starting concentration and finding the Log_{10} of the quotient. Experiments were repeated twice, in technical triplicate.

2.3.2 Biofilm persistence

Biofilms were formed for each isolate using the MBEC Assay® Biofilm Inoculator as above. Plates were incubated with shaking at 30°C for 72 h to allow formation of mature biofilms. The pegged lid with associated biofilms was rinsed in sterile PBS and transferred to a 96-well plate containing 200 µL of FW or MW for a starting concentration of ~1.5×10^4 CFU mL^{-1}. Pegs incubated in sterile media were used for sterility controls (SC). A subset of pegs was immediately collected, transferred to 200 µL sterile PBS for sonication, and serially diluted and spot-plated to confirm starting concentration. Plates were then incubated at 20, 25, or 30°C. At 1, 2, and 3 weeks post inoculation, respective pegs were collected and culturable organisms remaining quantified as above (Figure 3.1). Log survival was calculated by dividing the recovered CFU mL^{-1} by the averaged starting concentration for each isolate and finding the Log_{10} of the quotient. Experiments were repeated twice with two pegs per time point per isolate.

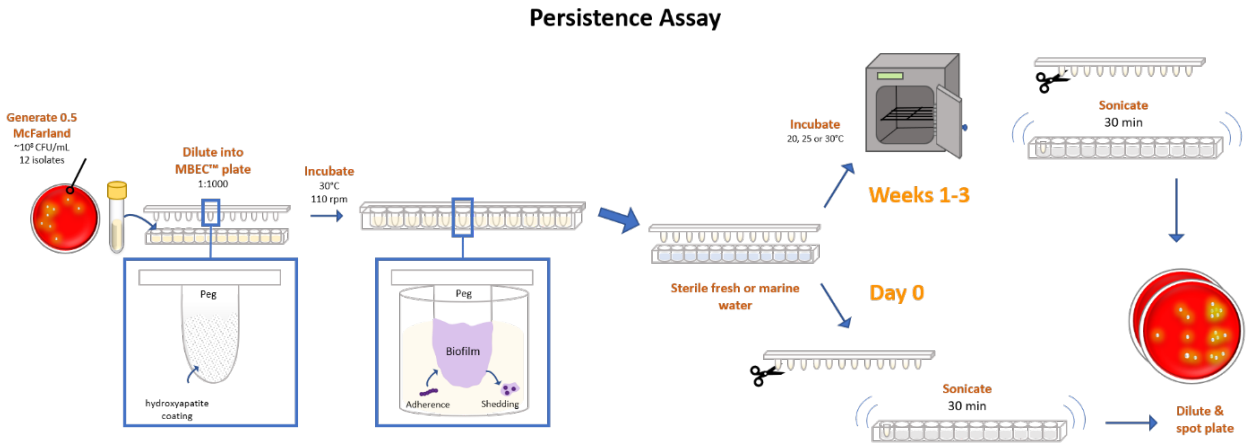

Figure 3.1: Experimental process for biofilm formation and persistence assays using the MBEC Assay®. Biofilms form on pegs when bacteria adhere to the surface under shear stress. Mature biofilms with adhered bacteria are rinsed and transferred into sterile wells of water and incubated. At each collection interval, pegs are transferred to a new 96-well plate with flame sterilized pliers and sonicated to dislodge biofilms into PBS for quantification. The figure is modified from the MBEC assay procedural manual, version 2.0 [41].

2.4 Disinfectant Resistance Assay

The resistance of *S. iniae* biofilms to common aquaculture disinfectants was investigated using an adapted version of the MBEC Assay® manufacturer procedure (Figure 3.2). Briefly, mature biofilms were formed as before. A subset of pegs was immediately removed and quantified as a biofilm growth check (BGC). The lid with remaining pegs was then transferred into a round-bottom 96-well "challenge" plate containing either sodium hypochlorite (bleach), povidone-iodine (Ovadine®), hydrogen peroxide, or Virkon® Aquatic, diluted in DD H_2O to concentrations recommended for disinfection in aquaculture (Table 3.2) [43]. Pegs exposed only to media were used as SC. Pegs with biofilms exposed only to sterile DD H_2O were used as growth controls (GC).

Table 3.2: Treatment conditions for testing biofilm susceptibility to common disinfectants selected from recommended protocols in aquaculture [43].

Disinfectant	**Active ingredient**	**Concentration**	**Contact time (m)**
Bleach	Sodium hypochlorite	200 mg L^{-1} available chlorine	30
Ovadine®	Povidone-iodine	50 mg L^{-1} free iodine	30
Virkon® Aquatic	21.4% potassium peroxymonosulfate 1.5% sodium chloride	10 g L^{-1}	15
Hydrogen peroxide solution	Hydrogen peroxide	3% H_2O_2	15

The biofilms were exposed for the recommended contact time then transferred to a "recovery plate" containing 200 µL sterile PBS. A 20 µL sample from the challenge plate was added to 180 µL BHI in a 96-well minimum bactericidal concentration (MBC) plate to determine biocidal activity of the disinfectant against planktonic bacteria. After 30 minutes incubation at room temperature, the recovery plate was sonicated. One hundred microliters of the suspension were transferred to a new 96-well "log recovery" plate and replaced with 100 µL BHI to generate the minimum biofilm eradication concentration (MBEC) plate. The suspensions in the log recovery plate were serially diluted and spot-plated to quantify remaining viable bacteria. Spot plates, MBC and MBEC plates were incubated at 30°C and checked for growth at 24 and 72 h. Absence of visible growth in the MBC or MBEC plates or colony growth on the SBA after 72 h indicated full susceptibility to the treatment. Experiments were repeated twice, in technical duplicate.

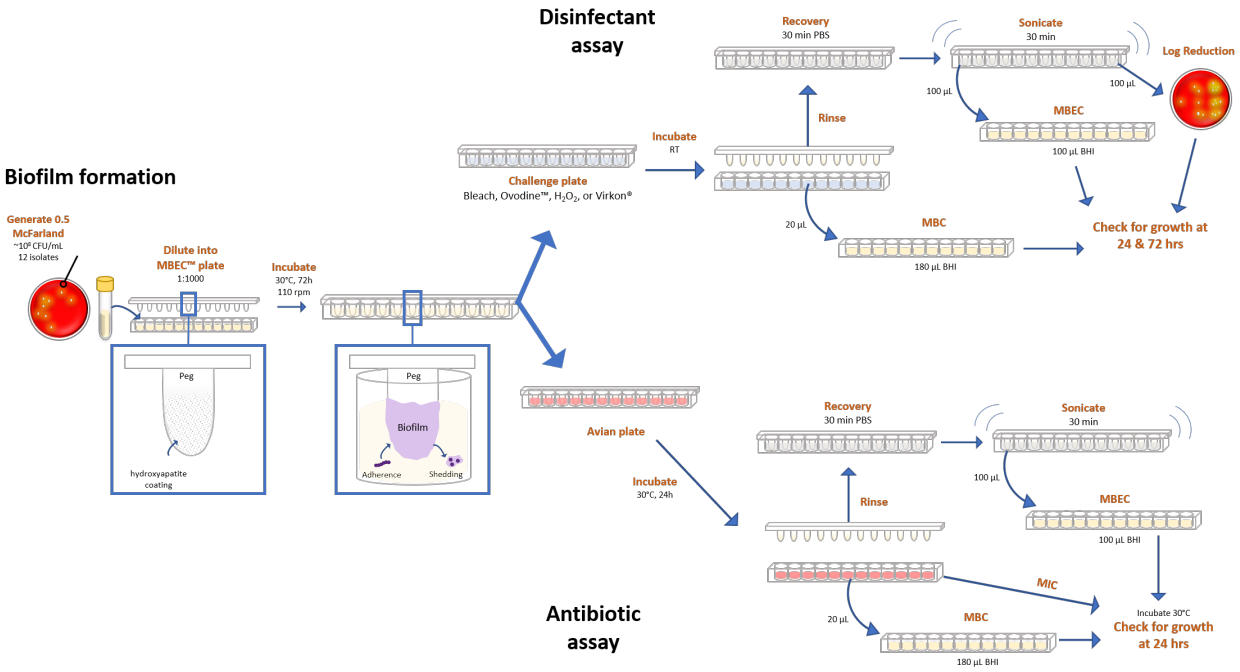

Figure 3.2: Experimental process for biofilm formation and susceptibility testing using the MBEC Assay®. Biofilms form on pegs when bacteria adhere to the surface under shear stress. Mature biofilms with adhered bacteria are rinsed and transferred into wells containing different types of antimicrobials. After exposure for a given time, the pegged lid is transferred to a recovery plate of PBS for sonication and quantification of remaining biofilm bacteria. Dispersed cells shed from the surface of the biofilm and serve as an inoculum for MIC and MBC determination. Figure adapted from the MBEC assay procedural manual, version 2.0 [41].

2.5 Antimicrobial Resistance Assay

The resistance of *S. iniae* biofilms to common antimicrobials was investigated using the MBEC Assay® and the Sensititre™ Avian AVIAN1F AST Plate (Thermo Fisher Scientific, USA) with some modifications (Figure 3.2). Avian plate antimicrobials were resuspended in 200 µL of cation-adjusted Mueller-Hinton broth with lysed horse blood (CAMHB; Thermo Fisher Scientific, USA) and transferred to a round bottom 96-well challenge plate. Mature biofilms for one randomly selected isolate per genogroup were formed as above, and a subset of pegs removed for BGC. The lid with remaining pegs was then transferred to the challenge plate for 24 h incubation at 30°C. The SC and GC pegs were exposed only to sterile CAMHB. After the

challenge, the pegged lid was transferred to the recovery plate containing 200 µL sterile PBS. A 20 µL sample from the challenge plate was added to 180 µL fresh CAMHB in a 96-well MBC plate. After 30 minutes incubation at room temperature, the recovery plate was sonicated, and 100 µL of suspension transferred into 100 µL fresh MHB in the MBEC plate. The challenge minimal inhibitory concentration (MIC), MBC, and MBEC plates were incubated at 30°C. The MIC, MBC, and MBEC for the different antibiotics were determined by absence of bacterial growth in the relevant wells after 24h incubation. The experiment was repeated twice for each representative isolate.

2.6 Statistics

Statistical tests were performed using GraphPad Prism (version 9.0.0, GraphPad Software, La Jolla, CA USA). Repeated measure two-way ANOVA or a Mixed-effect model with the Geisser-Greenhouse correction were used to determine significance. Tukey's multiple comparison test was used to compare biofilm formation between clades and the overall impact of temperature on planktonic and biofilm persistence for all isolates. Šidák's multiple comparisons test was used to compare planktonic and biofilm persistence in fresh versus marine water for all isolates. Individual variances were computed for each comparison.

3. Results

3.1 Biofilm Formation by *S. iniae*

All tested isolates were able to form biofilms using the MBEC™ system. Biofilm formation rate and extent differed by clade (Figure 3.3). At 24 and 48 hours the clade E isolates had formed significantly denser biofilms ($p < 0.05$). The clade A and D isolates reached similar concentrations of biofilm associated bacteria by 72 hours. Clade B isolates were less productive

biofilm formers and plateaued at significantly lower concentrations, even after 72 hours of incubation ($p < 0.05$).

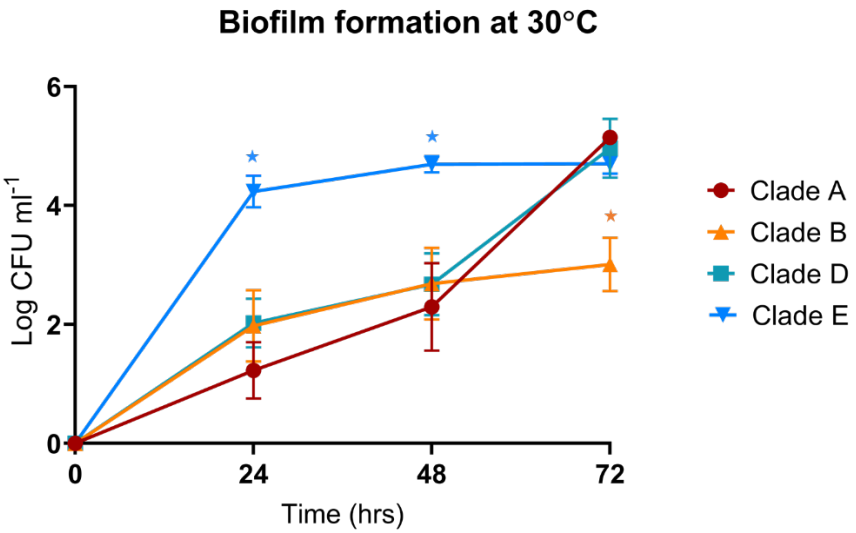

Figure 3.3: Biofilm formation presented in Log Colony Forming Units (CFU) per mL at 30°C of *Streptococcus iniae* isolates in brain heart infusion broth using the MBEC® system. A star designates statistical significance ($p < 0.0001$) for the clade of corresponding color, determined by a Mixed-effects model with the Geisser-Greenhouse correction and post-hoc Tukey's multiple comparison tests. Isolates are grouped by clade as determined by MLSA [5]. Experiments were preformed twice in technical triplicate. Error bars represent standard error.

3.2 Planktonic persistence

Streptococcus iniae does not persist long in the aquatic environment as planktonic, culturable bacteria. None of the isolates were recovered on SBA after a period of 5 days post inoculation under any tested condition (Figure 3.4). The persistence of isolates within that duration, however, was significantly impacted by temperature and salinity ($p < 0.05$). There was a trend of decreasing survival in increasing temperatures in marine water, and overall, survival in both marine and freshwater was lowest at 30°C ($p < 0.05$). Most isolates were unculturable after 24h at this incubation temperature. Higher salinity also supported longer periods of survival. The isolates persisted longest in marine water (5 days) incubated at 20°C compared to the maximum of 3 days reached in freshwater at any temperature ($p < 0.05$).

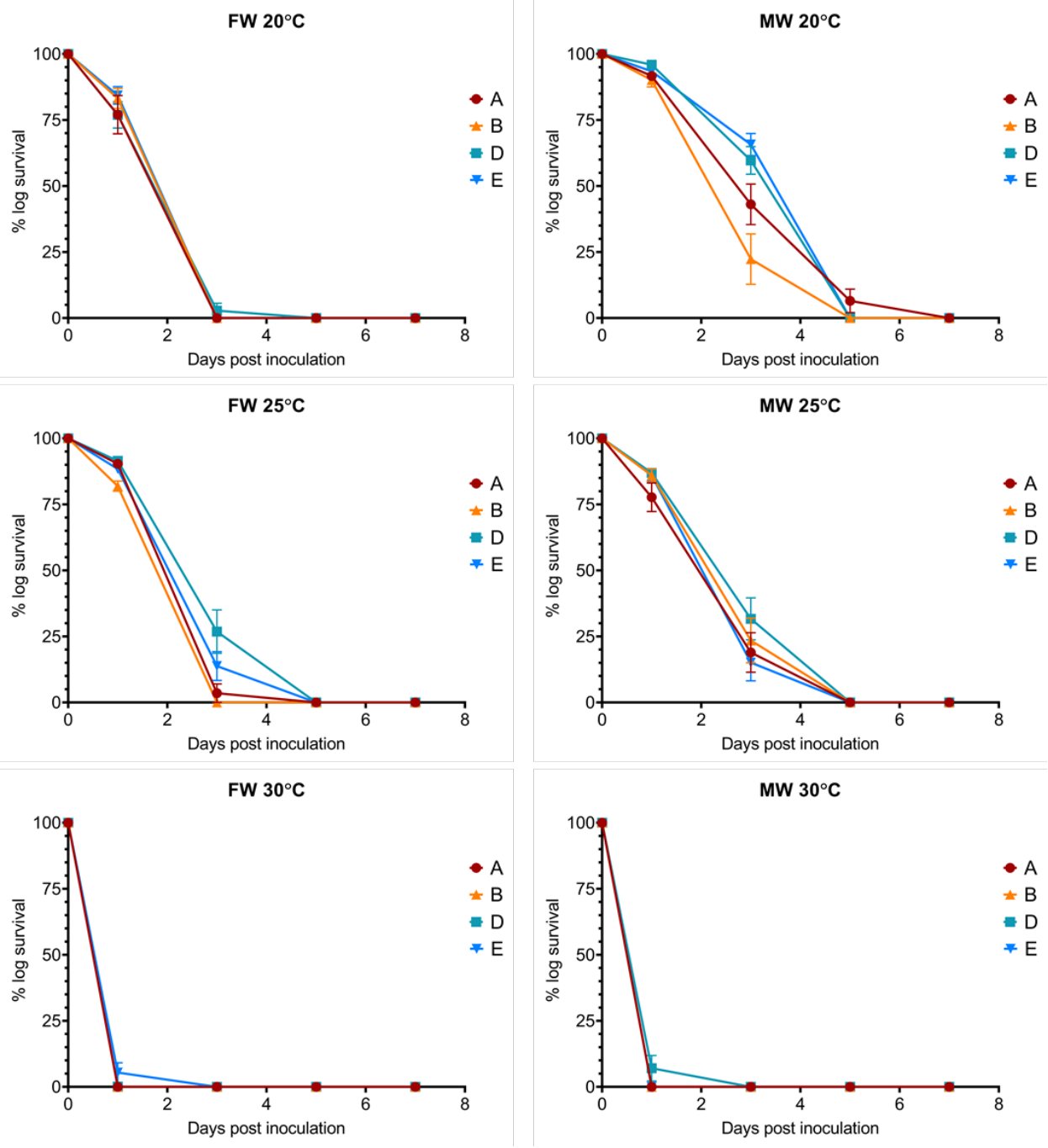

Figure 3.4: Percent log survival of *Streptococcus iniae* isolates in the planktonic form after suspension in filtered fresh or marine water. Isolates are grouped by clade as determined by MLSA [5]. Experiments were performed twice in technical triplicate. Error bars represent standard error.

3.3 Biofilm persistence

Streptococcus iniae's environmental persistence was greatly enhanced in the biofilm form (Figure 3.5). While bacteria in the planktonic form survived only to a maximum of 5 days, culturable bacteria continued to be recovered from biofilm pegs 3 weeks post-inoculation in all conditions tested. Biofilm persistence was similarly negatively correlated to temperature, with lower levels of bacteria persisting in warmer water. The pegs incubated in FW at 30°C had significantly fewer surviving bacteria at week 1 compared to 20°C ($p < 0.05$) as well as to 25°C in weeks 2 and 3 ($p < 0.05$). The numbers recovered for the 20°C and 25°C incubations were not significantly different. In marine water, incubation temperatures led to significant differences at weeks 2 and 3 – where 20°C conditions had higher numbers than both 25°C ($p < 0.0005$) and 30°C ($p < 0.0001$). The biofilms incubated at 25°C also had higher quantities of culturable bacteria than those at 30°C in weeks 2 and 3 ($p < 0.05$). Salinity played a role in biofilm persistence as well, though to the opposite effect of that observed in planktonic persistence. At 20°C both forms had high survival, and biofilms persisted in similar quantities regardless of salinity of microcosm. At 25°C and 30°C, however, there was overall higher persistence in the freshwater systems at weeks 2 ($p < 0.0001$) and 3 ($p < 0.05$). No evident trends in persistence between clades or between isolates was observed.

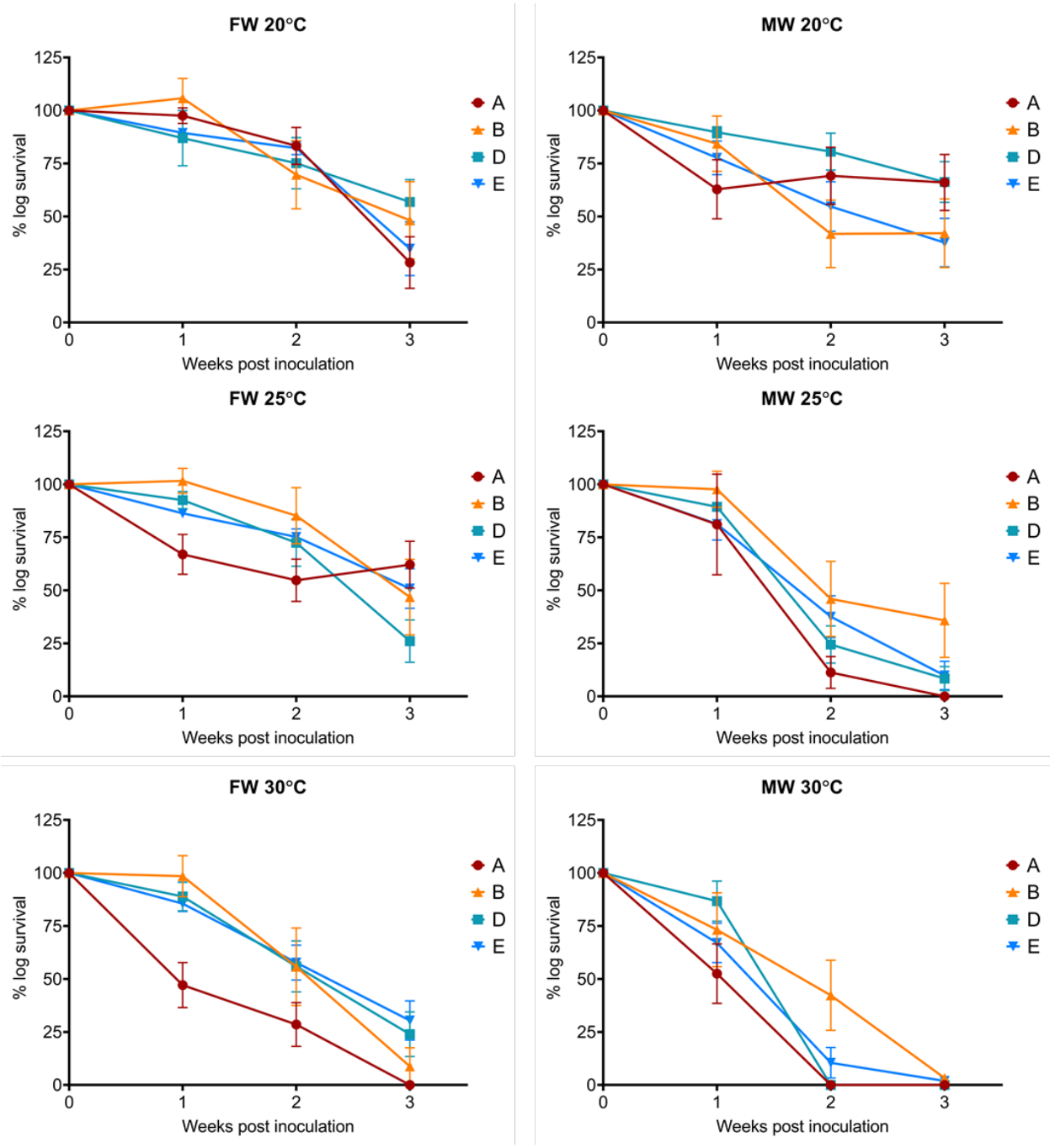

Figure 3.5: Percent log survival of *Streptococcus iniae* isolates in the biofilm form after submersion in filtered fresh (FW) or marine (MW) water at different temperatures. Isolates are grouped by clade as determined by MLSA [5]. Experiments were preformed twice in technical duplicate. Error bars represent standard error.

3.4 Susceptibility to common disinfectants

All disinfectants tested (Table 3.2) were sufficient to kill planktonic forms of all isolates, as determined by lack of growth in the challenge wells of the MBC plates. The treatments using Virkon® and hydrogen peroxide also completely eradicated all isolate biofilms. Bleach was mostly effective against biofilms as well, though growth of a few colonies on the log reduction plate for isolate LSU 96-034 was observed in one trial (Figure 3.6). Ovadine® (povidone-iodine) at the recommended concentration and exposure time was not effective for the eradication of *S. iniae* biofilms (Figure 3.6). Only biofilms from the weak clade B biofilm former WS-6B were completely eliminated. For the remaining 10 isolates, the number of viable bacteria was merely reduced. The second clade B isolate F15-4-3 had the highest percent log reduction (72.73%), compared to the most resistant isolate clade E isolate ARK PB03-62B (29.15%).

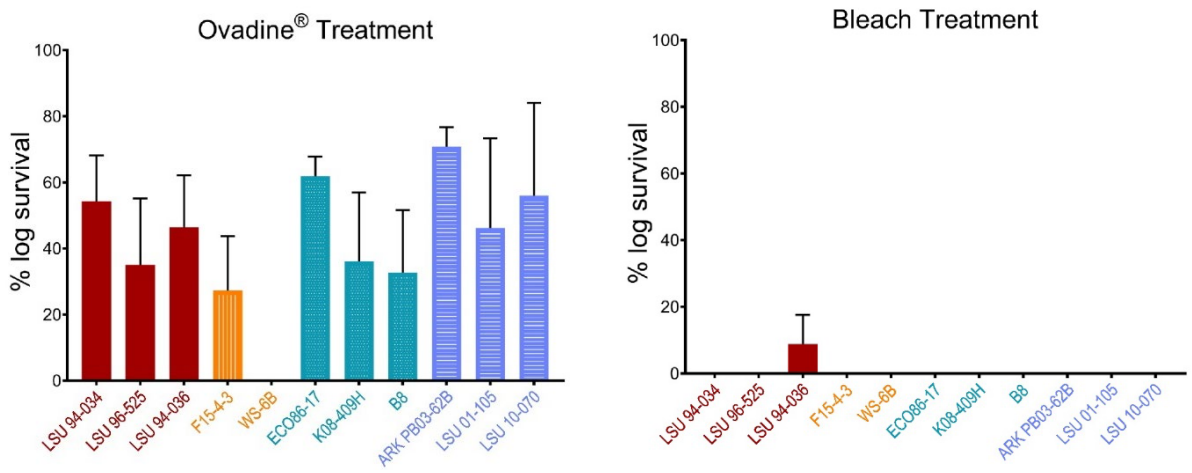

Figure 3.6: Percent log survival of *Streptococcus iniae* isolates in the biofilm form after challenge with the recommended treatment of Ovadine® (left) or bleach (right). Experiments were repeated twice in technical duplicate. Error bars represent standard error. Color and patterning reflect isolate phylogroup as determined by Heckman et al. [5]: Clade A: solid red; Clade B: orange with vertical stripes; Clade D: teal with dots; Clade E: blue with horizontal stripes.

3.5 Susceptibility to antimicrobials

Biofilm associated *S. iniae* demonstrated reduced sensitivity to most tested antimicrobials when compared to bacteria in the planktonic form (Table 3.3). The minimum concentration required to eradicate biofilms, or MBEC, was higher than the concentration required to inhibit planktonic cells (MIC) in at least one isolate for 15 out of 18 tested antibiotics. It was also higher than the concentration required to completely kill planktonic cells (MBC) for 11 out of 18, including oxytetracycline and florfenicol. There was no difference in the MIC, MBC and MBEC for three of the tested antibiotics: gentamicin, neomycin, and sulphadimethoxine. For gentamicin and neomycin, the MIC, MBC and MBEC were all greater than the tested concentration range in all isolates. The MIC, MBC and MBEC were all greater than the tested concentration range in 3 out of 4 isolates for sulphadimethoxine as well.

Table 3.3: Minimum antimicrobial concentrations in µg mL^{-1} to inhibit (MIC) or kill (MBC) planktonic bacteria or eradicate *S. iniae* biofilms (MBEC). Row shading indicates a difference in susceptibility between the planktonic and biofilm forms. Light blue indicates a higher MBEC than MIC, darker blue indicates the MBEC was higher than both MIC and MBC. Experiments were repeated twice for each isolate, data from the first trial is shown, similar results were obtained in the second trial. FDA approved antibiotics for treatment of *S. iniae* in aquaculture are in bold. Asterisks indicate treatments where MIC, MBC and MBEC were all above the tested concentration range.

	LSU 94-036 (A)			F15-4-3 (B)			B8 (D)			LSU 01-105 (E)		
Antimicrobials	**MIC**	**MBC**	**MBEC**	**MIC**	**MBC**	**MBEC**	**MIC**	**MBC**	**MBEC**	**MIC**	**MBC**	**MBEC**
Erythromycin	0.25	0.5	>1	0.0625	0.5	1	0.25	0.25	1	0.125	0.5	>1
Clindamycin	≤0.125	≤0.125	0.25	≤0.125	0.25	1	≤0.125	≤0.125	1	≤0.125	0.5	1
Penicillin	0.0625	0.125	0.125	≤0.015	≤0.015	0.0625	≤0.015	0.0625	0.25	≤0.015	0.03	0.0625
Amoxicillin	≤0.0625	≤0.0625	≤0.0625	≤0.0625	0.125	1	≤0.0625	0.25	1	≤0.0625	0.25	1
Tylosin tartrate	≤0.625	≤0.625	≤0.625	≤0.625	1.25	2.5	≤0.625	≤0.625	1.25	≤0.625	1.25	2.5
Trimethoprim/ sulfamethoxazole	≤0.13/2.38	0.5/9.5	>0.5/9.5	0.25/4.75	0.5/9.5	>0.5/9.5	0.5/9.5	>0.5/9.5	>0.5/9.5	≤0.13/2.38	>0.5/9.5	>0.5/9.5
Ceftiofur	0.125	0.5	0.5	0.125	0.25	1	0.125	0.25	0.125	0.125	1	>1
Oxytetracycline	1	1	>2	1	>2	>2	0.5	2	>2	>2	>2	>2
Florfenicol	2	>2	>2	2	>2	>2	2	2	>2	2	>2	>2
Enrofloxacin	0.25	0.5	0.5	0.25	0.25	0.5	>0.5	>0.5	>0.5	0.5	>0.5	>0.5
Streptomycin	32	32	32	32	32	32	32	32	32	32	32	64
Spectinomycin	16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
Sulphathiazole	64	>64	>64	>64	>64	>64	>64	>64	>64	64	>64	>64
Tetracycline	>2	>2	>2	>2	>2	>2	0.5	2	2	>2	>2	>2
Novobiocin	>1	>1	>1	1	>1	>1	>1	>1	>1	>1	>1	>1
Gentamicin*	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2
Neomycin*	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
Sulphadimethoxine	>64	>64	>64	64	64	64	>64	>64	>64	>64	>64	>64

4. Discussion

The ability to form biofilms is a near universal attribute of bacteria, affording them unique emergent properties that cannot be predicted by the study of planktonic cells [26]. While the importance of biofilms to human disease has received a great deal of attention, there is still a significant lack of research into their formation by animal pathogens like *S. iniae* [44]. In this study, all *S. iniae* isolates formed appreciable biofilms under the testing conditions, though rate and extent of formation differed between clades. The Clade E isolates formed dense biofilms

within 24 hours of incubation. Clade A and D isolates reached similar densities within 72 h, but Clade B isolate biofilms remained at a lower concentration of bacteria throughout the duration of the experiment (Figure 3.3). This reduced level of biofilm associated bacteria was somewhat surprising, as Clade B consists of unencapsulated isolates [5]. Previous investigation of biofilm formation by *S. iniae* indicated unencapsulated strains may form denser and thicker biofilms [33]. It is possible this discrepancy is due to the different experiemental systems used to measure biofilm development. Biofilm formation is impacted by the characteristics of the substrate and aqueous medium, such as surface roughness or pH and temperature [45]. Additionally, the MBEC™ system as used in this study quantifies viable adherent bacteria, compared to the crystal violet visualization techniques that look at the overall structure of the biofilm matrix. Differences in clade biofilm growth may also be due to other virulence factors predicted to impact adherence or cell-cell interactions, such as the polysaccharide deacytelase Pdi or putative adhesins [46,47]. Capsulation has been found to have contrasting effects on biofilm development in other streptococcal species. It is in general thought to inhibit bacterial adherence and biofilm formation, as with *S. pneumoniae* and *S. suis* [48–50]. However, the capsule is a key component of biofilm formation for the more closely related *S. agalactiae,* and has either a positive, or negligible effect on *S. pyogenes* biofilm structure [51–53]. Comparison of isogenic mutants differing singularly in capsular expression will be necessary to determine its role for *S. iniae.*

Biofilm formation by *S. iniae* greatly enhanced the ex-host persistence of the bacteria in aquatic microcosms. The planktonic form of *S. iniae* did not persist longer than 5 days in a culturable state (Figure 3.4). In the biofilm state, persistence was extended to three weeks or longer (Figure 3.5). The experiment was terminated at this time, but in many cases, high concentrations of viable bacteria were still being recovered, indicating the full range of survival

may be much longer. The assay also only quantified bacteria that could be recovered on SBA. It is possible that live bacteria were still present in the viable, non-culturable state [54]. In both the planktonic and biofilm forms, isolates generally survived best at colder temperatures. Planktonic bacteria rapidly vanished from the systems at 30°C in both fresh and marine water, and biofilms incubated at 30°C had significantly fewer remaining bacteria within 2 weeks of inoculation. The impact of temperature on persistence is interesting because of the association between warmer temperatures and more severe outbreaks of streptoccosis [22]. Warmer temperatures have also been shown increase biofilm formation in industrial and natural water systems [55–57]. The impact of temperature on biofilm development by *S. iniae,* in addition to environmental persistence, pathogen virulence and host response is complex, and should be investigated further to understand how these factors interplay. The role of salinity in persistence was somewhat contradictory. Marine water slightly improved planktonic persistence, while biofilms generally persisted longer in freshwater (Figures 3.4, 3.5). The *S. iniae* isolates used in this study originated from 5 different fish hosts, including warm and cold-water species from fresh and marine water environments (Table 3.1), but isolate persistence was not consistently correlated with genetic clade or origin.

While it is impossible and inadmissable to keep water systems free of microbes, disinfection of equipment and tank components to reduce levels of opportunistic pathogens is crucial for preventing disease in farmed fish [28]. Bleach, povidone-iodine, hydrogen peroxide and commercial solutions like Virkon Aquatic® are common disinfectants applied to the this end. The treatment concentrations and exposure times recommended for use of these disinfectants in aquaculture were effective againts *S. iniae* in the planktonic form, but not all were sufficient to eradicate biofilms [43]. Ovadine® is a popular povidone-iodine solution used for sanitization of

fish eggs, equipment, and general use surfaces. At the recommended dosage and contact time it did not eliminate biofilms formed by 10/11 isolates (Figure 3.6). An alternative disinfectant, or potentially a higher concentration and exposure time is suggested for disinfection of surfaces where presence of *S. iniae* is suspected. If the biofilm is not eradicated, it could potentially persist, allowing reintroduction to a new host.

Biofilms are well known for their ability to protect microorganisms from antimicrobials [26,35,36]. This trend extends to *S. iniae,* as demonstrated by the reduced susceptibility of biofilms to a range of antimicrobials used in food animal medicine (Table 3.3). The minimum concentration required to eradicate biofilms was higher than the MIC for 15 of the 18 antimicrobials included on the Avian™ plates. For 11 of these antibiotics the MBEC was also higher than the MBC. It is possible that the biofilm may have increased resistence for gentamicin and neomycin, but for these antibiotics the MIC, MBC and MBEC were all above the tested concentration range. Additionally, the MIC and MBC values determined using the MBEC® assay are for dispersed or detached bacteria – cells shed from the biofilm that may have different characteristics to both the established biofilm and truly planktonic cells [58,59]. The MIC and MBC of planktonic cells may therefore be slightly different than the concentrations aquired by this method. The demonstrated survival of biofilm associated bacteria in contrast to dispersed, however, is still cause for concern. There are few antibiotics approved for use in aquaculture [60], and both of the antimicrobials employed against *S. iniae* - oxytetracycline and florfenicol – were less effective against the biofilm form. Incomplete elimination of a pathogen from a host or system after antimicrobial treatment can lead to costly series of medication, and increased opportunities for antibiotic resistence developing [58,61]. If antibiotic resistant organisms can proliferate in a biofilm, the risk for recurrent and hard to treat disease outbreaks escalates.

The ability of diverse isolates of *S. iniae* to form *in vitro* biofilms, as demonstrated by this and previous studies [31–34], as well as the increased tenacity of the biofilm-associated bacteria to persist and resist antimicrobials should be translated into the aquaculture setting. There are several possible situations where biofilm formation would play a crucial role in mitigation of streptococcosis. *Streptococcus iniae* may be able to form biofilms on the pipes, tubes and other abiotic surfaces of a tank, or integrate into the existing biofilms formed by other environmental microbes. The levels of bacteria recovered from cage and farm water systems during disease outbreaks [22] are comparable to the inoculation dose used in our assays (~10^5 CFU mL^{-1}), suggesting that concentrations of bacteria shed by sick and dead fish are sufficient to form community aggregates. *Streptococcus iniae* may also associate with the existing aquatic biofilms, which are generally complex communities of bacteria and other microorganisms [26,28,62]. This incorporation of fastidious pathogens has been observed with other bacterial species, allowing enhanced survival and longer persistence of infectious organisms in a system [44,45,63]. The documented natural and experimental co-infections of *S. iniae* with other bacteria, viruses, fungi, and parasites [39,64] make this possibility of intraspecies communities intriguing. Investigation into the community composition of biofilms formed on tank components during the course of infection would be valuable for elucidating environmental harbors of *S. iniae.*

Alternatively, or in addition to contributing to ex-host persistence, biofilms may play a role in the pathogenesis of *S. iniae*. The closely related streptococcal pathogen *S. agalactiae* was shown to form biofilms in the brain tissues and surrounding meningeal surfaces of tilapia. These biofilms increased bacterial resistence to host immune defenses and penicillin, while facilitating the passage of bacteria across the blood brain barrier, potentially through the “Trojan horse”

mechanism [65]. The brain is a target organ in *S. iniae* infection, and while the mechanisms of pentetration are not fully resolved, the Trojan horse effect has similarly been suggested [66]. In addition, the brain can remain infected even when the bacteria is cleared from other organs, as the humoral response is key for eliminating *S. iniae* and antibodies cannot easily penetrate the blood brain barrier [4,67,68]. Formation of an *in vivo* biofilm, then, may be an integral step in *S. iniae* infection and transmission, allowing the colonization and continued residence of the bacteria in the brain. Biofilms formed in the intestine [22] or bone [33] are also possibilities for in-host havens. Carrier fish, or those with chronic, localized infections may serve as reservoirs [4,67], while fish who sucumb to infection provide an ideal site for proliferation of the bacteria [25]. Tank mates may be exposed through cannibalism of such fish or through the high levels of bacteria released into the environment, leading to episodic outbreaks [22,69].

5. Conclusion

This study is the first to demonstrate the potential impact of *Streptococcus iniae* biofilms on the ecology and pathogenesis of this widespread pathogen. Biofilm formation capability appears conserved across genetic groups of *S. iniae,* and allows the bacteria to persist outside of the host for weeks. The biofilms are also resistant to a widely used disinfectant, Ovadine® and a range of antimicrobials, including oxytetracycline and florfenicol. Facilities acting to treat and prevent *S. iniae* outbreaks should consider the possibility of *S. iniae* biofilms when developing management strategies.

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CHAPTER 4. Development and efficacy of *Streptococcus iniae* live-attenuated vaccines in Nile tilapia, *Oreochromis niloticus*

Abstract

Streptococcus iniae is a re-emerging bacterial pathogen in freshwater and marine aquaculture worldwide. There are no commercial vaccines available for *S. iniae* in the United States, and autogenous vaccines are restricted to inactivated whole-cell preparations with limited protection against heterogenous strains. Live-attenuated vaccines (LAV) represent an advantageous alternative to these bacterins, as they induce robust cellular and humoral immunity, and may provide longer lasting protection through less stressful routes of administration. We investigated whether accumulation of mutations in *S. iniae* by serial passage in the presence of rifampin can generate immunogenic LAV conferring protection against challenge with heterologous wild-type (WT) *S. iniae* strains in Nile tilapia (*Oreochromis niloticus*). Three lineages of rifampin-resistant *S. iniae* strains were generated from three genetically distinct parent strains ($n = 9$) by multiple passages in increments of Rifamycin SV sodium salt. Growth in liquid media, extent of capsulation, antimicrobial susceptibility, survival in Nile tilapia whole blood, and cytotoxicity in an *O. mossambicus* endothelial cell line were compared between the passaged and WT strains. Nile tilapia challenges were used to assess strain virulence, generation of anti-*S. iniae* IgM, and the protection conferred by LAV candidates against virulent *S. iniae*. Rifampin-resistant strains demonstrated changes in growth rate and cytotoxicity in endothelial cells, as well as significant reductions in whole blood survival ($p < 0.05$). Selected strains also showed attenuated virulence in the Nile tilapia challenge model, and anti-*S. iniae* IgM generated against these strains demonstrated cross-reactivity against heterologous bacteria. Immunization by intracoelomic

injection induced protection against a virulent WT strain of *S. iniae,* with relative percent survival up to 95.05%.

1. Introduction

Aquaculture plays an increasingly important role in global fish supply, particularly of food fish, where contributions from aquaculture are projected to exceed those of capture fisheries before 2030 [1,2]. Sustainable expansion of the sector has been a challenge, and effective control of infectious disease continues to limit progress and production yields [2–4]. Vaccines provide safe, environmentally friendly options for protecting against pathogenic microbes. Their implementation, particularly in the salmon industry, has seen reductions in fish loss, antimicrobial usage, and associated costs [5,6]. However, there are many important fish disease agents for which effective vaccines remain elusive. Streptococcosis is an invasive disease impacting an extensive range of fish species, including many farmed species of economic relevance [7–12]. As such, it is estimated to cost the global aquaculture industry billions of dollars (US$) annually [12–14]. One of the main etiologic agents of piscine streptococcosis is *Streptococcus iniae*, a species of high intraspecific diversity, which has made devising a cross-protective vaccine difficult [7,8,15]. In the absence of effective prophylactic measures, antimicrobials are frequently used to control outbreaks of streptococcosis in farmed fish. This is a falliable approach, as sick fish often become anorectic. Orally delivered antimicrobials can result in treatment failure if outbreaks have progressed to the point of population inappetence, necessitating repeat treatments and increasing the potential for emergence of antimicrobial resistance [7,16–20].

Traditional inactivated whole-cell vaccines, or bacterins, have been implemented against *S. iniae,* but have not proved to be viable, long-term solutions. Bacterins administered large-scale

or locally can provide protection for a time, but vaccine escape is not uncommon [7,21–23]. This may be due to failure of the tested vaccines to fully eradicate the pathogen from the population, where the selective pressures afforded by the vaccine lead to takeover by serologically distinct strains [21,22]. Differences in capsular composition between strains appear to be largely responsible for this lack of heterologous protection [21,23,24]. Bacterins are also associated with weaker immune responses and faster subsidence of antibody titers [7,25]. In addition, they tend to induce poor activation of cellular immunity and are less effective against intracellular bacteria [25–27]. *Streptococcus iniae* has demonstrated a facultatively intracellular lifestyle, the degree of which can vary between strains [28–32], and previous studies have shown that protection against *S. iniae* may not be solely antibody driven [33–35]. To provide sufficient protection, future vaccines must address the significant diversity of *S. iniae* and trigger a longer lasting response of both cellular and humoral immunity.

A range of experimental vaccines have been developed to this end [36–43], but no commercial vaccine is available for piscine streptococcosis in the United States. Homologous autogenous bacterins may be cheap and allow for site-specific flexibility, but in addition to the forementioned disadvantages, they will be efficacious only at sites where a particular strain is prevalent [7,23]. Development of a widely available, cross-protective vaccine is a highly desirable goal to control this industry-limiting disease. At present, there are only seven vaccines licensed for use in aquaculture in the US [44]. More than half of these are killed bacterins, but the remaining three are live-attenuated vaccines (LAVs), living organisms that have naturally low, or purposefully reduced, pathogenicity to a target host [5,25]. Although LAVs do not typically cause clinical disease, the attenuated organisms can still enter and proliferate within the host, stimulating a robust humoral and cell-mediated immune response at the local, mucosal and

systemic levels. Because of this, LAV generally do not require adjuvants or booster doses, and have high potential to afford protection through less labor-intensive oral and immersion routes [5,25,27].

Live-attenuated vaccines can be generated by modification of a virulent strain through chemical, physical, or genetic means. One of the most successful mutagenesis strategies used in veterinary medicine is passaging a pathogen in increasing concentrations of the antibiotic rifampin and screening mutants for virulence in target host species [5,25]. Freshwater LAVs developed by this method against enteric septicemia of catfish caused by *Edwardsiella ictaluri* and columnaris disease in catfish caused by *Flavobacterium columnare* have both been licensed in the US [45–47]. We applied this technique to *S. iniae* strains from distinct genetic groups causing disease in the North American continent and investigated changes in strain virulence and the immunogenicity and protectivity of candidate attenuated strains.

2. Materials and Methods

2.1 Attenuation process

Three discrete strains of *S. iniae* were used in this study, representing 3 major genetic groups circulating the North American continent and 3 host types relevant to fresh and marine water aquaculture (Table 4.1) [8]. The strains were revived from frozen stocks on trypticase soy agar with 5% sheep blood (SBA, University of California Davis, Biological Media Services, USA) incubated for 48 h at 30°C under ambient aerobic conditions. The rifampin minimum inhibitory concentration (MIC) of the strains was determined to be 0.312 µg/mL by examining growth of a ~1 x 10^5 CFU/mL inoculum in cation-adjusted Mueller Hinton broth with lysed horse blood (CAMHB, Fisher Scientific, USA) containing 0 – 0.64 µg/mL concentrations of Rifamycin SV (RIF) sodium salt (Sigma-Aldrich, USA). Attenuation of strains was achieved by successive

passage on SBA containing increasing concentrations of RIF (0.312-250 µg/mL), following methods adapted from Wise et al. (2015). Colonies grown on SBA were initially transferred to SBA plates containing 0.312 µg/mL of RIF and incubated for 72 h at 30°C. From the resulting growth, three colonies per strain were selected and these lineages (n = 9) were successively passaged to a final concentration of 250 µg/mL, first in increasing increments of 1 µg/mL, then 5, 10, and finally 25 µg/mL, resulting in a total of 15 passages and 135 strains. RIF-resistant (RIFr) strains at each increment were transferred to brain heart infusion broth (BHI; MP Biomedicals, USA), expanded for 24 h at 30°C, then frozen in 20% glycerol at -80°C.

Table 4.1: Wild-type (WT) strains selected for use in this study. Genetic clade was determined previously by multilocus sequence analysis (MLSA) (Heckman et al. 2020).

Strain	Previous designation	Host type	Geography	MLSA Clade
B-WT	WS-10A	White sturgeon (*Acipenser transmontanus*)	California, USA	B
D-WT	ECO86-17	Rose spotted snapper (*Lutjanus guttatus*)	Central America	D
E-WT	LSU 01-105	Nile tilapia (*Oreochromis niloticus*)	Minnesota, USA	E

2.2 *In vitro* evaluation of RIF-resistant strains

The RIFr strains from the 25 µg/mL and final 250 µg/mL concentrations (n = 18) were selected to assess changes in growth or *in vitro* virulence. Before each assay, strains were revived from frozen stocks on SBA for 48 h at 30°C. The 0.5 McFarland standard (~1.5×10^8 colony forming units (CFU)/mL) used for the *in vivo* and *in vitro* virulence assays corresponded to a bacterial suspension in phosphate-buffered saline (PBS) with an optical density (OD) measurement of 0.14−0.155 at 600 nm, read on a UV/Vis photometer (BioPhotometer Plus, Eppendorf AG).

2.2.1 Growth in liquid media

Growth of the RIFr lineages in liquid media compared to the wild-type (WT) strains was assessed by diluting a 0.5 McFarland standard of each strain into BHI for a final concentration of ~1.5 x 10^5 CFU/mL in a 96-well plate. Plates were incubated at 30°C with shaking at 150 rpm in a Cytation™ 5 Imaging Reader (BioTek, USA) and OD_{600nm} measured every hour for 96 h.

2.2.2 Buoyant density

Potential changes to the extent of capsulation were assessed by comparing the buoyant density of RIFr strains to the WT strains [48]. Buoyant density assays were performed as described in Heckman et al. [8]. Briefly, a standard isotonic Percoll solution (GE Healthcare, Sweden) was prepared by mixing 9 parts Percoll with 1 part 1.5 M NaCl. Each strain was grown in BHI for 24 h at 30°C and 1 mL pelleted by centrifugation (5250 x *g* for 10 min), washed, and resuspended in 500 µL PBS. The suspension was layered on top of 1 mL of the Percoll solution and centrifuged at 4000 x *g* for 90 min. Relative buoyancy reflective of capsulation was measured as the distance the bacterial suspension layer traveled through the Percoll gradient.

2.2.3 Survival in tilapia whole blood

The ability of RIFr strains to survive in whole blood was assessed using methods adapted from Locke et al. [49]. Whole, lithium-heparinized blood was collected from healthy, anesthetized Nile tilapia by caudal venipuncture. A 0.5 McFarland standard of each RIFr and WT strain was generated and diluted to ~1.5 x 10^5 CFU/mL in sterile PBS. Two microliters of suspension (~300 CFU) were transferred into 300 µL of blood and incubated for 1 h with shaking at 30°C. Positive controls for each strain consisted of 2 µL suspension in 300 µL PBS. Negative controls consisted of 2 µL PBS in 300 µL of blood. After incubation, six 25 µL aliquots of blood or PBS were spot-plated onto SBA, and plates incubated at 30°C for 72 h.

Percent survival was calculated by dividing colony counts from blood by respective colony counts from PBS and subsequently multiplying the quotient by 100.

2.2.4 Cytotoxicity to tilapia endothelial cells

The cytotoxicity of the RIFr strains was investigated by challenging the *O. mossambicus* bulbus arteriosus cell line (TmBs) [50,51] with the different bacterial lineages. The assay was performed following methods from Heckman et al. [8]. Briefly, TmBs were plated in 24-well dishes (~2.5×10^5 cells/well) in 500 µL of minimal essential media-2 + HEPES + 10% fetal bovine serum (MEM) and grown to confluence at 25°C. Approximately 10^7 CFU of bacteria were added to each respective well at a ratio of 1:100. Sterile PBS (100 µL) was added to uninfected control cells. Cells were exposed to the bacteria for 3 h at 30°C, washed 3x with 1 mL MEM to remove non-adherent bacteria, and re-incubated for 24 h. The colorimetric Cytotox96© Non-Radioactive Assay (Promega, USA) was used, following the manufacturer's instructions, to quantitate release of the stable cytosolic enzyme lactate dehydrogenase (LDH). The colored product signifying lysed cells was measured by reading adsorption at 490 nm with a Cytation™ 5 Imaging Reader (BioTek, USA). Adsorption values were standardized against negative controls and percent cytotoxicity calculated by dividing experimental treatment values by the positive lysed cell control value.

2.3 Evaluation of RIF-resistant strains

The strains resistant to 250 µg/mL RIF were selected for evaluation of virulence and generation of a host immune response based on evidence of attenuation from the *in vitro* assays. Prior to experimentation six fish were arbitrarily sampled from tilapia stocks and brain and spleen samples cultured on SBA to confirm absence of pathogens. All fish experiments were

conducted in compliance with the University of California, Davis School of Veterinary Medicine Institutional Animal Care and Use Committee.

2.3.1 Virulence in Nile tilapia

To investigate *in vivo* attenuation, Nile tilapia (average weight 46.4 g) were challenged with the WT and 250 µg/mL RIFr strains from each of the three clades, for a total of 12 exposed groups. A 0.5 McFarland standard was generated for each strain and 0.1 mL of the bacterial suspension injected intracoelomically into fish anesthetized with 100 mg/L buffered MS-222 (Syndel, USA). An additional group of negative control fish received 0.1 mL sterile PBS by the same route. Seventeen fish were challenged per treatment (n = 221). Fish were kept at 25-30°C in aerated recirculating systems and morbidity and mortality recorded daily for 21 d. Moribund fish exhibiting clinical signs of streptococcosis, such as abnormal swimming or buoyancy, lethargy, or exophthalmia, were euthanized with overdose of buffered MS-222 (500 mg/L) and necropsied. Brains of moribund or freshly dead fish were aseptically swabbed, plated on SBA, and incubated at 30°C. Whole moribund fish were fixed in 10% neutral buffered formalin (NBF, Sigma-Aldrich, USA) for histopathological examination. At the end of the 21 d challenge, all surviving fish were euthanized. Whole blood and brain swabs were collected from up to 6 survivors per treatment.

2.3.2 Evaluation of anti-*S. iniae* IgM in serum by enzyme-linked immunosorbent assay (ELISA)

Whole blood collected from surviving exposed and control fish was clarified by centrifugation at 2500 x *g* for 15 min and fractions stored at -20°C. An ELISA was preformed adapting protocols of Shahin et al. [52] to measure serum antibody levels against WT *S. iniae* from homologous or heterologous clades. Immulon® 2HB Flat Bottom microtiter® (Thermo

Scientific) 96-well plates were coated with 100 µL of 1% w/v poly-L-lysine in carbonate-bicarbonate buffer and incubated at room temperature (RT) for 1 h. Plates were washed three times with low salt wash buffer (LSWB; 0.02 mol/L Trizma base, 0.38 mol/L NaCl, 0.05% (v/v) Tween-20, pH 7.2). A bacterial suspension with an OD_{600nm} of 0.2 (~1.0×10^9 CFU/mL) was generated for each WT strain in PBS and 100 µL added to the respective plate wells. Plates were incubated overnight at 4°C. Fifty microliters of 0.05% v/v glutaraldehyde in LSWB were added to fix the bacteria to the wells, and plates were incubated at RT for 20 min before washing 3x with LSWB.

One hundred microliters of 3% hydrogen peroxide were added to each well and plates incubated at RT for 1 h before washing 3x with LSWB. A 250 µL suspension of blocking buffer (5% w/v skim milk powder in distilled water) was added to each well for further blocking, and plates were incubated at RT for 3 h before washing 3x with LSWB. Serum from fish challenged with the WT, RIFr strains, or from negative control fish injected with sterile PBS were diluted 1:200 in LSWB containing 1% bovine serum albumin (BSA; Sigma) and 100 µL of dilution added in triplicate to their respective plate wells. One hundred microliters of sterile PBS were added to blank control wells. Plates were incubated overnight at 4°C. Following incubation, plates were washed 5x with high-salt wash buffer (HSWB; 0.02 mol/L Trizma base, 0.5 mol/L NaCl, 0.01% (v/v) Tween-20, pH 7.7) with a 5 min soak on the last wash. Mouse anti-tilapia IgM monoclonal antibodies (Aquatic Diagnostics Ltd, UK) were diluted 1:75 in PBS and 100 µL added to each well. Plates were incubated at RT for 1 h before washing 5x with HSWB with a 5 min soak. Goat anti-mouse IgG with conjugated horseradish peroxidase (Sigma-Aldrich) was diluted 1/3,000 in LSWB with 1% BSA and 100 µL added to each well. Plates were incubated at RT for 1 h before washing 5x with HSWB with a 5 min soak. One hundred microliters of

substrate/chromogen (5 mL of substrate buffer [5.25 g citric acid, 2.05 g of sodium acetate, distilled water up to 15 mL, pH 5.4] containing 5 µL of hydrogen peroxide (Alfa Aesar) and 150 µL of trimethylbenzidine (TMB) dihydrochloride (Sigma-Aldrich) was added to the plates and incubated for 2.5 min at RT before the reaction was terminated with the addition of 50 µL 2M sulfuric acid per well. Absorbance at 450 nm was measured using a Cytation™ 5 Imaging Reader (BioTek, USA) and standardized against PBS controls.

2.3.3 Evaluation of vaccine candidates by intracoelomic administration

Three candidate vaccine strains – B2-r250, D2-r250 and E1-r250 – were selected based on *in vitro* assays, tilapia challenges and ELISA results. To assess the protection provided by these strains, 60 Nile tilapia fingerlings (average weight 28.1 g) were immunized by intracoelomic injection of 0.1 mL of a 0.5 McFarland suspension (~1.5 x 10^7 CFU) of each candidate strain (n=180). Two tanks of 60 control fish (n = 120) were injected with 0.1 mL of sterile PBS to serve as sham vaccinated controls. Fish were kept at 25-30°C in aerated recirculating systems and monitored for adverse effects from the injection procedure or the vaccine. Thirty days post vaccination (dpv), all tanks were split into replicates (2 tanks/treatment, 30 fish/tank). Fish immunized with candidate strains and the sham vaccinated positive controls were intracoelomically challenged with the virulent E-WT strain (0.1 mL of a 0.5 McFarland, ~1.5 x 10^7 CFU). Negative control fish were treated similarly but were injected with 0.1 mL sterile PBS. Moribund fish exhibiting clinical signs of streptococcosis were euthanized with an overdose of buffered MS-222 (500 mg/L) and necropsied. Brains of moribund or freshly dead fish were swabbed and plated on SBA incubated at 30°C. Whole moribund tilapia fingerlings were fixed in 10% neutral buffered formalin for histopathological examination. At the end of the 21 d challenge, all surviving fish were euthanized. Brain swabs were collected from 5 survivors

per treatment. Relative percent survival (RPS) was calculated using the equation RPS = (1- (% mortality in vaccinated fish / % mortality in non-vaccinated fish)) x 100% [53].

2.3.4 Antimicrobial susceptibility of vaccine candidates

Antimicrobial susceptibility was compared between the candidate and WT strains using the Sensititre™ Avian AVIAN1F AST Plate (Thermo Fisher Scientific, USA). *Escherichia coli* ATCC 25922 was used as a reference control strain. The experiment was performed following manufacturer's protocol and CLSI recommendations [54,55]. Briefly, a 0.5 McFarland suspension of each strain was diluted 1:1000 (~1.5 x 10^5 CFU/mL) into CAMHB and 50 µL of suspension distributed to the MIC plate wells. Plates were incubated at 37°C and the MIC for each antimicrobial determined by absence of bacterial growth in the relevant wells after 24 h incubation.

2.4 Histopathology

Fifteen whole fish specimens from the attenuation (n = 6) and vaccination (n = 9) challenges were fixed by immersion in 10% neutral buffered formalin for a minimum for 24 hours. Representative sections of the whole fish specimens were routinely processed to obtain 4 µm thick hematoxylin and eosin-stained sections. Sections of brain, spinal cord, eye, gill, heart, skin, skeletal muscle, liver, kidney, spleen, swim bladder, stomach, pancreas, fat, and intestine from all fish were examined.

2.5 Statistics

All statistical tests were performed in GraphPad Prism v9.1.0 (GraphPad Software, USA). The area under the curve (AUC) was computed for each strain growth curve using the mean OD_{600nm} at the first timepoint for the baseline. The RIFr AUCs were compared to their respective WT-parent's AUC by Brown-Forsythe and Welch ANOVA tests and Dunnett's T3 multiple

comparisons test, with individual variances computed for each comparison. Differences in buoyancy, whole blood survival and cytotoxicity between RIFr lineages and their WT parents were assessed Ordinary One-way ANOVA and Tukey's multiple comparisons test. Survival curves were compared with Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. A *p* value of ≤ 0.05 was considered statistically significant for all tests.

3. Results

3.1 Generation of RIF-resistant strains

All *S. iniae* WT strains were susceptible to rifampin, with an initial MIC of 0.312 µg/mL. Three lineages from each WT strain were successfully passed in increasing concentrations of RIF, to a final concentration of 250 µg/mL. The archived RIFr strains obtained from plates containing 25 and 250 µg/mL generally showed similar morphologies and shared the same capsulation phenotypes as their WT parent strains, with some minor variation (Figure 4.1). There were differences in growth rates between WT and RIFr strains. This was observed both on solid media, where some RIFr mutants required longer incubation times to achieve standard colony morphology (data not shown), and in BHI (Figure 4.2). RIFr strains derived from clade B and E plateaued at a lower OD_{600nm} at stationary phase, in some cases following an extended lag phase. All clade E RIFr strains had significantly lower ($p < 0.0001$) AUCs compared to E-WT. Strains B1-r25, B1-r250 and B2-r25 also had AUC values significantly lower ($p < 0.005$) than B-WT. Clade D RIFr strains showed more diversity in growth patterns, with some strains exhibiting slightly higher OD_{600nm} levels at stationary phase while others again showed longer lag phases and/or lower bacterial concentrations in comparison to the D-WT strain. The AUCs were only statistically different in the strains with the lowest OD_{600nm} readings at stationary phase – D2-r25, D3-r25 and D3-r250.

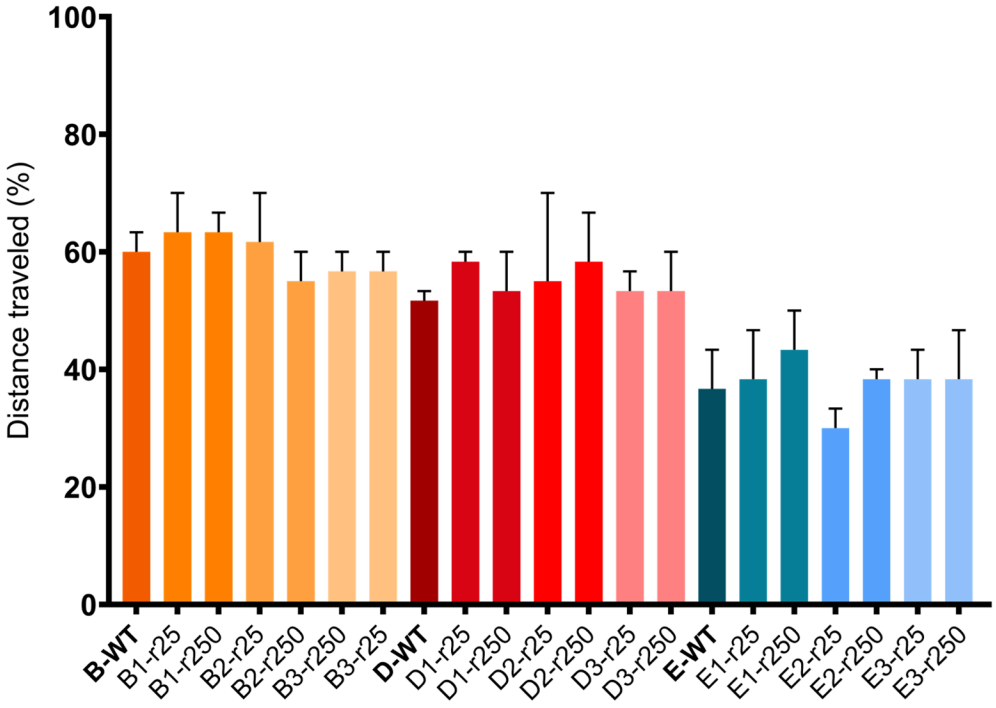

Figure 4.1: Extent of capsulation of wild type (B-WT, D-WT, E-WT) and rifampin-resistant (RIFr) strains as determined by buoyant density centrifugation. Capsulation is inversely proportional distance traveled through a standard Percoll density gradient. The experiment was preformed twice. Color reflects strain phylogroup: Clade B: orange; Clade D: red; Clade E: blue. RIFr strains from the same lineage have matching shading, with the WT parent in the darkest shade.

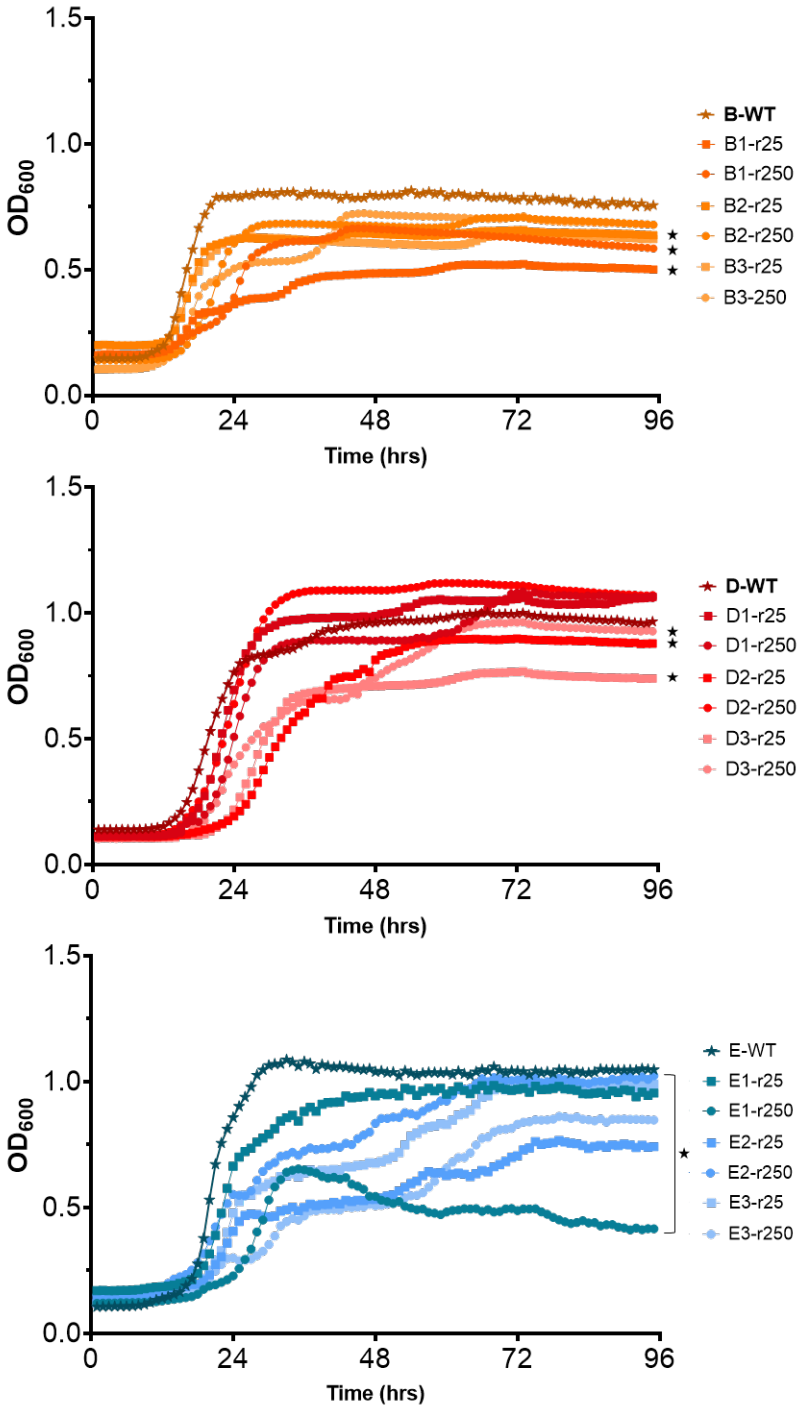

Figure 4.2: Growth curves of wild type (B-WT, D-WT, E-WT) and rifampin-resistant (RIFr) strains in brain heart infusion (BHI) media at 30°C. The experiment was completed three times in technical duplicate. Color reflects strain phylogroup: Clade B: orange; Clade D: red; Clade E: blue. Rifampin-resistant strains from the same lineage have matching shading, with the WT parent in the darkest shade. Stars represent RIFr curves where the area under the curve (AUC) was statistically different from that of the WT strain by Brown-Forsythe and Welch ANOVA tests with Dunnett's T3 multiple comparisons test.

3.2 Strain survival in tilapia whole blood

At least one RIFr strain in each clade showed significant differences from their WT parent in their ability to survive in tilapia whole blood (Figure 4.3). All strains, both 25 and 250 µg/mL RIFr isolates, derived from D-WT had significantly reduced levels of bacterial recovery from blood ($p < 0.0001$). Similarly, RIF-resistant strains derived from B-WT had comparatively reduced survival, although only two were significant (B1 and B2; $p < 0.05$). A single clade E RIFr strain (E1-r250), showed significantly lower survival in tilapia blood than the WT parent ($p < 0.05$).

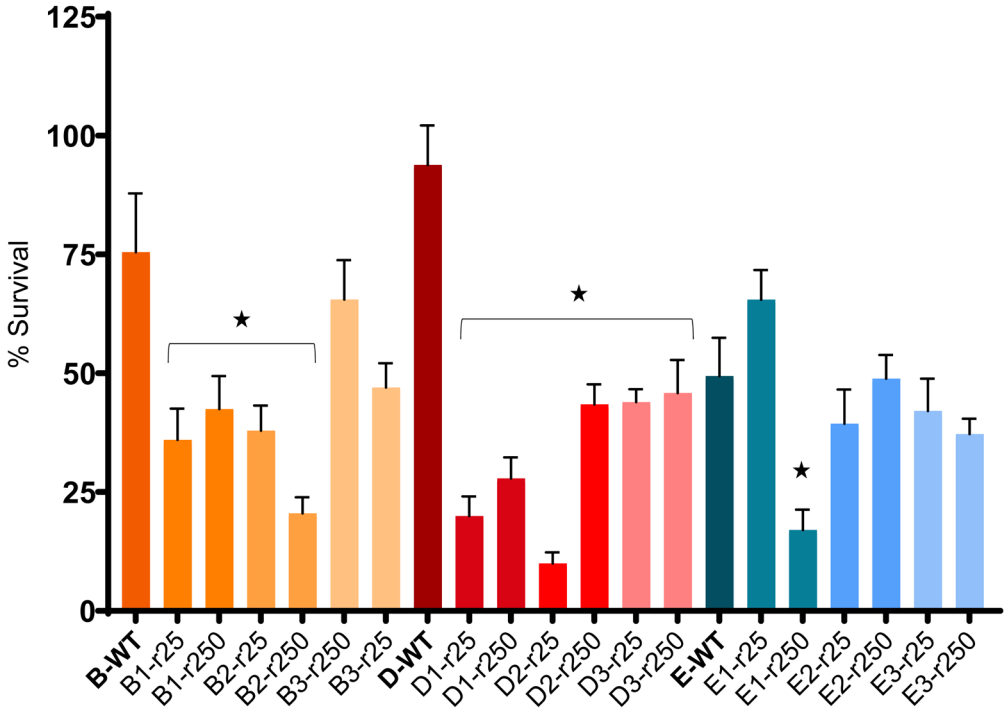

Figure 4.3: Percent strain survival after incubation for 1 h in whole heparinized blood from Nile tilapia (*Oreochromis niloticus*). The experiment was performed twice in biological duplicate using the same groups of fish. Error bars represent standard error (SEM). Color reflects strain phylogroup: Clade B: orange; Clade D: red; Clade E: blue. Rifampin-resistant strains from the same lineage have matching shading, with the wild-type (WT) parent in the darkest shade. Stars indicate statistical significance ($p < 0.05$) from the WT parent determined by One-Way ANOVA with Tukey's multiple comparison tests.

3.3 Cytotoxicity in tilapia endothelial cells

Wild-type representatives from clades D and E, and their derived RIFr strains caused damage or death in Tmb cells, as evidenced by the release of the cytosolic enzyme LDH (Figure 4.4). RIFr strains from clade D caused higher levels of LDH release, while those in clade E generally caused less cytotoxic effect than E-WT. These differences were not, however, significant ($p > 0.05$). Neither WT nor RIFr clade B strains caused LDH release above negative control levels, consistent with reports for other strains in that clade [8].

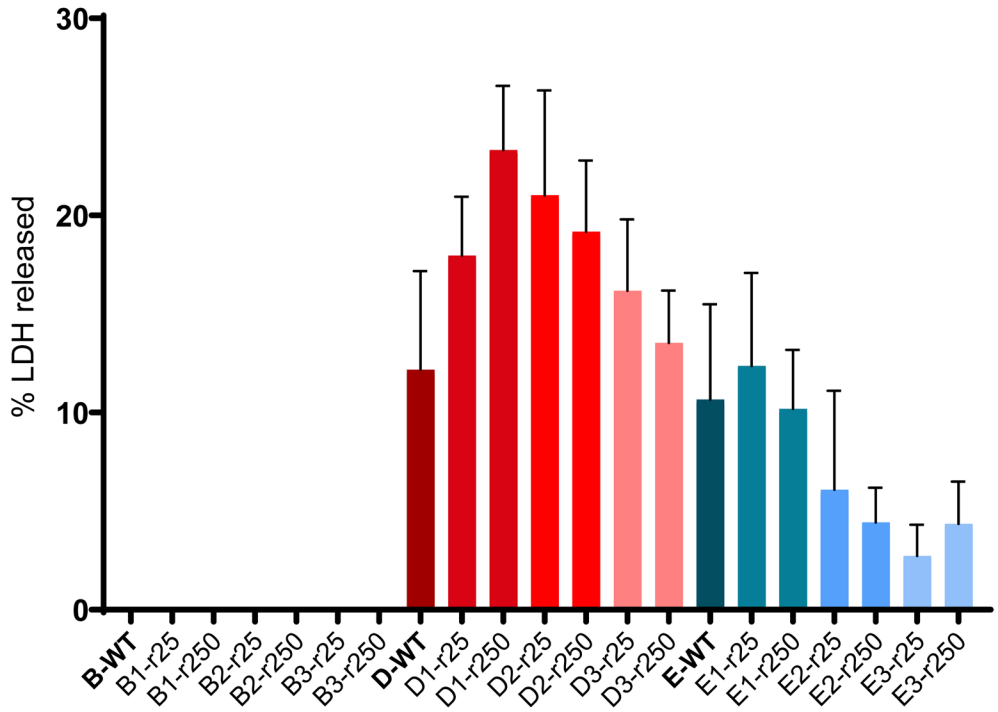

Figure 4.4: Percent cytotoxicity as measured in terms of lactate dehydrogenase (LDH) release from *Oreochromis mossambicus* bulbus arteriosus cell lines compared to lysed cells serving as a positive control. Cells were incubated with bacteria for 3 h, washed, and incubated for 24 h at 30°C. Experiments were carried out in technical triplicate and repeated twice. Error bars represent standard error (SEM). Color reflects strain phylogroup: Clade B: orange; Clade D: red; Clade E: blue. Rifampin-resistant strains from the same lineage have matching shading, with the wild-type parent in the darkest shade.

3.4 Virulence of attenuated *S. iniae* in tilapia

Strain E-WT was highly virulent in tilapia resulting in nearly 95% mortality (Figure 4.5). The primary clinical sign observed in moribund fish challenged with E-WT was unilateral exophthalmia, though fish often died without exhibiting signs, as previously reported [8,56]. Two of the RIFr strains derived from E-WT – E2-r250 and E3-r250 – were attenuated ($p < 0.005$) but still caused appreciable mortality of 41.2% and 58.3% respectively. Fish challenged with E2-r250 or E3-r250 displayed behavioral changes such as corkscrewing, spiraling, or floating and spinning vertically at the water surface. Uni- or bilateral exophthalmia with corneal opacity was also common and adhesion of internal organs were sporadically observed. Pure cultures consistent with *S. iniae* were consistently recovered from the brains of moribund or freshly dead fish challenged with E-WT, E2-r250, and E3-r250.

Histopathological findings were similar between moribund fish in these groups. All fish displayed mild to moderate granulomatous meningomyeloencephalitis, and moderate to severe multifocal to regionally extensive granulomatous and necrotizing chondritis with granulomatous cellulitis, myositis, and periostitis/osteomyelitis (Figure 4.6). Granulomatous coelomitis was also present to varying degrees in all examined animals, though inflammation was less prominent in the E2-r250 group compared to the E-WT. Intrahistiocytic and extracellular gram-positive coccobacilli were found in association with granulomas in selected opercular cartilage and intraceolomic tissue sections (Figure 4.6). The E-WT and E2-r250 groups also exhibited granulomatous epicarditis and enophthalmitis, again to a milder extent in fish challenged with the attenuated E2-r250. Inflammation in fish challenged with E3-r250 was not observed in these organs. In contrast to the E2 and E3 strains, E1-r250 caused no mortality or observable adverse effects. No bacteria were recovered from the brains of the survivors infected with this attenuated

strain. The WT strains from clade B and D are less virulent in tilapia [8], and only D-WT caused a mortality event where bacteria were recovered. There were single mortalities in fish challenged by D2-r250 and B1-r250, but bacteria were not recovered in either case.

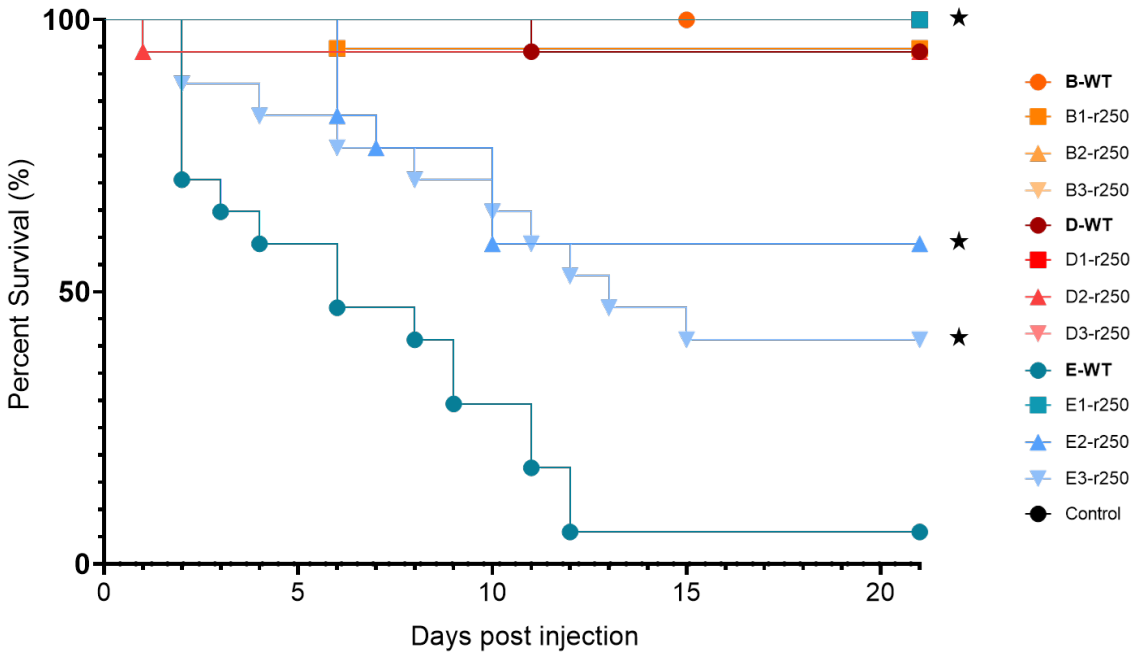

Figure 4.5: Survival curve for Nile tilapia (*Oreochromis niloticus*) intracoelomically challenged with *S. iniae* wild-type (WT) or strains resistant to 250 µg/ml of Rifamycin SV sodium salt (r250). For each strain 17 fish were challenged and kept in static tanks at 25-30°C for 21 days. Stars indicate statistical significance ($p < 0.005$) from the relevant WT survival curve, as determined by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.

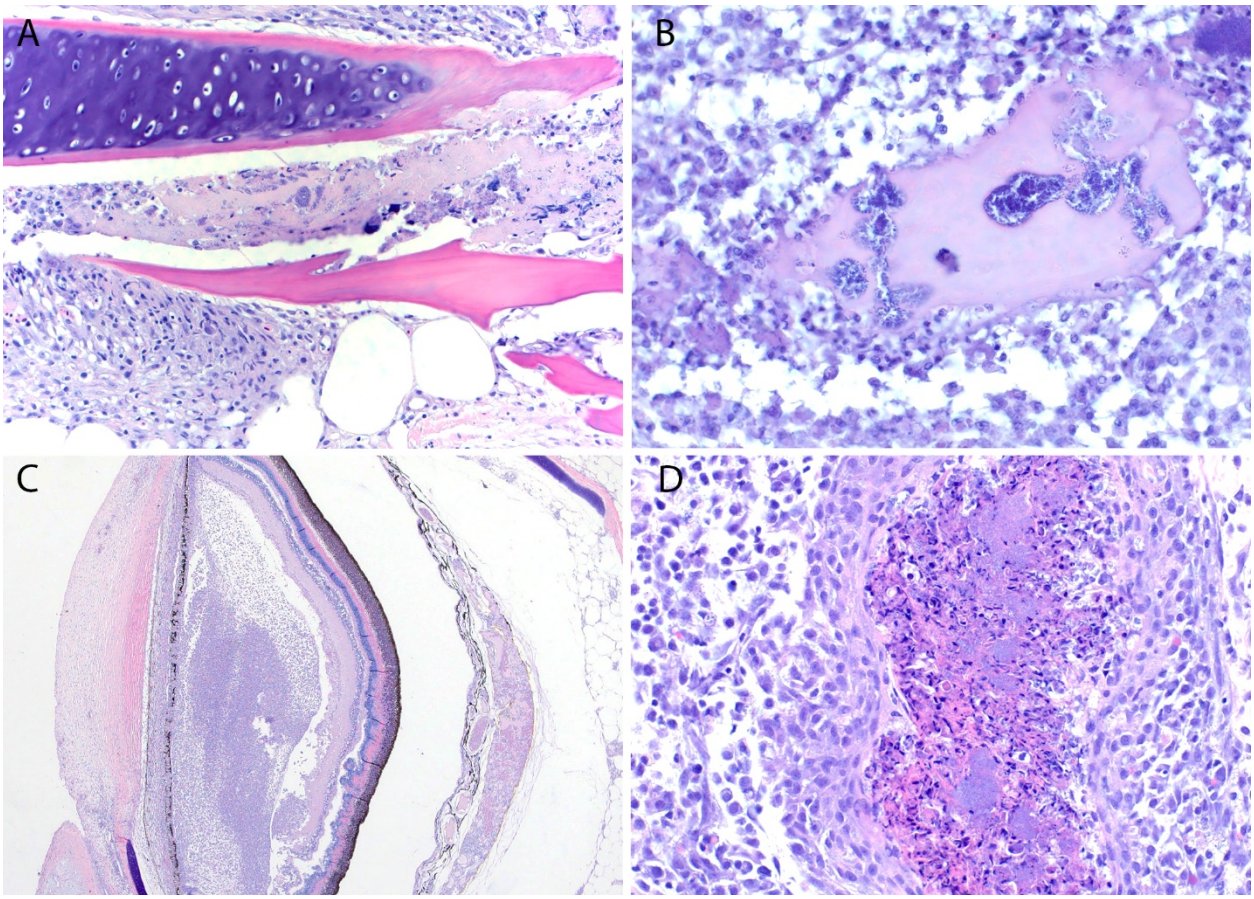

Figure 4.6: Microscopic pathology in Nile tilapia (*Oreochromis niloticus*) experimentally infected with *S. iniae*. (A) Severe necrotizing and granulomatous chondritis and granulomatous myositis with intralesional coccobacilli. Hematoxylin and eosin stain (H&E), 20x objective lens, 200x magnification. (B) Severe necrotizing and granulomatous chondritis with intralesional coccobacilli. H&E, 60x objective lens, 600x magnification (C) Severe unilateral granulomatous enophthalmitis and anterior uveitis. 4x objective lens, 40x magnification (D) Moderate to severe granulomatous coelomitis with extracellular and intrahistiocytic coccobacilli. 40x objective lens, 400x magnification.

3.5 Generation of cross-protective tilapia anti-*Streptococcus iniae* IgM

Infection by intracoelomic injection stimulated a humoral response in the Nile tilapia (Figure 4.7). The antibodies generated against the WT or RIFr strains showed activity against both the WT bacteria from their own clade, and often across clades. Median antibody levels against the semi-encapsulated D-WT and B-WT strains were generally higher than those for the fully capsulated E-WT bacteria. The B1-, B2-, and B3-r250 strains stimulated higher antibody titers

against B-WT than the wild-type bacteria itself, but these differences were not statistically significant ($p > 0.05$). Serum antibody titers against D-WT and E-WT generated by their respective RIFr derivatives were similar or lower to those generated by immunization with the WT strains, although having only a sole survivor in the LSU 01-105 WT challenge weakens comparisons against that strain.

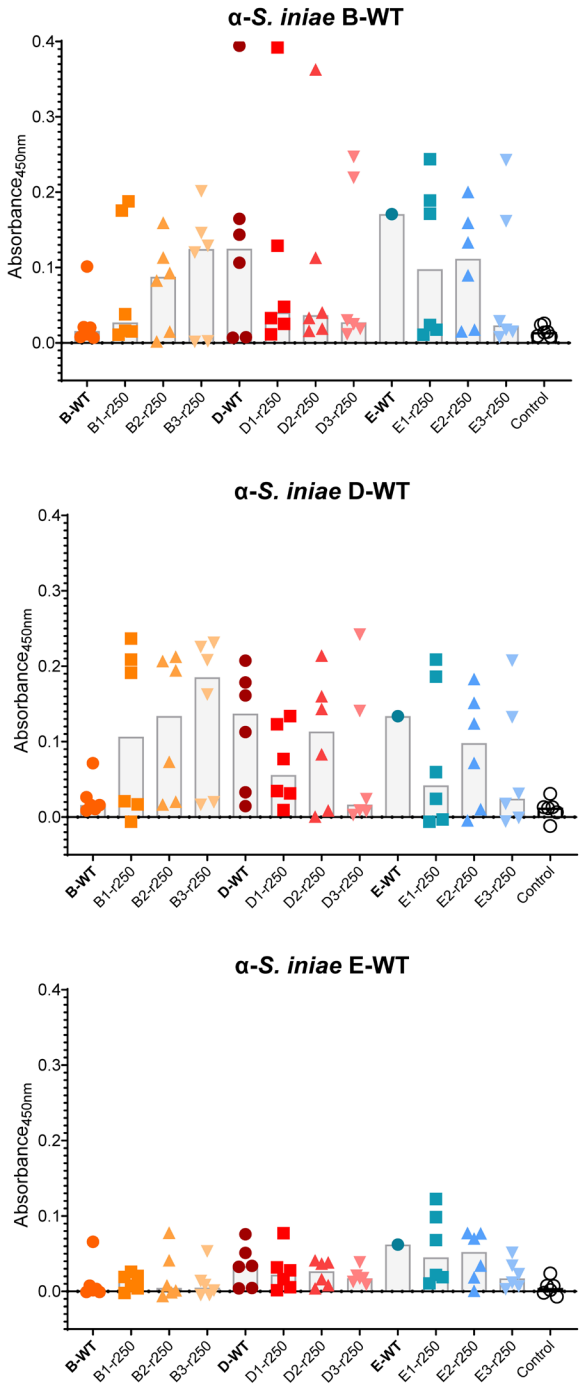

Figure 4.7: Serum antibody levels of Nile tilapia (*Oreochromis niloticus*) challenged with wild-type (WT) or strains resistant to 250 µg/ml of Rifamycin SV sodium salt (r250). Each point represents a single fish with the bar representing the median. Color reflects strain phylogroup: Clade B: orange; Clade D: red; Clade E: blue. Rifampin-resistant strains from the same lineage have matching shading, with the WT parent in the darkest shade. Control fish received sterile PBS and are represented by clear circles.

3.6 Vaccination by intracoelomic injection

Intracoelomic vaccination of tilapia fingerlings with LAV candidate strains B2-r250, D2-r250 and E1-r250 showed variable protection against challenge with E-WT (Figure 4.8). The strains derived from heterologous clades D and B did not provide strong protection against the E-WT bacteria. The homologous, attenuated E1-r250, however, conferred a high level of protection against infection with E-WT, with an RPS of 95.04%. Fish vaccinated with D2-r250 showed near identical mortality trends to the positive control fish, who received only PBS before WT challenge. The survival curve for B2-r250 was only significantly different ($p < 0.05$) from the positive control in the Gehan-Breslow-Wilcoxon test and not by log rank analysis. Clinical signs in moribund tilapia given this treatment were similar to those observed in the previous attenuation challenge with E2-r250 and E3-r250. Histopathological lesions in the positive control, B2-r250, and D2-r250 were also consistent with those previously discussed. The extent of lesions was milder in the B2-r250 group.

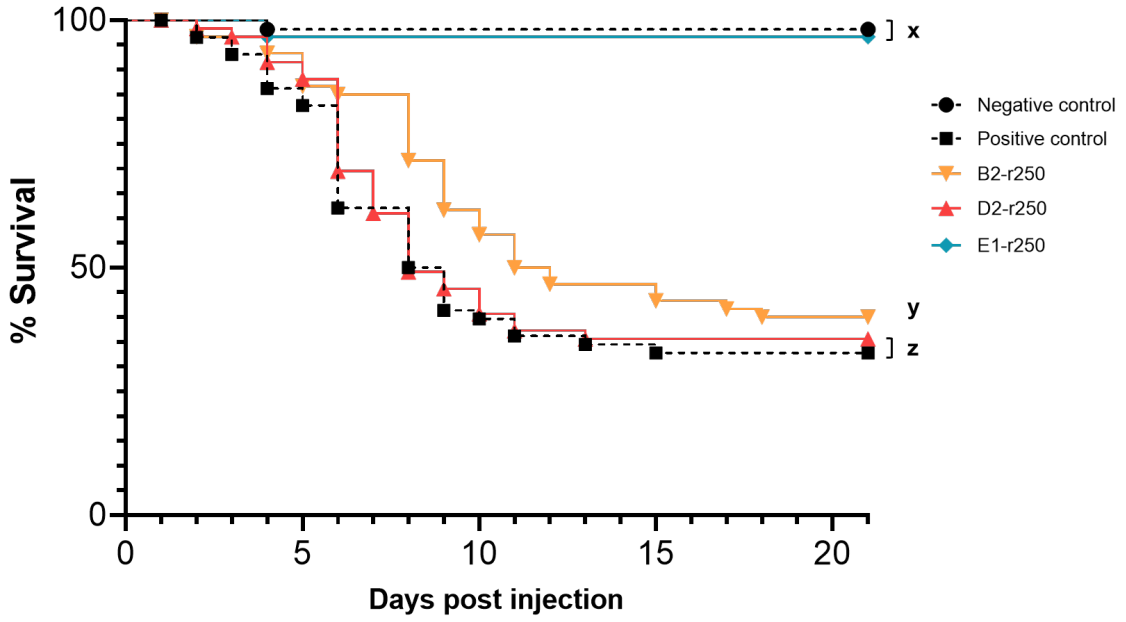

Figure 4.8: Survival curve of Nile tilapia (*Oreochromis niloticus*) vaccinated interperitoneally (IP) with live-attenuated vaccine (LAV) candidate strains then challenged with virulent E-WT by IP

injection 30 days post vaccination. Positive controls were challenged but received sterile PBS rather than a vaccine candidate. Negative controls received only PBS at the time of vaccination and challenge. Letters denote statistical significance as determined by Gehan-Breslow-Wilcoxon test (y; $p < 0.05$) and Log-rank (Mantel-Cox) tests (x and z; $p < 0.0001$).

3.7 Antimicrobial susceptibility of candidate strains

There were differences in antimicrobial susceptibility profiles between the WT strains from different clades, but the LAV candidates showed similar susceptibilities to their WT parent strains (Table 4.2). Small increases in the MIC of novobiocin (+1-3 µg/ml) were the only consistent changes in the RIFr strains.

Table 4.2: Minimum inhibitory concentrations (MIC) in µg mL^{-1} of common veterinary antimicrobials against *Streptococcus iniae* strains. Experiments were performed in duplicate with consistent MICs between plates.

Antimicrobials	**B-WT**	**B2-r250**	**D-WT**	**D2-r250**	**E-WT**	**E1-r250**
Enrofloxacin	0.5	0.5	1	0.5	0.5	0.5
Gentamicin	4	8	0.5	0.5	4	2
Ceftiofur	0.25	0.25	0.25	0.25	0.25	0.25
Neomycin	16	16	4	4	16	8
Erythromycin	0.12	0.12	0.12	0.12	0.12	0.12
Oxytetracycline	0.5	0.5	0.25	0.5	4	4
Tetracycline	0.5	0.5	0.25	0.5	8	8
Amoxicillin	0.25	0.25	0.25	0.25	0.25	0.25
Spectinomycin	16	8	8	8	16	16
Sulphadimethoxine	32	32	32	32	32	32
Trimethoprim / sulfamethoxazole	0.5 / 9.5	0.5 / 9.5	0.5 / 9.5	0.5 / 9.5	0.5 / 9.5	0.5 / 9.5
Florfenicol	1	1	1	1	1	1
Sulphathiazole	32	32	32	32	32	32
Penicillin	0.06	0.06	0.06	0.06	0.06	0.06
Streptomycin	8	8	8	8	8	8
Novobiocin	1	4	1	2	2	4
Tylosin tartrate	2.5	2.5	2.5	2.5	2.5	2.5
Clindamycin	1	0.5	0.5	0.5	0.5	0.5

4. Discussion

Prevention of streptococcosis through vaccination is the best option to overcome outbreaks in aquaculture. Successful vaccines reduce disease, animal losses, antibiotic dependence, and

associated costs [57]. Development of effective streptococcal vaccines, however, continues to be a challenge in both human and animal medicine [7,58–60]. The bacterins employed against *Streptococcus iniae* have failed to accommodate the species' diversity and pathogenesis, resulting in vaccine escape [8,15,23]. Alternative methods are necessary for addressing these deficiencies. Pathogen attenuation by selection for rifampin has been used for decades in the development of numerous veterinary vaccines, including several commercial vaccines used in the United States [45,46,61–66]. This method has almost exclusively been used to generate LAV for gram-negative pathogens, but our study demonstrates its applicability for gram-positive pathogens as well. The three parent strains selected for attenuation were initially susceptible to low levels of Rifamycin SV sodium salt (0.312 μg/mL). Successive passaging in increasing increments generated mutants up to a final tolerance of 250 μg/mL. The RIFr mutants differed from their parent strains noticeably in growth kinetics. Several mutant strains were observed to grow more slowly and form smaller colonies on solid media (data not shown). This was reflected in liquid media, where there were a range of divergences from WT growth curves (Figure 4.2). The curve of LAV candidate strain E1-r250 is particularly interesting as it has a slightly longer lag phase and notably shorter plateau before entering the death phase. Changes in growth kinetics are frequently reported alongside development of antibiotic resistance, so it is not an unprecedented finding [65,67]. Whether these altered kinetics contribute to attenuation by limiting growth or persistence of the pathogen in the host remains to be determined.

Complete immune clearance and low cytotoxicity are desirable characteristics in live vaccines [68]. While there were some trends observed in mutant cytotoxicity (Figure 4.4), the differences when compared to parent strains were not significant, suggesting the mutations did not strongly impact virulence factors involved in endothelial cell damage. Strain survival in host

whole blood is a better predictor of strain virulence, indicating ability to evade immune factors and establish bacteremia [8,29,49,69]. Whole blood survival was reduced in almost all RIFr strains, although the decrease was not always significant (Figure 4.3). Curiously, this change does not appear to be due to capsulation variation between WT and RIFr strains, as prominent differences were not observed by the buoyancy assay (Figure 4.1). However, it may have been impacted by altered growth (Figure 4.2). As E1-r250 was the only clade E strain to show significant reductions in whole blood survival, the mutants from the final passage (250 μg/mL resistance) were selected for virulence testing in Nile tilapia.

None of the RIFr mutants caused increased virulence in tilapia compared to the WT, which caused almost 95% mortality (Figure 4.5). The LAV candidate strain E1-r250 was completely attenuated and caused no mortality by IC injection. The E2-r250 and E3-r250 strains were attenuated but still caused mortality rates of 41.2% and 58.3%, respectively. Interestingly, the disease progressed differently in fish infected with these strains. The E-WT strain is highly virulent in tilapia and was originally isolated from that same host type (Table 4.1). Intracoelomic infection with E-WT leads to the acute form of streptococcosis, and onset of mortalities is rapid, with few clinical signs [56]. The fish injected with the E2-r250 and E3-r250 strains had a slower progression, allowing observation of dramatic behavioral changes such as corkscrewing, spiraling or periods of lethargy interspersed with frenzied bursts of activity. They also commonly displayed corneal opacity with severe uni- or bilateral exophthalmia, altogether indicating infection of the central nervous system [70]. These observations suggesting neurological infection were confirmed by histopathological assessment of moribund animals (Figure 4.6). All examined fish demonstrated some degree of granulomatous disease, involving the meninges with extension into the brain and spinal cord, and the cartilage, surrounding bone, skeletal muscle, and

tissue. Lesions were most severe in the head, but granulomatous inflammation was also found in the coelom, heart, and eye(s), though less commonly in E3-r250 fish and to a milder extent in E2-r250 fish compared to those infected with E-WT. These findings are consistent with previous reports of streptococcosis [71,72]. The attenuation of the B and D lineages is difficult to assess by this challenge model, as the WT strains themselves have low virulence in tilapia, despite causing outbreaks with high mortality in their original host species. This host specificity is not surprising, as it is known that isolates from one host are not necessarily pathogenic to other hosts, or even different varieties of the same fish [58]. To assess the attenuation of these strains, a different challenge model is required, such as an intramuscular injection of white sturgeon [73].

Regardless of their potential attenuation in their respective host species, the strains selected from clades B and D for initial efficacy testing did not provide cross-protection against virulent E-WT by IC vaccination (Figure 4.8). This was reflected in the ELISA results, where serum antibodies from fish challenged with B2-r250 and D2-r250 did not have a strong binding response to E-WT whole cells (Figure 4.7). This may be a result of capsulation differences, as clade B strains show intermediate capsulation and D strains are unencapsulated [8]. Both the capsule and shared surface proteins have been suggested to be antigenic in *S. iniae.* Vaccines developed from capsule-deficient strains generally do not protect against encapsulated strains, as the capsular polysaccharides can mask shared surface proteins [21,23,74]. The reverse, however, is not true, and capsulated strains have demonstrated cross-protection against the unencapsulated, raising antibodies against both CPS and protein antigens [23,74].

The overall levels of specific antibodies may appear low compared to mammalian titers, but teleosts have more moderate increases in IgM binding affinity following immunization, and neutralizing antibodies are often detected in only a fraction of fish [75]. Very low titers have

been found to be protective against *S. iniae* [33], and the antibodies generated against the LAV candidate E1-r250 were indeed sufficient for protection against E-WT. Antibodies raised against E1-r250 also showed similar or higher binding affinities for B-WT and D-WT cells, and it is likely that E1-r250 will provide additional protection against bacteria from these heterologous genetic groups. Further testing will be necessary in suitable host challenge models to evaluate this potential, as cross-reactivity does not guarantee cross-opsonization or the resulting protection [24,35]. If cross-protection is not achievable with a single strain, a combination approach could be considered. We have demonstrated the applicability of attenuation by rifampin passaging in *S. iniae* and could feasibly generate more LAV candidates targeting different serotypes. As vaccine failure is typically due to lack of heterogenous protection, a polyvalent vaccine may be more effective at limiting the possibility of vaccine escape. Such a combination of rifampin mutants has already been demonstrated to be possible and effective for vaccination against enteric septicemia of catfish [5].

The genomes of the three candidate LAV and WT strains have been submitted for whole genome sequencing. Analysis of these genomes will elucidate the nature and extent of the mutations induced by rifampin selection, as reversion to virulence is less likely when multiple genes contribute to attenuation rather than a single virulence gene [57]. Risk of reversion and other safety concerns are important considerations that will need to be addressed going forward. The RIFr candidate strains did not gain resistance to other antimicrobials, including the two FDA approved antibiotics for treatment of *S. iniae* in aquaculture – oxytetracycline and florfenicol (Table 4.2). They did, however, show small but consistent increases in the concentration of novobiocin required for inhibition. Novobiocin is an antimicrobial that targets the GyrB subunit of the bacterial DNA gyrase enzyme involved in replication and transcription. It is possible that

mutations in RNA polymerase to acquire RIF-resistance altered the transcription process or interactions between RNA polymerase and DNA gyrase [76–78]. Whole genome analysis may clarify whether these changes are due to pleiotropic effects of rifampin resistance or mutations specifically within the *gyrB* gene.

The E1-r250 LAV candidate that has demonstrated high vaccine potential will need to be back-passaged through tilapia to confirm no clinical or low-grade infections emerge. A 10x immunizing dose will also be tested to confirm no significant adverse effects at high bacterial concentrations [5]. Additionally, persistence of the bacteria in the host and environment will need to be determined. The safety and efficacy of the candidate strain will then be investigated by oral and immersion administration, as injection is time consuming and expensive. LAV are more likely to work as mucosal vaccines compared to bacterins, but previous studies have demonstrated that the high protection afforded by LAV injection does not always transfer to other routes [68,79].

There have been a handful of experimental LAV previously developed for *S. iniae*. Four were attenuated by disruption of specific virulence genes: the capsule synthesis (*cpsD*), M-like protein (*simA*), and sortase A (*srtA*) genes by allelic exchange, and phosphoglucomutase (*pgm*) by transposon mutagenesis [42,49,68,80,81]. Two others were generated by *in vitro* passaging in media with [34], or without [79], antibiotic selection. Each of these experimental vaccines showed high levels of protection against homologous strains in their target hosts, but without further experimentation, fall short of meeting the requirements for adaptation as an effective, commercial vaccine.

The Δ*cpsD* and Δ*pgm* mutants and the passaged strain YM011 show low levels of protection when delivered by immersion or oral gavage, and Δ*simA,* while offering complete protection by

injection and immersion, still causes disease and mortality itself by both routes [68,79]. The Δ*srtA* is also incompletely attenuated and has only been tested as an injectable vaccine [81]. These mutants developed by experimental genetic manipulation also have the disadvantage of negative industry and public perception of genetically modified organisms (GMOs) [82]. The attenuated strain ISNO [34], generated by selection for the antibiotic novobiocin, has perhaps the best potential for commercial adaptation in LAV developed to date. It shows high protectivity against homologous strains by injection and immersion (100 and 88% RPS respectively) and moderate to high protection by injection against heterologous strains [34]. Still, the strains were not characterized genetically or phenotypically, so the actual relatedness of these heterogenous strains is unknown. Unlike rifampin passaging, this method of attenuating bacteria is also relatively new and has not been used to generate any licensed vaccines.

In summary, serial passaging in rifampin is a viable method of attenuating *S. iniae* for development of prospective aquaculture vaccine candidates. The rifampin-resistant strain E1-r250 was attenuated in tilapia, causing no mortality at the administered dose, but affording high levels of protection against its virulent, wild-type parent when delivered by injection. This LAV candidate also has potential to offer cross-protection against heterologous strains, as antibodies raised against it showed appreciable activity against the WT strains from other genetic clades. Further development of this candidate, alone or in conjunction with other strains, could deliver a safe and effective vaccine for control of piscine streptococcosis.

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CONCLUSION

A combination of effective vaccination, antimicrobial treatment, and husbandry strategies will be required to have a substantial and enduring reduction in outbreaks of piscine streptococcosis. Current practices can be improved with a more comprehensive understanding of *S. iniae* strain diversity in relation to its behavior in the host and external environment. There are at least four genetic groups of *S. iniae* endemic to North America that differ in morphology and host virulence. Biofilm formation is conserved across the clades, with some variation in rate, and may play a role in the survival of the pathogen in the host or environment. Established disinfection and antimicrobial treatment protocols may not be sufficient to eliminate *S. iniae* biofilms following outbreaks of streptococcosis. Prevention of outbreaks may be possible by vaccination using cross-protective LAV, but further work to understand *S. iniae* will continue to improve the tools and treatments available for control of this widespread pathogen.