STREPTOCOCCUS: A WORLDWIDE FISH HEALTH PROBLEM

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Abstract

Streptococcus iniae and *S. agalactiae* are important emergent pathogens that affect many fish species worldwide, especially in warm-water regions. In marine and freshwater systems, these Gram-positive bacteria cause significant economic losses, estimated at hundreds of millions of dollars annually. Infection results in septicemia and neurotropic disease, with cumulative percent mortality between 30 to 50%. Streptococcal disease is very difficult to control because these pathogens are resistant or become resistant to most chemicals and drugs. The aim of this paper is to review current information on fish streptococcosis including aspects of epidemiology, diagnosis, pathogenesis, and prevention, treatment and control measures in farmed tilapia.

Keyword: *Streptococcus iniae, Streptococcus agalactiae,* epidemiology, diagnosis, pathogenesis, control

INTRODUCTION

Tilapia *(Oreochromis* sp.) are among the most cultured food fish worldwide (FAO 2004). Tilapia are likely to become the most important cultured food fish in the twenty-first century (Fitzsimmons 2000). Fitzsimmons reported that tilapia production has increased by 12% per year from 1984. Tilapia are raised commercially in about 100 countries with 96% of the production outside of the species native ranges (Shelton and Popma 2006). Farmed tilapia exceeded two million metric tons (MMT) in 2004 worldwide (FOA 2004). China was the leading producer, at 0.9 MMT. Taiwan produced 0.09 MMT. Other Asian countries produced about 0.0044 MMT. South, Central and North America also produce tilapia.

Tilapias are native to Africa and the Middle East. There are nine commercially important species, including Congo, Galilee, Black-Chinned, Mozambique, Zanzibar, Nile, Blue, Red hybrid, and White tilapia. Nile tilapia is among the most cultured and consumed tilapia, although some consumers in some regions prefer the Blue and Red tilapia.

Tilapia are cultivated in wide variety of aquaculture systems, including: ponds; raceways; tanks; and net pens. Net pets are used in ponds, lakes, rivers and bays. Under these culture conditions, tilapia have contact with native fish and transmission of pathogens between caged tilapia and native fish may occur. The culture units use

either fresh or brackish, flow–through or reused water. Tilapia are mouthbreeding fish that can be cultivated at water temperatures between 29 and 31°C. The upper lethal limits are near 42°C and the lower lethal limits are about 8 to 12°C. Tilapia tolerates a wide range of salinities equivalent to brackish water although most tilapia are cultivated in fresh water. Dissolved oxygen (DO) levels are usually maintained above 2 mg/L, but tilapia can tolerate DO levels of less than 0.5 mg/L. Frequent exposure to low DO is stressful and lowers disease resistance of tilapia.

At first, tilapia were considered to be more resistant to bacterial, parasitic, fungal and viral disease than other species of cultured fish. However, in more recent times, tilapias have been found to be most susceptible to both bacterial and parasitic diseases. Common tilapia pathogens are *Streptococcus* sp., *Flavobacterium columnare*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Ichthyophitirius multifillis*, *Trichodina* sp. and *Gyrodactylus niloticus*. Streptococcal disease in tilapia has become an increasing problem and is among the leading diseases that cause severe economical impact (Shoemaker and Klesius 1997). *Streptococcus iniae* and *S. agalactiae* are the major tilapia pathogens among the streptococcal species that affect fish (Evans *et al.* 2006a). Eldar *et al.* (1994) first described two new streptococcal species, *S. shiloi* and *S. difficile*, but later in a second study reported that *S. shiloi* was *S. iniae* (Eldar *et al.* 1995a). Further, Vandamme *et al.* (1997) demonstrated that *S. difficile* was *S. agalactiae*.

The estimated economic impact of both *S. iniae* and *S. agalactiae* in tilapia likely exceeds $250 million annually, although the exact economic monetary losses remain unknown. In 1997, losses were estimated at over $100 million annually for *S. iniae* infection (Shoemaker and Klesius 1997). The losses in production were reported to be 30 to 50% in tilapia and rainbow trout (*Oncorhynchus mykiss*) for S. iniae infections by Eldar *et al.* (1994). In intensive culture of susceptible species, severe losses of up to 75% mortality were reported for *S. iniae* disease in closed systems (Perera *et al.* 1994, Stroffregen *et al.* 1996). Among the unwanted economic impacts of both pathogens are severe mortality losses, decreased weight gains, wasted feed due loss of appetite, poor survival during transport of survivors, loss of labor, water and loan costs.

Streptococcal diseases have proven to be difficult to control with antibiotics; however vaccines have and are being developed to prevent *S. iniae* and *S. agalactiae* diseases. Further, management of these pathogens is likely to require a health management strategy that uses both antibiotics and vaccines, where appropriate, to both prevent and control streptococcal disease outbreaks. Excellent sanitation practices and quarantine of juvenile tilapia prior to entering the production system are

also needed to help break the cycles of repeating infections. The aim of this paper is to present a review of current information on fish streptococcosis including aspects of epidemiology, diagnosis, pathogenesis, and prevention, treatment and control measures in farmed tilapia. Recent reviews of the literature on both *S. iniae* and *S. agalactiae* are available and they should be consulted (Evans *et al.* 2006a, Shoemaker *et al...* 2006a, Klesius *et al.* 2006a, Agnew and Barnes 2007).

MORPHOLOGY AND CULTURE

Streptococcus iniae and *S. agalactiae* share many phenotypic and pathogenic characteristics with other streptococci. Both streptococci are encapsulated Gram-positive cocci, occurring in short and long chains, facultative anaerobic, non-motile and may or may not produce β-hemolysis on sheep blood agar (SBA). They have optimal growth temperatures of 28 to 30°C at pH of 5.5 to 8.5. They can grow at 10-45°C and tolerant salt up to 30%. The temperature, pH and salinity ranges are also optimal for tilapia cultivation. Tilapia are a perfect host for these Streptococcus. spp. Both streptococci also grow rapidly in rich media, such as brain-heart infusion (BHI) and trypticase-soy (TS) media. One of the major and important differences between *Streptococcus iniae* and *S. agalactiae* is that the former is non-typable and the latter is a Lancefield's group B (Facklam 2002). Also, group B *S. agalactiae* has nine capsular polysaccharides. They are serotypes Ia, Ib, II, III, III-2, V, VI, VII and VIII. The serotypes Ia to VI can be directly identified by PCR (Kong *et al...* 2002). The colony morphology of these streptococci are small, convex, opaque and mucoid. Some of the Brazilian isolates of *S. agalactiae* are non-mucoid and difficult to breakup on agar. Asian *S. iniae* isolates from humans have been reported to have larger colonies, and are more mucoid and more β-hemolytic than human isolates from North America (Lau *et al.* 2006).

Worldwide distribution of *Streptococcus iniae* and *S. agalactiae*

Streptococcus iniae was first isolated from a skin lesion of a captive Amazon river, *Inia geoffrensis.* in an American aquarium (Pier and Madin 1976). A second isolate was cultured from a skin lesion of another captive Amazon dolphin kept at another American aquarium in 1978 (Pier *et al.* 1978). A third isolate was cultured from a captive fresh water dolphin in another American aquarium (Bonar and Wagner, 2003). Since the isolation of *S. iniae* in 1976, the bacterium has been reported in many species of fresh, estuarine and marine fish species from 15 countries in 6 continents that include Africa, Asia, Australia, Europe, North and South America (Table 1). Kitao (1993) listed 22 species of fish that are susceptible to *S. iniae* infection (Kitao 1993). Since 1993, at least another six species have been identified as susceptible (Table 1).

Distinct genetic variation has been demonstrated in isolates of *S. iniae* from fish and humans (Nawawi *et al.* 2008). However, a good correlation between geographical location and genetic profiles has not been established. Recently, a new streptococcal species, *S. ictaluri* was isolated from channel catfish broodstock on four catfish farms in the Mississippi Delta (Shewmaker *et al.* 2007). This new species is genetically similar to *S. iniae*, but not to *S. agalactiae*.

Table 1. Sources of *S. iniae* isolates, country origins and corresponding references

Common fish name	Country	References
Ayu	Japan	Ohnishi and Jo 1981, Kitao *et al.* 1981
Barramundi	Australia	Bromage *et al.* 1999
Barramundi cod	Australia	Bromage and Owens 2002
Black margate	Grenadines	Ferguson *et al.* 2000
Chubb	Barbados	Ferguson *et al.* 2000
Coho salmon	Israel	Eldar *et al.*1995b
European seabass	Israel	Zlotkin *et al.* 1998
Gilthead sea bream	Israel	Zlotkin *et al.* 1998
Gold spot cod	Australia	Bromage and Owens 2002
Largescale mullet	Bahrain	Yuasa *et al.* 1999
Grey mullet	Israel	Eldar *et al.* 1995b
Grunt Hybrid striped bass	Barbados USA	Ferguson *et al.* 2000 Stoffregen *et al.* 1996, Perera *et al.* 1994, Shoemaker *et al.* 2001
Japanese flounder Lizard fish Lyretail grouper	Japan Israel Israel	Nakatsugawa 1983, Nguyen *et al.* 2002 Colorni *et al.*2002, Kvitt and Colorni 2004 Kvitt and Colorni 2004
Parrot fish	Barbados and Grenadines	Ferguson *et al.* 2000
Puffer fish	Australia	Bromage and Owens 2002
Rabbit fish	Singapore, Israel, Bahrain, Australia	Foo *et al.* 1985 Zlotkin *et al.* 1998 Yuasa *et al.* 1999 Bromage and Owens 2002
Rainbow trout	Israel, Japan	Eldar *et al.* 1994 ,Kitao *et al.* 1981
Red drum	Israel, China	Zlotkin *et al.* 1998, Eldar *et al.*1999b Shen *et al.* 2005
Silver bream Snapper	Australia Barbados	Bromage and Owens 2002 Ferguson *et al.* 2000
Striped piggy Russian and Serbian sturgeons Tilapia spp.	Israel Uruguay Canada, Coumbia, China, Israel, Philippines Taiwan, USA, Venezuela	Colorni *et al.* 2002, Kvitt and Colorni 2004 Klesius, personal communication Weinstein *et al.* 1997 Pulido *et al.* 1999 Shen *et al.* 2005 Eldar *et al.* 1995b Clark *et al.* 2000 Eldar *et al.* 1994, Ramesh *et al.* 1994, Bowser *et al.* 1998, Shoemaker *et al.* 2001, Klesius *et al.*2006 Conroy 2001
Yellowtail	Japan	Kaige *et al.*1984

In addition to its importance in aquaculture, *S. iniae* has been isolated from humans affected by bacteremia, cellulitis, meningitis and osteomyelitis (Weinstein *et al.* 1997, Durborow 1999, Lehane and Rawlin 2000, Lau *et al.* 2003, Facklam *et al.* 2005). The source of human infections has been associated with preparation of *S. iniae* infected tilapia for cooking (Lehane and Rawin 2000). Hence, *S. iniae* host

range and distribution is not limited to fish and to one continent. *Streptococcus iniae* has progressively spread to several continents and many aquatic organisms.

Streptococcus agalactiae is the only group B *Streptococcus*. It was first reported in captive freshwater shiners in 1966 (Robinson and Meyer 1966). This pathogen has been isolated from 17 fish species (Wilkinson *et al.* 1973, Plumb *et al.*.. 1974, Rasheed and Plumb 1984, Elliott *et al.*1990, Baya *et al.* 1990, Eldar *et al.* 1995, Vandamme *et al.* 1997, Evans *et al.* 2002, Duremdez *et al.* 2004, Suanyuk *et al.* 2005, Salvador *et al.* 2005, Evans *et al.* 2006a, Evans *et al.* 2006b and Kim *et al.* 2007, Garcia *et al.* 2008, Table 2).

Table 2. Sources of *S. agalatiae* fish isolates, country origins and corresponding references

Common fish name	Country	References
Asian sea bass	Thailand	Kasornchan *et al.* 1986
Atlantic croaker	USA	Cook and Lofton 1975, Plumb *et al.*.. 1974
Atlantic menhaden	USA	Plumb *et al.* 1974
Bluefish	USA	Baya *et al.* 1990
Golden shiner	USA	Robinson and Meyer 1966
Grey mullet	Israel	Eldar 1995
Grey weakfish	USA	Baya *et al.* 1990
Gulf menhaden	USA	Plumb *et al.* 1974
Hybrid striped bass	Israel	Garcia *et al.* 2008
	USA.	Garcia *et al.* 2008
Killifish	USA	Rasheed and Plumb 1984
Klunzinger mullet	Kuwait	Evans *et al.* 2002
Pinfish	USA	Plumb *et al.* 1974
Rainbow trout	Israel	Eldar *et al.* 1994
Sand goby	Thialand	Kasornchan *et al.* 1986
Sea catfish	USA	Plumb *et al.*1974
Silver pomfret	Kuwait	Durnmdez *et al.* 2004
Silver trout	USA	Plumb *et al.*1974
Spot	USA	Plumb *et al.* 1974
Striped bass	USA	Baya *et al.* 1990
Striped mullet	USA	Plumb *et al.* 1974, Cook and Lofton 1975
Tilapias	Brazil	Salvador *et al.* 2005.
	Honduras	Garcia *et al.* 2008
	Israel	Eldar *et al.* 1994
	Thailand	Suanyuk *et al.* 2005
	USA	Garcia *et al.* 2008
Yellowtail	Japan	Eldar *et al.* 1994

Susceptible cultured fish include rainbow trout, seabream, tilapia, yellowtail, catfish spp., croaker, killifish, menhaden spp., mullet spp. and silver pomfret. An aquatic mammal, the bottlenose dolphin is also susceptible to *S. agalactiae* infection (Zappulli *et al.* 2005, Evans *et al.* 2006b). Evans *et al.* (2006b) reported that *S. agalactiae* was isolated from the epaxial muscle of a recently deceased dolphin in Kuwait. The dolphin was collected during the *S. agalactiae* fish kill in the Kuwait Bay. *Lactococcus garvieae* was also isolated from the kidney of same dead dolphin. The *S. agalactiae* isolate was shown to cause disease in tilapia (90% mortality in experimental trials). Tilapia experimental infected with the *L. gariveae* isolate did not die. In an unpublished study, tilapia from Idaho, U.S. farms harbored both *S. agalactiae* and *S. iniae* (Klesius personal observation). Concurrent infections can

occur in fish raised in intensive systems. *Streptococcus agalactiae* has been reported in fish from 7 countries on 3 continents: United States (North America), Israel, Japan, Kuwait and Thailand (Asia), Honduras (Central America) and Brazil (South America).

In humans, *S. agalactiae* is the causative agent of neonatal meningitis, sepsis and pneumonia (Baker 1980). It is also a well known cause of bovine mastitis (Lämmler and Hahn 1994). In addition, it has been isolated from horses, monkeys, cats, dogs, rabbits, nutrias, guinea pigs, and mice, with occasional pathologic changes (Wibawan *et al.* 1993, Lämmler *et al.* 1998, Yildirim *et al.* 2002). Thus the host range and distribution of *S. agalactiae* is also not limited to fish and to one continent. The genetic variability of *S. agalactiae* isolates from fish was characterized by using single-strand conformation polymorphism (SSCP) analysis of the intergenic spacer region (ISR) and amplified fragment length polymorphism (AFLP) fingerprinting (Olivares-Fuster *et al.*.. 2008). A total of 46 *S. agalactiae* fish isolates were characterized into five distinct genotypes by SSCP. Genotype 1 included isolates from Kuwait while genotype 4 was from USA, Brazil and Honduras. Reference isolates from cattle and humans were grouped in genotype 2 and genotype 3. The reference isolate isolated in Israel from tilapia was in genotype 1 and a hybrid striped bass isolate also from Israel was in genotype 5. When data from ISP-SSCP and AFLP were combined a good correlation was found between geographical location and genotypes.

The initial geographical source of *S. iniae* and *S. agalactiae* is unknown for tilapia. However, these pathogens were most likely first transmitted from carrier fish to other countries from a region where conditions favored *Streptococcus* infection in tilapia. The importation of carrier tilapia from Thailand has been suspected, but not proven to account for arrival of both *S. iniae* and *S. agalactiae* in the U.S. Intensive culture and sale of tilapia fry within and outside the USA progressively spread the pathogens.

Transmission

The entry of newly introduced fish is the most frequent source of introduction of *S. iniae* and *S. agalactiae* into the fish farm. Asymptomatic carriers are the principal infection source. Both *S. iniae* and *S. agalactiae* can be isolated from the brain of tilapia, without disease signs. The stress associated with shipping, handling and changes in water temperature, dissolved oxygen, pH and other factors may induce active infections in carriers. Among the means to disseminate these pathogens, cannibalism of the external and internal organs of dead and dying carriers by the rest of the healthy fish seems to be the most likely mechanism of disease spread. Possible shedding of the pathogens into the water via the feces is another mechanism. These

pathogens can survive in the culture water and be infectious for healthy fish (Nguyen *et al.* 2002).

Another mechanism of introduction is feed contaminated with one or both of these pathogens. For example, trash fish used to feed cultured flounder in Korea was found to be contaminated with *S. iniae* (Kim *et al.* 2007). It is believed that that this was responsible for disease outbreaks in flounder. Experimentally, cohabitation of dead infected fish with healthy fish results in the infection of the healthy fish. Horizontal transmission of the pathogens between fish is very likely the mechanism of dissemination. Among the factors that promote horizontal transmission are low and high water temperature, dissolved oxygen levels, salinity, crowding, poor nutrition, injury and parasite infestion. Ndong *et al.* (2007) demonstrated that transfer of tilapia from 27°C to low temperatures of 19 and 23 °C after 12h and transfer to 19 and 35°C from 25°C reduce both their immune capacity and resistance to mortality from *S. iniae* infection. A low dissolved oxygen concentration (0.1 to 0.5 mg/L) was found to increase the susceptibility of tilapia to *S. agalactiae* infection (Evans *et al...* 2003). Dissolved oxygen levels of 1.0 mg/L or below for short duration was found to increase *S. agalactiae* mortality. Injured tilapia in elevated salinities at 25 and 30°C were found to be more susceptible to *S. agalactiae* infection (Chan and Plumb 1996).

In another experimental study, it was demonstrated that tilapia kept at a high stocking density were significantly more susceptible to *S. iniae* (Shoemaker *et al..* 2000). When tilapia were cultured at a densites of ≥11.2 g/L, mortality from *S. iniae* infection significantly increased. It is possible that fish become susceptible to streptococcal disease by direct infection through wounds and abrasions to their skin (Xu *et al.* 2008). A recent report demonstrates that concurrent infection of tilapia with *Gyrodactyus niloticus* and *S. iniae* resulted in a significant increase in susceptibility to *S. iniae* disease. (Xu *et al.* 2008). These authors also showed that *G. niloticus* can harbor *S. iniae* and may be a vector of infection for tilapia. Concurrent infection with *Trichodina* spp. and *S. iniae* or *S. agalactiae* also increased the susceptibility channel catfish to both of these streptococci (Evans *et al...* 2007). Further, neither *S. iniae* or *S. agalactiae* alone are considered to be natural pathogens of channel catfish. These results demonstrate that external parasites may play an important role in the susceptibility of fish to *S. iniae* and *S. agalactiae* infections.

Once *S. iniae* and/or *S. agalactiae* are introduced into a fish farm, they are likely to become permanent resident pathogens. Fish farms typically experience seasonal outbreaks, which usually occur during the months of hot water temperatures (30-35°C). However, fish farms using constant hot water also experience outbreaks of disease throughout the year. Pathogen contaminated water and sediments also

help sustain the presence of the streptococci and special care needs to be taken not to introduce contaminated water to pathogen-free water (Nguyen *et al.* 2002).

Tilapia and hybrid striped bass are susceptible to infection via their nares and gills (Evans *et al.* 2000; McNulty *et al.* 2003). These authors reported that after infection by these routes of exposure, the fish showed disease signs and mortality. They were able to recover *S. iniae* from nares, brain, eyes and head kidney of the dead fish and survivors. The survivors were asymptomatic carriers of *S. iniae*. The gills and nares are the likely the routes of infection and initial sites of colonization. It is reasonable to suspect that following the colonization of the gills that the microbes are disseminated by blood to the brain, heart, kidney, liver, spleen and eyes. Following colonization of the nares, the streptococci disseminate directly to the brain and then to the other organs.

The transmission of *S. iniae* and *S. agalactiae* between different species of wild and cultured fish within the same aquatic environment is likely to occur (Evans *et al.*.. 2002). Wild fish and fish cultured nearby were found to be infected with the same *S. iniae* strains in Israel (Zlotkin *et al.* 1998; Colorni *et al.* 2002). Bromage and Owens (2002) reported that fish cohabiting barramundi pens had the same *S. iniae* strain as the barramundi. Transmission between species of reef fish was reported in the Caribbean by Ferguson *et al.* (2000).

Fish to human transmission is known to occur, as previously discussed. The zoonotic transmission of *S. agalactiae* between fish and humans and other animals is thought to be rare and if it exists, it is of little significance. Human consumption of cooked infected fish is considered to be safe WHO (1998). Recently, a study reported that bovine mastitis isolates were not infectious for either tilapia or channel catfish (Garcia *et al.* 2008).

Pathogenesis

Diseases of fish caused by *S. iniae* infection include meningitis, panophthalmitis, necrotizing dermatitis and spleen and kidney destruction, which results in high levels of morbidity and mortality (Eldar and Ghittino 1999a). The disease associated with *S. iniae* infection starts with anorexia, lethargy, loss of orientation and erratic swimming. Later, the infected fish exhibit external signs of uni-or bilateral exophthalmia, deformed back and hemorrhages in the periorbital, intraocular area and base of fins and perianal region. However, the progression of disease is dependent on the virulence of the isolate, fish species, route of infection, fish age, water temperature and dissolved oxygen levels. These external signs are very similar to those produced by *S. agalactiae* (Evans *et al.* 2006a).

These pathogens are highly virulent to both wild and cultured fish. Relatively little information is known about virulence factors and genes which encode them. The virulence factors include presence of a capsule, a gene associated with an enzyme which regulates capsule production and genes which encode transport-associated or regulator proteins (Miller and Neely 2005, Barnes *et al.*. 2003, Jones *et al.*.. 2000). The capsule plays an important role in streptococcal virulence. A two component system, sivS-R regulating *S. iniae* capsule expression was identified and characterized (Bolotin *et al.* 2007a). In a second study, a sivS-R deletion-insertion mutant was shown to express reduced virulence (Bolotin *et al.* 2007b).

The enzyme phosphoglucomutase was identified as a virulence factor for *S. iniae* (Buchanan *et al.* 2005). This enzyme interconverts glucose-6-phosphate and glucose-1-phosphate, which play important role in *S. iniae* polysaccharide capsule production. A phosphoglucomutase–disrupted gene mutant was produced and demonstrated to be non-virulent in hybrid striped bass. The virulence factor(s) of *S. agalactiae* appear to be associated with regulatory protein or enzyme associated with cell surface metabolism (Jones *et al.* 2000). These authors found that the insertion of genes involved in these functions into mutants reduced their virulence. Additional virulence factors include genes associated with β-hemolysis (Spelleberg 2000, Fuller *et al.* 2002). Additional research is needed to identify and characterize virulence factors and genes that regulate their expression.

Diagnosis

Diagnosis should not be based on disease signs that appear to be characteristic of streptococcal infection because of the similarity of these signs between streptococcal species and other fish pathogens. Enrichment and selective media to isolate streptococci from a mixed bacterial species should be used. Further, fish tissue, water or sediment samples may have lower numbers of streptococci, making enrichment necessary to culture them. Samples may be taken from brain, kidney, nare and eye of fish using a sterile dry-cotton swab technique. The swab is put into a sterile tube that contains tryptic soy broth supplemented with 4-5 drops of defibrinated sheep blood and incubated for 4 to 6 h at 28 to 30°C. The swab is removed and an inoculating loop sample is streaked onto sheep blood agar (SBA). The plate is incubated for 18-20 h at 28 to 30°C and a single suspected pure colony is picked for characterization and storage. *Streptococcus* spp. are stored in freezer vials at -80°C. This enrichment technique was found to be very helpful in the isolation of streptococci from a variety of fish species (Shoemaker *el al.* 2001, Evans *et al.* 2002). Shoemaker *et al.* (2001) determined that the prevalence of tilapia and hybrid striped bass infected with *S. iniae* was 37 out of 970 (3.81%) and 30 out of 415 (7.23%),

respectively. The prevalence per farm ranged from 0 to 27.4% for tilapia and 0 to 21.6% for hybrid striped bass. Evans *et al.*.. (2002) determined that overall percentage of *S. agalactiae* infected mullet and seabream collected from outbreak in Kuwait was 82.1% and 60, respectively. The overall percentage for both species was 79.5. Brains from mullet and seabream yielded the best recovery of *S. agalactiae*. The head kidney and intestine yield the next best recovery of 40 and 25%, respectively.

Another selective agar and enrichment broth for isolation of *S. iniae* is thallium acetate-oxolinic acid-blood agar and Todd-Hewitt broth supplemented with thallium acetate, colistin and oxolinic acid (Nguyen *et al.* 2002). In this study, brain, kidney intestine, skin mucus, nares, gill and eye tissue were sampled from diseased Japanese flounder using a sterile swab. The swabs were placed into Todd-Hewitt supplemented broth and incubated for 72 h at 28ºC and then plated onto the selective medium. Suspected colonies grown on the selective medium were confirmed by slide agglutination tests (Nguyen and Kanai 1999). The detection results for brain and kidney were 6.1 and 5.7% from 230 cultured fish. The detection results for the gill, mucus, intestine, eye and nare were 26.6, 29.6, 10, 8.9, and 7.4%, respectively. Further, water and sediment samples were collected from the culture units. The detection result for water was 21.6% (8/37 units).

A single colony from a pure culture should be Gram-positive cocci, oxidase and catalase-negative and either non-hemolytic or β-hemolytic on SBA. The carbohydrate group antigen test should also be among the first presumptive test performed. The only group B streptococcal species is *S. agalactiae*. In contrast, *S. iniae* does not have a carbohydrate group antigen. If the streptococci hydrolyze starch, it is also presumptive test for *S. iniae* (Evans *et al.* 2004a). Biochemical and other identification tests are fully described elsewhere (Shoemaker *et al.* 2001, Evans *et al.* 2002).

Miniaturized systems such as API 20, API rapid strep 32 and API CH 50 should not be used to identify *S. iniae* because this microbe is not included in the systems' databases (Evans *et al.* 2006a). These miniaturized systems are suitable for the identification of *S. agalactiae* (Evans *et al.* 2006a). However, the miniaturized system results may not completely match with the classical biochemical test. Comparative evaluation between Vitek Gram-positive identification system and the API rapid strep 32 system revealed that 93% of *S. agalactiae* isolates were identified by both systems (Jayarsao *et al.* 1991). A comparison between API rapid strep 32 systems and Biology system using Gram-positive plates showed that both systems produced 100% identification of the *S. agalactiae* isolates (Evans *et al.* 2006b). The Biology system using Gram-positive plates was shown to correctly identify over 70% of S. iniae

isolates from disease fish collected in a fish kill in the Eastern Caribbean in 1999 (Roach *et al.* 2006). The Biology system results were confirmed by specific PCR methods.

Molecular diagnosis such as PCR techniques are useful tests to identify both species and a variety of PCR techniques are available. Many of the PCR techniques use the 16S rRNA gene as the molecular marker for identification of *S. iniae* (Zlotkin *et al.* 1998). Often the 16S based PCR technique lacks specificity. Mata *et al.* (2004) developed a PCR assay based on the lactate oxidase gene and demonstrated that this assay provided rapid and sensitivity identification of *S. iniae* from fish and other animals. This PCR assay had a sensitive between 31-62 cells. A PCR technique using 16S-23S ribosomal DNA intergenic spacers was found to be useful for the identification of *S. agalactiae* from fish (Berridge *et al.*.. 2001). A PCR assay targeting 23S rDNA sequences was also developed for identification of eight streptococcal species, including *S. agalactiae* (Kawata *et al.* 2004). This assay utilizes two tube reactions per isolate and appears to be an accurate diagnostic tool for the fish health laboratory. A rapid PCR assay was developed using 16S-23S rRNA sequences for *S. agalactiae* that may also have diagnostic potential for fish isolates (McDonald *et al.* 2005). The results of PCR assays for either species should be confirmed with other presumptive techniques to ensure the accuracy of the detection and identification.

An indirect fluorescent antibody technique (IFAT) based on a highly specific monoclonal antibody for *S. iniae* is suitable for the rapid detection and identification of *S. iniae* from experimentally or naturally infected tilapia (Klesius *et al.* 2006b). The olfactory epithelium of the nares of naturally infected tilapia was demonstrated to be a reliable, sensitive and non-lethal sample site for the detection and identification of *S. iniae*. This IFAT assay will also detect and identify *S. iniae* in carriers.

Prevention, Control and Treatment

A excellent health management plan needs to be establish for a fish farm and followed without variations on 24/7/12 basis. This plan should include managing the fish farm free of pathogens by purchasing of specific pathogen-free fish stock, quarantining new arrival fish stock, using lower fish densities, keeping separate water supplies for culture systems, removing dying and dead fish frequently, feeding pathogen-free rations, keeping excellent sanitary conditions and minimizing stressful conditions and parasitism. Further, periodic cleaning and disinfections of all production units and equipment will act to decrease the transmission of pathogens. The cleaning and disinfection should be performed according to the manufacturer's label and with the proper licensed products for a fish species. Maintaining good water quality and management of organic solids in the system is also needed. Since it is not

always possible to properly manage water temperatures and dissolved oxygen levels, beware of conditions that favor disease outbreaks and be ready to apply other means of disease control. For example, streptococcal outbreaks tend to occur at water temperatures higher than 15ºC.

Increased fish survival has been reported for chemotherapeutics such as amoxicillin (Darwish and Hobbs 2005). Chemotherapeutants can be a useful tool for treatment and short-term control of fish disease outbreaks, but are not useful for prevention. Indeed, in many countries antibiotics can not be used prophylactically to control fish diseases, nor should they be used this way. Prophylactic use promotes the development of antibiotic-resistant strains of bacteria, which reduces efficacy for treatment of both animal and human infections. In addition, effective treatment concentrations can be difficult to reach under field conditions. Moreover, antibiotic treatments can be only used after a prescribed number of dead and dying fish are reached in many cases.

Vaccination, combined with the prevention strategies discussed earlier, will greatly reduce the risk of fish disease outbreaks. Vaccination does not have many of the problems associated with chemotherapeutants. However, both vaccination and administration of antibiotics can work together under certain conditions. For example, antibiotics can be administered when a large pathogen load overwhelms vaccine efficacy or when the fish immune system is depressed under conditions such as suboptimal water temperatures or nutrition. An efficacious chemotherapeutic agent could be used to stop the outbreak. However, the interactions between vaccination and the application of a chemotherapeutic agent are unknown and needs to be determined.

A vaccine is a preventive tool used in a health management strategy for the control of infectious diseases (Klesius *et al.* 2006a). For more than 100 years, vaccines have proved to be effective for preventing infectious disease outbreaks in humans, poultry and other food animals. In aquaculture, the development and use of vaccines is now making rapid progress to achieve full potential as effective disease prevention tools. The objective of vaccination is to provide a strong immune response to an administered antigen able producing acquired long-term protection against a pathogen. To achieve this objective, either a killed or modified live vaccine needs to be developed and licensed for use in fresh or marine water aquaculture. The type of immunity needed, antibody and/or cell-mediated, against a particular pathogen is among the deciding factors in the development of a vaccine. Immunization with a killed bacterial vaccine produces an antibody-mediated protection, but not cell-mediated immunity. Further, antibody-mediated immunity provided by killed vaccines

is relatively short lived in terms of protection (3-5 months). The duration of protection may be increased by the addition of an adjuvant to the killed vaccine. An adjuvant is a substance that increases specific immune responses against the co-inoculated antigen. Killed vaccines are usually administered by intraperitoneal (IP) and/or intramuscular injection (IM) of individual fish. Injection is the least cost effective in terms of labor and time. Killed vaccines are considered to be safer than modified live vaccines which may revert to virulence. Consequently, future trends include oral delivery of vaccines, immersion delivery of killed vaccines, development of additional modified live vaccines and multivalent vaccines and improved vaccine adjuvants and immunostimulants. Vaccines prevent disease and mortality, but may not completely eliminate streptococci in surviving fish.

Formalin-killed vaccines have been developed against both of these pathogens over the past eleven years. The first autogenous vaccine was developed to prevent losses in trout due to *S. iniae* infection in Israel (Eldar *et al.* 1997). The mortality in rainbow trout IP immunized with this formalin-killed *S. iniae* vaccine was 5%, whereas in non-immunized fish the mortality exceeded 50% under field conditions. The duration of protection was at least 4 months.

A specific antibody response following immunization was associated with immunity to *S. iniae*. Further additional evidence for the role of specific antibody was demonstrated by Shelby *et al.* (2002). Previous studies have demonstrated the importance of antibody in the acquired immune response of tilapia to *S. iniae* either indirectly by measuring antibody in vaccinated and protected fish (Eldar *et al.* 1997; Klesius *et al.* 1999, 2000) or directly by passive immunization (Shelby *et al.* 2002). Vaccinated tilapia or tilapia that survives *S. iniae* infection are seroconvert and produce a protective antibody response with little or no effect on the growth performance (Shoemaker *et al.* 2006c; Whittington *et al.* 2005). Removal of tilapia showing overt signs of infection (i.e., difficulty in consuming feed) may improve overall production efficiency of water re-use systems (Shoemaker *et al.* 2006d).

A non-autogenous killed *S. iniae* vaccine supplemented with its extracellular products (ECP) was found to be effective in tilapia (Klesius *et al.* 1999). The mortality was reduced by 91.3% in tilapia immunized IP with this vaccine at 30 d post-experimental challenge with *S. iniae*. The molecular weight of the ECP was greater than 2 kD and the *S. iniae* isolated was from an infected hybrid striped bass. The relative percent survival (RPS) was 95% in 25 g tilapia and 84.2% to 94.7% in 100 g tilapia. In a second study, this vaccine was administered by IP and IM routes in tilapia (Klesius *et al.* 2000). The RPS result of heterogeneous isolate challenge was 93.8% in immunized tilapia. The RPS result in tilapia immunized by IM route was

17.7. A combination vaccine comprised of two *S. iniae* isolates gave RPS of 63% and 87% against each of these isolates. The ARS-60 vaccine provided protection against heterogeneous *S. inaie* isolate challenge. The vaccines stimulated *S. iniae* antibody response, which likely played a important role in immune protection. The efficacy of a Streptococcus vaccine is dependent on its antigen composition. In a third study, the ECP *S. iniae* vaccine was tested in a cohabitation model using calcein marked tilapia so that vaccinated and control fish could be discerned from each other but still cohabitated (Klesius *et al.* 2006c). The cumulative mortality of IP immunized tilapia was significantly less than for the non-immunized calcein marked tilapia in the same tank. Shin *et al.* (2007) reported that ECP S. iniae vaccine gave lower protection against a heterogeneous isolate challenge than for a homogeneous isolate challenge. Further, a significant difference in the amount ECP secreted between the two isolates was noted. Thus, It appears that some isolates secrete more ECP than other isolates. The ECP were also found to be more immunogenic compared to whole cell lysates in this study.

The ECP from *S. iniae* and *S. agalactiae* contain chemotactic factors for macrophages (Klesius *et al.* 2007). The chemotactic activities of ECP from both species are likely to be involved in proinflammatory responses of macrophages to *S. iniae* and *S. agalactiae*. Further, the secretion or excretion of *S. iniae* and *S. agalactiae* ECP are likely to be composed of immune regulatory molecules ncluding polysaccharides from their capsules. The immune regulatory molecules and capsular polysaccharides would play a important role in regulating immunity. Additional studies are necessary to determine the biological activities of ECP.

Whole cell and bacterial protein vaccines were produced against *S. agalactiae* (Eldar *et al.* 1995c). These authors reported that these vaccines were efficacious in tilapia. An ECP *S. agalactiae* vaccine was demonstrated to be efficacious in tilapia by IP injection (Evans 2004b). Immersion immunization of 5 and 30 g tilapia resulted in RPS valves that were two times lower than by IP immunization. Further, *S. agalactiae* immunized fish were not protected against *S. iniae* challenge.

Western blot analysis revealed predominant 54 and 55 kDa antigens in the ECP of *S. agalactiae* (Pasnik *et al.* 2005). The results of this study provide a correlation between protection and antibody production against ECP and for the importance of the 55 kDa antigen for vaccine efficacy against *S. agalactiae*.

In tilapia, bath immersion or oral route of immunization would be more cost-effective than injection. The ECP *S. iniae* vaccine was found to be efficacious in tllapia by bath immunization (Klesius *et al.* 2006d). In addition, the ECP *S. iniae* vaccine was shown to be efficacious when delivered orally using the commercial delivery system,

Oralject™ in tilapia (Shoemaker *et al.* 2006b). The Oralject™ delivery system consists of bioactive compounds and killed S. iniae vaccine. This technology relies on the temporary reduction of the digestive process by anti-protease and membrane permeability enhancers in combination with the vaccine. The RPS of this oral vaccine was 63 in tilapia fed 2×10^9 colony forming units/g feed for one day.

Bath immersion and oral vaccination offer attractive alternatives to the tradition method of vaccination by IP injection. The use of either of these routes for primary immunization does not guarantee complete protection, although further research may provide improved immersion and oral vaccines. The incorporation of immunostimulants may be able to booster the efficacy and duration of protection. Vaccination is the most effective method to prevent streptococcal disease. Overall, vaccination is still the most effective method to prevent streptococcal disease.

In conclusion, *S. iniae* and *S. agalactiae* are important emergent pathogens that affect many fish species world-wide. During the last decade, the number of reports describing disease outbreaks in many different species of fresh and marine fish has significantly increased. Tilapia are the perfect fish host for *S. iniae* and *S. agalactiae*. Although useful information of the mechanisms of transmission has increased, still more information need to learn and communicated to the fish producer. Rapid advances in the diagnosis of diseased and carrier fish have been made using rapid and accurate immunological and molecular techniques. Vaccines have and are being developed, licensed and use in many countries. Considerable evidence shows that anti-streptococci serum antibodies play a key role in immunity following vaccination. Mass vaccination strategies needs to be developed. However, vaccination against *S. iniae* and *S. agalactiae* needs to be part of health management plan to prevent and control infection and disease.

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