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In vivo and in vitro pathogenesis of Francisella asiatica in tilapia nilotica (Oreochromis niloticus)

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IN VIVO AND *IN VITRO* PATHOGENESIS OF *FRANCISELLA ASIATICA* IN TILAPIA NILOTICA (*OREOCHROMIS NILOTICUS*)

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ABSTRACT

Francisella asiatica is a Gram negative, facultative intracellular pathogen that causes fish francisellosis. In this project, homologues to the *F. tularensis iglABCD* genes were found present in *F. asiatica*. As few as 23 *F. asiatica* bacteria injected in the peritoneum were found capable of causing mortalities in tilapia nilotica (*Oreochromis niloticus*), and even fewer were enough to cause pathological changes. We examined the *in vivo* and *in vitro* interaction of *F. asiatica* wild type (WT) and a Δ*iglC* strain with tilapia and tilapia head kidney derived macrophages (HKDM). The Δ*iglC* was found to be attenuated following intraperitoneal and immersion challenges in tilapia. The WT was found to be able to invade HKDM and replicate vigorously within them, causing apoptosis and cytotoxicity in the macrophages. The Δ*iglC*, however is defective for survival, replication and the ability to cause cytotoxicity in HKDM. We further characterize the efficacy of the Δ*iglC* as a live attenuated vaccine against subsequent immersion challenge with the WT. Tilapia vaccinated by immersion with a suspension of the Δ*iglC* and subsequently challenged with WT were protected (90% survival) from the lethal challenges. *F. asiatica*-specific antibodies produced in response to immunization with the Δ*iglC* were subsequently found to protect tilapia against WT challenge in passive immunization experiments. The lack of effective treatments led us to investigate the efficacy of florfenicol for treatment of *F. asiatica in vitro* and *in vivo*. Addition of florfenicol to the medium at 10 µg/ml resulted in uptake of the drug by HKDM and significantly reduced bacterial loads *in vitro*. Fish fed medicated feed 1 and 3 days post infection showed significantly higher survival rates, and significantly lower numbers of bacteria than controls. Finally, a real time polymerase chain reaction assay was developed to rapidly and accurately detect and quantify *F. asiatica* from fish tissue. Probe specificity was confirmed by the lack of signal and cross-reactivity with twelve common fish pathogens, two

subspecies of *F. tularensis*, *F. noatunensis*, and tilapia tissue. The limit of detection was 50 fg of

DNA (~25 genome equivalents).

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

TILAPIA AND TILAPIA AQUACULTURE

Tilapia is a generic term used to designate a group of commercially important food fish belonging to the family Cichlidae. Cichlids are classified in the large order Perciformes, and inhabit the fresh and brackish waters of Africa, the Middle East, coastal India, Central and South America. Important commercial species include: the Mozambique or Java tilapia (*Oreochromis mossambicus*, also known as *Tilapia mossambica*), blue tilapia (*O. aureus*, a.k.a. *Tilapia aurea*), Nile tilapia (*O. niloticus*, a.k.a. *Tilapia nilotica*), Zanzibar or Wami tilapia (*O. hornorum*, a.k.a. *Tilapia urolepis*), and the redbelly tilapia (*O. zilli*, a.k.a. *Tilapia zilli*) (Chapman 1992; Sell 1993).

Tilapia are hardy, prolific, fast-growing tropical fish native to Israel, where they have been farmed for about 2,500 years. The suitability of tilapia for various types of aquacultures relates to their ease of propagation; handling tolerance; fast growth utilizing natural food or a variety of supplemental feeds under various degrees of intensification; tolerance of a wide range of environmental conditions, including resistance to poor water quality and disease; and being perceived as a palatable, marketable, and nutritious product (Balarin and Haler 1982; Lim et al. 2006). During the last two decades, production of tilapia has increased worldwide from 703 thousand tons in 1995 to 1.675 million tons in 2004, with *Oreochromis niloticus* and hybrid red tilapia making up most of the types cultured (Alston 2009). In 2004, China produced over 897 thousand tons of the world's production of tilapia, and Brazil had the fastest growth of its tilapia aquaculture industry from 1994 to 2004, with almost half the tilapia production in the Western Hemisphere in 2004; but small developing countries like Costa Rica had extensive growth in tilapia production and in 2004 grew almost 20 thousand tons of cultured tilapia (Alston 2009).

TILAPIA BACTERIAL DISEASES

Intensification of pond and indoor aquaculture practices utilizing water reuse systems with the concomitant reduction in water quality resulting in elevated stress levels; have resulted in an increase in incidence and severity of disease agents in tilapia. Amongst the most common and virulent pathogens found causing mortalities in cultured tilapia are the bacterial pathogens: *Flavobacterium columnare*, *Edwardsiella tarda*, *Aeromonas* sp., *Vibrio* sp., *Francisella* sp., *Streptococcus iniae*, and *Streptococcus agalactiae* (Grabowski et al. 2004; Figueiredo et al. 2005; Pirarat et al. 2007; Ruangpan et al. 1986; Maluping et al. 2005; Mauel et al. 2007; Pereira et al. 2010; Mian et al. 2009). Other bacteria described as rickettsia like-organisms have been reported on several occasions as tilapia pathogens, but since the organisms were not cultured and molecular characterizations were not performed, the true etiology of the pathogens could not be elucidated (Chen et al. 1994; Chern and Chao 1994; Mauel and Miller 2003).

RICKETTSIA LIKE-ORGANISMS AND PISCIRICKETTSIA LIKE-ORGANISMS

The first report of *Rickettsia*-like organisms (RLO's) as fish pathogens was given by Mohamed (1939), where examination of diseased *Tetrodon fahaka* from the Nile river in Egypt revealed microorganisms in stained tissue cells. The first RLO's to be isolated, recognized, characterized and demonstrated to cause disease in experimentally infected fish was *Piscirickettsia salmonis* (Fryer et al. 1992). This bacterium is now recognized as the causative agent of piscirickettsiosis or salmonid rickettsia septicaemia (SRS). Although it shares similarities to certain rickettsial species, like replication within cytoplasmic inclusions in host cells, a rippled cell wall and electron-lucent bodies, the 16S rDNA sequences of *P. salmonis* places it within the gamma subdivision of the class Proteobacteria, rather than within the alpha subdivision within the genus *Rickettsia* (McCarthy 2008). The bacterium can cause mortalities of up to 90% among salmonids, and it has been found in various countries with viable salmon

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culture industries, like Canada, Chile, Norway, and Ireland (Mauel and Miller 2002). In salmonids, infected fish often present with skin lesions and appear lethargic. Affected fish may be anemic and some present with ascites and darkened pigmentation. Internally, many organs appear pale and multiple whitish foci can be found in enlarged, gray kidneys and liver. In hematopoietic tissues like the spleen and kidneys, there is generalized necrosis, with edema and fibrosis (Fryer and Mauel 1997; Mauel and Miller 2002). Macrophages in these tissues are enlarged and contain *P. salmonis*; which has also been demonstrated *in vitro* to be capable of surviving and replicating in salmonid macrophages (McCarthy et al 2008).

Besides *P. salmonis*, there have been several reports of other RLO's causing disease in fish. Chen et al. (1994) reported RLO's in diseased Nile tilapia (*Oreochromis niloticus*) in southern Taiwan. In that report the authors collected diseased fish from outbreaks in 1992, with variable clinical signs. Clinical signs included: pale color, hemorrages and ulcers in the skin, and ascites in the visceral cavity, swelling of the liver, spleen and kidneys with diffuse white nodules. Upon histopathological analysis RLOs were found within the cytoplasm of degenerating hepatocytes and within macrophages. The authors were not able to isolate and grow the bacteria in tryptic soy agar (TSA) or TSA with 5% goat blood, brain heart infusion agar, Sabouraud's dextrose agar or Lowestein-Jensen medium, but utilizing chinook salmon embryo (CHSE)-214 cells, RLO's appeared in cytoplasmic vacuoles 2 days post-infection. No cytopathic effect was observed in the CHSE-214 infected cells, and no bacteria were observed in inoculated fathead minnow (FHM), bluegill fibroblast (BF)-2 or tilapia ovary (TO)-2 cells. Electron microscopy performed with tissues from infected fish revealed the presence of pleomorphic coccoid organisms usually enclosed within membrane-bound cytoplasmic vacuoles. The RLO's size ranged from 0.5-1.2 µm in length, and possessed a relatively thin cell wall with numerous cytoplasmic vesicles. Since the authors were not able to culture the organism in various

bacteriological media, it was characterized as an obligate intracellular bacterium. Similar

findings were observed by Chern and Chao (1994), where between 1992 and 1993 mass

mortalities of pond-reared tilapia occurred over the entire island of Taiwan. Similarly to the

description of Chen et al. (1994), the authors found Gram-negative RLO's, which grew in TO-2

and ephithelioma papulosum cyprini (EPC) cell cultures but not on brain heart agar (BHA), BHA

with 5% CO_2 , blood agar (BA), BA with 5% CO_2, chocolate agar, nutrient agar, Lowenstein-

Jensen medium, Austin's selective kidney disease medium, and mycoplasma medium at 28°C.

Also similar clinical signs, macroscopic and microscopic lesions, and electron-microscopic

findings were observed by both groups (Chen et al. 1994; Chern and Chao 1994).

Khoo et al. (1995) reported the presence of intracytoplasmic organisms resembling

rickettsia in tissues of two blue-eyed plecostomus (*Panaque suttoni*). As in previous reports,

gross lesions were renomegaly and splenomegaly, but upon histopathological examination

various tissues revealed the presence of numerous, small, discrete coccoid organisms in the

cytoplasm of monocytes and macrophages.

In a report by Chen et al. (2000a), hatchery reared juvenile white seabass (*Atractoscion*

nobilis) were also found to be affected by a *Piscirickettsia salmonis*-like organism (WSPSLO).

The fish exhibited no consistent external clinical signs, but internally presented with enlarged

kidneys, livers and spleens with multiple pale foci. Microscopic lesions often included multifocal

hepatic, renal and splenic necrosis and intralesional macrophages containing the WSPSLO.

Smears from tissues stained with Wright-Giemsa revealed the presence of an intracytoplasmic

coccoid organism, that ranged in size from 0.5 to 1 μm. The bacterium was also found to possess

antigenic differences from *P. salmonis*, yet was found to be as virulent to salmon as *P. salmonis*

strains. The authors were not able to recover the suspected isolate on bacteriological agar media.

Also in 2000, another group reported the presence of *Piscirickettsia*-like organisms (PSLO) as

the causative agents of disease in grouper (*Epinephelus melanostigma*) in Taiwan. As with other

reports, the fish exhibited external dark lesions, and upon histopathological analysis, there was a

widespread congestion and splenomegaly with white nodules. Infected cells contained enlarged

vacuoles and numerous, polymorphic, basophilic intracytoplasmic organisms. The authors were

also able to infect CHSE-214 cells, but not FHM, BF2, GF and TO2 cells, and were not able to

isolate the bacteria using TSA with 5% goat blood, brain heart infusion agar, Sabouraud's

dextrose agar and Lowenstein-Jensen medium (Chen et al. 2000b).

Fukuda et al. (2002) reported sporadic mortalities occurring in Kyushu and Shikoku

districts in Japan since 1999 in cultured three-line grunt (*Parapristipoma trileneatum*). The fish

had been imported from China, and presented similar clinical signs, gross pathology and

histopathological lesions as the ones reported as caused by RLOs in cultured fish in Taiwan. The

white foci found in the internal organs were composed of granulomas; in which coccoid Gram-

negative intracellular organisms were observed, but could not be isolated (Fukuda et al. 2002).

During 2001 and 2002, Qionglin et al. (2004) reported the presences of RLO's in freshwater

cultured snakehead fish (*Ophiocephalus argus*) in China. As in the previous report of RLO

infected fish, the main pathological findings were the presence of white nodules in swollen

kidneys and spleens, that when observed microscopically revealed a severe granulomatous

inflammatory response with the presence of intracytoplasmatic inclusions that were composed of

small pleomorphic rods found enclosed in membrane-bound cytoplasmic vacuoles in

mononuclear cells. No isolation was attempted in this study (Qionglin et al. 2004).

Mauel and Miller (2002) and Mauel et al. (2003), reported the presence of PLOs causing

severe mortalities since the 1990s in cultured Hawaiian tilapia (*Oreochromis mossambicus* and

Sarotherodon melanotheron) on the island of Oahu. As with the previous reports, tilapia

suffering with infection by PLO and RLO showed few external lesions (darkening and

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ulcerations), gill epithelial hyperplasia; but when internal organs analysis was performed, multiple granulomas were observed in the gills, spleen, head kidney and choroid glands. The crater-form liver lesions diagnostic of *P. salmonis* infected salmon were not observed. Mortalities were found to occur mainly in the cooler months (October to April), and mortalities exceeded 60% of the farmed tilapia on the island. As with the previous reports, no bacterial species was consistently grown on any of the artificial media. Interestingly cohabitational studies performed demonstrated the virulence of the bacteria, causing more than 70% mortalities in exposed tanks after only 23 days. It was also concluded that the pathogenesis of the disease in cultured tilapia was higher in cooler temperatures (<25°C, than in temperatures closer to 30°C) (Mauel et al. 2003). The authors also reported that examined tissue from cultured tilapia from Jamaica, Indonesia, southern California, South Carolina and Florida, presenting similar clinical signs and mortality patterns were also found to contain histological lesions similar to those previously reported in PLOs epizootics; but in none of the cases were bacterial pathogens consistently isolated (Mauel and Miller 2002). In another report, Mauel et al. (2005) reported another epizootic of PLO causing mortality in culture tilapia during 2001 to 2003 in farms in Florida, California and South Carolina. Similar lesions, microscopic and bacteriological results were found as to those reported previously in Taiwan, and Hawaii, but again no bacterium was consistently isolated.

GENUS *FRANCISELLA*

Francisella is the only genus within the family *Francisellaceae*, and after analysis of the 16S rRNA the genus is placed in the γ subclass of the Proteobacteria (Forsman et al. 1994; Nano and Elkins 2006). The organism most closely related to *Francisella* is *Wolbachia persica*, a tick endosymbiont (Forsman et al. 1994). *Francisella* species are small Gram-negative, pleomorphic, non-motile, obligatory aerobic bacteria that do not produce spores (Foley and Nieto 2009).

Historically there were only three recognized species in the genus *Francisella*: *F. tularensis*, *F. novicida*, and *F. philomiragia*. All three species can cause human infections, although *F. novicida* and *F. philomiragia* rarely do (Nano and Elkins 2006). *Francisella tularensis* is a highly infectious member of this genus and is the causative agent of tularemia, also known as rabbit fever or deer-fly fever (Foley and Nieto 2009). Tularemia is a potentially fatal, multi-systemic disease of humans and animals, which can be transmitted by ticks, biting flies, water exposure, food and aerosols (Petersen et al. 2009).

Several different subspecies of *F. tularensis* have been reported. In older classifications these were *F. tularensis* biovar. *tularensis* (Jellison Type A), *F. tularensis* biovar. *palaeartica* (Type B), and *F. tularensis novicida* which has often been regarded as a separate species, *F. novicida*. In newer classifications, the subspecies *F. tularensis holarctica* and *mediaasiatica* have been proposed from *F. tularensis* subsp. *palaeartica* and *F. tularensis* also contains the subspecies *F. tularensis* subsp. *tularensis* (Keim et al. 2007; Foley and Nieto 2009). Differences among biovars were described on the basis of pathogenicity and geographical distribution. *Francisella tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* cause most human illness. *Franciella tularensis* subsp. *tularensis* is confined to North America, whereas subspecies *holarctica* is found in many countries of the Northern Hemisphere (Santic et al. 2010).

F. tularensis subsp. *tularensis* is considered a potential bioweapon because of its extreme virulence, low infectious dose, ease of aerosol dissemination, and capacity to cause severe illness and death. Indeed, inhalation of as few as 10 colony-forming units (CFU) is sufficient to cause disease in humans and 30% to 60% of untreated infections can be fatal (McLendon et al. 2006).

Interestingly Anda et al. (2001) identified *F. tularensis* biovar *palaearctica* in an outbreak of ulceroglandular tularemia in humans, associated with crayfish (*Procambarus clarkii*) fishing in a contaminated freshwater stream. It was also found that the bacterium only colonized

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the crayfish transiently since the bacterium was cleared from the digestive tracts of the crayfish within 4 weeks of collection (Anda et al. 2001).

FISH FRANCISELLOSIS

As reported by Fukuda et al. (2002), mortalities were found in cultured three line grunts in Japan since 1999, and clinical signs, gross lesions, and histopathological analysis of tissues were consistent to those previously described from a disease caused by RLOs (Mauel and Miller 2003). Kamaishi et al. (2005) utilized spontaneously diseased three line grunts collected from aquaculture farms from 1999-2000, and extracted the DNA from frozen kidneys. The DNA was utilized for PCR amplification and sequencing of the 16S rDNA utilizing universal eubacterial primers (Kamaishi et al. 2005). After PCR amplification and sequencing, the 16S rDNA sequence was aligned with other eubacterial 16 rDNA sequences and high similarities (>97%) was shown between the fish pathogen and *Francisella* species. Amongst these species, *F. philomiragia* was the closest relative (Kamaishi et al. 2005). Utilizing culture media commonly used to artificially grow *Francisella* in the laboratories, the authors were able to isolate bacteria from spleen samples utilizing Cysteine heart agar with 1% hemoglobin (CHAH) and then culturing at 25°C for 2 weeks. The authors observed smooth green-gray colonies in the CHAH and confirmed its identity utilizing PCR and sequencing analysis. They also proved Koch's postulates by injecting healthy fish with dilutions of the bacterial isolated, and producing lesions and mortalities as those found in nature (Kamaishi et al. 2005). This was the first report demonstrating the presence of a member of the genus *Francisella* causing disease in culture fish.

After this initial report of *Francisella* species causing mortalities in fish, several reports were made worldwide identifying members of the genus *Francisella* causing diseases and mortalities in cultured marine and freshwater fish. Nylund et al. (2006), Olsen et al. (2006), Ottem et al. (2007), Mikalsen et al. (2007), and Ottem et al. (2007 b) described and characterized

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a novel member of the genus *Francisella* causing systemic granulomatous inflammatory diseases and mortalites in farmed Atlantic cod (*Gadus morhua* L) on the coasts of Norway. The diseased fish presented similar clinical signs, gross pathological lesions and histopathological findings to descriptions of fish infected with *P. salmonis* and RLOs. Affected cod had white granulomas in the visceral organs and skin, and a species of *Francisella* was isolated on blood agar plates with addition of 0.1% cysteine and 1% glucose, and cysteine heart agar plates with the addition of 5% sheep blood, that were incubated at temperatures ranging from 6-22°C, but not in buffered charcoal yeast or bacto chocolate agar. The isolates showed 16S rDNA gene sequences similarities (>99%) to *F. philomiragia*. After complete sequence of 16S rDNA, 16S-23S intergenic spacer, 23S rDNA, 23S-5S intergenic spacer, 5S rDNA, FopA, lipoprotein TUL4 (LpnA), malate dehydrogenase and hypothetical lipoprotein (LpnB), Ottem et al. (2007) demonstrated and supported the close relationship between the *Francisella* sp. causing mortalities in cod and *F. philomiragia*. This group also demonstrated that the bacterium is catalase positive, indole positive, oxidase-negative, does not produce H_2S in Triple Sugar Iron agar, and does not hydrolyze gelatin. His group also reported the isolate produced acid from D - glucose, maltose, sucrose (weak) but not from lactose or glycerol. The isolate grew on both MacConkey agar and in nutrient broth (6% NaCl) and was resistant to trimethoprim-sulfamethoxazole, penicillines, cefuroxime and erythromycin, but was susceptible to ceftazidime, tetracycline, gentamicin, ciprofloxacin (Ottem et al. 2007). The bacteria was found to be present in most farmed cod from most parts of Norway when utilizing two real-time PCR assays, targeting the 16S rRNA gene and the FopA gene of *Francisella* sp. The bacteria was detected in wild populations of cod from all counties examined south of Sogn og Fjordane in southern Norway (overall prevalence 13%, n = 221) whereas wild cod north of Sogn og Fjordane were negative for the bacterium (n = 201). The authors concluded that the apparent absence of

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the bacterium in wild populations of cod in the northern parts of Norway and its widespread

occurrence in wild cod from southern parts of Norway is believed to relate to differences in

seawater temperatures (Ottem et al. 2009).

An isolate sharing 100% 16S rRNA sequence identity to the *Francisella* sp. isolated from

Norwegian cod was isolated and grown on cysteine heart agar with 5% sheep blood from spleen

and kidneys of moribund Atlantic salmon (*Salmo salar* L.) parr reared in Lake Llanquihue,

Chile. The isolate shared genetic and phenotypic characteristics to that described in Norway, but

Koch's postulates were not completed (Birkbeck et al. 2007; Bohle et al. 2009)

Ostland et al. (2006) and Mauel et al. (2007) also described emerging diseases associated

with aquatic *Francisella* – like bacteria that caused mortality in intensively cultured hybrid

striped bass (*Morone chrysops* X *M. saxatilis*) in southern California and cultured nile tilapia

(*Oreochromis niloticus*) in Latin America, respectively. Fish showed loss of appetite, dark

coloration, were lethargic, and presented similar gross pathology and histopathological findings

as previously described in Japanese three line grunts. In this study the authors were not able to

isolate the bacteria, since they utilized media not suitable for the growth of *Francisella* sp.; but

upon amplification and sequence analysis of the 16S rRNA gene from total genomic DNA

preparations of infected head kidney and spleen tissues, a 100% identity was obtained to partial

16S rRNA gene sequences of a un-described intracellular bacterium (*Francisella* sp.) isolated

from tilapia in Taiwan, and to the isolated *Francisella* sp. recovered from three line grunt by

Kamaishi et al. (2005).

During that same time, Hsieh et al. (2006, 2007) published two papers reporting the

isolation of 10 strains of *Francisella* sp. recovered from visceral granulomas of diseased tilapia

cultured in Taiwan between the years of 2001-2004; and the use of PCR and *in situ* hybridization

for the detection of the bacteria in ornamental cichlids. The clinical signs, pathology and

microscopic analysis performed on diseased fish were similar to that reported by Chen et al. (1994), and Chern and Chao (1994) in cultured tilapia from the same area. Hsieh et al. (2006) in an analysis of the 16S rRNA gene, revealed high sequence similarities to *F. philomiragia* and *F. tularensis*, 98.6 and 97.4% respectively. Moreover, ultrastructural analysis done by electron microscopy, revealed that the intracellular organisms were extremely irregular, pleomorphic, with a size 0.35 x 0.35± 0.15µm, similar to that previously described in RLOs (Hsieh et al. 2006; Chern and Chao 1994; Chen et al. 1994). *In situ* hybridization analysis of infected tissue demonstrated brown precipitates localized mainly in large foamy cells of the granulomas, which also correlates to histopathological and electron microscopic analysis previously reported in RLOs infected fish (Chern and Chao 1994; Chen et al. 1994; Mauel and Miller 2002; Hsieh et al. 2006). They further utilized the technique to examine archived formalin-fixed, paraffin-embedded tissues from 28 diseased ornamental cichlid fish associated with visceral granulomas, in which culture of an intracellular bacterium resembling RLOs failed. Positive hybridization signals were observed in tissues of kidney, spleen, GI tract, heart, brain and gills of all the ornamental fish analyzed, demonstrating the use of this diagnostic tool to study presence of fastidious organism in fixed tissue samples (Hsieh et al. 2007).

Recently, Vojtech et al. (2009), utilized several *Francisella* sp. isolates from hybrid striped bass and tilapia to establish a zebrafish/Francisella comparative model of pathogenesis and host immune response. The authors were able to induce acute mortalities in zebrafish, but only after injecting 10^6 CFU per fish. This level of infection resulted in 100% mortality in 5 days, while 3.45 x 10^5 CFU's only resulted in ~ 2%. In the same work, it was found that the zebrafish mounted a significant tissue-specific pro-inflammatory response to infection as measured by the upregulation of interleukin-1β, gamma interferon, and tumor necrosis factor alpha mRNA beginning by 6 h postinfection and persisting for up to 7 days postinfection. In

addition, exposure of zebrafish to heat-killed bacteria demonstrated that the significant induction of IL-1 β was highly specific to live bacteria. Further, using immunohistochemical analysis, bacterial antigens were localized in lymphoid tissues and livers of zebrafish following infection by intraperitoneal injection. The authors concluded that the model showed enough similarities to those reported in mammals, highlighting the usefulness of it for addressing both general and specific questions about *Francisella* host-pathogen interactions via an evolutionary approach (Vojtech et al. 2009).

TAXONOMIC CLASSIFICATION OF *FRANCISELLA* SP. ISOLATED FROM FISH

As previously stated, the genus *Francisella* contains three recognized species, *F. tularensis*, *F. novicida,* and *F. philomiragia* (Thomas et al. 2003). Molecular diagnostic techniques and biochemical testing methods have been used to identify *Francisella* causing similar pathological lesions in fish species (Ostland et al. 2006; Mauel et al. 2007). The *Francisella* spp. identified as emerging pathogens of fish are poorly characterized due to the fastidious nature of the bacteria and the resulting difficulties in culturing the organism from fish tissues. Sequence comparison of the 16S rDNA from pathogenic *Francisella* sp. has placed the fish isolates at 97-99% identity to other isolates from water and soil environmental samples and mammalian isolates of *F. tularensis* and *F. philomiragia* (Kamaishi et al. 2005; Mailman and Schmidt 2005; Hsieh et al. 2006; Ostland et al. 2006; Birkbeck et al. 2007; Mauel et al. 2007; Mikalsen et al. 2007).

Genetic, phenotypic and biochemical differences have been found between the isolates recovered from cultured tilapia, three line grunt, and hybrid striped bass in brackish and fresh warm waters of Latin America, USA, Taiwan and Japan, and the ones isolated from cultured cod and Atlantic salmon in cold water of Norway and Chile (Mikalsen et al. 2007; Ottem et al. 2009; Mikalsen and Colquhoun 2009).

Different research groups have given different names to the *Francisella* sp. isolates

recovered from fish. Kay et al. (2006) performed comparative analysis of the LPS of *Francisella*

sp. isolates recovered from tilapia and those of *F. tularensis* and due to the degree of differences

between the two named the tilapia isolate *Francisella victoria*. Ottem et al. (2007) characterized

strain GM2212 (isolated from Norwegian cod) by sequence comparison of the complete 16S

rDNA, 16S-23S intergenic spacer, 23S rDNA, 23S-5S intergenic spacer, 5S rDNA, FopA,

lipoprotein TUL4 (LpnA), malate dehydrogenase and hypothetical lipoprotein (LpnB)

sequences, by comparison to *F. philomiragia* by DNA-DNA hybridization and fatty acid

analysis, and biochemical and phenotypic characters and proposed the name *Francisella*

piscicida. At the same time, a different group of researchers in Norway characterized seven

bacterial isolates from farmed Atlantic cod displaying chronic granulomatous disease by

phenotypic and molecular taxonomic methods. Comparison of 16S rRNA gene sequences and

six partial housekeeping gene sequences (groEL, shdA, rpoB, rpoA, pgm and atpA) confirmed

the organism as a member of the genus *Francisella*, with *F. philomiragia* as its closest relative.

Despite the close relationship with *F. philomiragia*, isolates from Atlantic cod could be

readily distinguished phenotypically and genetically from *F. philomiragia* ATCC 25015. Thus

on the basis of phenotypic and molecular genetic evidence, they proposed that the strains isolated

from Atlantic cod should be recognized as *F. philomiragia* subsp. *noatunensis* subsp. nov.

Recently, Ottem et al. (2009) utilized isolates recovered from warm and cold water fish

species, and compared them through sequencing of the 16S rRNA-gene, several housekeeping

genes and determination of biochemical and phenotypic properties. After analysis they proposed

to elevate *F. philomiragia* subsp. *noatunensis* to species rank as *F. noatunensis* comb. nov.,

while *F. piscicida* was considered a heterotypic synonym of *F. noatunensis* comb. nov. Also

they proposed that *Francisella* sp. Ehime-1 (isolated from three line grunt in Japan) represented

a novel subspecies of *F. noatunensis*, for which the name *F. noatunensis* subsp. *orientalis* subsp. nov. was proposed (Ottem et al. 2009). During the same period of time, Mikalsen and Colquhoun (2009) characterized bacterial isolates from diseased farmed tilapia (*Oreochromis* sp.) from Costa Rica (PQ 1104), Atlantic salmon (*Salmo salar*) from Chile (PQ 1106) and three-line grunt (*Parapristipoma trilineatum*) from Japan (Ehime-1) by phenotypic and molecular taxonomic methods, and on the basis of molecular genetic evidence, they proposed that isolates PQ 1104 and Ehime-1 should be recognized as *Francisella asiatica* sp. nov. In the same study no differences between *F. piscicida* and *F. philomiragia* subsp *noatunensis* were identified by the same methods and these species were found to constitute heterotypic synonyms for which the epithet *noatunensis* has priority. Finally, given the increased evidence of ecological differentiation within the *F. philomiragia* group and the existence of a specific fish pathogenic clade, they proposed that the *F. philomiragia* subsp *noatunensis* be elevated to species level as *F. noatunensis* comb. nov., sp. (Mikalsen and Colquhoun 2009).

FRANCISELLA TULARENSIS PATHOGENESIS

Francisella tularensis is the most important species belonging to the genus *Francisella* (Dennis et al. 2001; Sjostedt 2007). Besides being an important animal pathogen, *F. tularensis* is a zoonotic agent which has received considerable study as a potential bioterrorism agent. The organism has a high infectivity rate and multiple infectious routes (Keim et al. 2007; Nano and Shmerck 2007). It has been found that human exposure to as few as 10-50 colony forming units of *F. tularensis* subsp. *tularensis* can cause fatality rates as high as 30% in non treated patients (Foley and Nieto 2009). Moreover there are a wide variety of clinical presentations of the disease in humans, usually depending on the mode of transmission of the bacteria. Six forms have been described: ulceroglandular, oculoglandular, pneumonic, oropharyngeal, gastrointestinal, and thypoidal (Dennis et al.2001). The most common (80% of cases) is ulceroglandular tularemia,

which is usually acquired through the bite of an arthropod vector (usually a tick) (Dennis et al. 2001; Foley and Nieto 2009; Parmely et al. 2009). The pulmonary and oropharyngeal/gastrointestinal tularemia may be acquired following inhalation of the bacterium or oral exposure (potentially from ingestion of contaminated water or meat), are the most dangerous of all, with fatality rates between 40-60% (Dennis and Foley 2009).

The clinical signs and symptomatology of humans infected with *F. tularensis* depends on the mode of acquisition of the pathogen. Human patients generally will present a history of high fever, headache and signs of toxicity (myalgia, anorexia, prostration) for several days. Pneumonia with nonproductive cough and liver damage indicated by elevated liver transaminases often follow (Parmely et al. 2009). The histological appearance of tularemia in humans is characterized by irregular microabscesses, pyogranulomatous inflammation in the liver, spleen, and lymph nodes, and necrotizing pneumonia, indicating a highly cytotoxic process that results from damage to both inflammatory and parenchymal cells (Parmely et al. 2009; Twenhafel et al. 2009). Localized lesions develop that consist of walled granulomatous structures with central necrosis suggestive of tuberculosis (Dennis et al. 2001; Foley and Nieto 2009).

The ability to survive intracellularly is crucial in the pathogenesis of several bacteria after they invade their eukaryotic target cell. Bacterial pathogens like *Listeria monocytogenes*, *Shigella flexneri*, *Burkholderia pseudomallei*, *Rickettsia*, *Legionella*, *Salmonella*, *Mycobacterium*, and *Francisella* species, evade host cell killing by surviving and replicating inside phagocytic or non-phagocytic cells. Many of them do so by surviving in membrane-bound compartments, whereas others will ultimately reside free in the cytoplasm of host cells. These pathogens have evolved three main strategies to avoid killing by phagocytic host cells. *Legionella* sp. will modulate the phagosome biogenesis at distinct stages in the endosomal-

lysosomal degradation pathway; *Coxiella* sp. will adapt to the harsh acidic environment within phagolysosomes; while *Listeria, Shigella, Rickettsia, Burkholderia pseudomallei, Rickettsia* and *Francisella tularensis* escape from the phagosome into the cytoplasm after degradation of the phagosomal membrane (Santic et al. 2006; Clemens et al. 2007; Oyston 2008; Ray et al. 2009).

Previous studies have described the intracellular localization, survival, replication and escape of *F. tularensis* subspecie*s* from a wide variety of cells including: type II alveolar cells epithelial cells, neutrophils, mammalian monocytes, mammalian monocyte derived macrophages, adherent mouse peritoneal cells, a mouse macrophage-like cell line J774A.1, and a human macrophage cell line THP-1, as well as arthropod derived cells and *Acanthamoeba castellanii* (Baron and Nano 1998; Abd et al. 2003; Golovliov et al 2003; de Bruin et al. 2007; Vonkavaara et al. 2008; Santic et al. 2009); but *in vivo* its primary target appears to be the macrophage (Fortier et al. 1994; Oyston 2008). Macrophages are generally ubiquitous mononuclear phagocytes responsible for numerous homeostatic, immunological, and inflammatory processes. It has been described that uptake of *F. tularensis* by human macrophages occur by looping phagocytosis. Uptake by mammalian mononuclear cells has been found to require complement factor C3-derived opsonins (Nasr and Kimple 2008). With the *F. tularensis* live vaccine strain (LVS), optimal phagocytosis by dendritic cells (DC) is dependent on complement factor C3-derived opsonins and the major complement receptors expressed by DC, the integrins CR3 (CD11b/CD18) and CR4 (CD11c/ CD18) (Nasr and Kimple 2008). Further work with *F. tularensis* (Balagopal et al. 2006; Shulert and Allen 2006) demonstrated that monocyte-derived macrophages (MDM) phagocytose more *Francisella* than monocytes, with a major contribution from the mannose receptor on MDM. Other receptors demonstrated to be involved in the uptake of *F. tularensis* by mammalian macrophages, include Fc γ receptors, pulmonary collectins, surfactant proteins, and type I and II class A scavengers (Balagopal et al.

2006; Shulert and Allen 2006). Following uptake, the bacterium resides in a spacious vacuole at the pheriphery of the cell, which will rapidly shrink in size (Clemens and Horwitz 2007). *Francisella tularensis* subspecies will arrest maturation of the phagosome at a late endosomal-like stage, followed by acidification and acquisition of the late endosomal markers Lamp1 and Lamp2. This late endosome-like compartment acquires the proton vacuolar ATPase pump and becomes transiently acidified but does not associate with lysosomal markers, such as cathepsin D (Clemens et al., 2004; Santic et al., 2005, 2008) for the first 4 h post infection (Clemens et al. 2004; Santic et al. 2005; Santic et al. 2006). Eventually, the phagosomal membrane will be degraded and the bacteria escape into the cytoplasm where they multiply vigorously. The infective process eventually leads to host cell death, whereby the bacteria are freed to infect neighboring cells (Clemens et al. 2004; Santic et al. 2005, 2006; Oyston 2008; Schmerck et al. 2009).

FRANCISELLA TULARENSIS PATHOGENICITY ISLAND

The genetic basis of *F. tularensis* virulence is still poorly understood although several virulence determinants have been identified (Golovliov et al. 2003; Nano et al. 2004; Barker and Klose 2007). Some of the most interesting genes identified in *F. tularensis* are the genes of the intracellular growth locus (*iglA, iglB, iglC*, and *iglD*) present as part of a 30 Kb pathogenicity island described by Nano et al. (2004) and Barker and Klose (2007). Depending on the *Francisella* biotype, the FPI consists of 16 to 19 genes and contains four large open reading frames (ORF's) which range from 2.5 kb to 3.9 kb, with a %GC content considerably lower in relation to the rest of the *F. tularensis* subsp. genome. The FPI genes show extensive conservation amongst *Francisella* subspecies, but *in silico* analysis has been unsuccessful in uncovering any significant homolog of many pathogenicity island proteins within the available database (Nano et al. 2004; Barker and Klose 2007; Ludu et al. 2008). Expression of FPI

encoded genes is mediated by the global regulatory proteins MglA and MglB, which are homologues of the *Escherichia coli* stringent starvation transcription regulators, SspA and SspB (Lauriano et al. 2004; Hansen et al., 2005; Brotcke et al., 2006, Santic et al., 2006). Unlike SspA, whose expression peaks during stationary phase, MglA expression peaks during the exponential growth phase and is induced within 90 minutes of macrophage infection (Lauriano et al. 2004; Hansen et al., 2005; Brotcke et al., 2006, Santic et al., 2006).

The functions of the conserved proteins corresponding to the genes of the FPI are elusive. Overall, Igl proteins appear to be essential for the ability of *F. tularensis* to survive inside the macrophages and cause disease (Golovliov et al. 1997; Nano et al. 2004; Lai et al. 2004; Lauriano et al. 2004; Santic et al. 2005; Brotcke et al. 2006; de Bruin et al. 2007). Recent data have shown that IglA and IglB are part of a novel *Francisella* Pathogenicity Island (FPI) encoded Type Six Secretion System (T6SS) (Nano and Schmerk, 2007; Ludu et al. 2008-b). Mutations of these four genes in *F. tularensis*, have shown decreased pathogenicity of the bacterium both *in vivo* and *in vitro* in mammalian and insect tissues and cell lines (Lauriano et al. 2003; Nano et al. 2004; de Bruin et al. 2007; Vonkavaara et al. 2008). Barker et al. (2009) demonstrated that proteins encoded on the FPI (VgrG and IglI), also share homology with type VI secretion gene clusters from *Vibrio cholerae* and *Pseudomonas aeuroginosa*. The proteins are secreted into the cytosol of infected macrophages, and are required for *F. tularensis* phagosomal escape, intramacrophage growth, inflammasome activation and virulence in mice (Barker et al. 2009).

The intracellular growth locus C protein (IglC) was one of the first of the FPI-encoded proteins found to be highly induced following *F. tularensis* infection of macrophages (Golovliov et al. 1997). As with other genes mutants in the FPI, *iglC* mutants fail to grow in macrophages, are deficient in ability to escape from phagosomes, and fail to down-regulate the

proinflammatory response in macrophages (Golovliov et al. 2003; Nano et al. 2004; Lindreng et al. 2004; Ludu et al. 2008). Sun et al. (2007) determined the crystal structure of IglC at 1.65 Å resolution, and described a β-sandwich conformation that exhibits no similarity with any known protein structure. The authors revealed a very low degree of structural similarity between the *F. tularensis* IglC and 1) pyridoxamine 5′-phosphate oxidase, 2) with a chain C of the largest subunit of RNA polymerase II, 3) the sigma C capsid protein from avian reovirus, and 4) gp27, a cell-puncturing device component of bacteriophage T4 (Sun et al. 2007). The role of IglC is not completely understood, by it could be direct, or indirect through its interactions with one or several other proteins like PdpD, VgrG, IglI, amongst others (Ludu et al. 2008; Barker et al. 2009). There is some published evidence that IglC primarily localizes to the cytoplasm, with a small proportion of IglC localizing to the outer membrane, thus it is possible that IglC could play a role in formation of a secretion channel in the outer membrane of *Francisella*, or alternatively, it could play a role in forming a channel in a host cell membrane structure (Golovliov et al. 1997, 2003, Ludu et al. 2008; Barker et al. 2009).

OBJECTIVES

Due to its emergent nature as a fish pathogen, and its genetic, phenotypic and biochemical similarities to the human pathogen *F. tularensis*, the main objectives of this dissertation were to: 1) Characterize a member of the genus *Francisella* isolated from diseased tilapia in Costa Rica; 2) Describe suitable challenge models to study the disease progression in indoor laboratory challenge rooms and report the lethal dose 50 of the recovered isolate in tilapia nilotica fingerlings; 3) Describe and characterize virulence genes present in a novel *Francisella* pathogenicity island in the Costarican fish isolate; 4) Describe a mutation methodology to attenuate *Francisella* sp. by insertional mutagenesis in the *Francisella* sp. *iglC* gene; 5) Describe the interaction of *F. asiatica* strains with the tilapia immune system; 6) Develop and demonstrate

the usefulness of an attenuated live vaccine to prevent fish francisellosis; 7) Develop a real-time PCR assay for the identification and quantification of *F. asiatica*; and 8) Compare the *in-vitro* and *in-vivo* efficacy of Florfenicol for treatment of *F. asiatica* infection in tilapia.

Although it has been previously shown to be highly virulent in a wide variety of fish species, very little is known about the pathogenesis of members of the genus *Francisella* in fish. Moreover, higher stocking densities, poor water quality, and overall high levels of stress present in aquaculture, makes it necessity to have a complete understanding of the mechanisms employed by this pathogenic bacteria to infect fish. Research is necessary to develop rapid diagnostic techniques, efficacious treatments using chemotherapeutics, and a highly desirable efficacious vaccine to actually prevent the disease.

In the second chapter, the causative agent of a high mortality episode in cultured tilapia in Costa Rica is identified as a member of the genus *Francisella* by bacteriological, histopathological, and molecular analysis. In the third chapter, the presence of homologues to the intracellular growth loci (*igl*) virulence genes in non-tularensis fish pathogenic *Francisella* sp is reported, also in this chapter, there is a description of a useful method for allelic exchange using PCR products to mutate *Francisella* sp., and the creation of an attenuated *igl*C mutant upon intraperitoneal and immersion challenges in tilapia (*Oreochromis* sp.). Finally, intraperitoneal and immersion infectivity trials, to induce francisellosis in tilapia (*Oreochromis* sp.) are described and the dose required to cause mortality in 50% of the fish (LD$_{50}$) of this important emergent fish pathogen is reported. In the fourth chapter, F. *asiatica* LADL 07-285A was used to investigate the interaction between this emergent pathogen and innate immunity in tilapia. It is demonstrated that the *F. asiatica* wild type isolate is resistant to serum killing, is able to enter, survive, and replicate in tilapia head kidney derived macrophages, and ultimately kills the cell by inducing apoptosis. It was also found that a mutation of the *iglC* gene, makes *F*.

asiatica defective for intramacrophagic survival and replication, as well as for induction of apoptotic caspase 3 and 7 cleavage and cytotoxicity, but does not affects its ability to survive in serum. Finally is demonstrated that complementation of the IglC protein restores virulence, the pro-apoptotic features of the defective mutant, and cytotoxicity. The fifth chapter study goal was to characterize the efficacy of a defined *F. asiatica* mutant ($\Delta iglC$) as a live attenuated vaccine against subsequent immersion challenge with the wild-type organism. The results show that the $\Delta iglC$ stimulated a systemic antibody response as demonstrated by the high antibody titers found in serum and skin mucus of vaccinated fish. The $\Delta iglC$ was then tested to determine its ability to protect tilapia against challenge with high doses (lethal dose 80) of wild-type bacteria. Naive tilapia vaccinated by immersion with a suspension of the $\Delta iglC$ and subsequently challenged with wild-type (WT) *F. asiatica* were protected (90% survival) from the lethal challenges. *F. asiatica*-specific antibodies produced in response to immunization with the $\Delta iglC$ were subsequently found to protect naive tilapia against high-dose *F. asiatica* challenge in passive immunization experiments. Significant protection (p<0.001) was obtained when fish were passively immunized and challenged with 10^4 and 10^5 CFU/fish of WT *F. asiatica*; but not when challenged with 10^6 CFU/fish. In Chapter 6 a quantitative real-time PCR assay was created using the previously described *iglC* gene of the fish pathogen *F. asiatica* as a target. The chapter contains a complete description of a highly sensitive, specific and reliable molecular diagnostic technique for identification and quantification of *F. asiatica* from diseased fish. The aim of Chapter 7 is to determine the ability of florfenicol medicated feed to control experimentally induced *F. asiatica* infection in tilapia. Additionally an evaluation of the capacity of florfenicol to eliminate intracellular *F. asiatica* from THKDM *in vitro* is included.

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CHAPTER 2

FRANCISELLA SP., AN EMERGING PATHOGEN OF TILAPIA (*OREOCHROMIS NILOTICUS*) IN COSTA RICA*

INTRODUCTION

Several emerging diseases have been described in the past ten years causing significant mortalities and economic losses in a wide variety of freshwater and marine fishes. According to Woolhouse and Dye (2001), "an emerging pathogen can be defined as the causative agent of an infectious disease whose incidence is increasing following its appearance in a new host population or whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology". Some of the current emergent fish pathogens include organisms from different taxonomic groups and etiology. Included among the most pathogenic are: the viral hemorrhagic septicemia (VHS) virus in the Great Lakes of North America, the zoonotic nematode *Anisakis* spp. in South America, the fungus-like protistan *Ichthyophonus,* and bacterial infections by *Lactococcus garvieae* and Rickettsia-like organisms (RLO) including *Piscirickettsia* spp. and *Francisella* spp. in several cultured fish species worldwide (Chen et al. 1994; Chern and Chao 1994; Mauel and Miller 2002; Fryer and Hedrick 2003; Athanassopoulou et al. 2004; Cabrera et al. 2004; Kokan et al. 2004; Pereira et al. 2004; Corbeil et al. 2005; Snow et al. 2005; Nylund et al. 2006; Hsieh et al. 2007). It is unclear in many of these cases if the increase in incidence is the result of spread of the pathogen to new hosts and geographic locations or the increased technology available to detect them.

Francisellosis is an acute to chronic disease caused by different *Francisella* species. It has been diagnosed in a wide variety of animals, including more than 200 mammals (including humans), as well as birds, reptiles, crustaceans, and ticks.

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Members of the genus have also been found to be present in soil and water samples (Scoles 2004; Barns et al. 2005; Birkbeck et al. 2007; Keim and Wagner, 2007; Müller et al. 2007). The most significant animal pathogen species belonging to this genus is the type species, *Francisella tularensis,* the causative agent of tularemia. Besides being an important animal pathogen, *F. tularensis* is a zoonotic agent which has received considerable study as a potential bioterrorism agent. The organism has a high infectivity rate and multiple infectious routes (Keim et al. 2007; Nano and Shmerck 2007).

The genus *Francisella* contains four recognized species, *F. tularensis*, *F. novicida, F. philomiragia*, and recently *F. piscicida*, an isolate recovered from cod cultured in Norway (Thomas et al. 2003; Otten et al. 2007). Molecular diagnostic techniques and biochemical testing methods have been used to identify *Francisella* causing similar pathological lesions in fish species (Ostland et al. 2006; Mauel et al. 2007). The *Francisella* spp. identified as emerging pathogens of fish are poorly characterized due to the fastidious nature of the bacteria and the resulting difficulties in culturing the organism from fish tissues. Sequence comparison of the 16S rDNA, from pathogenic *Francisella* sp. has placed the fish isolates at 97-99% identity to other isolates from water and soil environmental samples and mammalian isolates of *F. tularensis* and *F. philomiragia* (Kamaishi et al. 2005; Mailman and Schmidt 2005; Hsieh et al. 2006; Ostland et al. 2006; Birkbeck et al. 2007; Mauel et al. 2007; Mikalsen et al. 2007).

From infections in fish, only on rare occasions have researchers been able to successfully isolate the bacterium (Kamaishi et al. 2005; Mikalsen et al. 2007).

The purpose of this study is to report and describe the clinical pathology, histopathology, microbiology, and molecular characteristics of the emergent fish pathogen *Francisella* sp., affecting tilapia aquaculture in Costa Rica.

MATERIALS AND METHODS

Fish History. During the months of August and September 2007 a tilapia aquaculture farm in Alajuela, Costa Rica experienced increased morbidity and mortality in several freshwater-pond culture tilapia. According to the producer, around 50-60% (~ 6000 fish) of the cultured tilapia died during this period of time, with mortalities as high as 200 fish/tank/day.

Approximately 50 tilapia (*Oreochromis niloticus*) cultured in the province of Alajuela, Costa Rica were received and analyzed in the Pathology Service of the School of Veterinary Medicine of the Universidad Nacional de Costa Rica during August -October 2007. A complete necropsy showed consistent gross and microscopic lesions similar to those reported during high mortality event in cultured tilapia in Taiwan, Hawaii, the continental USA, and Latin-America since 1994 (Chern and Chao 1994; Mauel et al. 2003; Mauel et al. 2005; Hsieh et al. 2006; Mauel et al. 2007).

Fifteen euthanized fish were sent to the Louisiana Aquatic Diagnostic Laboratory (LADL) at Louisiana State University - School of Veterinary Medicine (LSU-SVM) for further analysis.

Histological Analysis. The gill, spleen, kidney, liver, heart, brain, ovary, testis, and muscle were fixed in neutral buffered 10% formalin; processed by standard methods, and stained with hematoxylin and eosin and Giemsa stain, and examined by light microscopy.

Isolation, Media and Growth Conditions. Fish tissues (spleen, anterior kidney, and liver) were aseptically collected and used for bacteriological analysis by streaking on different agar media. Commercially available media tested for primary recovery of bacteria from fish tissue smears included: Trypticase Soy agar with 5% sheep blood (TSA), Cystine Heart Agar (CHA) with rabbit blood and antibiotics, Chocolate Agar/Improved Thayer-Martin biplate (Remel, Lenexa, KS, USA), Chocolate II Agar (GC II Agar with Hemoglobin and Isovitalex), and Modified

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Thayer-Martin Agar, (BD BBL, Sparks, MD, USA). Two types of agar plates used as primary isolation media were prepared in the media preparatory lab at LSU-SVM: Cystine Heart Agar supplemented with bovine hemoglobin solution (BD BBL, Sparks, MD, USA) (CHAH) and Mueller-Hinton base supplemented with 3% fetal bovine serum, 1% glucose, and 0.1% cystine. Polymixin B 100 units/ml and/or ampicillin 50 µg/ml were added to the media to select against secondary contaminants, since they are widely use as selective agents for *Francisella* sp., and fish diseases diagnosis (Hawke and Thune 1992; Petersen et al. 2004).

Plates were incubated at 22-25 °C for 2-5 days. Colonies observed from primary isolation agar plates were re-plated for purity of culture under the same conditions. Once single colonies were observed and purity of the isolate determined, the isolate was re-suspended in liquid medium reported by Baker, et al. 1985 with modifications. The liquid medium consisted of a modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL, Sparks, MD, USA) and 0.1% glucose (MMH). Broth cultures were grown overnight at 22 °C in a shaker at 175 rpm, and bacteria were frozen at -80 °C in the broth media containing 20% glycerol for later use.

Three different isolates (obtained from three different fish) were tested at different culturing temperatures; 15, 20, 22, 25, 28, 30, 32, 35 and 37°C on CHAH for a period of 7 days to find the in vitro optimal growth temperature of the bacteria.

DNA Extraction. Two isolates (07-285A and 07-285B) recovered from fish cultured in different systems and submitted to the LADL LSU-SVM were used for molecular analysis. A loop of the bacterium was suspended in 400 µl of sterile water, washed and centrifuged at 3000 g for 5 min, and re-suspended in 200 µl PBS. The bacterial suspension was subjected to DNA

extraction and purification as per the manufacturer's protocol using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). DNA was stored at 4°C until further use.

PCR and 16S rRNA Gene Sequence. Two different sets of primers were used during the study to amplify gene sequences important in identification of *Francisella*. The 50 µl *Francisella* sp.-specific PCR reaction was composed of 0.2 µM of each primer (F11, 5′-TAC CAG TTG GAA ACG ACTGT-3′ and, F5, 5′-CCT TTT TGA GTT TCGCTC C-3′) developed by Forsman et al. (1994), 0.2 mM of dNTPs, 2.5 mM MgCl$_2$, 5 U of *Taq* DNA polymerase (Applied Biosystems-Roche), 1X PCRx Amp buffer (Invitrogen), 1X PCRx Enhancer solution (Invitrogen) and approximately 200 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 60s at 60°C, and 60s at 72° C, with a final extension step of 5 min at 72°C performed in a Perkin Elmer GeneAmp PCR System 2400 (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA, USA).

The 50µl universal eubacterial 16S rRNA PCR reaction was composed of 0.5 µM of each primer (F1, 5'-GAG TTT GAT CCT GGC TCAG-3' and R13,5'- AGA AAG GAG GTG ATC CAG CC-3') (Dorsch & Stackebrant 1992) 0.2 mM of dNTPs, 2.5U of *Taq* DNA polymerase, 1X buffer H (Invitrogen), and approximately 200 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 30s at 94°C, followed by 30 cycles of 30s at 94°C, 60s at 58°C, and 90s at 72°C, with a final extension step of 7 min at 72°C in a Perkin Elmer GeneAmp PCR System 2400. The PCR products were subjected to electrophoresis on a 1% agarose gel and stained with SYBR® Safe DNA gel stain (Invitrogen, Eugene, OR, USA).

Amplicons for sequencing were purified with the QiaQuick PCR Cleanup Kit (Qiagen) as directed by the manufacturer, and were sequenced on an Applied Biosystems 3130 Genetic Analyzer using PCR primers (F11 - F5) and (F1 – R13).

The sequence was compared with those stored in GenBank using the BLASTN program from the National Center for Biotechnology Information. Eubacterial 16S rRNA sequences of members of the genus *Francisella* and representative warmwater fish pathogens were obtained and aligned using the Clustal W application of the MEGA (Molecular Evolutionary Genetic Analysis) package (version 4; Tamura, et al. Kumar 2007) and used in the construction of phylogenetic tree. The alignment was performed using neighbor- joining and distance analysis within the MEGA package. The evolutionary history in the phylogenetic tree was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1163 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

Experimental Challenges. In order to fulfill Koch's postulates, experimental infections were performed by intraperitoneal injection (IP) and gill spraying (GS) with *Francisella* sp. Costa Rica isolate LADL07-285A. Isolate LADL07-285A, recovered from cultured infected tilapia in Costa Rica was grown in CHAH at 25 °C for 72 h. Cells were harvested, suspended in 5 ml of MMH broth, and incubated in a shaking incubator overnight at 22°C to obtain a final optical density at 600 nm (OD$_{600}$) of 0.48. Enumeration of the bacteria was done by the drop plate method with 50 μl drops of each 10-fold dilution placed on cystine heart agar with hemoglobin. Resulting colony forming units per ml CFU/ml were determined.

Experimental infection of naïve tilapia nilotica (average length ~ 9.0 cm and average weight ~ 18.9 g) was tested by intraperitoneal injection (IP) and the gill spraying exposure route (GS). The fish were obtained from a source considered to be free of *Francisella* infection and were found to be negative for Francisellosis by culture of spleen and head-kidney smears and by PCR, prior to use in the study. Fish were maintained in 3 different tanks (10 fish per tank), representing the 2 different challenge methods and a control tank at 23-25 °C. Prior to challenge, all fish were anesthetized with MS-222 (100 mg/l). The IP challenge fish received a 0.1ml injection of the bacterial suspension (~10^7 CFU/fish). The GS challenge fish were sprayed with 0.1-0.2 ml of the bacterial suspension, and left out of the water for approximately 15s. Control fish were treated in a similar manner, but received 0.1ml of sterile MMH broth used in the study.

Following each challenge exposure, the fish were placed in the respective tanks and mortality was recorded every 12 h for 10 d. Dead and moribund fish were subjected to a complete clinical, bacteriological, and histopathological examination. The identity of isolated bacteria was confirmed by PCR.

RESULTS

Clinical Signs and Histopathology. Affected tilapia fingerlings ranged between 6.5-9.0 cm in length and 13-20 g in weight. Except for lethargy, abnormal swimming behavior, rare exophthalmia, and anorexia, no clear external clinical signs were observed in the fish. Examination of the gills and skin in wet mounts revealed the presence of light *Ichthyobodo* sp., *Ambiphyra,* monogenean trematodes, and heavy *Trichodina* sp. infestations. Internally, the most significant gross pathological change observed was the presence of widespread, multifocal white nodules dispersed in the anterior kidney, posterior kidney, and spleen (Fig. 2.1). Also, fish had marked splenomegaly and renomegaly. In some cases white nodules were observed in gills, liver, choroid gland, and sporadically in the gastrointestinal walls and mesenteric fat.

Histopathologically, the most severe changes involved the gills, spleen, and kidneys, but pathologic changes were also present in the liver, heart, eye, central nervous system, and gastrointestinal tract. Granulomatous inflammation was present in almost every organ, with large numbers of macrophages containing small pleomorphic coccobacilli. In addition to the granulomatous response, the gills exhibited primary and secondary lamellar fusion due to epithelial hyperplasia (Fig. 2.2a). In severe cases, a widespread cellular infiltrate and presence of granulomas were observed in pericardium and myocardium (Fig. 2.2b). Granuloma formation was not observed in the brain; instead a massive macrophagic inflammatory infiltrate was found in severely infected fish. When special stains (Giemsa) were used, the presence of the small, pleomorphic coccobacilli were visible inside and outside the cells (Fig. 2.3).

Figure 2.1 Splenomegaly and renomegaly with widespread multifocal white nodules in infected nile tilapia (*Oreochromis niloticus*).
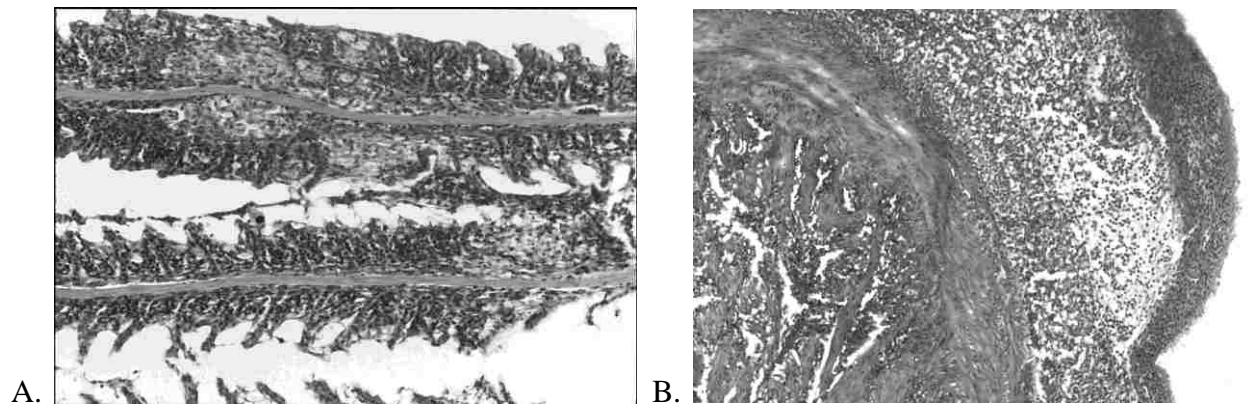
Figure 2.2 Histopathological findings of Francisellosis in nile tilapia (*Oreochromis niloticus*) (a) Hyperplasic and hypertrophic primary and secondary gill lamella (b) Pericarditis with granulomatous cellular infiltrate
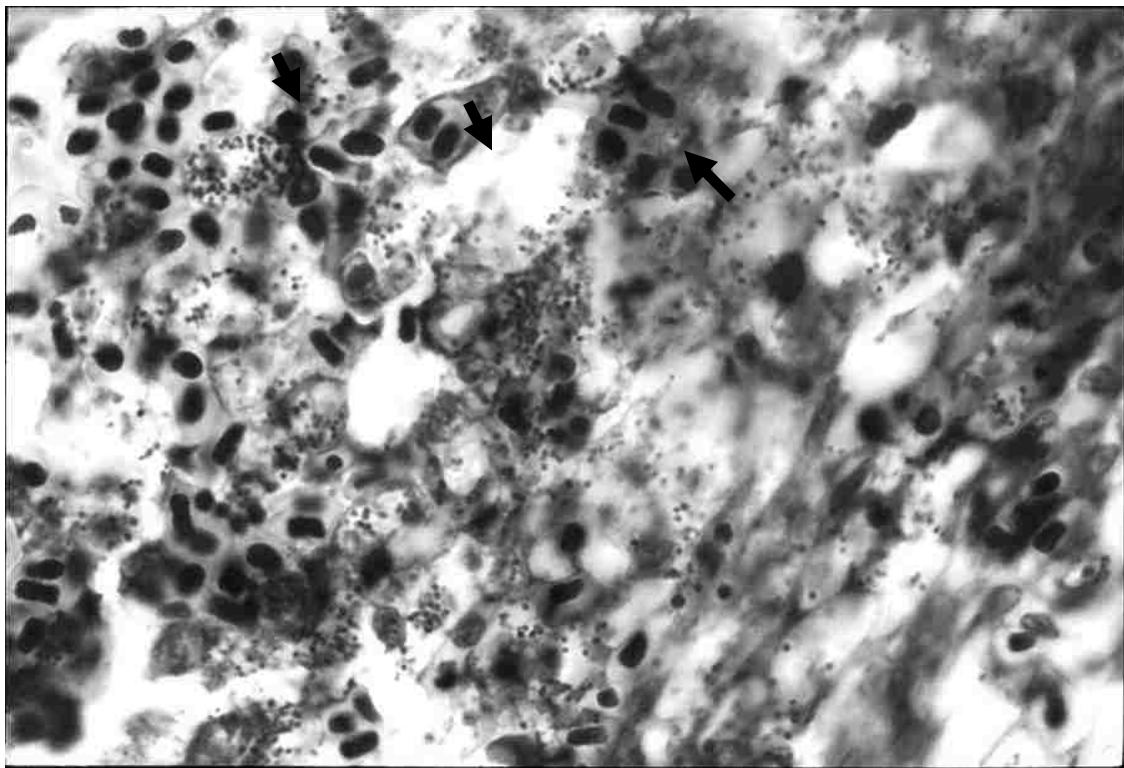
Figure 2.3 *Francisella* sp. bacteria seen intra and extracellularly with Giemsa stain (100X). Arrows indicate location of bacteria.

Media and Growth Conditions. Cystine Heart Agar supplemented with bovine hemoglobin solution and antibiotics, the Modified Thayer-Martin Agar, and CHA with rabbit blood and antibiotics were useful for the primary isolation of *Francisella* sp. from the spleen and kidneys of diseased fish. The Chocolate Agar/Improved Thayer-Martin biplate, Chocolate II Agar, and the Mueller Hinton base supplemented with 3% fetal bovine serum, 1% glucose, and 0.1% cystine were not suitable for primary isolation although sub-culture could be successfully performed on these agars. The *Francisella* sp. failed to grow on TSA agar with 5% sheep blood, however, other secondary and contaminating organisms, including: *Plesiomonas shigelloides, Aeromonas* sp., and *Pseudomonas* sp., were isolated. The strains of *Francisella* sp. isolated from tilapia from Costa Rica by the LADL were designated as strains LADL07-285A and LADL07-285B.

Growth of *Francisella* sp. was visible on CHAH, 36-48 h post inoculation and colonies were gray, smooth, and convex. Optimal growth of *Francisella* sp. occurred at 28-30°C, but growth was present from 20-28°C after four days of incubation. Growth at 22-25°C was slower than at 28°C, and no growth was observed at 15°C or at 33°C. By light microscopy, the morphology of the bacterium was extremely pleomorphic, non-motile, and very small in size (~ 0.5-1μm wide).

Molecular Analysis. The isolates recovered from the infected spleen and kidneys yielded the appropriately amplified PCR products of 1150 bp using the *Francisella* genus-specific primers F11 and F5 (Fig. 2.4a). When using the universal eubacterial 16S rRNA primers F1 and R13, a 1384 bp product was amplified from LADL07-285A and LADL07-285B. The sequence for isolate LADL07-285A was deposited in GenBank under the accession number EU672884 (Fig.2.4b).

The 16S rRNA sequence obtained was compared with those stored in GenBank using the BLASTN program. The Clustal W program was used to create a neighbor-joining tree based on

the 1384 bp sequence of 16S ribosomal RNA and shows the phylogenetic relationship of

Francisella sp. LADL07-285 (Alajuela, Costa Rica) with other *Francisella* sp., and other fish

pathogens (Fig. 2.5).
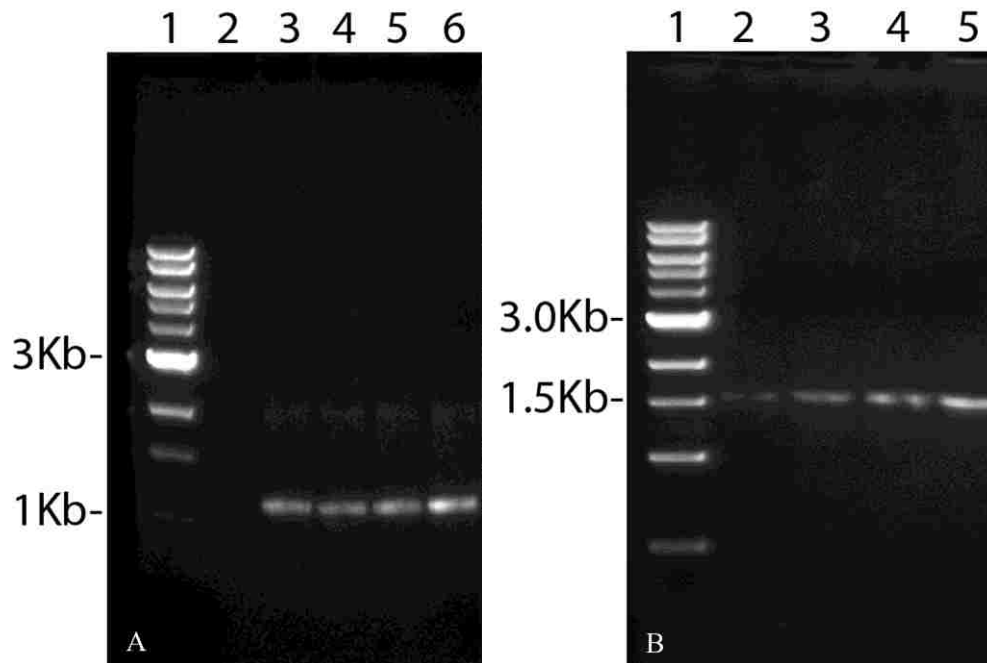
Figure 2.4 PCR amplification of *Francisella* sp. isolates recovered from tilapia (*Oreochromis niloticus*). (A) Amplification using genus specific primers. Lane 1. 1kb ladder. Lane 2. Negative control. Lane 3. Isolate 07-285A wild type recovered from cultured tilapia. Lane 4. Isolate 07-285B wild type recovered from cultured tilapia. Lane 5. Isolate 07-285A recovered from intraperitoneal challenge. Lane 6. Isolate 07-285A recovered from gill exposure challenge. (B) Amplification using Eubacterial 16s rRNA primers. Lane 1. 1kb ladder. Lane 2. Isolate 07-285A wild type recovered from cultured tilapia. Lane 3. Isolate 07-285B wild type recovered from cultured tilapia. Lane 4. Isolate 07-285A recovered from intraperitoneal challenge. Lane 5. Isolate 07-285A recovered from gill exposure challenge

A 97-99% identity was shown between the isolate recovered from tilapia in Alajuela,

Costa Rica and other *Francisella* species sequences stored in GenBank. Interestingly, all the

Francisella sp. isolated from fish shared around 99% identity to our isolate, while the

mammalian pathogens *Francisella tularensis* and *Francisella philomiragia* shared around 97%

and 98% homology with our isolate. Estimates of evolutionary divergence between isolate

LADL07-285 16S rRNA with other *Francisella* sp., and other fish pathogens are given in Table 2.1.
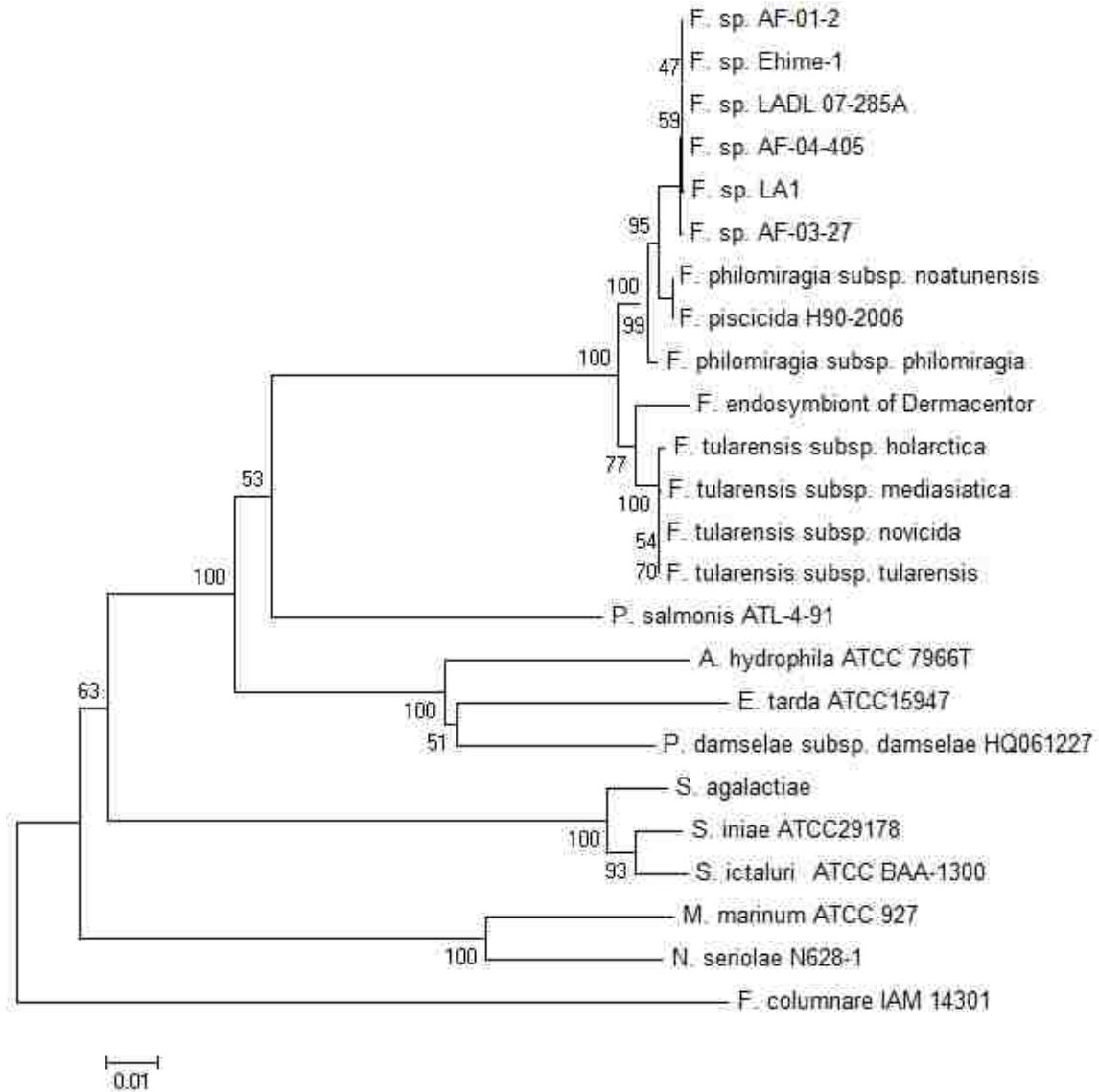
Figure 2.5 Evolutionary relationships of 24 taxa based on partial 16S rRNA sequences of members of the genus *Francisella* and representative warmwater fish pathogens.

Experimental Challenge. Intraperitoneal injection of ~10^7 CFU/fish caused 100% mortality in naïve tilapia by 72 h post inoculation. Tilapia exposed to bacteria by gill immersion also exhibited high mortality (80%), but this occurred gradually over the duration of the study (10

days). The clinical signs presented in the experimentally challenged fish were consistent with those found in the naturally infected cases. In the IP injection group, a more acute onset of the disease was seen and most fish died in a short period of time (<48 h post challenge).

Table 2.1 Estimates of evolutionary divergence between 16S rRNA sequences of members of the genus *Francisella* and representative warmwater fish pathogens and isolate LADL 07-285A. The number of base differences per site from analysis between sequences is shown. All results are based on the *p*-distance parameter model with a pairwise distance calculation.

Strain	GeneBank Accesion Number	% Distance from LADL 07-285A	% Identity with LADL 07-285A
Francisella **sp. AF-01-2**	AY928388	0	99
Francisella **sp. Ehime-1**	AB194068	0	99
Francisella **sp. AF-04-405**	DQ007455	0	99
Francisella **sp. LA1**	DQ473646	0.001	99
Francisella **sp. AF-03-27**	AY928393	0.001	99
Francisella philomiragia subsp. noatunensis **strain 2006/09/130**	EF490217	0.007	99
Francisella piscicida **isolate H90-2006**	EF685350	0.007	99
Francisella philomiragia **subsp.** *philomiragia* **ATCC 25015**	AJ698862	0.009	99
Francisella tularensis subsp. novicida **strain FSC 040**	AY968237	0.020	97
Francisella tularensis subsp. tularensis **strain FSC 199**	AY968225	0.020	97
Francisella tularensis subsp. mediasiatica **strain FSC 148**	AY968235	0.021	97
Francisella tularensis subsp. holarctica **strain UT01-1901**	AY968232	0.021	97
Francisella endosymbiont **of** *Dermacentor variabilis* **strain 2040460**	AY805306	0.028	96
Piscirickettsia salmonis **ATL-4-91**	U36915	0.144	84
Photobacterium damselae subsp. damselae **HQ061227**	EF635307	0.164	82
Aeromonas hydrophila **ATCC 7966T**	X74677	0.176	81
Edwardsiella tarda	EF467289	0.187	82
Streptococcus agalactiae **strain ATCC 13813**	DQ303183	0.217	79
Streptococcus iniae **29178**	DQ303187	0.221	79
Streptococcus ictaluri **BAA-1300**	DQ462421	0.222	79
Mycobacterium marinum **ATCC 927**	AF456240	0.233	78
Nocardia seriolae **N628-1**	EF513204	0.233	82
Flavobacterium columnare **IAM 14301**	AB010951	0.261	75

The clinical signs in the acutely infected fish were; bloody ascites, slight swelling of the spleen and kidney, with increased number and size of melanomacrophage centers were present but no granulomas were seen. Numerous small cocco-bacilli were present both intracellularly and extracellularly in the tissues. Fish exposed by gill immersion presented with a more subacute to chronic form of the disease, showing signs of anorexia and erratic swimming behavior. At necropsy, splenomegaly and renomegaly were pronounced and granulomas were numerous in both organs. Numerous intra and extracellular bacteria were observed microscopically in gills, spleen, and anterior and posterior kidney. *Francisella* sp. was re-isolated from both challenged groups by inoculating homogenates of spleen and posterior kidney on CHA supplemented with bovine hemoglobin solution and antibiotics. The isolates were confirmed by PCR as members of the genus *Francisella* sp.

At the completion of the experimental challenge, all control fish were alive, and no bacterial infection was detected by bacteriological, histopathological, or molecular analysis.

DISCUSSION

Since the first diagnosis of "rickettsia-like" bacteria (*Piscirickettsia salmonis*) in the 1980s, different cultured freshwater and marine fish worldwide have been described with intracellular microorganisms causing high mortalities and granulomatous lesions (Fryer et al. 1992; Chen et al. 1994; Hsieh et al. 2006; Ottem et al. 2007; Mauel et al., 2007). Diagnosis in many of these cases has been challenging due to various issues, namely: highly fastidious microorganisms, low numbers of bacteria, non-culturable microorganisms, multiple infectious agents, antibiotic treated hosts, and /or emergent diseases in the same case. Molecular techniques such as PCR and sequencing of 16S rRNA has demonstrated its potential as a diagnostic tool, and has been used for definitive diagnosis of controversial and problematic cases (Ostland et al., 2006; Mauel et al., 2007).

During the past 5 years, the use of molecular techniques has helped to clarify the presence of an emerging group of fish pathogens that are members of the genus *Francisella*, family *Francisellaceae*, gamma subclass of the class Proteobacteria. This group of pathogens causes high mortalities in important cultured fish like cod (*Gadus morhua*) in Norway, three line grunt (*Parapristipoma trilineatum*) in Japan, hybrid striped bass (*Morone chrysops* x *M. saxatilis*) in the USA, Atlantic salmon (*Salmo salar* L.) in Chile, and tilapia (*Oreochromis* sp.) in Latin America, Taiwan, and now Costa Rica (Kamaishi et al. 2005; Hsieh et al. 2006; Olsen et al. 2006; Ostland et al. 2006; Birkbeck et al. 2007; Mikalsen et al. 2007). The existence of one or more species or subspecies in this group is still in question and will have to be addressed by taxonomists in the future. Certainly phenotypic and genetic differences exist between the cool water *Francisella* pathogens of marine fish such as the cod and warm water pathogens of fresh water fish species such as the tilapia.

The clinical signs, gross pathological changes, and histopathological findings described from the affected tilapia from Alajuela, Costa Rica are in agreement with previous reports by Hsieh et al. (2007) and Mauel et al. (2007) in cultured tilapia. Interestingly, during this event, the predominant clinical sign was the high mortality present among the fingerlings. According to farmers, affected fish will swim erratically for 5-10 minutes and then will succumb and die. This clinical sign may be related to the amount of granulomatous inflammatory cellular infiltration present in the central nervous system, as the most severely affected fish are those that exhibit this behavior.

Only rarely have fish health diagnosticians been able to recover the causative bacterium from affected fish, and culture it in bacteriological media (Kamaishi et al. 2005; Hsieh et al. 2006; Olsen et al. 2006; Birckbeck et al. 2007). In our case, we strongly recommend the use of a selective medium such as CHAH with the addition of Ampicillin and Polymixin B when

Francisella sp., is suspected as the causative agent of disease. This proved to be the most reliable medium of all those evaluated in this case providing good growth of the pathogen and inhibiting secondary and contaminating bacteria. In an i*n vitro* growth temperature comparison, we found that isolate LADL07-285A from tilapia differs in optimal temperature from the *Francisella* sp. diagnosed from farmed Atlantic cod and recently re-named *F. philomiragia* subsp. *noatunensis* (Mikalsen et al. 2007). The optimal temperature for the tilapia isolate was found to be 28-30°C, whereas the cod isolated was 22°C. These results are consistent with the environmental temperatures at which the fish are cultured. Further research is under way to determine the optimal temperature for disease development in tilapia (Soto, unpublished).

When comparing the 1384 bp partial 16S rRNA sequence of isolate LADL07-285A to other fish and mammalian pathogens, a high identity (more than 99%) was found between all the fish *Francisella* sp. sequences. Interestingly, a different tree branch can be observed amongst the warmwater cultured fish species showing nearly 100% similarities and the marine cold water isolates of cod (Fig. 2.5). Recent comparison of the 16S rRNA gene sequences, six partial housekeeping gene sequences (*groEL, shdA, rpoA, rpoB, pgm* and *atpA*), and DNA-DNA hybridization gave enough evidence to name the isolate from cod *F. philomiragia* subps. *noatunensis* subsp. nov. (Mikalsen *et al*. 2007). Kay et al. (2006) characterized the lipopolysaccharide and β-glucan from an isolate recovered from moribund tilapia by chemical and spectroscopy methods and confirmed the isolate as a relative of *F. tularensis*, with enough similarities to be placed in the same genus. Whether this isolate and the isolates from other fish species worldwide are the same is still to be determined.

In order to begin understanding the causal relationship of the microorganism and the disease, Koch's postulates were fulfilled with the isolate recovered from Costa Rican tilapia. High mortality rates were observed over a period of 10 days in fish challenged by intraperitoneal

injection and gill exposure, causing both acute and chronic presentations of disease respectively. Acute disease caused by IP injection was characterized by rapid mortality with no clearly observable clinical signs or gross pathological changes. Chronic disease followed gill immersion exposure and was typified by the presence of multiple white nodules in various organs. After clinical, histopathological, bacteriological and molecular analysis, the *Francisella* sp. isolate was recovered and shown to be the responsible agent for the disease.

In conclusion, the causative agent of a high mortality episode in cultured tilapia in Costa Rica has been identified as a member of the genus *Francisella* by bacteriological, histopathological, and molecular analysis. Research in the areas of diagnostic tools, virulence mechanisms and virulent factors, immune response and vaccines, prevention methods and treatments are urgent and necessary for aquaculture industries to be able to prevent and control this emergent pathogen.

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CHAPTER 3

ATTENUATION OF THE FISH PATHOGEN *FRANCISELLA* SP. BY MUTATION OF THE *IGLC* GENE*

INTRODUCTION

Tilapia is one of the most important cultured species in the world. Worldwide tilapia aquaculture production, mainly Nile tilapia (*Oreochromis niloticus*), has been increasing in exponential proportions during the last decade, to greater than 2.5 million tons in 2005. The main producing countries are China, Ecuador, Egypt, Israel, Indonesia, Singapore, Philippines and Thailand, but Mexico, Costa Rica, Honduras and other Latin-American countries have more than doubled their production in the past five years. The United States of America is the country that imports the highest amount of tilapia, receiving more than 80% of worldwide tilapia exports (Josupetit 2008). As the tilapia aquaculture industry expands, tilapia farms are often challenged with disease outbreaks, which in several cases have caused severe economic loses, due to high mortality events, decreased weight gain, antibiotic and treatment expenses, etc.

Francisella sp. is an emergent bacterial pathogen that causes acute to chronic disease in warm and cold water cultured and wild fish species. During the past 5 years the bacterium has been implicated as the cause of mortalities in tilapia and other important warm and cold water species cultured in the USA, Taiwan, Costa Rica, Latin America, Hawaii, Norway, Chile, and Japan (Kamaishi et al. 2005; Hsieh et al. 2006; Ostland et al. 2006; Birckbeck et al. 2007; Mauel et al. 2007; Mikalsen et al. 2007; Ottem et al. 2007; and Soto et al. 2009). Infected fish present with non-specific clinical signs such as erratic swimming, anorexia, anemia, exophthalmia and high mortality. Upon gross and microscopic examination, several internal organs (mainly spleen and kidney) are enlarged and contain widespread multifocal white nodules.

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Histological examination reveals the presence of multifocal granulomatous lesions, with the presence of numerous small, pleomorphic, cocco-bacilli (Soto et al. 2009).

The *Francisella* spp. that have been identified as emerging pathogens of fish, are poorly characterized due to the fastidious nature of the bacteria and difficulties in culturing the organism from fish tissues. For this reason many have not been properly classified. In the majority of the cases, PCR and sequence comparison of the 16S rRNA have made it possible to place the organism at 97% similarity to *F. tularensis,* 98% similarity to *F. philomiragia*, and 99% to other strains isolated from fish species (Kamaishi et al. 2005; Hsieh et al. 2006; Ostland et al. 2006; Mauel et al. 2007; Mikalsen et al. 2007; Ottem et al. 2007; Soto et al. 2009). *Francisella philomiragia* subsp. *noatunensis* and *F. piscicida* were recovered from moribund farmed Atlantic cod in Norway (Mikalsen et al. 2007; Ottem et al. 2007) displaying chronic granulomatous disease. Strains from cod in Norway have been characterized by phenotypic and molecular taxonomic methods as closely related members of *F. philomiragia* subsp. *philomiragia* (Mikalsen et al. 2007; Ottem et al. 2007).

Currently it is not known if the isolate recovered from diseased tilapia in Costa Rica by Soto et al. (2009), and the cod isolates represent two different species or different subspecies of the same species of bacterium. The strain to be utilized in this research project, LADL 07-285A, was isolated from tilapia from Costa Rica, Central America, at the Louisiana Aquatic Diagnostic Laboratory, LSU School of Veterinary Medicine, and was confirmed by molecular analysis as *Francisella* sp. and exhibited 99% identity with other fish pathogenic *Francisella* sp. (Soto et al. 2009).

Francisella tularensis is the most important species belonging to this genus (Dennis et al. 2001; Sjostedt 2007). Besides being an important animal pathogen, *F. tularensis* is a zoonotic agent which has received considerable study as a potential bioterrorism agent. The organism has

a high infectivity rate and multiple infectious routes (Keim et al. 2007; Nano and Shmerck 2007). The genetic basis of *F. tularensis* virulence is still poorly understood although several virulence determinants have been identified (Golovliov et al. 2003; Nano et al. 2004; Barker and Klose 2007). Previous studies have described the intracellular localization, survival, replication and escape of *F. tularensis* subspecies, in adherent mouse peritoneal cells, a mouse macrophage-like cell line J774A.1, and a human macrophage cell line THP-1. (Baron and Nano 1998; Golovliov et al 2003; de Bruin et al. 2007). Some of the most interesting genes identified in *F. tularensis* are the genes of the intracellular growth locus (*iglA, iglB, iglC*, and *iglD*) present as part of a 30 Kb pathogenicity island described by Nano et al. (2004) and Barker and Klose (2007). The functions of the conserved proteins corresponding to the genes are elusive. Overall, Igl proteins appear to be essential for the ability of *F. tularensis* to survive inside the macrophages and cause disease (Golovliov et al. 1997; Nano et al. 2004; Lai et al. 2004; Lauriano et al. 2004; Santic et al. 2005; Brotcke et al. 2006; de Bruin et al. 2007). Recent data have shown that IglA and IglB are part of a novel *Francisella* Pathogenicity Island (FPI) encoded Type Six Secretion System (T6SS) (Nano and Schmerk, 2007; Ludu et al. 2008-b). Mutations of these four genes in *F. tularensis*, have shown decreased pathogenicity of the bacterium both in-vivo and in-vitro in mammalian and insect tissues and cell lines (Lauriano et al. 2003; Nano et al. 2004; de Bruin et al. 2007; Vonkavaara et al. 2008).

The present research aims to study the presence of homologues to the Igl virulence genes in non-tularensis fish pathogenic *Francisella* sp. The study also describes a useful method for allelic exchange using PCR products to mutate *Francisella* sp., and the creation of an attenuated *igl*C mutant upon intraperitoneal and immersion challenges in tilapia (*Oreochromis* sp.). Finally, we examine intraperitoneal and immersion infectivity trials, to induce francisellosis in tilapia

(*Oreochromis* sp.), and report the dose required to cause mortality in 50% of the fish (LD_{50}) of this important emergent fish pathogen.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Strains, plasmids and primers used in this study are listed in Table 3.1. *Francisella* sp. LADL 07-285A was isolated from cultured tilapia (*Oreochromis* sp.) by our laboratory and described in previous work (Soto et al. 2009). *Francisella* LADL 07-285A was grown in Cysteine Heart Agar supplemented with bovine hemoglobin solution (BD BBL, Sparks, MD, USA) (CHAH) for 48 h at 28°C. A liquid culture medium consisted of a modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL, Sparks, MD, USA) and 0.1% glucose (MMH) (Soto et al. 2009). Broth cultures were grown overnight at 25 °C in a shaker at 175 rpm, and bacteria were frozen at -80°C in the broth media containing 20% glycerol for later use. Polymixin B (100 units/ml), and ampicillin (50 μg/ml) were added when needed to make the primary isolation media selective to aid in recovery of the bacteria from fish tissues and kanamycin (15 μg/ml) was used for recovery of transformed bacteria following electroporation. *Escherichia coli* XL1 Blue MRF′ was grown using Luria-Bertani broth or agar for 16 - 24 h at 37°C and supplemented with kanamycin (50 μg/ml) when needed to recover the plasmid containing bacteria after electroporation.

Identification of *F. tularensis* Operon i*glABCD* Homologue in *Francisella* sp. 07-285A. The complete genome sequences of *F. philomiragia* subsp. *philomiragia* ATCC 25017 (GeneBank accession number CP000937)*, F. tularensis* subsp. *novicida* U112 (GeneBank accession number CP000439), and partial genome sequences of F. *piscicida* strain GM2212 (GeneBank accession number EU492905), available from the National Center for Biotechnology Information (NCBI), were used to compare the Igl ABCD regions.

Table 3.1 Description of strains, plasmids and primers used in this study

Characteristics		
Bacterial Strain		
Francisella sp. 07-285A	Isolated from tilapia nilotica	Soto et al., 2008
E.coli XL1 Blue MRA		
Plasmids		
pEN1	Km^R	Ludu et al., 2008-a
Pbs	High copy number plasmid	Stratagene
pBS*iglC*	High copy number plasmid – wild type *igl*C	This work
pBSΔ*iglC*	High copy number plasmid with Δ*iglC* , Km^R	This work
Primers used for mutagenesis		
F-40 (*igl*C-XhoI)	5' aatt*ctcgag*tgttggtgctgagcaaattc 3'	This work
F-41(*igl*C-SpeI)	5' aattta*actagt*cagcacagcatacaggcaag 3'	This work
F-46 (*igl*C)	5' tgttggtgctgagcaaattc 3'	This work
F-47 (*igl*C)	5' cagcacagcatacaggcaag 3'	This work
F-12 FA1451-1	5' ttttgggttgtcactcatcgt 3'	Liu et al., 2007
F-13 FA1451-2	5' cgctataaccctcttcattt 3'	This work
Plasmids used for amplification of *igl*ABCD homologues		
F36*igl*A	5' gggaagatcggtagatgcaa3'	This work
F37*igl*A	5' cgagtagtgctctgatttctgg 3'	This work
FA22*igl*B	5' gtcagaagagtaaataatggtgt 3'	Liu et al., 2007
FA23*igl*B	5' ggctctatactaatactaaaagc 3'	Liu et al., 2007
F30*igl*Binternal	5' tttagttattattcgcaccg 3'	This work
F31*igl*Binternal	5' caggaagtttgtcaagatga 3'	This work
FA26*igl*C	5' gagtttgaaggaatgaatactacaatga 3'	This work
FA27*igl*C	5' gagccatcttcccaataaatcctt 3'	This work
F38*igl*D	5' gctggagctattgcctttctt 3'	This work
F39*igl*D	5' tgctatcctctatctttgcaggt 3'	This work

Previously published *F. tularensis* primers to these genes were also compared and were

used as a template to design primers to amplify homologous regions from the *Francisella* sp.

LADL 07-285A chromosomal DNA by polymerase chain reaction (PCR). PCR amplicons for

sequencing were purified with the QiaQuick Minelute PCR Cleanup Kit (Qiagen, Valencia, CA,

USA) as directed by the manufacturer, and were sequenced on an Applied Biosystems 3130

Genetic Analyzer using the PCR primers in Table 3.1. The sequences from the *Francisella* sp.

LADL 07-285A *igl*ABCD genes and the corresponding amino acid sequences were compared

with those stored in the NCBI database using the BLASTN and BLASTP program, with default settings.

Electroporation. Electrocompetent *E.coli* and *Francisella* sp. LADL 07-285A were prepared following Maier et al. (2004) with some modifications. Briefly, *E. coli* was aerobically grown until mid-logarithmic stage (OD_{600} 0.7), and the cells were prepared by washing 2 times in water followed by 1 wash in 10% glycerol. The electrocompetent *E. coli* were electroporated using a BioRad Gene Pulser Controller, in a 2 mm electroporation cuvette (BTX Harvard apparatus, Holliston, MA). The pulser was set at a voltage of 2.5kV, a capacitance of 25uF, and a resistance of 200 Ω. Immediately after electroporation, cells were suspended in 1 ml of LB-broth, and incubated with shaking for 1 h at 37°C. After the 1 h incubation period, *E. coli* was plated on LB agar with kanamycin (50ug/ml).

     Francisella sp. LADL 07-285A was grown aerobically until late-logarithmic stage (OD_{600} 0.6), and the cells were prepared by using 0.5 M sucrose. The electrocompetent *Francisella* sp. were electroporated using the Gene Pulser in a 2mm cuvette. The pulser was set at a voltage of 2.5kV, a capacitance of 25uF, and a resistance of 600 Ω. Immediately after electroporation, cells were suspended in 1 ml of MMH-broth, and incubated with shaking for 4 h at 28°C. After the 4 h incubation period, *Francisella sp.* was plated on CHAH with Kanamycin (15ug/ml).

Mutant and Plasmid Construction. A fragment of approximately 850 base pairs corresponding to a portion of the *igl*B and *igl*C genes from *Francisella* sp. LADL-07-285A was PCR amplified using primers F-40 and F-41 (Table 3.1), which contain XhoI and SpeI sites, respectively. All enzymes used during the study were supplied by New England Biolabs, and were used under the conditions recommended by the manufacturer. The PCR product was cleaved with these two endonucleases and ligated into the high copy number plasmid pBluescript SK (pBS), resulting in

plasmid pBS-*igl*C. The plasmid was electroporated into *E. coli*, amplified, and then purified

from the bacterium using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA)

following the manufacturers protocol.

Plasmid pEN1, constructed and donated by Ludu et al. (2008-a), contains a Tn903

Kanamycin cassette linked to *Francisella novicida* promoter derived from the region upstream of

gene FTN_1451 (Km-P) (Gallagher et al. 2007). Purified pEN1 plasmid was digested with PstI

to release the Km-P cassette.

For the construction of pBS-Δ*igl*C, plasmid pBS-*igl*C was digested with PstI

endonuclease, which cuts once in the *igl*C gene. The 1100 bp KmP cassette was ligated into the

unique PstI site in pBS-*igl*C, resulting in pBS-Δ*igl*C. The resulting insertion was verified by

sequencing.

Tilapia LD-50 Virulence Assays. *Intraperitoneal Injection (IP)*. *Francisella* LADL 07-285A

was cultivated in MMH in a shaking incubator at 175 rpm overnight at 25 °C. The bacteria were

then pelleted and the concentrations were adjusted to ~ 2.3 x 10^9 CFU/ml in PBS.

The LD_{50} of the wild type organism LADL 07-285A in naïve tilapia (average length ~ 9.0

cm and average weight ~ 18.9 g) was determined experimentally by IP injection   The fish were

obtained from a source believed to be free of *Francisella* infection and a sub-sample of the

population was confirmed as negative for *Francisella* sp. bacteria by culture on CHAH and PCR,

prior to use in the study. The fish were allowed to acclimate for at least 2 weeks prior to

challenge. At challenge, all fish were anesthetized with MS-222 (100 mg/l). Groups of 10 fish

were placed in 40 L tanks with recirculating water flow (one tank per treatment). From an initial

bacterial concentration of 2.3 x 10^9 CFU/ml of PBS, 10 serial dilutions in PBS were prepared.

Each fish per treatment was injected with 0.1 ml of the bacterial suspension. Fish in the control

tank were injected with 0.1 sterile PBS. Bacterial suspensions were inoculated on CHAH plates

and resulting colonies were enumerated, as described above. Water temperature was maintained in the range of 23-25°C.

Immersion Challenge (IC). The LD_{50} of wild type strain of *Francisella* sp. LADL 07-285A was determined in naïve tilapia (average length ~ 9.0 cm and average weight ~ 18.9 g) following immersion exposure. *Francisella* sp. LADL 07-285A and fish were prepared as described above. The fish were obtained from a source with no history of *Francisella* infection and a sub-sample of the population was confirmed as negative for *Francisella* sp. bacteria by culture on CHAH and PCR, prior to use in the study. Prior to challenge all fish were anesthetized with MS-222 (100 mg/l) to reduce the stress of handling. Ten fish were placed in each 40 L tank with recirculating water flow (one tank per treatment). Immersion challenge was carried out in 8 different dilutions of the bacterial suspension. The IC fish were immersed in 10 L of static water containing 2.3×10^8, 2.3×10^7, 2.3×10^6, 2.3×10^5, 2.3×10^4, 2.3×10^3, 2.3×10^2, 2.3×10^1 CFU/ml of tank water for 3 h. After 3 h, fish were moved to a clean 40 L tank with biofiltered recirculating water. Actual bacterial counts delivered were determined by plate counts from the inoculums, as described above. Control fish were treated with sterile PBS in a similar manner. Water temperature was maintained in the range of 23-25°C.

Analysis of Dead and Surviving Fish After Challenge. Dead and surviving fish were subjected to a complete clinical, bacteriological, and histopathological examination. Molecular analysis by PCR was performed following the protocol of Soto et al. (2009), from bacterial cultures recovered from moribund and dead fish, as well as from DNA extracted from spleen tissue of fish surviving challenge. Histopathological analysis was conducted in splenic, hepatic and renal tissue, and the severity of the lesions was recorded.

The LD_{50} was calculated by the method of Reed-Muench (Anderson 1984), at days 20 and 40 in both the intraperitoneal and the immersion challenges.

***In Vivo* Challenge of *Francisella* sp. LADL 07-285A Wild Type and Δ*igl*C.** *Francisella* sp.

LADL07-285A wild type and Δ*igl*C isogenic strains, were grown in CHAH at 25°C for 72 h.

Cells were harvested, suspended in 1 liter of MMH broth, and incubated in a shaking incubator

overnight at 24°C to obtain a final optical density at 600 nm (OD_{600}) of 0.75. Enumeration of the

bacteria was done by serial dilution utilizing 50 μl drops of each 10-fold dilution placed on

CHAH.

Experimental infection of naïve tilapia (average length ~ 9.0 cm and average weight ~

18.9 g) was tested by IP and IC challenges. The fish were obtained from a source with no

history of *Francisella* infection and a sub-sample of the population was confirmed as negative

for bacteria by culture on CHAH prior to use in the study. Fish were maintained at 10 fish per

tank. Three tanks were used per treatment, and one tank was used as a control. Prior to

challenge all fish were anesthetized with MS-222 (100 mg/l). Intra-peritoneal challenged fish

received a 0.1 ml injection of bacterial suspension (~$3x10^8$ CFU/fish, or ~$1.5x10^8$ CFU/fish).

The IC fish were immersed in 8 L of static water containing approximately $3.7x10^7$ CFU/ml in

tank water or $1.8 x10^7$ CFU/ml of tank water for 3 h, and then the volume of the tanks was

adjusted to 20 liters with clean water. Control fish were treated in a similar manner, but received

sterile PBS.

Following each challenge exposure, mortality was recorded every 12 h for 30 d. Dead

fish and survivors from each challenge were subjected to a complete clinical, bacteriological, and

histopathological examination. The mean number of granulomas found in a 10X microscopic

field was used to correlate with the severity of the diseases Table 3.2. Polymerase chain reaction

was performed on DNA from bacterial cultures recovered from moribund and dead fish to

confirm the presence of wild type or Δ*igl*C.

Statistical Analysis. Data (both original and inverse sine transformed) obtained from IC and IP challenges with the *Francisella* sp. LADL 07-285A wild type and Δ*igl*C strains were compared in an analysis of variance of a factorial arrangement of treatments with the SAS® statistical program (version 9.1.3). Where significance was found, post hoc pairwise comparisons were conducted with t tests of least squares means. Differences were considered significant at P ≤ 0.05.

RESULTS

Identification of *igl*ABCD Operon. The deduced amino acid products of the *Francisella* sp. LADL 07-285A *igl*A gene have 95, 92 and 88% similarities to the intracellular growth locus protein A of *F. philomiragia* subsp. *philomiragia*, *F. piscicida*, and *F. tularensis* subspecies, respectively. The amino acid sequences of the *Francisella* sp. LADL 07-285A proteins IglB, IglC and IglD showed identity of 97, 95 and 92% (IglB), 93, 90 and 89% (IglC) and 94, 92 and 80% (IglD) respectively to the intracellular growth locus proteins found in *F. philomiragia, F. piscicida,* and *F. tularensis* species, respectively. The G+C content found in the *igl*ABCD operon from LADL 07-285A (GeneBank accession number FJ386388) was 31%. Overall DNA comparison between *Francisella* LADL 07-285A, *F. philomiragia subsp. philomiragia* and *F. tularensis* subsp. novicida U112 *igl*ABCD operon, showed that the fish pathogen shares 94% identity to *F. philomiragia* and 83% identity with *F.tularensis subsp. novicida.* The *igl*ABCD operon of the three members of the genus *Francisella* sp. used, were in the same orientation and arrangement.

Generation of a *Francisella* sp. LADL 07-285A *igl*C Mutant. *Francisella tularensis* IglC protein has been shown to be up-regulated and important for intramacrophage survival and growth in *F. tularensis* subspecies (Nano et al. 2004; de Bruin et al. 2007). An insertion mutation made in the *igl*C gene of LADL 07-285A by allelic exchange using Km-P was found to

have approximately 400 base pairs of flanking sequences on either side of the insertion site.

Insertion of Km-P was confirmed by PCR using 2 different set of primers and DNA sequencing.

Primer sets F46-F47 and F31- F38 were used to verify the insertion and position of the 1100 bp

Km-P cassette in *igl*C (Figure 3.1). Primers used for amplification of the FA-1451 promoter

region, were also used to sequence the inside region of the insertion, and verify the presences of

the promoter in the mutant.
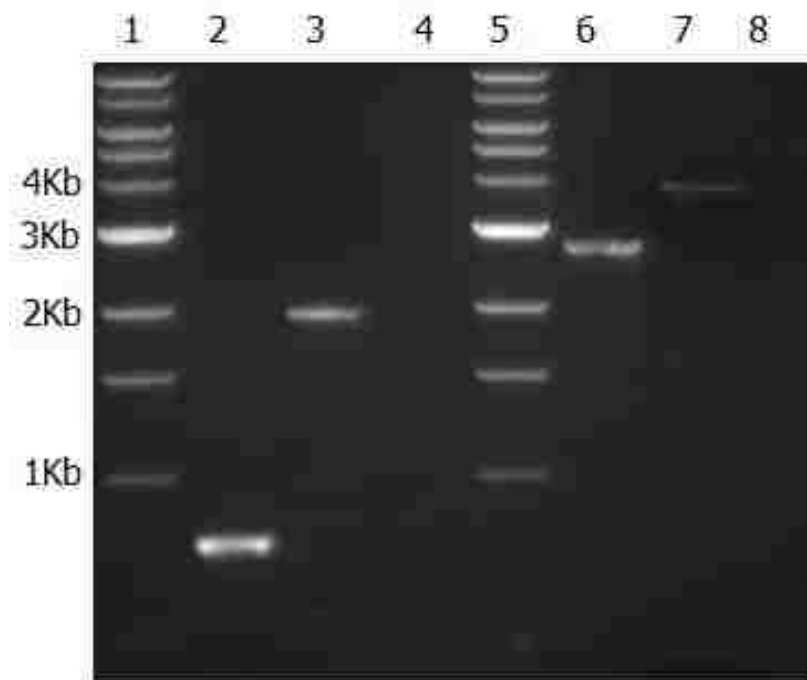
Figure 3.1 PCR amplification of iglC from wild type and isogenic mutant *Francisella* sp. LADL
07-285A. Lanes 1 and 5, 1kb Ladder. Lanes 2 and 6, PCR amplification of the *igl*C gene from
Francisella sp. LADL 07-285A using primer sets F46-F47 and F31- F38, respectively. Lanes 3
and 7, PCR amplification of isogenic mutant strain *Francisella* sp. LADL 07-285A Δ*igl*C using
primer sets F46-F47 and F31- F38, respectively. Lanes 4 and 8, Water.

The resulting *Francisella* sp. LADL 07-285A Δ*igl*C strain had no obvious morphological

differences from the wild type strain and growth characteristics were identical to those of the

parental strain in broth and on agar media.

Tilapia LD$_{50}$ Virulence Assays. Mortalities of tilapia challenged by IP or IC are shown in

Figure 3.2 and 3.3 respectively. Based on the cumulative mortalities found at day 20 and at day

40, the observed median lethal dose (LD$_{50}$) for the IP challenged tilapia infected with *Francisella*

sp. LADL 07-285A was $10^{-5.1}$ (~18269 CFU/fish), and $10^{-5.3}$ (~11527 CFU/fish) respectively.

On the other hand, the observed median lethal dose (LD$_{50}$) for the IC tilapia at day 20 and at day

40, were $10^{-0.52}$ (~6.9 x 10^7 CFU/ml), and 10^{-1} (~2.3 x 10^7 CFU/ml) respectively. The least

amount of bacteria required to cause mortality in the IP challenged tilapia was 23 CFU, whereas

for the IC, 2.3 x 10^2 CFU/ml of tank water was necessary to cause mortality (Table 3.2).
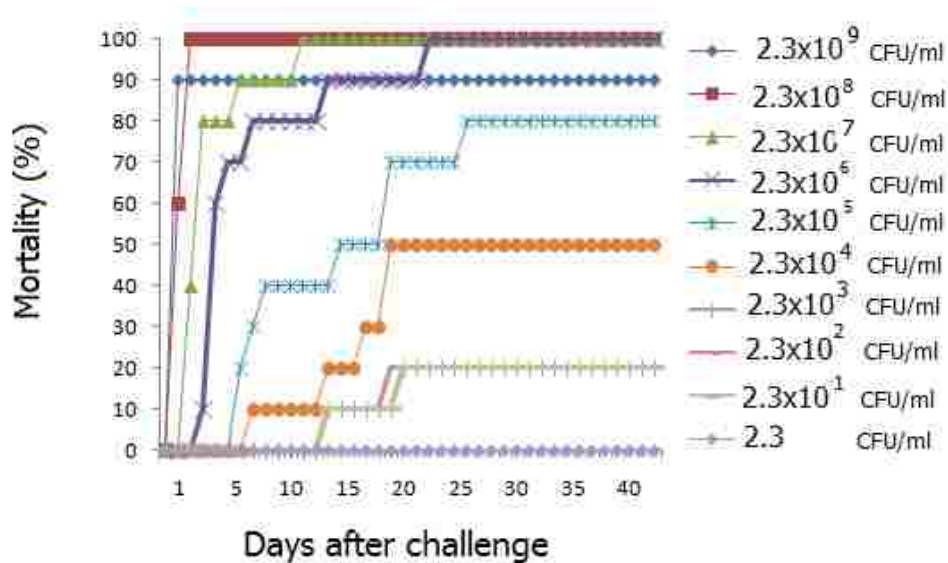
Figure 3.2 Mortality of tilapia challenged with *Francisella* sp. LADL 07-285A by intraperitoneal injection (10 fish were infected per treatment)

The dead and moribund fish presented the same clinical signs and histopathological

lesions as described in Soto et al. (In Press). No obvious external clinical signs were observed in

the fish. Internally, the most significant gross pathological change observed was the presence of

widespread, multifocal white nodules dispersed in the anterior kidney, posterior kidney, and

spleen, with a marked splenomegaly and renomegaly. Histopathologically, granulomatous

inflammation was present in the spleen and kidneys with large numbers of macrophages containing small pleomorphic coccobacilli.
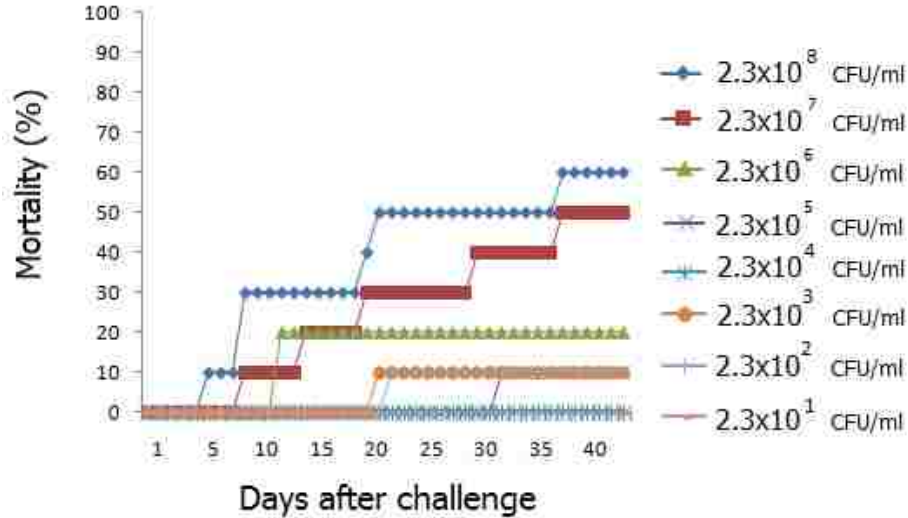
Figure 3.3 Mortality of tilapia challenged with *Francisella* sp. LADL 07-285A by immersion (10 fish were infected per treatment)

Francisella sp. LADL 07-285 A was isolated from the spleen and kidney of dead and moribund fish from both treatments. Bacteriological recovery, histopathological and molecular analysis (PCR) from the internal organs of surviving fish from both IP and IC are shown in Table 3.2.

***In Vivo* Challenge of *Francisella* sp. LADL 07-285A Wild Type and Δ*igl*C.** To examine the role of IglC in a fish model of infection, we measured survival rates of tilapia infected with *Francisella* sp. LADL 07-285A wild type and Δ*igl*C by two different routes of inoculation. After 48 h following IP injection of 0.1 ml of bacterial suspension (~3x10^8 CFU/fish, or ~1.5x10^8 CFU/fish), all the infected tilapia with the WT had died, while only one fish infected with the Δ*igl*C died 30 days post challenge. The dead fish recovered from the challenge with the Δ*igl*C IP injection, was not examined since it was in an advanced stage of decomposition. The

62

difference in dosages didn't show significance, while the percent mortality between wild type

and mutant injected fish was significantly different ($P < 0.0001$) (Figure 3.4).

Table 3.2 Summary of *Francisella* sp. LADL 07-285A LD_{50} Virulence Assays and % mortalities calculated by the method of Reed-Muench

Challenge dose	Percent Mortality	Bacterial isolation from dead fish	Bacterial isolation from survivors	Survivors Spleen PCR	Mean value of granulomas in 10X microscopic field		
Intraperitoneal Challenge (CFU/ml of PBS)					Spleen	Head kidney	Liver
2.3×10^9 CFU/ml	98.3	Pos	Pos	Pos	Moderate	Mild	Mild
2.3×10^8 CFU/ml	98	Pos	N/A	Pos	N/A	N/A	N/A
2.3×10^7 CFU/ml	97.5	Pos	N/A	Pos	N/A	N/A	N/A
2.3×10^6 CFU/ml	96.6	Pos	N/A	Pos	N/A	N/A	N/A
2.3×10^5 CFU/ml	86.3	Pos	Pos	Pos	Severe	Severe	Mild
2.3×10^4 CFU/ml	57.8	Pos	Pos	Pos	Mild	Mild	Mild
2.3×10^3 CFU/ml	27.7	Pos	Pos	Pos	Severe	Severe	Mild
2.3×10^2 CFU/ml	14.2	Pos	Pos	Pos	Severe	Moderate	Mild
2.3×10^1 CFU/ml	5.8	Pos	Pos	Pos	Severe	Moderate	Mild
2.3×10^0 CFU/ml	0	N/A	Neg	Pos	Moderate	Mild	Neg
2.3×10^{-1} CFU/ml	0	N/A	Neg	Pos	Mild	Mild	Neg
2.3×10^{-2} CFU/ml	0	N/A	Neg	Neg	Mild	Neg	Neg
Control	0	N/A	Neg	Neg	Neg	Neg	Neg
Immersion Challenge (CFU/ml of tank water)					Spleen	Head kidney	Liver
2.3×10^8 CFU/ml	78.9	Pos	Neg	Pos	Severe	Severe	Mild
2.3×10^7 CFU/ml	50	Pos	Neg	Pos	Severe	Severe	Mild
2.3×10^6 CFU/ml	19	Pos	Neg	Pos	Severe	Severe	Mild
2.3×10^5 CFU/ml	6.8	Pos	Neg	Pos	Severe	Moderate	Mild
2.3×10^4 CFU/ml	5.1	Neg	Neg	Pos	Moderate	Moderate	Mild
2.3×10^3 CFU/ml	4.1	N/A	Neg	Pos	Moderate	Moderate	Mild
2.3×10^2 CFU/ml	1.7	N/A	Neg	Neg	Mild	Mild	Neg
2.3×10^1 CFU/ml	0	N/A	Neg	Neg	Mild	Mild	Neg
Control	0	N/A	Neg	Neg	Neg	Neg	Neg

Legends:
   a) Pos = Positive
   b) Neg = Negative
   c) N/A = Not Applicable
   d) Severe = $X > 20$
   e) Moderate = $7 < X < 20$
   f) Mild = $X < 7$

- Surviving fish from both challenges were subjected to complete clinical, bacteriological examination at 40 days post challenge. Selected tissue for histopathological analysis was added to fixative at 40 days post challenge.
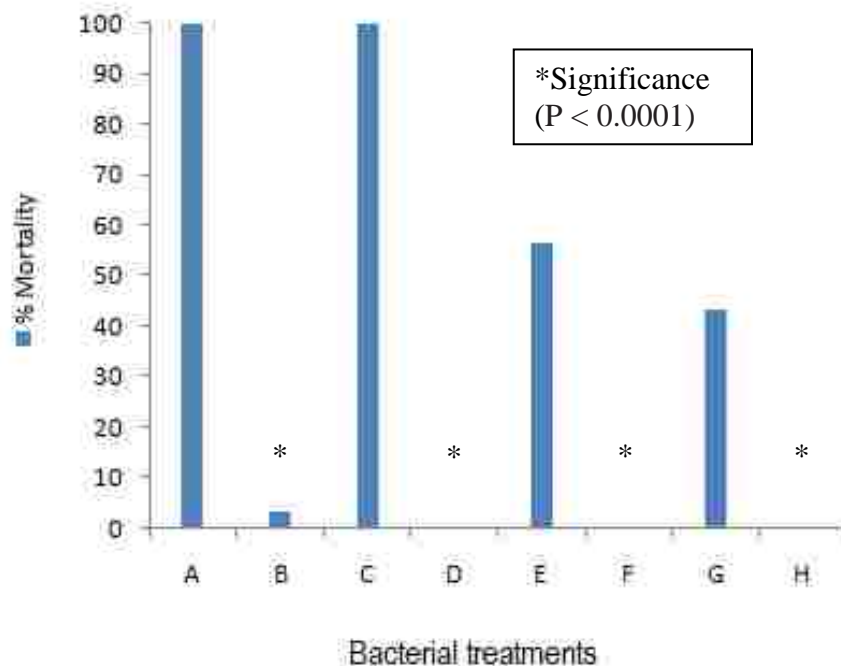
Figure 3.4 Percent mortality of tilapia (*Oreochromis* sp.) challenged by immersion and intraperitoneal routes with *Francisella* sp. LADL 07-285 A wild type and Δ*ig*lC.
Bacterial treatments:
   A. IP challenge of Wild type (~3x10^8 CFU/fish)
   B. IP challenge of Δ*ig*lC (~3x10^8 CFU/fish)
   C. IP challenge of Wild type (~1.5x10^8 CFU/fish)
   D. IP challenge of Δ*ig*lC (~1.5x10^8 CFU/fish)
   E. IC challenge of Wild type (~3.7x10^7 CFU/ml)
   F. IC challenge of Δ*ig*lC (~3.7x10^7 CFU/ml)
   G. IC challenge of Wild type (~1.8 x10^7 CFU/ml)
   H. IC challenge of Δ*ig*lC.Wild type (~1.8 x10^7 CFU/ml)

The fish immersed with ~3.7x10^7 CFU/ml of wild type bacteria in tank water had a survival percentage of 43.3%, and survival was 56.6% with the groups immersed with 1.8 x10^7 CFU/ml. The dosages did not result in significantly different mortality ($P \leq 0.05$). On the other hand, the fish challenged with the mutant strain had a 100% survival when challenged with ~3.7x10^7 CFU/ml and 1.8 x10^7 CFU/ml of tank water. Percent mortality was significantly different between groups challenged with the wild type and mutant strains ($P < 0.0001$) (Figure 3.4).

The histopathological analysis of the fish challenged with the wild type, showed the same lesions as previously described in the LD_{50} challenge, with widespread granulomas and granulomatous inflammation in the spleen and head kidney. Upon gross and histopathological analysis, the fish challenged with the Δ*igl*C by immersion challenge did not show any lesions in the analyzed tissues. The fish challenged with the Δ*igl*C strain by IP injection, presented higher numbers of activated melanomacrophages in the head kidney and the spleen than the control group of fish injected with PBS after 30 days of challenge. The control fish immersed with PBS did not display any lesions in the tissues and organs.

These data indicate that the loss of IglC activity results in a less virulent pathogen, which is presumably due to decreased intramacrophage survival. This will be assessed in future in vitro studies.

DISCUSSION

Members of the genus *Francisella* sp., are fastidious facultative bacteria that have been found to infect a great variety of animals (including humans), but very little is known regarding the virulence mechanisms and virulence factors of this genus (Barker and Klose 2007; Keim et al. 2007). The different subspecies of *F. tularensis* have been found to exist within macrophages in different vertebrate hosts, arthropods, and in amoebae (Keim and Wagner 2007; Abd et al. 2003; Vonkavaara et al. 2008). Several genes provide the pathogen with properties for survival in the extracellular compartment and also for survival and multiplication inside of potent phagocytes like neutrophils and macrophages (Baron and Nano 1998; Allen 2003; Nano et al. 2004). During previous histopathological analysis of infected tissue from tilapia, we observed the presence of large numbers of bacteria inside the macrophages, implying that the organism was a facultative intracellular pathogen.

The first aim of this study was to investigate the presence of homologous virulence factors in the emergent *Francisella* sp. pathogen from fish, and the other members of the *Francisella* genus. By comparing different *Francisella* spp. genomes, including *F. tularensis* and *F. philomiragia* genomes available at the NCBI website, we constructed primers to amplify the *igl*ABCD operon from the tilapia isolate.

The *igl*ABCD operon was described by Nano et al. (2004) as part of the *F. tularensis* pathogenicity island. The ability to survive inside macrophages and the presence of a Type VI secretion system are two of the most important virulence factors described for this important human pathogen present in the *F. tularensis* pathogenicity island.

In this study, we target a homologue of the highly expressed intracellular protein IglC, in *F. tularensis* species. The insertional mutagenesis protocol followed in this study, allowed us to select for a double recombination in the *Francisella* sp. LADL 07-285A *igl*C gene. Kanamycin was used as the selective antibiotic resistance marker due to the natural kanamycin susceptibility of the *Francisella* sp. strain used in this study (data not shown). As in *F. tularensis* subspecies, the *igl*C mutation significantly attenuates the pathogen upon in vivo challenges, and increases the survival rates of the mutant infected fish when compared with the wild type infected fish after both IP and IC challenges. We suspect that the presence of activated melanomacrophages in the spleen and head kidney of IP injected fish with the mutant strain is due to a normal immune reaction of fish targeting the injected bacterium, and it is not due to intracellular survival of the bacterium in macrophages, as observed in the wild type infected fish. Current work is being done in our laboratory to determine the pathogenesis of the tilapia isolate following experimental infection including the fate of cells following phagocytosis by the macrophage. Also we would like to assess the role of proteins, whose expression may be upregulated in tilapia macrophages after *in vitro* cell challenges.

We also compared two different administration routes (IP and IC) for challenging tilapia with *Francisella* sp, and reported the LD_{50} at 20 and 40 days post-challenge. The IP challenge was chosen since it was an easy and quick method to accurately administer suspended bacteria, but several problems developed when administering the bacteria by this method, including the lack of exposure of the bacteria to innate immune protection present in the skin, gills and other mucosa. As was expected, an acute onset of the disease was observed, with high mortalities and few clinical signs in the fish receiving the higher dosage. The low dose of bacteria, ~2 CFU injected into the peritoneum of the fingerlings, was able to cause mortalities. Even more surprising, was the amount and severity of lesions (granulomas) caused by a very low number of bacteria (~1 CFU/fish) in important hematopoietic and osmoregulatory organs like the spleen and the anterior kidney. Survivors of this treatment were observed with significant lesions in spleen, head kidney and liver, which will not only impair the fish's ability to osmoregulate, but will also immunosuppress them by direct damage of their hematopoietic organs making them more susceptible to other important and common tilapia diseases seen in culture facilities such as streptococcosis and columnaris disease.

The IC challenge route was chosen because it more closely resembles a natural infection. The fact that the bacteria have to come into close contact with the innate immune system present in skin, gills, gastrointestinal mucosa, etc., more closely resembles the way the disease progresses in nature. As expected, the amount of bacteria needed to cause mortality was higher than in the IP treatment, and the onset of the disease was more sub-acute to chronic, presenting anorexia, change in coloration, and pale gills. A dose of 2.3×10^2 CFU/ml of tank water was needed to cause mortality in the immersed fish, but when analyzing histopathological lesions of the survivors, it was evident that even a dose of 23 CFU/ml of tank water was able to cause significant lesions in the spleen and head kidney (Figure 3.5). The experiment was terminated

40 days after exposure to the bacterium, but we suspect that the survivors of this trial may

become carriers of the pathogen as in seen in natural infections.
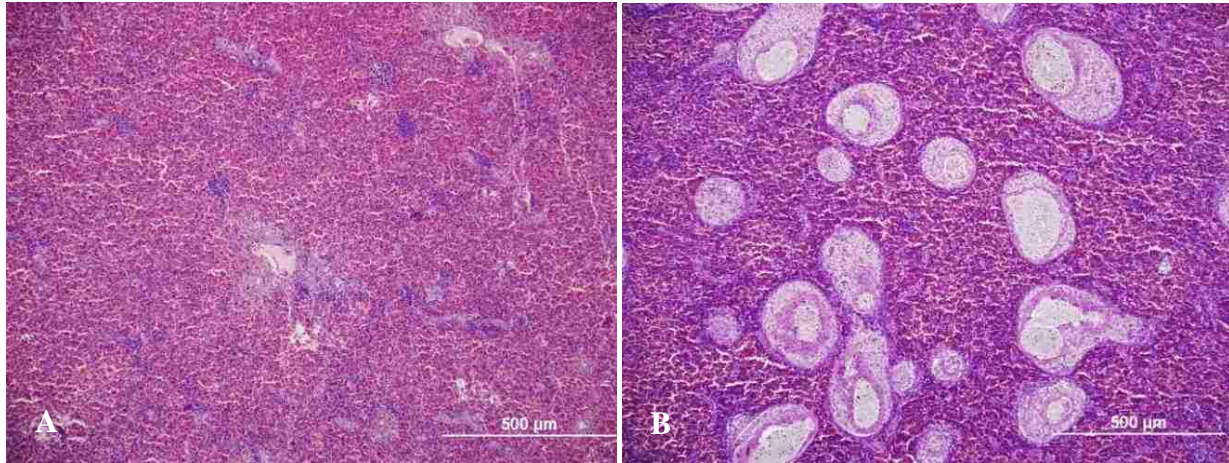
Figure 3.5 Histological microphotograph of un-infected and infected tilapia spleen 40 days post infection. (A) Normal splenic parenchyma and stroma in a non-infected tilapia spleen. (B) Severely infected tilapia spleen presenting widespread multifocal granulomatous lesions with mixed inflammatory infiltrates.

This study provides the first identification of homologous genes of the *F. tularensis*

pathogenicity island in the *Francisella* sp. members isolated from tilapia tissue. The data

gathered in this study provides useful information of potential target genes for future virulence,

pathogenesis, diagnosis, and immunological studies for this emergent worldwide pathogen. It

also provides an easy and reliable method for mutagenesis of this fastidious pathogen. We also

show important data regarding the virulence capacity of the bacterium in tilapia, and provide

examples of two challenge methods to infect tilapia.

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CHAPTER 4

INTERACTION OF *FRANCISELLA ASIATICA* WITH TILAPIA (*OREOCHROMIS NILOTICUS*) INNATE IMMUNITY*

INTRODUCTION

Francsiella asiatica and *F. noatunensis* are recently described members of the genus

Francisella sp. (Mikalsen et al. 2007; Mikalsen and Colquhoun 2009). *Francisella noatunensis*

isolates were recovered from diseased cultured cod in Norway (Mikalsen et al. 2007; Mikalsen

and Colquhoun 2009). *Francsiella asiatica* (isolate Ehime-1) was recovered from diseased three

line grunt in Japan, and was the isolate used to describe the new subspecies (Kamaishi et al.

2005; Mikalsen et al. 2009). In the last five years the bacterium caused substantial mortality in

tilapia and other important warm and cold water species cultured in the USA, Taiwan, Costa

Rica, Latin America, Hawaii, Norway, Chile, and Japan (Birkbeck et al. 2007; Hsieh et al. 2006;

Kamaishi et al. 2005; Mauel et al. 2007; Mikalsen et al. 2008; Ostland et al. 2006; Ottem et al.

2007; Soto et al. 2009a). In Taiwan, reports of rickettsia-like organisms causing diseases in fresh,

brackish and salt pond-culture tilapia can be tracked to the early 90's, and in recent years, several

farms have report mortalities up to 95% due to this pathogens (Hsieh et al. 2006). In Latin

America, Hawaii, and Costa Rica, a similar situation has been present since 2004, when

mortalities up to 90% were reported in brackish and fresh water cultured tilapia (Mauel et al.

2007; Soto et al. 2009a). Moreover, the bacterium has not only being isolated and found causing

diseases in important worldwide culture species like tilapia, three line grunt, cod and Atlantic

salmon; but have been found in wild fish like the Guapote in Latin America and Costa Rica and

wild mackerel and cod in Norway (Birkbeck et al. 2007; Hsieh et al. 2006; Kamaishi et al. 2005;

Mauel et al. 2007; Mikalsen et al. 2008; Ostland et al. 2006; Ottem et al. 2007; Soto et al.2009a).

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Fish francisellosis is an emergent disease of a wide variety of fish species. The diseases can present as an acute syndrome with few clinical signs and high mortality, or as a sub-acute to chronic syndrome with non-specific clinical signs, including anorexia, exophthalmia, and anemia. Upon macroscopic and microscopic examination, internal organs are enlarged and contain widespread multifocal white nodules. Histological examination reveals the presence of multifocal granulomatous lesions containing numerous small, pleomorphic, cocco-bacilli (Soto et al. 2009a). In the majority of the cases, polymerase chain reaction (PCR) and sequence comparison of the 16S rRNA place the organism at 97% similarity to *F. tularensis* and 98% similarity to *F. philomiragia* (Birkbeck et al. 2007; Hsieh et al. 2006; Kamaishi et al. 2005; Mauel et al. 2007; Mikalsen et al. 2008; Ostland et al. 2006; Ottem et al. 2007; Soto et al. 2009a).

Francisella tularensis is the most important species belonging to this genus (Abd et al. 2003; Dennis et al. 2001; Sjostedt 2007). Besides being an important animal pathogen, *F. tularensis* is a zoonotic agent that has received considerable study as a potential bioterrorism agent because it has a high infectivity rate and multiple infectious routes (Keim et al. 2007; Nano and Schmerk 2007). The genetic basis of *F. tularensis* virulence is still poorly understood, although several virulence determinants have been identified (Barker and Klose 2007; Nano et al. 2004). Previous studies described the intracellular localization, survival and replication of *F. tularensis*, in polymorphonuclear leukocytes (PMNs), macrophages, adherent mouse peritoneal cells, a mouse macrophage like cell line J774A.1, and a human macrophage cell line THP-1, and include the ultimate escape from the phagolysosome into the cytoplasm (Abd et al. 2003; Allen 2003; Anthony et al. 1991; Baron and Nano 1998; de Bruin et al. 2007; Golovliov et al. 2003; Ray et al. 2009). Some of the most interesting genes involved in this process are the genes of the intracellular growth locus, *iglA, iglB, iglC*, and *iglD,* present as part of a 30 Kb pathogenicity

island (Barker and Klose 2007; Nano and Schmerk 2007). The functions of the conserved proteins corresponding to these genes are elusive, although the Igl proteins appear to be essential for the ability of *F. tularensis* to survive inside macrophages and cause disease (Bonquist et al. 2008; Brotcke et al. 2006; Clemens and Horwitz 2007; de Bruins et al. 2007; Golovliov et al. 1997; Lai et al. 2004; Lauriano et al. 2004; Nano et al. 2004; Santic et al. 2005). Recent data showed that IglA and IglB are part of a novel *Francisella* Pathogenicity Island (FPI) encoded Type Six Secretion System (T6SS) (Ludu et al. 2008; Nano and Schmerk 2007). Mutations of the *iglABCD* genes in *F. tularensis* resulted in decreased pathogenicity both *in vivo* and *in vitro* in mammalian and insect tissues and cell lines (de Bruins et al. 2007; Lauriano et al. 2003; Nano et al. 2004; Vonkavaara et al. 2008).

Homologues to the *F. tularensis iglA, iglB, iglC*, and *iglD* genes are present in *F. asiatica* strain LADL 07-285A, isolated from diseased tilapia. DNA sequence comparison between *F. asiatica* LADL 07-285A, *F. philomiragia* subsp. *philomiragia* and *F. tularensis* subsp. novicida U112 *igl*ABCD operon, revealed 94% identity to *F. philomiragia* and 83% identity with *F. tularensis* subsp. *novicida*. It was previously demonstrated that as few as 23 *F. asiatica* bacteria injected in the peritoneum are capable of causing mortalities in tilapia nilotica (*Oreochromis niloticus*), and that even fewer are enough to cause serious pathological lesions in important organs like the head kidney and spleen (Soto et al. 2009b), but the pathogenic mechanisms that underlie its remarkable infectivity and its capacity to cause disease in a broad range of fish hosts are poorly known. In previous work, however, an insertion mutation in the *iglC* gene of *F. asiatica* LADL 07-285A was constructed by allelic exchange, and the Δ*iglC* mutant was found to be attenuated following intraperitoneal and immersion challenges in tilapia (Soto et al. 2009b).

In the present study we use *F. asiatica* LADL 07-285A to investigate the interaction between this emergent pathogen and innate immunity in tilapia. We demonstrate that the

F. asiatica wild type isolate is resistant to serum killing, is able to enter, survive, and replicate in tilapia head kidney derived macrophages, and ultimately kills the cell by inducing apoptosis. Mutation of the *iglC* gene, however, makes *F. asiatica* defective for intramacrophagic survival and replication, as well as for induction of apoptotic caspase 3 and 7 cleavage and cytotoxicity, but does not affects its ability to survive in serum. Finally we demonstrate that complementation of the IglC protein restores virulence, the pro-apoptotic features of the defective mutant, and cytotoxicity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Francisella asiatica* LADL 07-285A was isolated from cultured tilapia (*Oreochromis* sp.) and was described in previous work (Soto et al. 2009a). The Δ*iglC* mutant isolate was made by homologous recombination using a PCR product, and its attenuation was demonstrated *in vivo* (Soto et al. 2009b). *Francisella asiatica* was grown on Cystine Heart Agar supplemented with bovine hemoglobin solution (CHAH) (Becton Dickenson (BD) BBL, Sparks, MD, USA) for 48 h at 28°C, or in Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL, Sparks, MD, USA) and 0.1% glucose (MMH) (Baker et al. 2005). Broth cultures were grown overnight at 25 °C in a shaker at 175 rpm, and bacteria were frozen at -80 °C in the broth media containing 20% glycerol for later use.

Escherichia coli QC 779 serum sensitive isolate and *E. coli* strain DH5*a* were grown using Luria-Bertani broth or agar for 16 - 24 h at 37°C. When needed, kanamycin and/or tetracycline were added to the agar and broth media at a concentration of 15 ug/ml and 10 ug/ml, respectively.

Growth curves of the *F. asiatica* wild-type and Δ*iglC* mutant strains were determined by inoculating duplicate culture tubes containing 50 ml MMH broth with 500 μl of overnight broth cultures adjusted to an optical density of 0.8 at 600 nm of each strain. The cultures were

incubated at 25 °C for 24 hours on an orbital shaker (200 rpm) and growth was monitored every two hours by measuring the optical density at 600 nm.

Construction of Complementing IglC Plasmid. Briefly, for complementation, plasmid pKEK894 (Zogaj et al. 2008) was used to clone the *iglC* gene. The *iglC* gene was amplified by PCR from *F. asiatica* LADL 07-285A genomic DNA with primers FAcoI-*iglC*-Comp-F (5'AACGCGCCATGGGTATGAATGAAATGATAACAAGAC-3') and FEcoRI-*iglC*-Comp-R (5'GCGCGGAATTCCGATCTTACTATGCAGAT-3'). The PCR fragment was digested with *NcoI* and *EcoRI*, and ligated into *NcoI* and *EcoRI* digested pKEK894, to form pKEK-Fa*iglC*. The pKEK-Fa*iglC* plasmid was then electroporated into *E.coli* DH5*a*, reisolated, and electroporated into *F. asiatica* LADL 07-285A wild type and Δ*iglC* mutant as previously described (Soto et al. 2009b).

Fish. Adult tilapia (*Oreochromis niloticus*) (mean weight 342 g) were obtained from an inland farm with no previous history of fish francisellosis. Fish were acclimated for a minimum of 2 months in a recirculating system at 25°C under optimum water quality conditions. Ten fish were euthanized using 100 mg/ml of tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, Washington), and analyzed for evidence of *Francisella* by clinical examination, bacteriological isolation, and polymerase chain reaction (Soto et al. 2009a).

Bactericidal Activity of Normal and Heat-Inactivated Serum. Blood was collected from 10 adult tilapia by caudal venipuncture using 3 ml red top Vacutainers tubes (BD Vacutainer Systems, Franklin Lakes, New Jersey, USA). Prior to bleeding, the fish were anaesthetized with 100 mg/ml MS-222. Blood was allowed to clot for 4 h at 4°C before serum was collected by centrifugation at 3000 × *g* for 10 min. A sub-sample of the collected sera was heated in a water bath at 55°C for 30 min to inactivate complement.

Francisella asiatica wild type, the Δ*iglC* mutant, and *E. coli* isolate QC 779 (serum sensitive), were cultured as previously described. Bacteria were adjusted to a concentration of

1×10^7 CFU/ml in PBS, and equal volumes of the bacterial isolates and either normal or heat inactivated tilapia sp. serum were combined and incubated at room temperature. At 0, 1, and 2 h sub-samples were collected, serially diluted in PBS, and spotted onto either CHAH (*F. asiatica*) or LB (*E. coli*) plates for determination of CFU numbers.

Macrophage Media. An optimal medium for culture of tilapia macrophages was designed based on previously published media for cultivation of channel catfish and hybrid striped bass macrophages (Booth et al. 2006; Elkamel et al. 2003), but with an osmolality of 320 mosmols/kg H_2O to match tilapia serum osmolality. The complete tilapia macrophage medium (CTMM) consisted of Roswell Park Memorial Institute (RPMI) medium 1640 (GIBCO, Invitrogen Corp., Carlsbad, California) with 14mM Hepes Buffer (GIBCO, Invitrogen Corp.), 0.3% sodium bicarbonate (GIBCO, Invitrogen Corp.), 0.05 mM 2-beta mercaptoethanol (Sigma Chemical Co., St Louis, Missouri), and 5% heat-inactivated, pooled tilapia serum.

Growth of *F. asiatica* in Macrophage Culture Media. To evaluate the reliability of using various media for an *in vitro* intramacrophagic survival assay, growth of *F. asiatica* LADL 07-285A was compared in Dulbecco modified Eagle medium containing 10% tilapia serum (DMEM), CTMM, CTMM with the addition of 10 ug/ml of gentamicin, and MMH. Triplicate wells of a 96-well microtiter plate were inoculated with 200 µl each of media containing approximately 2.3×10^6 CFU/ml. Bacteria growth was measured over a period of 24 h by plating serial dilutions on CHAH.

Collection and Cultivation of Head Kidney Derived Macrophages. Previous protocols were modified for the collection and culture of tilapia macrophages (Booth et al. 2006; Miles et al. 2001; Secombes et al. 1990). Briefly, fish were anesthetized with MS-222 and bled from the caudal vein to collect autologous serum. Anterior kidneys were aseptically removed, and the cells were dissociated by passage through a double stainless steel mesh (280 and 140 µm) cell

dissociation sieve (Sigma Chemical Co.). Dissociated cells from individual fish were suspended

in CTMM. An isosmotic Percoll gradient was prepared following previous published protocols

with several modifications (Gesssani et al. 2000). The isosmotic Percoll consisted of 9.25 parts

of Percoll (Amersham Bioscience, Sweden) and 0.750 parts of 10X phosphate buffer solution

(ph 7.1). A Percoll density gradient was prepared in a centrifuge tube by layering a 51% Percoll

solution (51% isosmotic Percoll, 49% CTMM) below a 34% Percoll solution (34% isosmotic

Percoll, 66% CTMM). The macrophage cell suspension was layered on top of the gradient and

was subjected to centrifugation at 400 x g for 25 min at 4°C with medium acceleration and low

deceleration. The macrophages were collected from the gradient interface, washed twice in

CTTM at 400 x g, and viability counts were determined using Trypan blue dye exclusion.

Purification of HKDM was confirmed by non-specific esterase and Sudan Black staining

(Ellsaesser et al. 1984). Purity of the samples were >90% head kidney derived macrophages.

Cells were adjusted to 1 x 10^7 cells/ml, and 100 μl of the suspension were aliquoted into each

well of 96-well microtitre plates coated with poly-D-lysine (BD Biosciences, Bedford, MA,

USA). Macrophages were allowed to adhere for 4 h (4h-HKDM) or 5 days (5d-HKDM) at 25°C

with 5% CO_2, after which non-adherent cells were removed with three washes of warm CTMM,

and fresh CTMM was added. In certain experiments soluble mannan was used to block the

mannose receptors (MR) on HKDM as previously described (Bagalopal et al. 2006). When used,

mannan (5 mg/ml) was incubated with HKDM for 30 min at 25°C prior to the addition of

bacteria.

Intramacrophage Survival Assays. To infect tilapia HKDM, a modification of previous

protocols was used (Brotcke et al. 2006; Nano et al. 2004; Santic et al. 2005). Briefly, 4 h or 5

day cultures of tilapia head kidney macrophages in 96 well plates containing 1-5 x 10^5 cells/well

were used. *Francisella asiatica* LADL 07-285A was grown for a period of 8 h in MMH at 25°C.

Optical density (OD_{600}) of the culture was determined and the cells were adjusted to an estimated

final concentration of 5 x 10^8 CFU/ml, based on an OD/CFU standard curve. One ml aliquots of

the bacterial suspension were pelleted at 10,000 x g for 5 minutes in an Eppendorf 5415 D

centrifuge (Eppendorf-Brinkman, Westbury, New York), and the pellet was resuspended in

either 1ml of normal autologous serum (NS), 1ml of heat- inactivated autologous serum (HINS),

or 1ml of PBS. Ten-fold serial dilutions were plated on CHAH after incubation to determine

actual CFU/ml. After a 30 min incubation, the 96 well plate was inoculated with 10 µl of

opsonized bacteria per well to achieve a multiplicity of infection (MOI) of 50 bacteria: 1

macrophage. The plates were centrifuged for 5 min at 400 x g to synchronize bacterial contact

with macrophages. Following 2 h incubation at 25°C with 5% CO_2, the cells were washed three

times with warm media (25°C), and further incubated with fresh media for 0, 12, 24, or 36 hours.

Cells in three wells were lysed by the addition of 100 µl of 1% Saponin in PBS at each time

point. The lysates were serially diluted and spread onto CHAH plates to determine viable

counts. Experiments were performed in triplicate on a minimum of three separate occasions to

affirm the reliability of the results.

Detection of *F. asiatica*-Mediated Cytotoxicity. Cytotoxicity was assessed by measuring the

release of cytosolic lactate dehydrogenase (LDH) into the supernatant, which reflects a loss of

plasma membrane integrity in infected cells. Cytosolic LDH levels were measured using the

colorimetric Cytotox 96 Kit (Promega, Madison, WI, USA) according to the manufacturer's

instructions. The percentage of cytotoxicity was calculated as $100 \times$ [(experimental release −

spontaneous release)] / [total release − spontaneous release)], where spontaneous release is the

amount of LDH activity in the supernatant of uninfected cells and the total release is the activity

in cell lysates.

Caspase Activity Assay. The Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) was used to measure the activity of caspase-3 and -7 in infected and uninfected HKDM following the manufacturer's instructions. Members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in eukaryotic cells (Lai et al. 2001; Lai et al. 2005). The Apo-ONE® Homogeneous Caspase-3/7 Assay provides a profluorescent substrate with an optimized bifunctional cell lysis/activity buffer for caspase-3/7 (DEVDase) activity assays. The percentage of apoptosis was calculated as $100 \times$ [(experimental release – spontaneous release)] / [total release – spontaneous release)], where spontaneous release is the amount of caspase-3/7 activity in the supernatant of uninfected cells and the total release is the activity in cell previously exposed to etoposide (MBL International Corporation, Boburn, MA, USA) following the manufacturers recommendations to induce 100% apoptosis in the cells.

Electron Microscopy. Tilapia head kidney derived macrophages were attached to 13-mm tissue-culture treated Therminox cover slips (Nalge Nunc, Rochester, New York), infected at an MOI of 50:1, incubated for 2, 6 or 12 h, and processed for TEM. Briefly, primary fixation was for 6 h at RT in 1.25% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cells were post-fixed for 1 h in 1% osmium tetraoxide (OsO4) in distilled water and stained for 2 h with 2% uranyl acetate in 0.2 M sodium acetate buffer, pH 3.5. Ethanol dehydrated cells were infiltrated and then embedded in epoxy resin. Ultra-thin sections were cut on a Sorvall Model MT600 ultra microtome, mounted on 300-mesh copper grids, and stained for 10 min with 5% uranyl acetate in distilled water. Sections were washed three times with double-distilled water and then stained for 2 min with lead citrate. Stained sections were examined on a Zeiss model EM 10C microscope at various magnifications.

Statistical Analysis. The experimental design was completely randomized, with a factorial arrangement of treatments. Data were analyzed by the general linear models procedure (PROC GLM) in the Statistical Analysis System after a $\log_{10}$ transformation of the numbers of CFU recovered per well (SAS Institute, Inc. 2003). When the overall model indicated significance (P <0.05), Scheffe´s test was used for pairwise comparison of main effects and a least-squares means procedure was used for pairwise comparison of interaction effects.

RESULTS

*Francisella asiatica **∆igl*C** Mutant Grows at Same Rate as Wild Type in Media.** There were no significant differences in the growth curves of the *F. asiatica* 07-285A wild type and mutant ∆*igl*C when grown in MMH broth at 25°C (Fig. 4.1), indicating that in vivo effects were not due to a growth defect in the *ΔiglC* mutant.
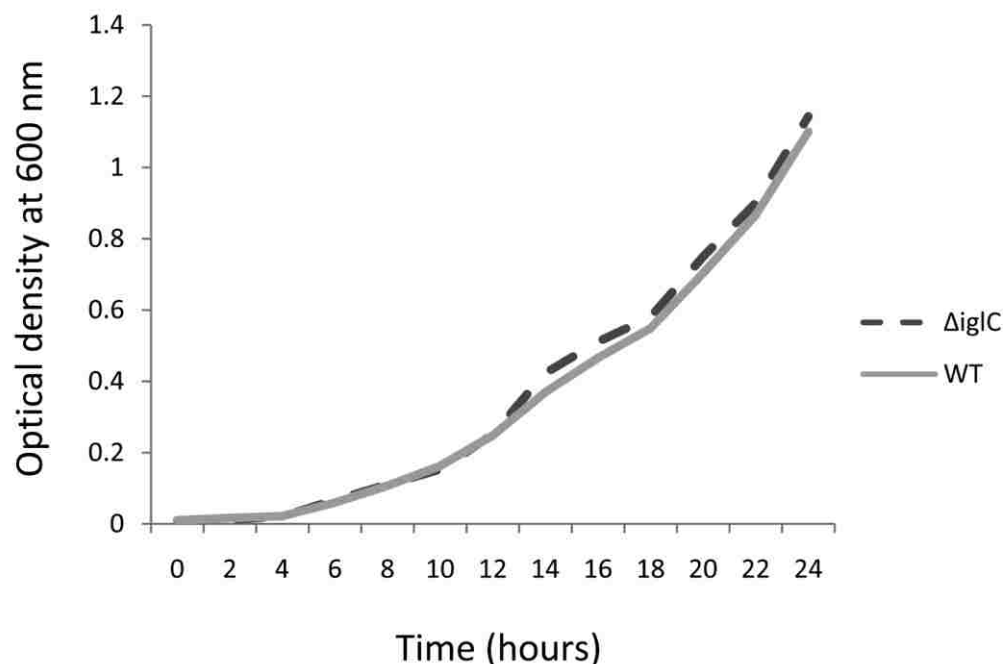
Figure 4.1 Growth curves of *Francisella asiatica* LADL 07-285A wild type and the ∆*igl*C strains. Each strain was grown in MMH broth 25 °C and growth was monitored by determining the optical density at 600 nm.

Complete Tilapia Macrophage Media (CTMM) Does Not Support the Growth of *F.*

asiatica. As demonstrated in Figure 4.2, neither DMEM media with the addition of 10% heat

inactivated heterologous tilapia serum, nor CTMM were favorable environments for *Francisella*

growth. *Francisella asiatica* incubated in MMH showed exponential growth after the same

incubation period. This finding is in congruence with previous work done with *F. tularensis*

isolates, in which the relative inability of *Francisella* spp. to grow extracellularly in macrophage

cultures allowed the use of CTMM or DMEM in an *in vitro* assay without the presence of

antibiotics in the medium (Anthony et al. 1991; Brotcke et al. 2006; Nano et al. 2004; Santic et

al. 2005).
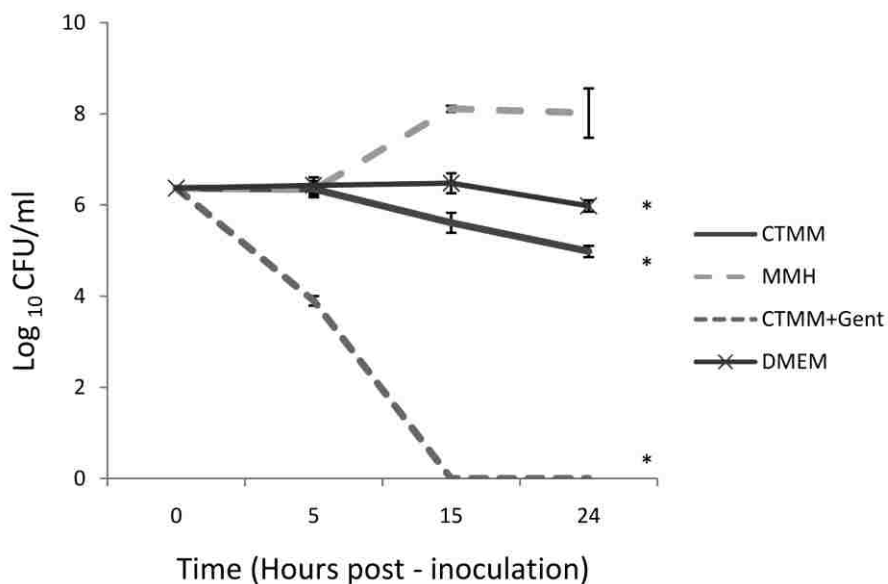
Figure 4.2 *In vitro* growth of *Francisella asiatica* LADL 07-285A. LADL 07-285A cultured in modified Mueller-Hinton broth (MMH), Complete Tilapia Macrophage media (CTMM), Dulbecco's Modified Eagle's medium (DMEM) and CTMM with 10ug/ml Gentamicin for 24 h with 5% CO2. Samples were removed at 5, 15 and 24 h post inoculation and then assayed for bacterial CFU by serial dilution in PBS and plating on CHAH plates. The error bars represent standard error of triplicate samples and the results shown are representative of three independent experiments. Significant differences between bacterial growth in CTMM, DMEM and CTMM with gentamicin, and growth in MMH are marked (*, *P<0.05*)

Both the *F. asiatica* Wild Type and *ΔiglC* Mutant Strains Are Resistant to Serum Killing.

Both *F. asiatica* wild type and mutant ΔiglC demonstrated complete resistance to serum killing by both heat-inactivated and normal serum, as the number of bacteria re-isolated from the wells was similar or significantly higher than he number inoculated (p<0.001) (Fig. 4.3). The serum sensitive *E.coli* isolate used in the assay was undetectable after only 1 h of incubation with the normal serum. When incubated with heat-inactivated serum, no killing was observed, suggesting that the killing is due to the action of complement.
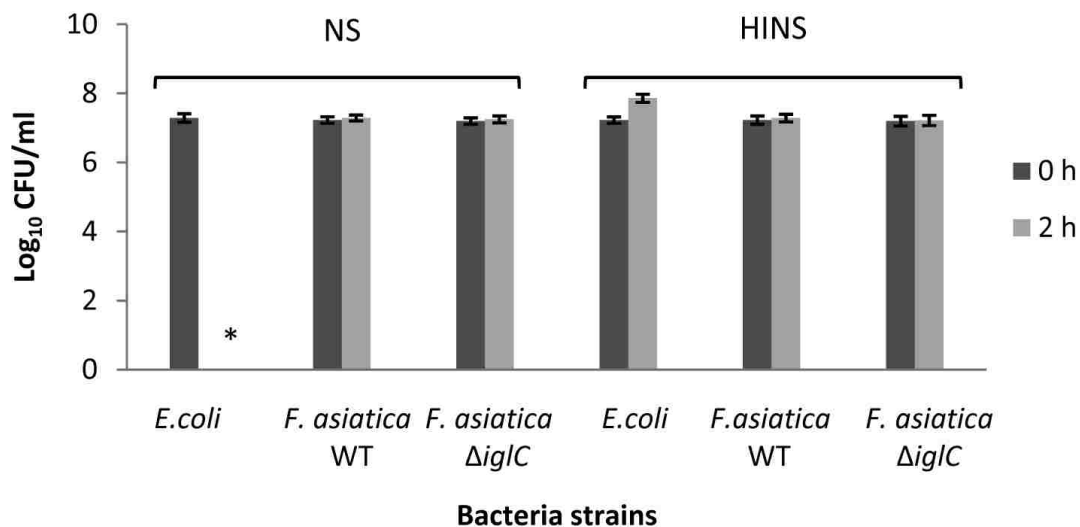
Figure 4.3 Survival and growth of *Francisella asiatica* LADL 07-285A wild type and *ΔiglC* and *E. coli* in normal serum (NS) and normal serum that have been heat inactivated at 55°C for 30 m (HINS). The error bars represent standard error of triplicate samples and the results shown are representative of three independent experiments. Statistically significant differences between different treatments are marked (*, *P*<0.001)

A Heat-Sensitive Serum Component and Mannose Receptors Are Necessary for Efficient Uptake of *F. asiatica* Isolates by Tilapia HKDM. The uptake of *F. asiatica* by tilapia HKDM was assessed in the presence or absence of normal serum and mannan (competitive inhibitor of

macrophage mannose receptors) in the media in order to provide insight into the receptors that are involved in the recognition and uptake of *F. asiatica*. In both the 4h and 5d HKDM uptake of *F. asiatica* was significantly greater when NS was used, indicating that a heat sensitive component of the serum, most likely complement, was an important mediator of uptake (Fig. 4.4). Bacteria opsonized with HINS showed a 59 and 96.5% decrease in internalization in 4h and 5d-HKDM, respectively, compared to bacteria treated with NS. Similar results were obtained when using PBS to opsonize the bacteria (Data not shown). Once inside the macrophage, both NS and HINS treated bacteria increased equally in numbers after 12 h of incubation. Although NS increased the up-take of *F. asiatica* with both 4h and 5d HKDM, bacteria were also taken up efficiently in the absence of complement (Fig. 4.4).
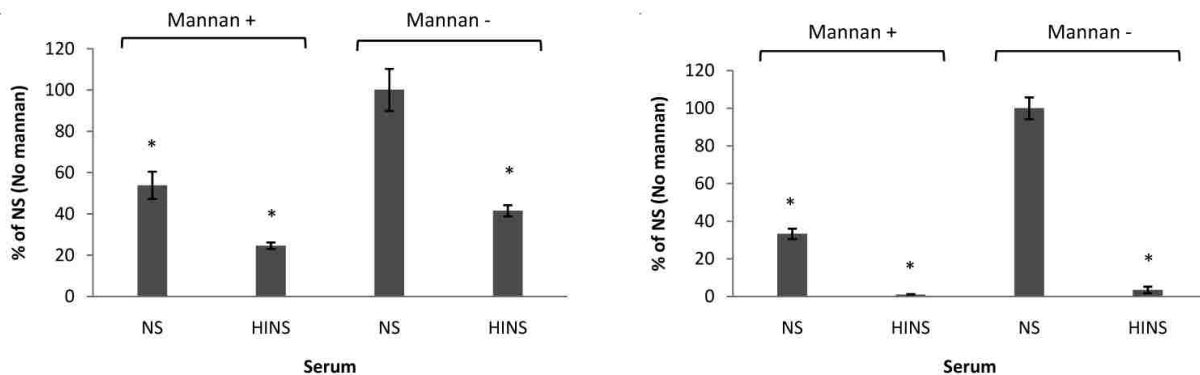
Figure 4.4 Phagocytosis of *F. asiatica* LADL 07-285A by tilapia head kidney derived macrophages (HKDM) is partially mediated by heat-stable serum components and mannose receptors. The 100% level for 4h-HKDM opsonized with NS without mannan pre-treatment is 2.65×10^6 CFU/ml. The 100% level for 5d-HKDM opsonized with NS without mannan pre-treatment is 4.33×10^6 CFU/ml. 4h (A) and 5d (B) Tilapia HKDM were assessed for their ability to phagocytose *F. asiatica* as described in Materials and Methods. HKDM were incubated with *F. asiatica* (MOI 1:50 for 2 h) pretreated with either tilapia autologous normal serum (NS), or heat-inactivated normal serum (HINS). HKDM were pre-incubated with either CTMM containing 5ug/ml of mannan (Mannan +) or no mannan (Mannan -) 30 m before addition of bacteria. The error bars represent standard error of triplicate samples and the results shown are representative of three independent experiments. Statistically significant differences between bacterial uptake in different treatments 4 and 5 d-HKDM opsonized with NS without mannan pre-treatment are marked (*, P<0.001)

To determine the contribution of the MR in *F. asiatica* recognition, 4h and 5d-HKDM were pre-incubated with soluble mannan, a competitive inhibitor of the MR (6). Mannan pre-treatment of HKDM populations significantly decreased internalization of *F. asiatica,* in both NS and HINS opsonized bacteria. Pre-incubation of 4h-HKDM with mannan decreased the uptake of *F. asiatica* by 46 and 75% in NS and HINS opsonized bacteria, when compared to uptake of bacteria opsonized with NS in non-mannan treated HKDM (Fig. 4.4a). On the other hand, pre-incubation of 5h-HKDM with mannan decreased the uptake of *F. asiatica* by 66 and 99% in NS and HINS opsonized bacteria, when compared to uptake of bacteria opsonized with NS in non-mannan treated HKDM (Fig. 4.4b). Although not significantly different, uptake of NS opsonized bacteria by 5d-HKDM was greater than that of 4h-HKDM (Data not shown).

***Francisella asiatica* Wild Type Survives, Replicates and Is Cytotoxic in Tilapia HKDM, but the Δ*iglC* Mutant Fails to Replicate.** To determine whether *F. asiatica* wild type and *ΔiglC* mutant were able to survive and replicate in tilapia 4h and 5d-HKDM, the numbers of viable bacteria internalized were monitored over a 36 hour period in six different experiments. The number of wild type bacteria recovered from 4h-HKDM after 12, 24 and 36 h increased significantly by 5, 45 and 61 fold, respectively, compared to time 0. As shown in figure 4.5, the Δ*iglC* mutant failed to grow (P<0.001), which is consistent with observations of *F. tularensis* Δ*iglC* mutants in mammalian macrophages. Although the macrophages internalized the mutant and wild type equally, the mutant was unable to replicate, but did persist for more than 36 h with only a slight decline. Similar results were found in 5d-HKDM (data not shown).

By electron microscopy, it was possible to observe heavily infected cells after 6 h post-inoculation for the wild type, but after 12 h a large numbers of macrophages detached from the plate. Two hours post-inoculation, *F. asiatica* were located inside a membrane-bound tight

phagocytic vacuole (Fig. 4.6A-C). After 12 h the majority of the bacteria were observed inside

spacious vacuoles, although some appeared to have escaped to the cytoplasm (Fig. 4.6D-F).

Figure 4.5 Growth of *Francisella asiatica* LADL 07-285A wild type (WT), *iglC* mutant (Δ*igl*C),
wild type complemented with IglC (WT:IglC) and *iglC* mutant complemented with IglC
(Δ*igl*C:IglC), in tilapia head kidney derived macrophages. The error bars represent standard error
of triplicate samples and the results shown are representative of six independent experiments.
Statistically significant differences between different treatments are marked (*, $P<0.001$).

Cytotoxicity of the wild type and Δ*iglC* mutant was examined by monitoring cell

morphology and LDH release of the tilapia HKDM. The course of the infection was associated

with a progressive cellular degeneration following wild type challenge. The amount of LDH

released by HKDM infected with the wild type was significantly greater than the amounts

released in HKDM challenged with the mutant. As expected citotoxicity was time-dependent

when infected with the wild type, as the amount of LDH released by infected HKDM was

significantly greater at 48 h post-inoculation than at 0 or 12 h post-inoculation (Figure 4.7).

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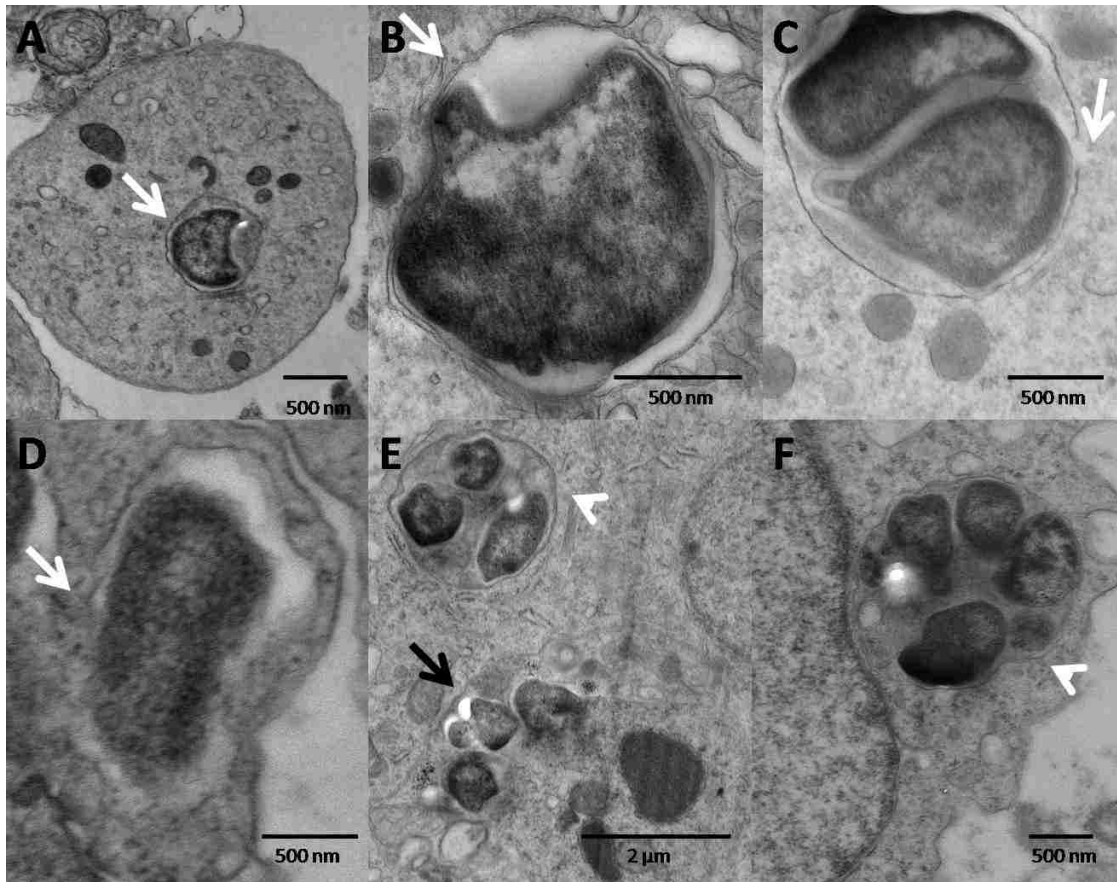
Figure 4.6 Transmission electron micrographs of tilapia head kidney derived macrophages infected with *Francisella asiatica* LADL 07-285A. (A and B). After uptake the bacteria is located inside a membrane-bounded tight phagocytic vacuole (white arrow) within the macrophage. (C and D) Breakdown of the phagosomal membrane (White arrows) appears to allow *F. asiatica* access to the cytoplasm 6-12 h post-infection. (E and F) After 12 h several bacteria are found inside a spacious vacuole (arrowhead) and some appear to have escaped to the cytoplasm (black arrow).

Complementation of the Δ*iglC* Mutant of *F. asiatica* Restores the Intramacrophage Growth Ability, Cytotoxicity, and Proapoptotic Features. Intracellular growth, cytotoxicity, and caspase 3/7 activity were all restored in the Δ*iglC* mutant strain upon complementation (Figure 4.5, 4.7, 4.8). The IglC complemented Δ*iglC* mutant, as well as an IglC complemented wild type, showed no statistical differences in HKDM intracellular growth, cytotoxicity or pro-apoptotic features than that of the wild type.
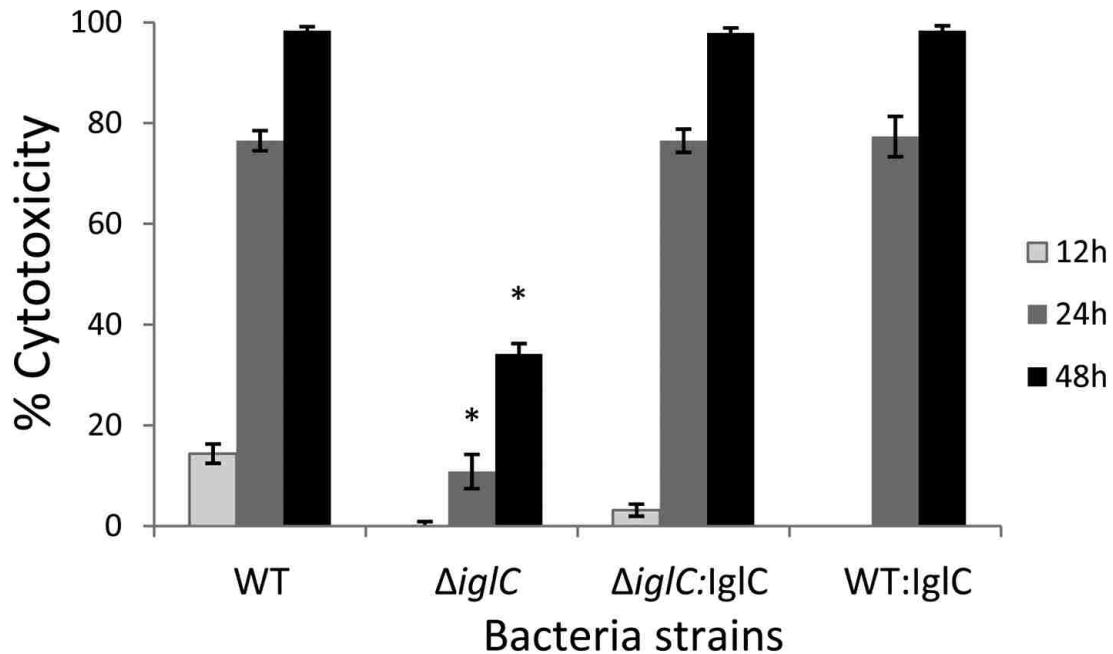
Figure 4.7 Cytotoxicity of *Francisella asiatica* LADL 07-285A wild type (WT), *iglC* mutant (Δ*igl*C), wild type complemented with IglC (WT:IglC) and *iglC* mutant complemented with IglC (Δ*igl*C:IglC), in tilapia head kidney derived macrophages 12, 24 and 48 h post inoculation. Cytotoxicity was assayed by release of LDH from infected cells as described in materials and methods. The error bars represent standard error of triplicate samples and the results shown are representative of three independent experiments. Statistically significant differences between different treatments are marked (*, $P<0.001$).

***F. asiatica* Infection Is Proapoptotic in Tilapia Head Kidney Derived Macrophages.** To

determine if infection-induced cytotoxicity is associated with apoptosis, we measured the activity

of caspases 3 and 7 in infected tilapia HKDM in both wild type and mutant *iglC* strains. Active

caspases participate in a cascade of cleavage events that disable key homeostatic and repair

enzymes and bring about systematic structural disassembly of dying cells. Thirty-six hours post-

infection, tilapia HKDM infected with the Δ*iglC* mutant had similar levels of caspase 3 and 7

activity and behaved similarly to the uninfected control cells, whereas the wild type showed a

significant increase in caspase 3 and 7 activity, which are hallmarks of apoptosis (Figure 4.8).

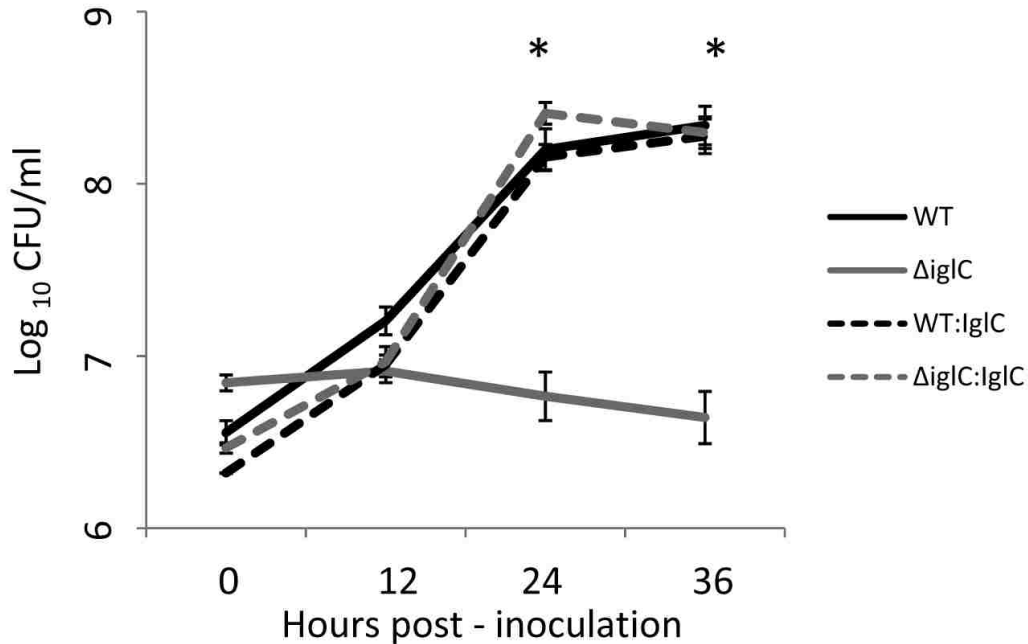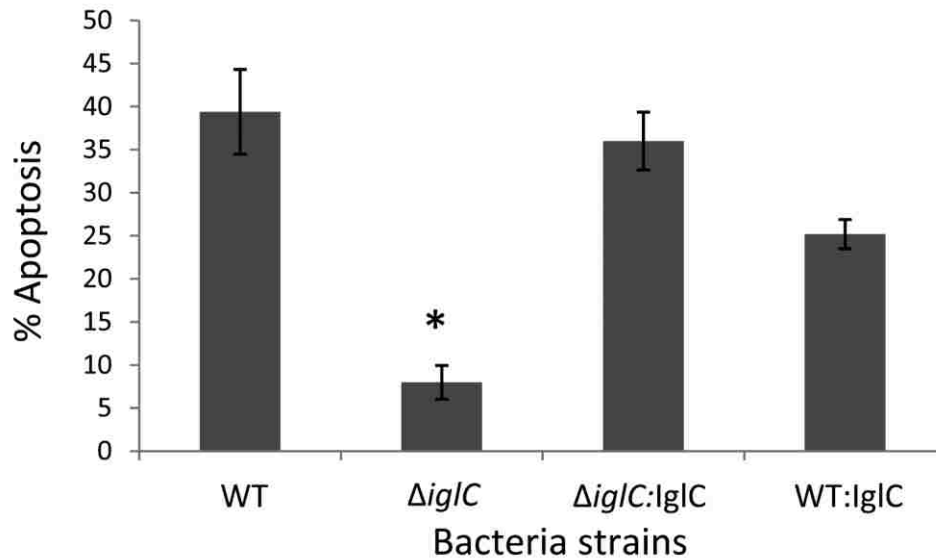**Figure 4.8** Tilapia head kidney derived macrophages display apoptosis 36 h post-infection with F*rancisella asiatica* LADL 07-285A wild type (WT), *iglC* mutant (Δ*iglC*), wild type complemented with IglC (WT:IglC) and *iglC* mutant complemented with IglC (Δ*iglC*:IglC). Percent apoptosis was determined by measuring caspase 3/7 activity from infected cells 36 h post inoculation as described in materials and methods. The error bars represent standard error of triplicate samples and the results shown are representative of three independent experiments. Statistically significant differences between different treatments are marked (*, $P<0.001$).

DISCUSSION

Francisella asiatica was recently described as a new member of the genus *Francisella*, and the clinical isolate used in this study LADL 07-285A recovered from moribund tilapia in Costa Rica (Soto et al. 2009a) was found to share more than 99% homology with the *F. asiatica* by sequence comparison of the 16S rDNA.

As previously described by several authors, a wide variety of mammalian and fish bacterial pathogens are resistant to normal serum killing, whereas non-virulent strains of Gram-negative bacteria and capsule and/or lipopolysacharide mutants are generally susceptible to the bactericidal activity of the serum (Acosta et al. 2006; Barnes et al. 2002; Barnes et al. 2003; Gomez and Balcazar 2008; Wiklund and Dalsgaard 2002). In this study, it was demonstrated that both the *F. asiatica* wild type and a Δ*iglC* mutant are resistant to the action of the

complement in tilapia serum. Recent work on the human pathogen *F. tularensis* demonstrated that the bacterium is resistant to serum killing, but requires complement factor C3-derived opsonins for uptake by phagocytic cells and subsequent intracellular growth (Ben Nasr et al. 2006). Their data suggests that important virulence factors for *F. tularensis* are its ability to bind the complement regulatory glycoprotein factor H and inactivation of C3b to iC3b, which culminates in opsonin-induced uptake for subsequent intracellular growth. The C3b inactivation also leads to inefficient membrane attack complex assembly, which contributes to the ability of this bacterium to resist complement lysis. While it is clear that *F. asiatica* isolates are able to survive killing by serum, it is still unknown if all *F. asiatica* and *F. noatunensis* isolates share the same mechanism of survival as *F. tularensis*.

The capability of *F. tularensis* to multiply intracellularly is well documented in insects, as well as in a broad range of mammals, including rabbits, rodents, beavers, and man (Clemens and Horwitz 2007). In the case of *F. asiatica,* the ability to survive inside a wide variety of fish cells was hypothesized, but this conclusion was based only on histopathological analysis of infected tissue in natural cases (Soto et al. 2009a).

Macrophages are generally a population of ubiquitous mononuclear phagocytes responsible for numerous homeostatic, immunological, and inflammatory processes (Clemens and Horwitz 2007; Ray et al. 2009). The ability to survive intracellularly is crucial for several bacterial fish pathogens after invasion of their eukaryotic target cells (Booth et al. 2006; Elkamel et al. 2003; Mccarthy et al. 2008). Distinct sub-populations of macrophages derived from goldfish (*Carassius auratus*) kidney leukocyte cultures were previously characterized. The sub-populations had distinct morphological, cytochemical and flow cytometric profiles, and also differed in their anti-microbial functions after activation with macrophage activation factors (MAF) and bacterial lipopolysaccharide (LPS) (Barreda and Belosevic 2001; Neumann et al.

2000; Stafford et al. 2001). Similar results were found when analyzing tilapia HKDM by flowcytometry and light microscopy (Data not shown). Five day old HKDM were bigger, and morphologically similar to mature tissue macrophages of mammals, while the 4 h-HKDM appeared as round cells with eccentrically placed nuclei that resembled more a mammalian monocyte (Barreda and Belosevic 2001; Neumann et al. 2000; Stafford et al. 2001).

In the present study, the *F. asiatica* wild-type was found capable of intracellular survival and replication within both 4h and 5d-HKDM from tilapia. Effective internalization by both cell types was partially mediated by a heat-sensitive serum component, presumably complement. Complement and/or complement receptors have been associated for efficient internalization of many mammalian and fish pathogens, including *F. tularensis*, *Mycobacterium* sp., *Listeria monocytogenes*, and *Edwardsiella ictaluri* (Balagopal et al. 2006; Ben Nasr and Klimpel 2008; Ben Nasr et al. 2006; Booth et al. 2006; Elkamel et al. 2003). With the *F. tularensis* live vaccine strain (LVS), optimal phagocytosis by dendritic cells (DC) is dependent on complement factor C3-derived opsonins and the major complement receptors expressed by DC, the integrins CR3 (CD11b/CD18) and CR4 (CD11c/ CD18) (Ben Nasr et al. 2006).

Uptake of *F. asiatica* by both 4h-HKDM and 5D-HKDM following opsonization with tilapia NS was significantly greater than uptake following pre-treatment with HINS (Fig 4.4), indicating involvement of complement and the complement receptor. In 4h-HKDM uptake remained at 40% of NS uptake when HINS was used to pre-treat the bacteria (Fig. 4.4), indicating that uptake was only partially mediated by complement, similar to the situation for *F. tularensis* (Balagopal et al. 2006; Ben Nasr and Klimpel 2008; Ben Nasr et al. 2006). In 5D-HKDM with HINS pre-treatment, however, uptake was significantly lower than for 4h-HKDM with HINS, at only 5% of NS uptake, indicating that either 5D-HKDM increased expression of the CR or there is increased affinity for complement components compared to 4h-HKDM.

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Further work with *F. tularensis* (Balagopal et al. 2006; Schulert and Allen 2006) demonstrated that monocyte-derived macrophages (MDM) phagocytose more *Francisella* than monocytes, with a major contribution from the mannose receptor on MDM. When using NS to opsonize *F. asiatica*, pretreatment of 4h-HKDM with mannan reduced uptake to 55% of NS, indicating a substantial involvement of the MR, with an even larger decline with mannan treated 5D-HKDM, at 30% of NS. This is similar to *F. tularensis*, where mannan pre-treatment had a greater effect on uptake by MDM than on monocytes, although the effect of mannan pretreatment of tilapia 4h HKDM on *F. asiatica* uptake was greater than seen for *F. tularensis*. Uptake of *F. asiatica* by tilapia 4h-HKDM was reduced to 20% of NS when mannan pre-treatment was applied and bacteria were pre-treated with HINS, indicating that CR and MR are not the only receptors involved in uptake. Other receptors demonstrated to be involved in the uptake of *F. tularensis* by mammalian macrophages, include Fc γ receptors, pulmonary collectins surfactant proteins, and type I and II class A scavangers (Balagopal et al. 2006; Pierini et al. 2006; Schulert and Allen 2006). The difference between tilapia HKDM and the human blood-borne monocytes could be a result of the differential maturation of HKDM, but a clear involvement of both the CR and the MR was observed in both 4h- and 5D-HKDM. The combination of mannan pretreatment and opsonization with HINS reduced uptake of *F. asiatica* in 5D-HKDM by 99.7 % of uptake following NS treatment, indicating that the primary receptors involved are the CR and MR. This is in contrast to *F. tularensis*, where additional receptors were suspected for MDM (Schulert and Allen 2006) (Fig. 4.4).

Our results are consistent with the involvement of the MR in phagcytosis of *F. tularensis*, particularly in 5d-HKDM (Fig. 4.4). The ligands that engage the MR of *F. tularensis* are unknown, but the LPS is a proposed candidate, but the only mannose residues present in the *F. tularensis* LPS are in the core region, which is presumably covered-up by the O-antigen repeats.

The mannose-containing capsule of *F. tularensis* is also a candidate (Gunn and Ernst 2007; Hood 1977). The LPS of *F. asiatica* also contains mannose in the core, also covered up by the O-antigen repeats (Kay et al. 2006), but a capsule has not yet been described for *F. asiatica*. Analysis of the *F. asiatica* genome revealed high sequence homology to the *F. tularensis capB* and *capC* sequences indicating that a capsule might be present as well in this bacterium (Soto et al. unpublished data).

Differences between the uptake of *F. asiatica* by 4h and 5d-HKDM indicates that the presence or absence of receptors, like the MR, plays a role in the uptake of bacteria by fish phagocytes. In mammals, uptake of *F. tularensis* by MDM was greater than by monocytes, presumably because the MR are more abundantly or newly present on mature macrophages compared to monocytes. More research is needed to elucidate the role of different bacterial receptors in fish mononuclear cells. After uptake, regardless of whether the bacteria were pre-opsonized with normal serum, heat inactivated serum, intracellular replication was equal in either HKDM population (Data not shown), similar to the situation in *F. tularensis* (Ben Nasr et al. 2006).

Previously, we identified the *iglABCD* operon in the fish isolate *F. asiatica* LADL 07-285A, and demonstrated that *iglC* is required for virulence in the fish host (Soto et al. 2009b). In this study we show that *iglC* is required for intracellular survival and growth in tilapia HKDM. Similar results have been obtained in *F. tularensis,* where Δ*iglC* mutant strains are defective for survival and replication within mammalian macrophages. Expression of *iglC* was induced during growth of *F. tularensis* in macrophages, and was required for intracellular multiplication in macrophages and for virulence in mice (Golovliov et al. 1997; Keim et al. 2007; Lindgren et al. 2004; Santic et al. 2005). Inactivation of the *iglC* and *mglA* gene of *F. tularensis* also abolishes its capacity to escape from the phagosome into the cytoplasm and to multiply

intracellularly in mouse peritoneal exudate macrophages (Lindgren et al. 2004; Santic et al. 2005).

After 24 h of infection with *F. tularensis*, the murine macrophage-like cell line J774.A1 underwent apoptosis and pronounced cytopathogenesis. Further work by the same group demonstrated that a *F. tularensis* Δ*iglC* mutant did not induce apoptosis in infected cells, suggesting an involvement of IglC in the induction of apoptosis in *F. tularensis* infected macrophages (Lai et al. 2004). Similar results were found for *F. asiatica* in this study, with significantly greater LDH levels in supernatants of tilapia HKDM infected with the wild type and the IglC complemented *Francisella* strains than in the Δ*iglC* mutant. The Δ*iglC* mutant strain also induced significantly lower caspase 3/7 activity, similar levels as that of the uninfected cells.

Apart from the advantages that microbes gain from controlling host cell apoptosis, it has been suggested that apoptosis functions as a host defense mechanism by depriving microorganisms adapted to the intracellular environment of their preferred habitat (Lai et al. 2001; Lai et al. 2004). As previously described in *F. tularensis, F. asiatica* mediated apoptosis occurred at a later stage of *in vitro* infection in macrophages than that described for *Salmonella*, *Yersinia*, *Shigella*, or *Legionella* (Lai et al. 2001; Lai et al. 2004). As previously suggested, the delayed apoptosis induce by *Francisella* sp. would allow the bacteria to replicate within the target cells, and that subsequent induction of apoptosis allows them to escape when nutrients become limiting (Lai et al. 2004).

The initial uptake of *F. tularensis* occurs by looping phagocytosis, in which the bacterium is engulfed in a spacious, asymmetric, pseudopod loop (Clemens et al. 2005; Clemens and Horwitz 2007). A similar process was not observed for *F. asiatica*, but only limited cells were observed. After uptake, *F. asiatica* appear to reside within a tight membrane bound phagocytic vacuole (Fig. 4.6 A and B). As previously described, *F. tularensis* resides within

membranes containing discrete, easily identifiable lipid bilayers measuring between 25-34 nm immediately after infection (Clemens et al. 2005; Clemens and Horwitz 2007). Although not fully characterized, a clear, tight membrane bound phagocytic vacuole surrounds internalized *F. asiatica* (Fig. 4.6 A and B). Similar to *F. tularensis*, the phagosomal membrane of some vacuoles containing *F. asiatica* is disrupted, allowing *F. asiatica* to escape to the cytoplasm, but some bacteria replicate in spacious vacuoles (Fig. 4.6 D-F). Eight to twelve hours post-infection most *F. tularensis* containing vacuoles are fragmented and the majority of the bacteria are free in the cytoplasm (Anthony et al. 1991; Clemens et al. 2005; Clemens and Horwitz 2007). Although some of the *F. asiatica* were observed free in the cytoplasm 12 h post-inoculation, the majority of bacteria were found in spacious vacuoles (Fig. 4.6 D-F). Further work is needed to completely elucidate the location of *F. asiatica* in HKDM at later time points of infection.

In conclusion, the results indicate that *F. asiatica* is able to resist complement mediated-lysis, and to survive and efficiently replicate in 4h and 5d-HKDM, whereas a Δ*iglC* mutant was deficient for intra-macrophage growth. The mutant remained resistant to complement, but failed to release significant amounts of LDH or to induce significant activity of caspases 3 and 7.

The pathology and immune response to acute *Francisella* infection in zebrafish was recently described, and it was demonstrated that there are many features in common with infections in mammals (Vojtech et al 2009), suggesting the zebrafish system as a model for studying *Francisella* sp. infection. Infection in the zebrafish, however, required intraperitoneal injection of 10^6 CFU to cause 100% mortality in 5 days, while 3.45×10^5 CFU's only resulted in ~ 2% (Vojtech et al 2009). In contrast as few as 23 bacterium injected in the peritoneum are capable of causing mortalities in tilapia, and that even fewer are enough to cause serious pathological lesions in important organs like the head kidney and spleen (Soto et al. 2009b). Macrophage studies are difficult in zebrafish because of their small size, so comparative analysis

of intracellular pathogenesis cannot be done. Given the highly virulent infection in tilapia,

similar to *F. tularensis* in mammals, the similarity of intracellular replication, and the high

degree of homology between *F. tularensis* and *F. asiatica* virulence gene sequences, including

the *iglABCD* operon and type VI secretion genes (*dotU*, *vgrG*, *iglAB*), we suggest that *F. asiatica*

infection in tilapia could be used as a model for tularemia in mammals.

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CHAPTER 5

ATTENUATED *FRANCISELLA ASIATICA IGLC* MUTANT INDUCES PROTECTIVE IMMUNITY TO FRANCISELLOSIS IN TILAPIA

INTRODUCTION

Francisella asiatica is a Gram-negative facultative intracellular bacterium, and the causative agent of francisellosis in fish. The bacterium is an emergent pathogen of tilapia (*Oreochromis niloticus*), hybrid striped bass (*Morone chrysops* x *M. saxatilis*), atlantic salmon (*Salmo salar* L.), three line grunt (*Parapristipoma trilineatum*), and many other important cultured fish species (Kamaishi et al. 2005; Hsieh et al. 2006; Ostland et al 2006; Kay et al 2006; Nylund et al. 2006; Olsen et al. 2006; Mikalsen et al. 2007; Ottem et al. 2007; Mauel et al. 2007; Hsieh et al. 2007; Ottem et al. 2007; Birkbeck et al 2007; Ottem et al. 2008a; Ottem et al. 2008b, Soto et al. 2009a; Ottem et al. 2009). Infected fish present with non-specific clinical signs such as erratic swimming, anorexia, anemia, exophthalmia and high mortality. Upon gross and microscopic examination, several internal organs (mainly spleen and kidney) are enlarged and contain widespread multifocal white nodules. Histological examination reveals the presence of multifocal granulomatous lesions, with the presence of numerous small, pleomorphic, cocco-bacilli (Soto et al. 2009a; Soto et al. 2010).

In previous work, it was found that as few as 23 *F. asiatica* colony-forming units (CFU) injected in the peritoneum were capable of causing mortalities in tilapia, and that even fewer were enough to cause serious pathological lesions in important organs like the head kidney and spleen. Nevertheless, the pathogenic mechanisms that underlie its remarkable infectivity and its capacity to cause disease in a broad range of fish hosts are not known (Soto et al. 2010; Soto et al. 2009b). Some of the most interesting virulence genes identified in *F. tularensis* are the genes of the intracellular growth locus (*iglA, iglB, iglC*, and *iglD*) present as part of a 30 Kb

pathogenicity island previously described (Nano et al. 2004). The functions of the conserved proteins corresponding to the genes of the Francisella Pathogenicity Island (FPI) are elusive. Overall, Igl proteins appear to be essential for the ability of *F. tularensis* to survive inside the macrophages and cause disease (Nano et al. 2004; Nano and Schmerck 2007; Golovliov et al. 1997). The intracellular growth locus C protein (IglC) was one of the first of the FPI-encoded proteins found to be highly induced following *F. tularensis* infection of macrophages (Golovliov et al. 1997). As with other genes mutants in the FPI, *iglC* mutants fail to grow in macrophages, are deficient in ability to escape from phagosomes, and fail to down-regulate the proinflammatory response in macrophages (Nano and Schmerck 2007; Golovliov et al. 2003; Lai et al. 2004; Ludu et al. 2008).

In previous work, we identified homologues to the *F. tularensis iglA, iglB, iglC*, and *iglD* genes in *F. asiatica* strain LADL 07-285A isolated from diseased tilapia (Soto et al. 2009b). An insertion mutation in the *iglC* gene of *F. asiatica* LADL 07-285A was constructed by allelic exchange, and the Δ*iglC* mutant was found to be attenuated following intraperitoneal and immersion challenges in tilapia (Soto et al. 2009b). Recently, *F. asiatica* wild type (WT) and Δ*iglC* mutant were found to be resistant to killing by normal and heat-inactivated serum. It was also found that the WT bacterium was able to invade the tilapia head kidney derived macrophages (HKDM) and replicate vigorously within them; causing significant apoptosis and cytopathogenicity at 24 and 36 h post infection (Soto et al. 2010). The Δ*iglC* mutant was found to be defective for survival and replication within HKDM, and its cytotoxicity was significantly reduced *in vitro* (Soto et al. 2010).

Here we investigated the ability of an attenuated *F. asiatica* Δ*iglC* mutant, given by immersion as a vaccine, to stimulate adaptive immune protection against fish francisellosis in tilapia fingerlings. Our results show that the attenuated strain may be used as an effective

immersion vaccine against *F. asiatica* and that the vaccine-mediated protection is partially dependent on antibodies.

MATERIALS AND METHODS

Bacteria. *Francisella asiatica* LADL 07-285A WT was isolated from cultured tilapia (*Oreochromis* sp.) and was described in previous work (Soto et al. 2009a). The Δ*iglC* mutant isolate was made by homologous recombination using a PCR product, and its attenuation was demonstrated *in vivo* and *in vitro* (Soto et al. 2009b; Soto et al. 2010). *F. asiatica* isolates were grown in cystine heart agar supplemented with bovine hemoglobin solution (CHAH) (Becton Dickenson (BD) BBL, Sparks, MD, USA) for 48-72 h at 28°C. The liquid medium consisted of Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL, Sparks, MD, USA) and 0.1% glucose (MMH) (Baker et al. 1985). Broth cultures were grown overnight at 25 °C in a shaker at 175 rpm, and bacteria were frozen at -80 °C in the broth media containing 20% glycerol for later use. *Escherichia coli* DH5α was grown using Luria-Bertani broth or agar for 16 - 24 h at 37°C.

Preparation of Sonicated *F. asiatica* Lysate for ELISA. Approximately 1 x 10^{12} CFU of *F. asiatica* were harvested from 500 ml of broth culture by centrifugation at 1,500 x *g* for 10 min at 4°C in a GSA rotor in an accuspin 3R refrigerated Centrifuge (Fisher Scientific). The pellet was washed three times with Dulbecco's phosphate-buffered saline (PBS; Gibco/Invitrogen, Carlsbad, CA), followed by centrifugation at 1,500 x *g* for 10 min. Following the final wash, the pellet was resuspended in 4 ml of 20 mM Tris-Cl (pH 8.0) with a protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). The bacteria were sonicated on ice for a 30-s pulse, followed by a 30-s rest, ten times using a Sonic Dismembrator Model 500 (Fisher Scientific) at a power of 70%. The samples were then centrifuged for 1 h at 16,000 x *g* at 4°C in an Eppendorf

centrifuge 5415 R (Fisher Scientific). Protein concentration of the sonicate was determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Fish. Adult and fingerling tilapia nilotica (*Oreochromis niloticus*) used during the trial were obtained from a source with no history of *Francisella* infection. For verification, a sub-sample of the population was tested for bacteria by complete clinical, bacteriological, serological and molecular analysis as previously described, to ensure that they were free of *F. asiatica* (Soto et al. 2009a). Fingerlings were maintained at 15 fish per tank in 40 L tanks containing 30 L of water flowing through at 25°C, and fed commercial tilapia feed daily (Burris Aquaculture Feeds, Franklinton, LA) at 3% fish body weight per day. The mean weight of the fish was 6.4 g. Adults weighing an average of 346 g were acclimated for a minimum of 2 months in a flow through water system at 25°C. Five adult fish were maintained in a 100 L tanks containing 80 L of water/tank with constant oxygenation.

Immunization and Challenge. Vaccination trials were conducted using tilapia fingerlings. Four different Δ*iglC* mutant vaccination treatments and a mock immunized control treatment were evaluated. Each treatment consisted of eight tanks (15 fish in each tank). The first group of fish was vaccinated by addition of 10^7 CFU/ml of the Δ*iglC* mutant to 10 L of static water and incubation for 180 min. The second group received a dose of 10^7 CFU/ml of the Δ*iglC* mutant but for 30 min. The third group received a dose of 10^3 CFU/ml of the Δ*iglC* mutant for 180 min; and the fourth group received a dose of 10^3 CFU/ml of the Δ*iglC* mutant for 30 min. The control tanks received 100 ml of 1x PBS into 10 L of static water for a period of 180 min. After either 30 or 180 min, the flowing water in each tank was restored to a final volume of 30 L of water/tank. Four weeks following a single immersion immunization with the Δ*iglC* mutant or PBS (control tanks), tilapia fingerlings were challenged by immersion following previously established protocols (Soto et al. 2009b). Briefly, water volumes in each tank were adjusted to 10 L of

water/tank, and 100 ml of PBS containing *F. asiatica* suspension was added to each tank for a final concentration of 10^8 CFU/ml of WT *F. asiatica*. The fish colonization and infection was allowed to progress for 180 minutes in static water with oxygenation, after which flowing water was re-assumed to a final volume of 30 L/tank. Three tanks per treatment were utilized for monitoring mortality every 12 h for 30 days. The remaining tanks were utilized for mucus and serum collection and analysis. The protective index was calculated according to the formula (Aned 1981): RPS=100% (1-(% mortality in vaccinated fish/% mortality in control fish).

Mucus and Serum Collection. Mucus and serum collection was performed following previously published protocols (Grabowski et al. 2004). Mucus was sampled from 5 tilapia fingerlings from each immunized or mocked-immunized group at 0, 2, 4, 6, 8 and 10 weeks post-vaccination. Fish were euthanized with an overdose of MS-222 and mucus collected by swabbing both sides of the fish 10 times from head to tail with a cotton applicator. Swabs were placed in 1.5 ml microcentrifuge tubes containing 0.9 mL of PBS supplemented with 0.02% (w/v) sodium azide. Tubes were stored overnight at 4°C. The next morning, tubes and swabs were vigorously vortexed for 2 min and liquid removed from the swabs by pressing against the side of the tube. The resulting liquid was centrifuged at 3000 x g for 10 min and the supernatant collected and frozen at -20°C in polypropylene tubes until analyzed by ELISA.

Blood samples were obtained from the same fish at each time point, by caudal venepuncture and collection with plain Fisherbrand micro-hematocrit capillary tube (Fisher Scientific, Pittsburg, PA). Blood was held at 25°C for 1 h and then separated with centrifugation at 400 x g for 5 min and then stored at -20 °C for later analysis by ELISA.

ELISA. An ELISA was developed to quantify anti-*F. asiatica* antibody produced by tilapia in serum and mucus. Immulon II 96-well flat-bottom microtitre plates (Thermo Labsystems, Franklin, MA, USA) were coated overnight at 4 °C with a 7 ug protein mL^{-1} solution of

sonicated *F. asiatica* whole cell antigen in 0.05M carbonate coating buffer, pH 9.6, at 100 µL per

well. Plates were then washed three times in PBS containing 0.05% Tween-20 (PBST). The

wells were blocked for 1 h at room temperature (RT) with filter-sterilized PBS containing 0.05%

Tween 20 (Sigma) and 1% bovine serum albumin (BSA; Fraction V, Sigma) (PBST-BSA).

Tilapia serum and mucus samples were diluted 1:1000 or 1:50 (respectively) in PBST-BSA, and

100 µL of the resulting solution was added to three replicate wells of the microtitre plate. The

plate was incubated at 25 °C for 2 h and washed 3x with PBST. Mouse anti-tilapia IgM heavy

chain specific monoclonal antibody kindly provided by Richard Shelby (USDA/ARS, Auburn,

AL) was diluted 1:100 in PBST and 100 µL of this solution added to each well (Shelby et al.

2002). The plate was incubated at 25 °C for 1 h and washed 3x with PBST. Peroxidase-

conjugated goat anti-mouse IgG (Pierce Biotechnology, Rockford, Illinois, USA) was diluted

1:10,000 in PBST and added to each well. After incubation at 25 °C for 1 h, the plate was

washed again 3x in PBST and 100 µL of ABTS Peroxidase Substrate System (KPL,

Gaithersburg, MD) was added to each well. The ELISA reaction was stopped after 30 min with

100 µL 1% sodium dodecyl sulfate (SDS), and the optical density (OD) of the reactions was read

at 405 nm with a SpectraMax M2 / M2e Microplate Readers (Molecular Devices, Sunnyvale,

CA). The relative amount of specific antibody was measured as the OD value.

Adoptive Transfer Studies. Twenty adult fish serum donors were immunized by

intraperitoneal (IP) vaccination with the Δ*iglC* mutant. Prior to challenge the fish were

anesthetized with MS-222 (100 mg/l). Intra-peritoneal vaccinated fish received a 0.1 ml

injection of bacterial suspension (10^7 CFU/fish). Serum collected from the 20 immunized fish at

4, 5, and 6 weeks post-immunizations were pooled together and was analyzed by ELISA.

Endpoint titers were reported as the reciprocal of the last dilution yielding an OD more than

twice that of the serum from naïve control fish. Normal fish serum was obtained from 20 adult

naïve fish injected with 1x PBS and processed the same way as the immunized fish. Heat-inactivated immunized serum (HIIS) and heat-inactivated normal serum (HINS), were obtained by incubating serum obtained from immunized and mocked immunized fish at 56°C for 30 min. Two hundred and forty naïve tilapia fingerlings (20/tank) were injected IP with either 200 µl of pooled HIIS, HINS or PBS 24 h before IP challenge with *F. asiatica* WT. Three tanks per treatment were challenged with either10^3, 10^4, 10^5, or 10^6 CFU *F. asiatica*/fish by IP injection. During the subsequent 21-d challenge period, fish were monitored daily for clinical signs of disease and mortality. Moribund and dead fish were removed twice daily, and bacterial samples were aseptically obtained from the spleen of morbid and dead fish to confirm the presence of *F. asiatica*. The LD_{50} for each treatment was calculated by the method of Reed-Muench (Reed and Muench 1938), at day 21 post-injection.

Direct Complement Lysis. *F. asiatica* WT and *E. coli* DH5α, were cultured as previously described. Bacteria were adjusted to a concentration of $1x10^7$ CFU/ml in PBS. A 1:1 ratio of the bacterial isolates to either PBS, normal (NS), immunized (IS) tilapia sp. serum was combined and the samples were incubated for 2 h at room temperature. Following incubation sub-samples of the bacteria/serum mixtures were collected at 0, 1, and 2 h, serially diluted in PBS and spotted onto either CHAH (*F. asiatica*) or LB (*E. coli*) plates.

Opsonophagocytosis Assays. To examine the opsonic potential of the immune sera, an opsonophagocytosis assay was established. Tilapia head-kidney derived macrophages (HKDM) were collected and purified following previously established protocols (Neumann et al. 1998; Secombes 1992). Modifications to previously established protocols were used to infect tilapia HKDM (Lauriano et al. 2003; Lauriano et al. 2004). Briefly, 5 day cultures of tilapia HKDM in 96 well plates containing 1-5 x 10^5 cells/well were used. *F. asiatica* was grown for a period of 8 h in MMH at 25°C. Optical density (OD_{600}) of the culture was determined and the bacteria were

adjusted to a final concentration of 5 x 10^8 CFU/ml. One ml aliquots of the bacterial suspension was pelleted at 10,000 x g for 5 minutes in an Eppendorf 5415 D centrifuge (Eppendorf-Brinkman, Westbury, New York), and the pellet was resuspended in either 1ml of HINS or HIIS. Ten-fold serial dilutions were plated on CHAH after incubation to determine total bacterial cell viability. After 1h incubation, the 96 well plate was inoculated with 10 μl of opsonized bacteria per well to achieve a multiplicity of infection (MOI) of 50 bacteria: 1 macrophage. The plates were centrifuged for 5 min at 400 x g to synchronize bacterial contact with macrophages. Following 2 h incubation at 25°C with 5% CO_2, the cells were washed three times with warm media (25°C), further incubated with fresh media and lysed for 15 min at time 0, 24, and 48 h by the addition of 100 μl of 1% Saponin in PBS. The lysates were serially diluted and spread onto CHAH plates to determine viable counts. Experiments were performed in triplicate on a minimum of three separate occasions with similar results.

Statistical Analysis. The Statistical Analysis System (SAS Institute, Inc. 2003) was used with the general linear models procedure (PROC GLM) to conduct analysis of variance (ANOVA) of a factorial arrangement of treatments. When the overall test indicated significance, pairwise comparisons of main effects were calculated with Tukey's test. Interaction effects were examined with pairwise t-test comparison of least - square means. For the mortality studies the percent mortalities were transformed with an arcsine transformation to normalize the data. To ensure overall protection level of Type I error, only probabilities associated with pre-planned comparisons were used. All comparisons were considered significant at ($P < 0.05$).

RESULTS

Immersion Vaccination with Δ*iglC* Protected Tilapia Fingerlings Against Homologous *F. asiatica* Immersion Challenge. To evaluate the efficacy of Δ*iglC* in protecting tilapia fingerlings against virulent *F. asiatica* immersion challenge, tilapia fingerlings were vaccinated

by immersion by four different treatments. Vaccination with a dose of 10^7 CFU/ml of water for a period of 30 or 180 min conferred 68.75 % and 87.5 % relative percent survival (RPS) respectively, against otherwise lethal (80% mortality) immersion challenge with the WT isolate during a period of 30 days. Vaccination with a dose of 10^3 CFU/ml of water for a period of 30 min or 180 min conferred 56.25% and 62.5 % RPS respectively, against immersion challenge with the WT isolate. Mock (PBS)-vaccinated fish succumbed to the infection by day 7, presenting clinical signs of the disease, including ascites and widespread granulomas in spleen and kidney. Fish vaccinated with either treatments of $\Delta iglC$ had significantly higher survival rates than those mocked vaccinated with PBS after challenge with WT *F. asiatica* ($p<0.05$) (Fig. 5.1).
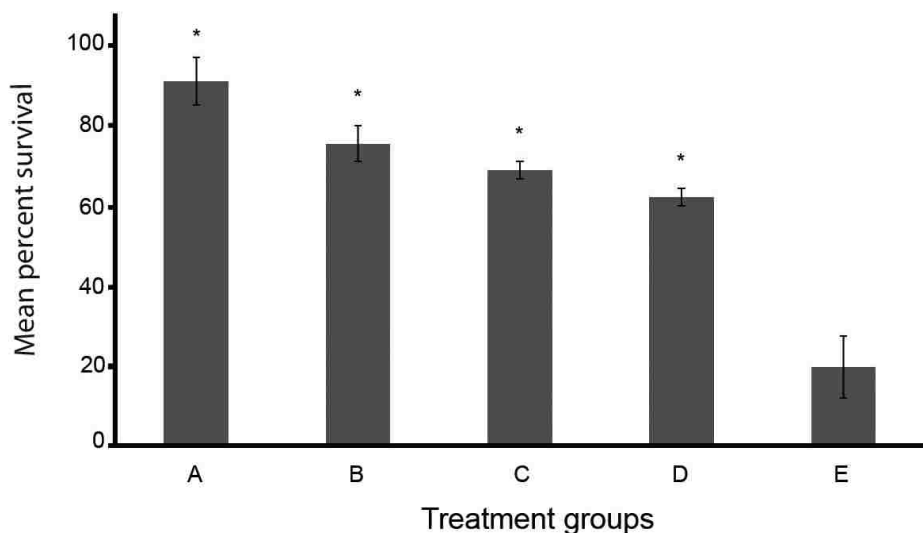
Figure 5.1 Mean percent survival of tilapia vaccinated with different treatments of a *F. asiatica* $\Delta iglC$ by immersion, or mock vaccinated with PBS (Controls) and challenged 4 weeks later with WT *F. asiatica*. Fish were vaccinated with: A. 10^7 CFU/ml of the $\Delta iglC$ mutant for 180 min. B. 10^7 CFU/ml of the $\Delta iglC$ mutant for 30 min. C. 10^3 CFU/ml of the $\Delta iglC$ mutant for 180 min. D. 10^3 CFU/ml of the $\Delta iglC$ mutant for 30 min. E. PBS for 180 min. Four weeks post-immunization fish were challenged with 10^8 CFU/ml of WT *F. asiatica* for 180 min. Mean percent survival was calculated 30 days post-challenge with WT. Each bar represents the mean percent survival ± standard error of three tanks (15 fish/tank). * Denotes significant differences, $P<0.05$ with respect to the control group by a Student's *t*-test.

Juvenile tilapia vaccinated with either treatment of Δ*iglC* generated a weak serum and mucosal antibody response that wasn't significantly different than that of controls at 2, 4 and 6 weeks post-vaccination (Fig. 5.2, Fig. 5.3). However, after the WT immersion challenge, the serum and mucosal samples from *iglC* vaccinated fish with a dose of 10^7 CFU/ml of water for a period of 30 and 180 min, resulted in a significantly greater secondary antibody response at week 8 and 10 post-initial vaccination ($p<0.05$) (Fig. 5.2, Fig. 5.3). The non-immunized fish showed an increased primary antibody response after WT challenge when compared to antibodies levels at week 0 (Fig. 5.2, Fig. 5.3).
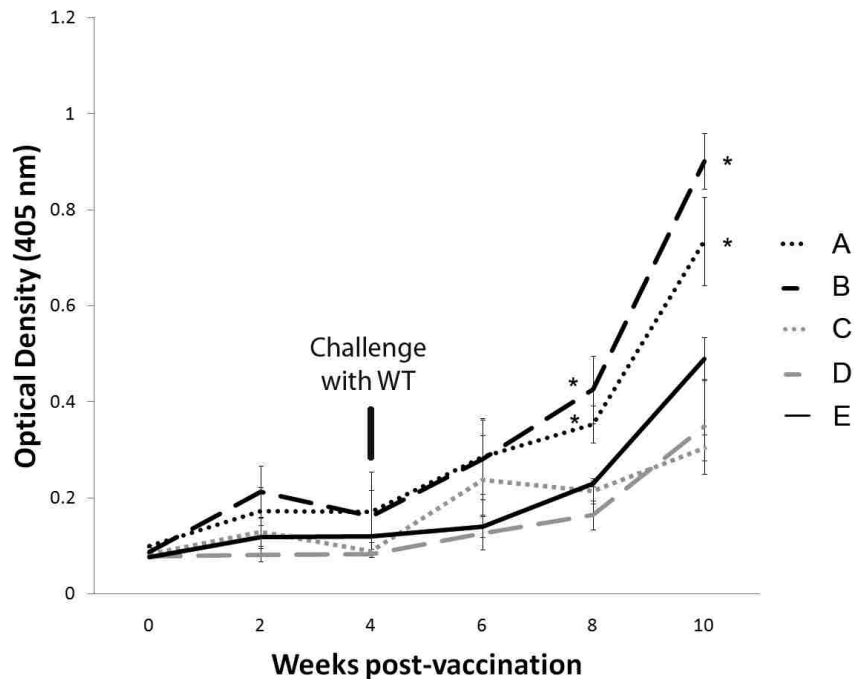
Figure 5.2 Serum anti- *F. asiatica* antibody response in actively immunized tilapia fingerlings. Fish were vaccinated with: A. 10^7 CFU/ml of the Δ*iglC* mutant for 180 min. B. 10^7 CFU/ml of the Δ*iglC* mutant for 30 min. C. 10^3 CFU/ml of the Δ*iglC* mutant for 180 min. D. 10^3 CFU/ml of the Δ*iglC* mutant for 30 min. E. PBS for 180 min. Four weeks post-immunization fish were challenged with 10^8 CFU/ml of WT *F. asiatica* for 180 min. Antibodies were measured during 10 weeks post-vaccination (every 2 weeks) as described in materials and methods. Serum was diluted 1:1000. Mean OD values were calculated for each treatment every two weeks. Each point represents the mean OD value ± standard error of 5 fish samples (serum). * Denotes significant differences, $P<0.05$ with respect to the control group by a Student's *t*-test.
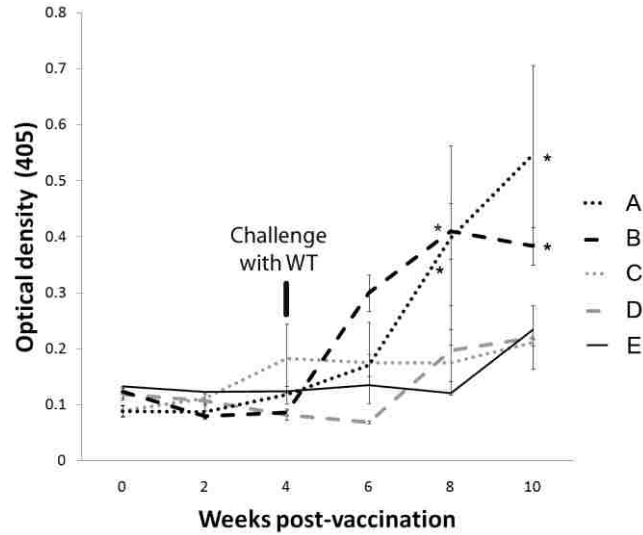
Figure 5.3 Mucus anti- *F. asiatica* antibody response in actively immunized tilapia fingerlings. Fish were vaccinated with: A. 10^7 CFU/ml of the $\Delta iglC$ mutant for 180 min. B. 10^7 CFU/ml of the $\Delta iglC$ mutant for 30 min. C. 10^3 CFU/ml of the $\Delta iglC$ mutant for 180 min. D. 10^3 CFU/ml of the $\Delta iglC$ mutant for 30 min. E. PBS for 180 min. Four weeks post-immunization fish were challenged with 10^8 CFU/ml of WT *F. asiatica* for 180 min. Antibodies were measured during 10 weeks post-vaccination (every 2 weeks) as described in materials and methods. Mucus was diluted 1:50. Mean OD values were calculated for each treatment every two weeks. Each point represents the mean OD value ± standard error of 5 fish samples (mucus). * Denotes significant differences, P<0.05 with respect to the control group by a Student's *t*-test.

Antibodies Partially Contribute to the Protection Conferred by Vaccination with the *F. asiatica* $\Delta iglC$ Mutant. Intraperitoneal injection with the *F. asiatica* $\Delta iglC$ mutant induced a strong humoral response in adult tilapia and enhanced the production of antibodies. The pooled immunized sera presented antibody titers >52,000. To test the functional ability of such antibodies, we performed opsonophagocitic and killing assays; as well as passive immunization trials.

F. asiatica susceptibility to direct effects of IS was compared with that of *E.coli*, after mixing the bacteria strains with PBS, NS or IS for a period of 2 h. Both IS and NS completely inhibited growth of the *E.coli* isolate. In contrast, neither IS or NS had an effect on the growth of *F. asiatica in vitro* (data not shown).

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To test the functional ability of antibodies against *F. asiatica* in the HIIS to mediate phagocytic uptake of *F. asiatica* WT, we utilized a complement-independent opsonophagocytic assay using HKDM (Fig. 5.4). Heat-inactivated sera prepared from tilapia immunized with the *iglC* mutant efficiently mediated phagocytosis of the WT *F. asiatica*, whereas HINS opsonophagocytosis ability was significantly lower ($p<0.05$). Bacteria taken up by the HKDM efficiently grew regardless of being opsonized or not with antibodies (Fig. 5.4).
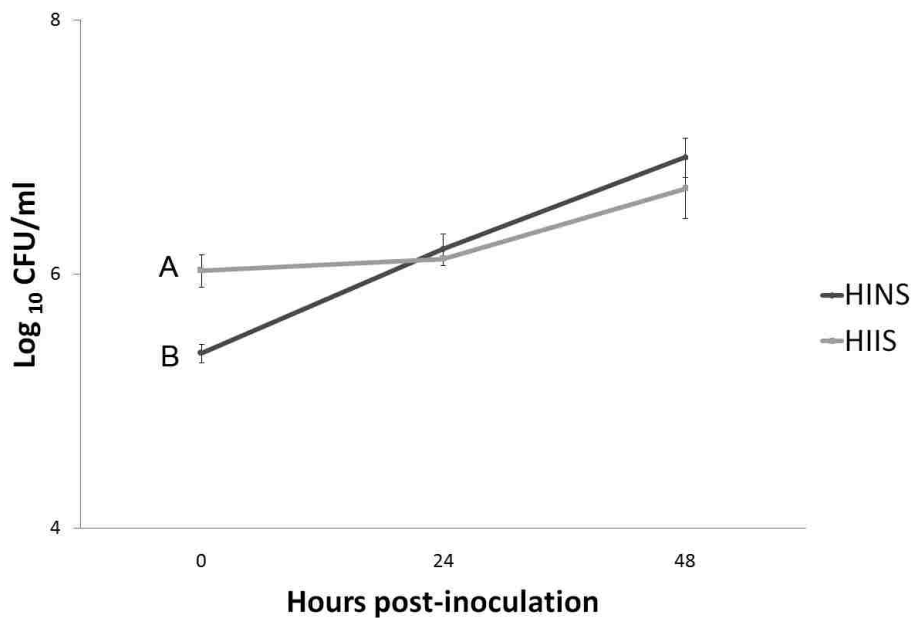
Figure 5.4 Enhanced antibody- dependent phagocytosis of *F*. asiatica by tilapia head kidney derived macrophages (HKDM). *F. asiatica* was opsonized with heat-inactivated immunized (HIIS) or heat-inactivated normal (HINS) sera obtained from adult tilapia. Phagocytosis assays were performed with tilapia HKDM (MOI 1:50) as described in material and methods. Results are shown as mean Log_{10} CFU/ml of *F. asiatica* uptake in HKDM at 0, 24, and 48 h time point. The error bars represent standard error of triplicate samples and the results shown are representative of three independent experiments. Different letters denote significant differences between treatments, $P<0.05$.

Finally, due to the strong antibody response observed in immunized fish, a series of passive transfer experiments were performed to determine whether these antibodies could prevent infection *in vivo*. Naive tilapia fingerlings received IP injections of PBS, HINS or HIIS

sera (200 µL) collected from adult tilapia immunized with 10^7 CFU/fish. The tilapia fingerlings were then challenged (IP) with either 10^3, 10^4, 10^5 or 10^6 CFU/fish of WT *F. asiatica* and were monitored daily for health and survival for a total of 21 days post challenge. Although passive immunization of HIIS did not protect against high doses of the bacterium (10^6 CFU/fish) injected in the peritoneum of naïve fingerlings, significant (p<0.05) reductions in mortality were observed in HIIS immunized fish when challenged to 10^4 and 10^5 CFU/fish and compared to those immunized with PBS or HINS (Fig. 5.5).
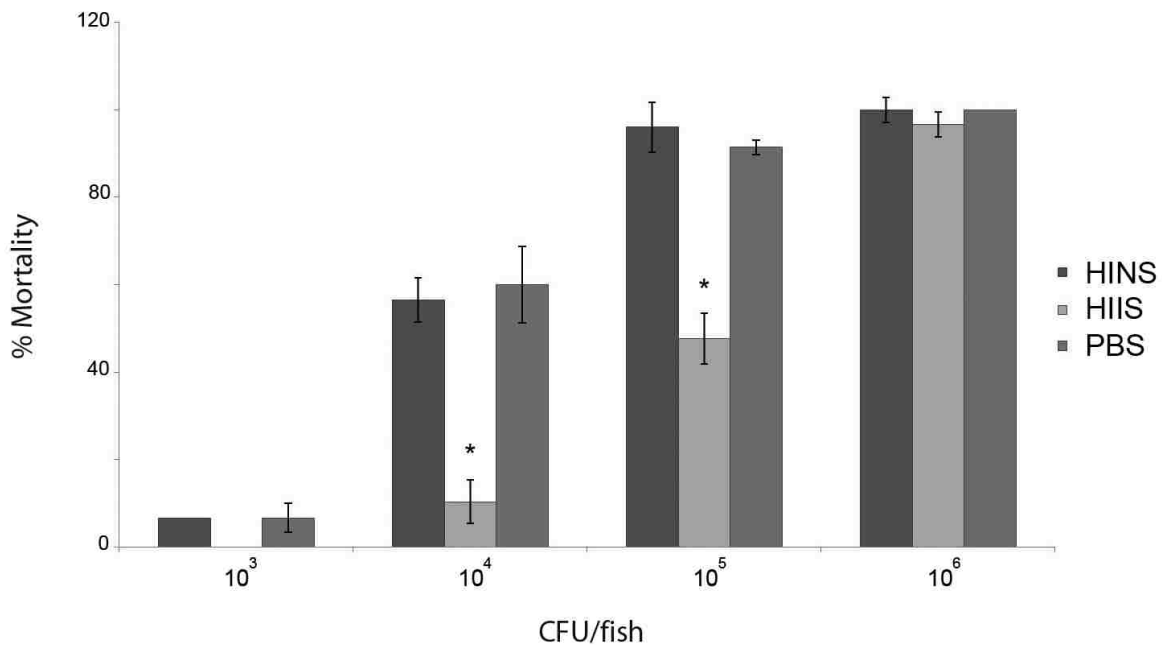
Figure 5.5 Adoptive transfer of heat-inactivated normal serum (HINS), heat-inactivated immunized serum (HIIS) or PBS to naïve tilapia fingerlings. Immune sera was collected from 20 adult tilapia vaccinated by intra-peritoneal injection (IP) with the Δ*iglC* mutant 4, 5 and 6 weeks post-vaccination. Sera were pooled and antibodies titers were measured before passively immunized the fingerlings. Normal sera were collected and pooled form 20 adult tilapia injected with PBS 4, 5 and 6 weeks post-injection. Naïve fingerlings (60 fish/treatment) were injected IP with 200 µl of pooled HINS, HIIS or PBS 24 h before IP challenge with 10^3, 10^4, 10^5 or 10^6 CFU/fish of *F. asiatica* WT. Animals were monitored daily for morbidity and mortality. Results are representative of two independent experiments. Mean percent mortality for each treatment was calculated 21 days post-challenge with WT. Each bar represents the mean percent mortality ± standard error of three tanks (20 fish/tank). * Denotes significant differences, P<0.05 with respect to the control group (PBS) by a Student's *t*-test.

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DISCUSSION

A live attenuated vaccine given to the fish by the immersion route, has the advantage of directly targeting the natural routes of attachment and penetration of the bacteria into the fish and hence inducing protective immunity at the primary site of infection. Results showed that an immersion vaccination with four different treatments of a $\Delta iglC$ mutant significantly ($p<0.05$) protects tilapia fingerlings against homologous *F. asiatica* immersion challenge (Fig. 5.1). Results of immunization trials indicated that when the $\Delta iglC$ mutant vaccine was delivered for either 30 or 180 m at a dose of 10^7 CFU/ml, relative percent survival (RPS) values of 68.75 % and 87.5 % were obtained, demonstrating the potential of the vaccine to prevent francisellosis in tilapia. During the first 4 weeks post-vaccination, a relatively small antibody response was observed in immunized fish, and they weren't significantly different to those observed in the control groups. However, upon exposure to WT *F. asiatica*, a significantly higher ($p<0.05$) mucosal and humoral antibody response was evident in the fish vaccinated with a dose of 10^7 CFU/ml (Fig. 5.2, Fig. 5.3). The importance of antibody-mediated protection at the mucosal or systemic level has yet to be determined for *F. asiatica*. However, antibody has been shown to correlate with protection for *Flavobacterium columnare* (Grabowski et al. 2004); *F. psychrophilum* (LaFrentz et al. 2003), *Vibrio* spp. (Akhlaghi et al. 1999), *Yersinia ruckeri* (Furones et al. 1993), *Streptococcus iniae* (Shelby et al. 2002), *A. hydrophila* (Ruangpan et al. 1986), *Edwardsiella tarda* (Gutierrez et al. 1993), and *E. ictaluri* (Klesius et al. 1995) in cultured fish species.

The passive immunity studies described here demonstrate that *F. asiatica*-specific antibodies mediate protection after IP injection of different concentration of *F. asiatica* WT (Fig. 5.5). Thus we believe that the *F. asiatica*-specific antibody response is a useful component of the protective immune response to lethal *F. asiatica* infection in fish. Since *F. asiatica* is a

facultative intracellular organism, the bacteria can exist in an extracellular form in the tilapia, thus it is conceivable that antibodies are able to prevent the systemic spread of bacteria. Since mucosal responses observed in this study demonstrated that vaccinated fish presented higher amount of anti- *F. asiatica* antibodies at 6, 8 and 10 weeks post-vaccination, it can also be speculated that mucosal antibody might serve to inhibit the damage or possible external bacterial colonization in fish tissues (Fig. 5.2, Fig. 5.3).

In many Gram-negative bacteria, the protective antibody may act in conjunction with complement *in vivo* in a direct bactericidal response, or aid phagocytes in their ability to engulf and kill the pathogen. Antibodies may directly influence the phagocytic activity of Fc receptor-bearing cells, such as macrophages and NK cells, to enhance phagocytosis of intracellular pathogens. Our results demonstrate that neither NS nor IS obtained from adult tilapia, damaged the bacteria *in vitro*, however sera from *ΔiglC* mutant-vaccinated animals exhibited enhanced antibody-mediated phagocytosis of *F. asiatica* in HKDM (Figure 5.4). In *F. tularensis*, antibody-mediated bacterial clearance and *in vitro* analysis of opsonophagocytosis revealed that IFN-γ treatment of macrophages induced rapid killing of intracellular bacteria; but opsonophagocytosis via FcγRs in the absence of IFN-γ failed to control bacterial replication (Pammit et al. 2006; Kirimanjeswara et al. 2008). These results suggested that FcγR-mediated cellular activation synergizes with IFN-γ for intracellular bacterial killing (Pammit et al. 2006; Kirimanjeswara et al. 2008). In this study, non-activated HKDM from naïve fish failed to control intracellular bacterial replication, even thought they were opsonized with antibodies against *F. asiatica* (Fig. 5.4). The role of cell mediated immunity has yet to be determined in the control of fish francisellosis; but activation of fish macrophages have been shown to play a role in the defense against important fish pathogens like *Aeromonas salmonicida* and *Renibacterium salmoninarum* (Ellis 2001; Ellis 1999). In *F. tularensis*, cell mediated immunity has long been believed to be

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critical for protection, furthermore, synergy between antibodies, T cell-derived cytokines, and

phagocytes appears to be critical to achieve sterilizing immunity against the pathogen. Defined

F. novicida mutant strains like *ΔiglB* and *ΔiglC,* protected mice against homologous challenges

via the participation of IFN-γ producing cells such as natural killers (NK) and T cells (Pammit et

al. 2006; Cong et al. 2009).

In summary, an attenuated strain of *F. asiatica* (*ΔiglC*) was characterized as a potential

live-vaccine for fish francisellosis. Immunization of tilapia nilotica with this strain by

immersion delivery provided long lasting protective immune responses (p<0.05), as

demonstrated by antibodies levels, and the antibodies directed to *F. asiatica* were protective as

shown in passive immunity trials.

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CHAPTER 6

DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR IDENTIFICATION AND QUANTIFICATION OF THE FISH PATHOGEN *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS**

INTRODUCTION

Francisellosis is an emergent disease in fish caused by Gram negative facultative intracellular bacteria that are members of the genus *Francisella*. In tilapia the disease can be observed as an acute syndrome with few clinical signs and high mortality, or as a sub-acute to chronic syndrome with non-specific clinical signs like anorexia, exophthalmia, and anemia. Upon macroscopic and microscopic examination, internal organs are enlarged and contain widespread multifocal white nodules. Histological examination often reveals the presence of multifocal granulomatous lesions, with the presence of numerous small, pleomorphic, cocco-bacilli (Hsieh et al. 2006; Mauel et al. 2007; Soto et al. 2009a).

During the past five years, bacteria of the genus *Francisella* have caused significant mortalities in cultured tilapia (*Oreochromis spp.*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar* L), hybrid striped bass (*Morone chrysops* × *M. saxatilis*), three line grunt (*Parapristipoma trilineatum*), and ornamental cichlids; both in warm and cold water environments (Kamaishi et al. 2005; Nylund et al. 2006; Olsen et al. 2006; Hsieh et al. 2007; Ostland et al. 2006; Birkbeck et al. 2007; Mauel et al. 2007; Mikalsen et al. 2007; Ottem et al. 2007; Soto et al. 2009a).

The identification and taxonomic characterization of the *Francisella* spp. identified as worldwide emerging pathogens of fish has been difficult due to the fastidious nature of the bacteria and the small number of isolates recovered from fish (Soto et al. 2009a). In the majority of the cases, PCR and sequence comparison of the 16S rRNA have made it possible to place the

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organism at 97% similarity to *Francisella tularensis,* 98% similarity to *F. philomiragia*, and 99%

to other *Francisella* spp. strains isolated from fish species (Kamaishi et al. 2005; Hsieh et al.

2006; Ostland et al. 2006; Mauel et al. 2007; Mikalsen et al. 2007; Ottem et al. 2007; Soto et al.

2009a).   The *Francisella* strain utilized in this research project, LADL 07-285A, isolated from

tilapia *Oreochromis* spp. from Costa Rica, at the Louisiana Aquatic Diagnostic Laboratory, LSU

School of Veterinary Medicine, was confirmed by molecular analysis as *Francisella noatunensis*

subsp. *orientalis* (Soto et al. 2009a) and exhibited 99% identity with *F. noatunensis* subsp.

noatunensis isolated from diseased atlantic cod in Norway (Mikalsen and Colquhoun 2009;

Ottem et al. 2009) by sequence comparison of the 16S rRNA.

The diagnosis of this highly virulent fish pathogen has many constraints including the

fastidious nature of the bacterium, and the lack of biochemical, molecular, and serological tests

specific for this aquatic animal pathogen.

Previous diagnosis of francisellosis in fish species has been made with the aid of

histopathology, electron microscopy, conventional culture assays, conventional polymerase

chain reaction (PCR) using *Francisella* sp. specific primers, 16S rRNA sequencing, and in-situ

hybridization (Kamaishi et al. 2005; Hsieh et al. 2006; Ostland et al. 2006; Mauel et al. 2007;

Ottem et al. 2007; Soto et al. 2009a). However, the diagnosis of the pathogen remains a

challenge, and some of the current techniques are difficult, time consuming, expensive, require

specialized personnel and are prone to show false negatives due to low sensitivity, or false

positives due to low specificity. Moreover, studies with *F. tularensis* have shown that diagnosis

based on isolation by culture is prone to show false-negative results (Fujita et al. 2006).

Real-time PCR is a well known molecular technique that is currently used in many

laboratories for diagnosis of microbial pathogens including the fastidious bacteria

Mycobacterium spp., *Bacillus anthracis*, *F. tularensis*, and organisms that are non-culturable on

cell free media, the *Rickettsia* spp. and viruses (Bode et al. 2004; Kocagoz et al. 2005; Kidd et al. 2008; Tomaso et al. 2007; Abril et al. 2008; Takahashi et al. 2007). In recent years, fish disease diagnosticians have used this technique to identify and quantify bacterial, viral and parasitic fish pathogens such as: *Aeromonas salmonicida*, *Flavobacterium columnare*, *Renibacterium salmoninarum*, *Henneguya ictaluri*, Largemouth bass virus, and recently *Francisella piscicida* in Norwegian cod (Balcazar et al. 2007; Getchell et al. 2007; Panangala et al. 2007; Suzuki and Sakai 2007; Griffin et al. 2008; Ottem et al. 2008). The high sensitivity, high specificity, and short turnaround time for results, make this technique an attractive replacement method for conventional diagnostic techniques (Espy et al. 2006).

The genes of the intracellular growth locus (*iglA, iglB, iglC*, and *iglD*) are some of the most interesting genes identified in the genus *Francisella.* These genes are present as part of a 30 Kb pathogenicity island described by Nano et al. 2004 and Barker and Klose 2007 in *F. tularensis.* The *Francisella* pathogenicity island (FPI) is a cluster of 16–19 genes, that has been found duplicated in some *F. tularensis* genomes, but as a single copy in the *F. philomiragia* subsp. *philomiragia* ATCC 25015 isolate; a close relative of the tilapia pathogen *F. noatunensis* subsp. *orientalis* strain LADL 07-285A (Nano and Schmerk 2007; Soto et al. 2009b).

The functions of the conserved proteins corresponding to the genes are elusive. Overall, Igl proteins appear to be essential for the ability of *F. tularensis* to survive inside the macrophages and cause disease (Golovliov et al. 1997; Nano et al. 2004; Lai et al. 2004; Lauriano et al. 2004; Santic et al. 2005; Brotcke et al. 2006; de Bruin et al. 2007). Homologues of the *F. tularensis iglABCD* genes in the tilapia pathogenic *F. noatunensis* subsp. *orientalis* strain LADL 07-285A were identified and described in a previous study (Soto et al. 2009b). The presence of a single *iglC* gene in the completely sequenced and closely related isolate *F. philomiragia* subsp. *philomiragia* ATCC 25017 suggests that the gene is also present in single

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copy in *F. noatunensis* subsp. *orientalis* strain LADL 07-285A. The presence of a single copy of

the *iglC* gene in *F. noatunensis* subsp. *orientalis* makes the gene an excellent target for

developing a highly specific diagnostic test and will provide a means to quantify with a high

degree of confidence the amount of bacterial DNA present in a sample.

The aim of this study was to develop a quantitative real-time PCR assay using the

previously described *iglC* gene of the fish pathogen *F. noatunensis* subsp. *orientalis* as a target.

We describe a highly sensitive, specific and reliable molecular diagnostic technique for

identification and quantification of *F. noatunensis* subsp. *orientalis* from diseased fish.

MATERIALS AND METHODS

Bacterial Species and Strains. The bacterial strains used in this project were chosen because

they represent common bacterial fish pathogens, or are members of the genus *Francisella.* Strain

LADL 07-285A, isolated from diseased cultured tilapia (*Oreochromis* spp.) was chosen as a

representative of the warm water strain of fish pathogenic *F. noatunensis* subsp. *orientalis*. The

majority of the isolates tested were recovered by the Louisiana State University-School of

Veterinary Medicine (LSU-SVM) Louisiana Aquatic Diagnostic Laboratory (LADL), from

diseased fish, while others were acquired from the American Type Culture Collection (ATCC).

Francisella tularensis subsp. novicida U112 and *F. tularensis* subsp. *holarctica* (LVS isolate)

DNA were kindly donated by Dr. Bernard Arulanandam and Dr. Jieh-Juen Yu, from the

Department of Biology, University of Texas at San Antonio. *Francisella noatunensis* subsp.

noatunensis is a recently described member of the genus *Francisella* isolated from farmed

Atlantic cod displaying chronic granulomatous disease (Mikalsen et al. 2007; Ottem et al. 2009;

Mikalsen & Colquhoun 2009), was kindly donated by Dr. Anne-Berit Olsen, National Veterinary

Institute, Bergen, Norway. *Francisella* sp. isolates recovered from moribund hybrid striped bass

(Ostland et al. 2006) and tilapia (Kay et al. 2006) and showing >99% identity with *F.*

noatunensis subsp. *orientalis* after 16S rDNA sequence comparison, were kindly donated by Dr. John Hansen, Interdisciplinary Program in Pathology, University of Washington, Seattle, Washington, USA. Following previous published molecular techniques and protocols (Soto et al. 2009b), PCR and sequence comparison of the *iglABCD* operon (Figures 6.1, 6.2) and 16S rDNA sequences, as well as phenotypic characteristics, temperature requirements, and host range analysis, demonstrated that isolated *Francisella* sp. #1, *Francisella* sp. #2, *Francisella* sp. #3 and *Francisella victoria* are in fact members of the recently described family *F. noatunensis* subsp. *orientalis* (Figures 6.1, 6.2) (Soto et al. 2009b). *Francisella noatunensis* subsp. *orientalis* strain LADL 07-285A was grown in Cystine Heart Agar with hemoglobin (CHAH) supplemented as outlined in Soto et al. 2009a, for 48 h at 28°C. *Francisella noatunensis* subsp. *noatunensis* was grown in a similar manner but was incubated at 20°C for 5 d. *Flavobacterium columnare* was grown on dilute Mueller Hinton Agar for 48 h at 28°C. *Mycobacterium marinum* and *Nocardia seriolae* were grown on Lowenstein Jensen slants for one week at 28°C. All the other bacteria used in the study were grown on blood agar (BA) 5% sheep blood plates for 48 h at 28°C. The complete list of the isolates used in this study is shown in Table 6.1.

Template DNA Preparation. Bacterial cultures grown on agar media were suspended in 1 ml of 1X phosphate buffer saline (PBS) and 200 μl was used for nucleic acid isolation following the manufacturer's protocol in the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). Nucleic acid was also extracted from a negative control consisting of 1X sterile PBS alongside of the unknowns to ensure no cross- contamination occurred during the extractions.

Table 6.1 Bacterial DNA used in this study

Bacteria species	Source	qPCR result[a]
Francisella noatunensis subsp. *orientalis* isolate LADL 07-285A	Tilapia (*Oreochromis spp.*) LADL	+
Francisella noatunensis subsp. *orientalis* isolate LADL 07-285B	Tilapia (*Oreochromis* spp.) LADL	+
Francisella noatunensis subsp. *orientalis*	Hybrid striped bass (*Morone chrysops* x *M. saxatilis*) (UW)	+
Francisella noatunensis subsp. *orientalis*	Hybrid striped bass (*Morone chrysops* x *M. saxatilis*) (UW)	+
Francisella noatunensis subsp. *orientalis*	Hybrid striped bass (*Morone chrysops* x *M. saxatilis*) (UW)	+
Francisella noatunensis subsp. *orientalis*	Tilapia nilotica (Oreochromis niloticus) (UW)	+
Francisella noatunensis	Atlantic Cod (*Gadus morhua L.*) NVI	-
Francisella tularensis subsp. *novicida* U112	UTSA	-
Francisella tularensis subsp. *holarctica* LVS	UTSA	-
Edwardsiella tarda	Tilapia (*Oreochromis* spp.) LADL	-
Edwardsiella ictaluri (ATCC 33202)	Channel catfish (Ictalurus punctatus)	-
Photobacterium damselae subsp. piscicida (ATCC 17911)	White perch (*Roccus americanus*)	-
Aeromonas hydrophila	LADL	-
Aeromonas salmonicida	LADL	-
Streptococcus iniae (ATTC 29177)	Amazon fresh water dolphin (*Inia geoffresis*)	-
Streptococcus agalactiae	Tilapia (*Oreochromis* spp.) LADL	-
Flavobacterium columnare (ATCC 23463)	Chinook salmon (Oncorynchus tshawytscha)	-
Vibrio anguillarum	Hybrid striped bass LADL	-
Vibrio mimicus	Crayfish LADL	-
Mycobacterium marinum	Florida pompano (*Trachinotus carolinus*) LADL	-
Nocardia seriolae	Pompano *(Trachinotus blochii)* LADL	-

[a] A negative Ct value (-) represents no logarithmic amplification detected within 40 amplification cycles.

LADL: Louisiana Aquatic Diagnostic Laboratory, LSU School of Veterinary Medicine
NVI: National Veterinary Institute, Bergen Norway
UTSA: Dept. of Biology, University of Texas at San Antonio.
ATCC: American Type Culture Collection
UW: University of Washington
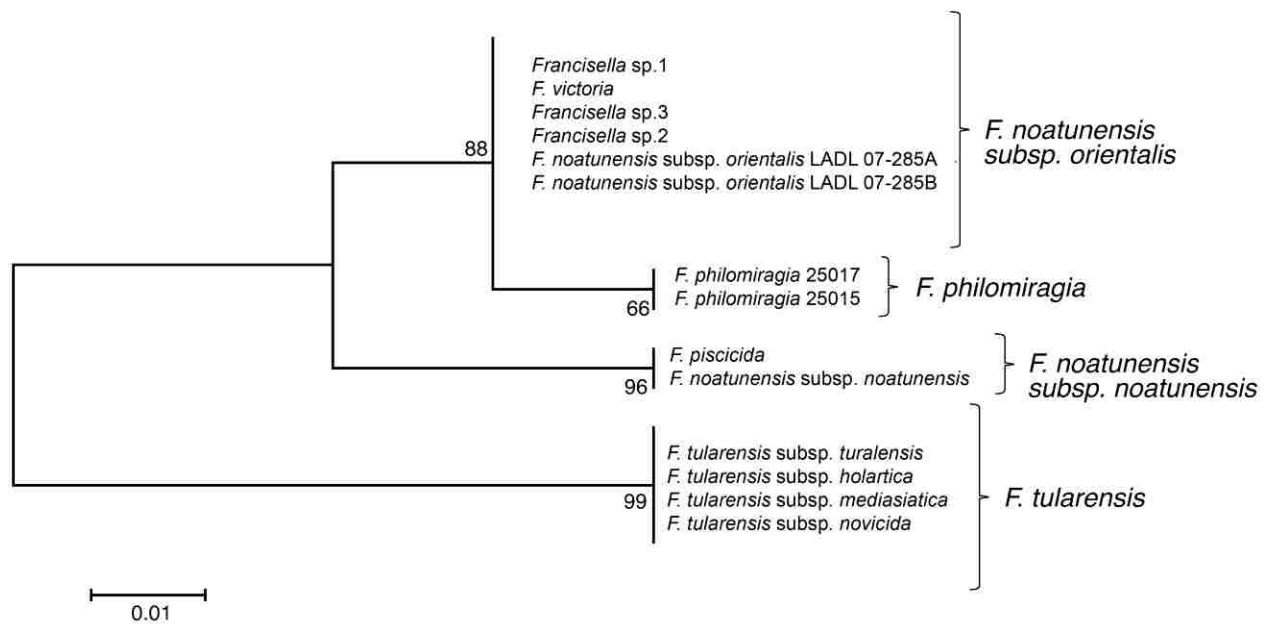
Figure 6.1 Evolutionary relationships of 14 members of the genus *Francisella* based on partial *iglABCD* sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.14285714 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 70 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 (Tamura et al. 2007).

The primers and probe were designed following the real-time qPCR Assay Design Software (Biosearch Technologies, San Francisco, CA, USA). Primers and probe concentration were optimized at the beginning of the study, to determine the minimum primer concentrations giving the maximum ΔRn, and the minimum probe concentration that gave the minimum C_T. The optimization was done according to the TaqMan Universal PCR Master Mix manufacturer (Applied Biosystems, Foster City, CA, USA).
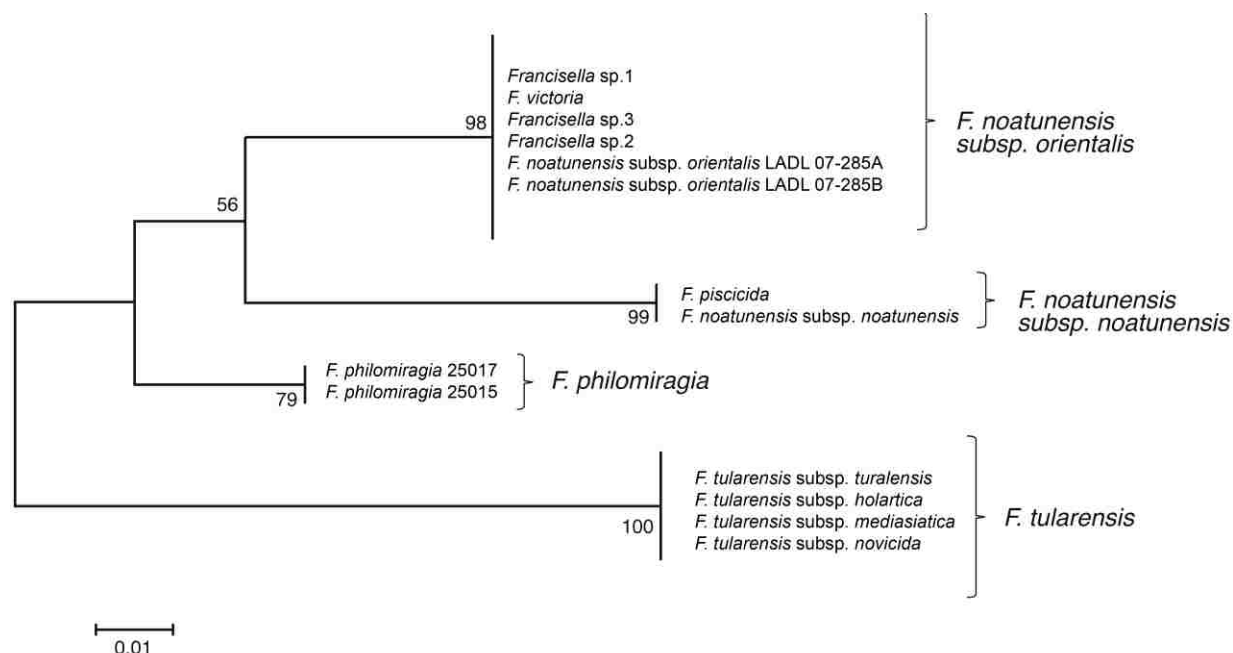
Figure 6.2 Evolutionary relationships of 14 members of the genus *Francisella* based on partial *iglC* sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.23571429 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 70 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Table 6.2 TaqMan primers and probe used in this study

Primers and Probes	5′-3′ Sequence	Melting temperature (C°)
iglC forward	Gggcgtatctaaggatggtatgag	66.36
iglC reverse	Agcacagcatacaggcaagcta	66.63
iglC probe	FAM atctattgatgggctcacaacttcacaa BHQ-1	68.34

Real-time TaqMan PCR Assays. The real-time PCR assays were conducted and analyzed

within the Applied Biosystems 7500 Fast Real-Time PCR Systems (Applied Biosystems). The

25 µl reaction mixture consisted of a TaqMan Universal PCR Master Mix (Applied Biosystems),

containing 10 pmol of each primer, 3 pmol of probe and 5 µl of DNA extracted sample.

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Template controls containing PCR grade water and seven serial dilutions of 100 ng μl^{-1} of *F. noatunensis* subsp. *orientalis* isolate LADL 07-285A diluted in PCR grade water and measured in a NanoDrop Spectrophotometer ND-1000 V3.5 (Nanodrop Technologies Inc., USA) were included in each run.

The unknown samples, as well as the diluted standards and negative controls were run in triplicate. Cycling conditions were 2 min at 50°C, 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 60 s at 60°C.

Sensitivity of the Real Time PCR Assays. For sensitivity determination, the TaqMan assays were evaluated by two different independent methods. Three separate extractions of *F. noatunensis* subsp. *orientalis* DNA were adjusted to a concentration of 100 ng μl^{-1} NanoDrop Spectrophotometer ND-1000 V3.5 (Nanodrop Technologies Inc., USA), and ten fold dilutions were made in PCR grade water until reaching a concentration of 1 fg μl^{-1}. Genome equivalent (GE) calculation was based on assuming a 2-MB genome size for *F. philomiragia* and several subspecies of *F. tularensis* (Takahashi et al., 2007; Tomaso et al. 2007; Abril et al., 2008; Ottem et al., 2008). For determination of colony forming units (CFU), several isolated colonies of *F. noatunensis* subsp. *orientalis* were picked from a fresh CHAH culture and suspended in 1ml of phosphate buffered saline (PBS) pH 7.2, until an OD$_{600}$ of 0.75 was reached and measured in a DU-640 Spectrophotometer (Beckman Coulter Inc., USA). Ten fold serial dilutions in PBS were made from this sample, and colony counts were performed on CHAH by the drop plate method to verify bacterial numbers. Extraction of DNA from 200 μl of each dilution was used for CFU quantification in the real time PCR assay. Amplification efficiencies were determined. All assays were run in triplicate.

Sensitivity of the Real Time PCR Assay in Fish Spleen. In order to determine the sensitivity limit of the assay, triplicate samples of one gram of uninfected tilapia spleen (recently acquired

fresh tissue) were homogenized with a Kontes PELLET PESTLE® Micro Grinder (A. Daigger and Company, Inc. 620 Lakeview Parkway, Vernon Hills, IL, USA) in a 4 ml suspension of early stationary phase *F. noatunensis* subsp. *orientalis* cells diluted in PBS to a final concentration of 2, 20, 200, $2x10^3$, $2x10^4$, $2x10^5$, $2x10^6$, $2x10^7$ CFU g tissue^{-1}. Two hundred microliters of the homogenates containing approximately 50 mg of spleen, were centrifuged at 12 000 g for 1 min and DNA extracted following the manufacturers protocol "Isolation of Nucleic Acids from Mammalian Tissue", High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). Enumeration of *F. noatunensis* subsp. *orientalis* by real-time PCR was compared with plate count values, taking into account dilution/concentration factors due to volumes used in DNA extraction and final elution volumes. Amplification efficiencies were determined. All assays were run in triplicate.

Experimental Infectivity Trial. The tilapia fingerlings used during the trial were obtained from a source with no history of *Francisella* infection and a sub-sample of the population was confirmed as negative for bacteria by complete clinical, bacteriological and molecular analysis as described in Soto et al. 2009a, to ensure that they were negative for francisellosis. Fish were maintained at 10 fish per tank and fed commercial tilapia feed daily (Burris Aquaculture Feeds, Franklinton, LA) at ~3% fish body weight per day. The mean weight of the fish was 9.1 g and the mean length was 18 cm. Three tanks were used per treatment, and one tank was used as a control. Fish were immersed in 8 L of static water containing approximately $3.7x10^7$ CFU/ml in tank water for 3 h at 23-25°C, and then the volume of the tanks was adjusted to 20 liters with clean oxygenated water. Control fish were treated in a similar manner, but received sterile PBS.

Following each challenge exposure, mortality was recorded every 12 h for 30 d. Prior to collection of spleen, moribund and survivor fish were euthanized with an overdose of MS-222. The spleens from dead, moribund and survivor fish were collected aseptically in 1.5

microcentrifuge tubes (Fisherbrand, Fisher Scientific, USA), weighed, and DNA was extracted from ~20 mg of spleen following the manufacturers protocol "Isolation of Nucleic Acids from Mammalian Tissue", High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). The rest of the tissue was homogenized in ~ 50 µl PBS and plated on CHAH. The eluted DNA was stored at 4°C until used.

RESULTS

Specificity. The assay was found to be specific for the warm water fish pathogen, *F. noatunensis* subsp. *orientalis* (Table 6.1) and no evidence of crossreactivity was detected (no significant elevated signal was observed with any of the other tested bacterial DNA) (Figure 6.3).

Sensitivity. The sensitivity of the assay was determined using triplicate dilution series from 0.5 fg reaction^{-1} to1.4 mg reaction^{-1} of *F. noatunensis* subsp. *orientalis* genomic DNA. The lowest amount of detection was determined to be 50 fg of DNA (equivalent to ~25 GE). Threshold cycle (Ct) determined by TaqMan real-time PCR amplification of DNA, extracted from serial dilutions of pure *F. noatunensis* subsp. *orientalis* bacterial culture, showed a linear (R^2 =0.994) relationship with log numbers of CFU from 2.5 x 10^7 to 2.5 x 10^1 CFU ml^{-1} based on plate counts (Figure 6.4). Ten fold serial dilutions of nucleic acid extracted from the initial dilutions of the pure bacterial culture also showed a linear relationship between the log amount of nucleic acid and the TaqMan real-time PCR Ct from 1.4 mg to 50 fg (Figure 6.4). Linear detection of amplified product was also revealed in serially diluted *F. noatunensis* subsp. *orientalis* spiked spleen homogenates (R^2 =0.985) (Figure 6.4 and Table 6.3). This indicates that the presence of tissue homogenate did not impede the sensitivity of the real-time PCR assay within this range of CFUs. Uninfected tilapia spleen and water controls showed no signal after 40 cycles.

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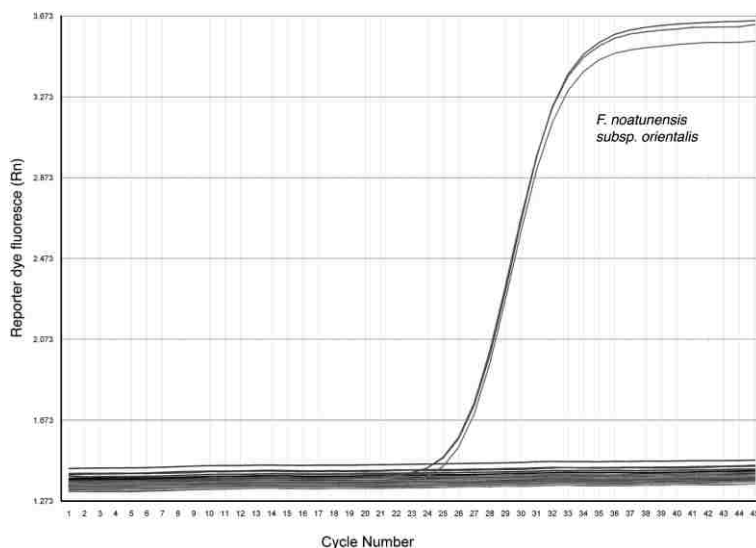
Figure 6.3 Detection of the *igl*C gene by the TaqMan Probe real time PCR assay. Five hundred pg per reaction of DNA from 21 different strains of fish pathogens or member of the genus *Francisella* sp. were used. The amplification plot displays normalized reporter dye fluoresce (R_n) as a function of cycle.

Table 6.3 Quantification of *Francisella noatunensis* subsp. *orientalis* from inoculated PBS and spleen

Inoculum (log CFU ml^{-1})	PBS counts * (log CFU ml^{-1} ± SD)	Spiked spleen homogenate counts * (log CFU ml^{-1} ± SD)	PBS extracted DNA qPCR Ct ± SD) **	Spiked spleen extracted DNA qPCR Ct ± SD) **
7.3	7.59±0.061	7.43±0.13	18.35±0.028	18.53±0.228
6.3	6.62±0.12	6.69±0.12	25.70±0.017	25.12±0.117
5.3	5.52±0.13	5.46±0.15	29.65±0.059	29.33±0.125
4.3	4.86±0.12	4.88±0.12	32.80±0.112	32.1±0.134
3.3	3.59±0.33	3.5±0.0.39	36.54±0.083	36.74±0.121
2.3	2.43±0.19	2.58±0.2	38.74±0.016	38.32±0.026
1.3	0.33±0.35	1.03±0.43	25.70±0.118	26.12±0.418

* *Francisella noatunensis* subsp. *orientalis* numbers were determined by plate counts on CHAH plates as described in materials and methods.
** Real-time PCR determination of *F. noatunensis* subsp. *orientalis* concentrations was based on the mean of triplicate samples. Concentrations were derived from a standard curve using the mean of triplicate Ct values of serial tenfold dilutions of DNA extracted from known concentrations of bacteria
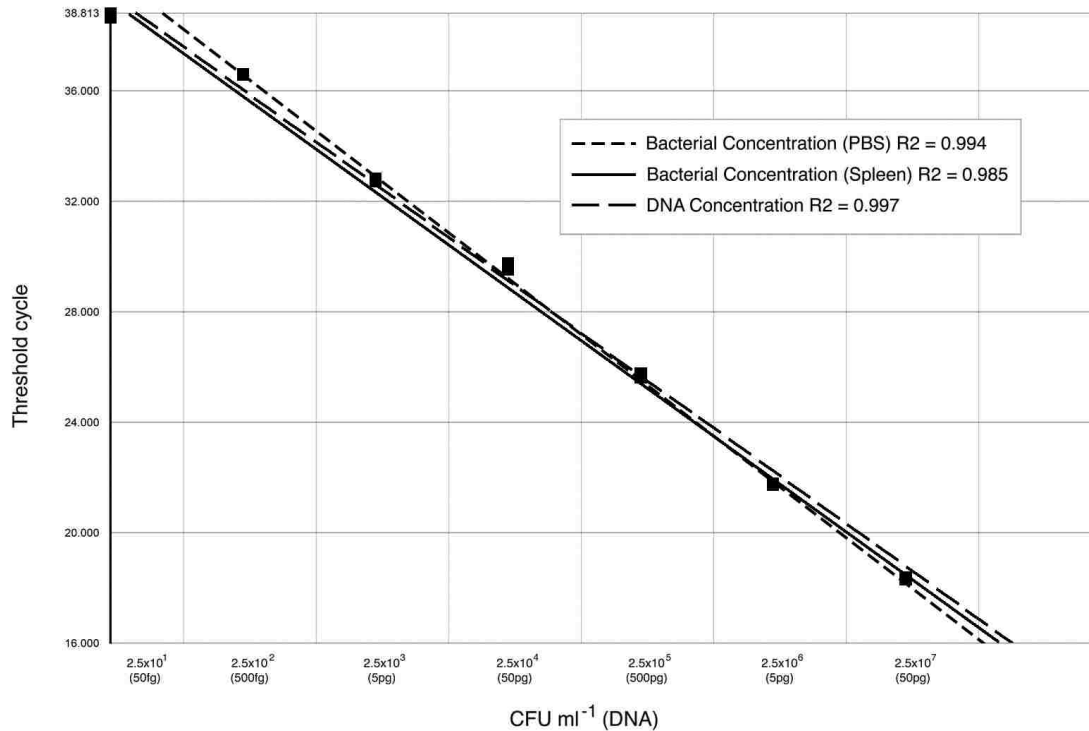
Figure 6.4 Standard curve denoting the sensitivity limits of detection with 10-fold serial dilutions of *F. noatunensis* subsp. *orientalis* isolate LADL 07-285A DNA, colony-forming units (CFU) added to phosphate-buffered saline (PBS), and CFU added to spiked spleen homogenates. Threshold cycle values plotted against DNA or CFU added to PBS or spleen homogenates ranging from 2.5×10^1 to 2.5×10^7 CFU ml^{-1} show a linear correlation of R2 = 0.994 for CFU in PBS (short-dashed line), R2 = 0.985 for CFU in spleen homogenates (black line), and R2 = 0.997 for DNA (long-dashed line). Results are from triplicate samples run simultaneously

Detection of *Francisella noatunensis* subsp. *orientalis* in Experimentally Infected Fish. After 30 days of challenge, the mean mortality in the tanks was 56.6 %. In order to test the ability of the *iglC* TaqMan assays to identify *F. noatunensis* subsp. *orientalis* in tilapia tissue, spleens from infected fish were analyzed. One hundred percent of the morbid and survivor (challenged) fish were positive by the assay, and all non-challenged fish were negative. Detection of the bacteria by culture on CHAH agar media was possible in 58% of the dead fish, and in 38% of the survivors. The mean amount of *F. noatunensis* subsp. *orientalis* GE detected in spleens from dead fish analyzed by real time PCR was 1.8 x 10^5 GE ng^{-1}of spleen tissue, while surviving fish presented a mean amount of 1.5 x 10^3 GE ng^{-1}of spleen tissue.

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DISCUSSION

In the present study, we developed a TaqMan real-time quantitative PCR assay for the rapid identification and quantification of the emergent fish pathogen *F. noatunensis* subsp. *orientalis*. The development of this highly sensitive diagnostic method will enhance the diagnosis of this fastidious organism that could be present at low levels in fish tissue, may require specialized media to grow, and may possibly be overgrown by secondary contaminants following attempts at primary isolation. Molecular diagnostic tests have been used to alleviate some of these problems, and have been used along with DNA sequencing to give definitive diagnosis in complicated cases (Hsieh et al. 2006; Mauel et al. 2007; Soto et al. 2009a). As with many other diagnostic techniques, conventional PCR has certain limitations. It is time consuming, results are based on band size discrimination, results are measured at End-Point (plateau), and there is often low sensitivity, low resolution, and no quantification. On the other hand, real time PCR collects data in the exponential phase, has increased dynamic range of detection, and has reduced time in post PCR processing (Espy et al. 2006). It is not surprising that the technique has increasingly been used in the past five years for the detection and quantification of important human and veterinary fastidious bacterial pathogens like, *Mycobacterium tuberculosis, Francisella tularensis, Bacillus anthracis*, etc (Bode et al. 2004; Kocagoz et al. 2006; Tomaso et al. 2007; Abril et al. 2008; Takahashi et al. 2007). The disadvantages of real-time PCR include the cost of the assay, which is substantially higher than that of either microscopy or conventional PCR, and the need for specialized real-time PCR analyzers, which are currently beyond the means of many laboratories. Also the presence of specific bacterial DNA in a tissue sample may not always indicate disease or viable bacteria. Thus is important to correlate the findings of this molecular technique with the history and macro

and microscopic findings of the clinical isolate (Bode et al. 2004; Espy et al. 2006; Kocagoz et al. 2006; Tomaso et al. 2007; Abril et al. 2008; Takahashi et al. 2007).

The assay developed in this study is directed against the previously identified *iglC* gene in *F. noatunensis* subsp. *orientalis* isolate LADL 07-285A (Soto et al. 2009b). The homologous gene in *F. tularensis* subspecies has been found to be upregulated *in-vivo* and *in-vitro* when the bacterium is infecting and colonizing macrophages, and has been found to be essential for the survival of the bacterium inside the cells (Nano et al. 2004; Santic et al. 2005).

The specificity of the TaqMan probe real-time *iglC* PCR assay was assessed with other strains of the genus *Francisella* (*F. tularensis* subsp. *novicida* U112 and *F. tularensis* subsp. *holarctica* LVS), clinically relevant cold and warm water fish pathogens (*F. noatunensis* subsp. *noatunensis*, *Streptococcus spp., Edwardsiella spp., Aeromonas spp., Vibrio spp., Mycobacterium spp., Photobacterium spp., etc*), and non-infected tilapia splenic tissue. After 40 cycles, DNA samples from these strains failed to show amplification using the real time PCR assay, and the assay showed no cross reaction of the chosen primers and probe with fish tissue or opportunistic fish pathogens listed above . This is particularly important with francisellosis since moribund and dead fish are commonly found with secondary infections, and attempts to isolate *Francisella* spp. can be very difficult due to the fastidious nature of the organism. There is evidence that the presence of other bacteria in clinical specimens may inhibit the growth of *Francisella spp.* and may impair the ability to isolate *F. tularensis* (Petersen and Schriefer 2005). The high specificity achieved by the TaqMan real time PCR assay did not amplify the closely related cold water pathogen *F. noatunensis,* but it did amplify representative *F. noatunensis* subsp. *orientalis* isolates recovered from warm water cultured tilapia and striped bass. We have previously described that after comparison of the 16S ribosomal RNA sequence, the *F. noatunensis* subsp. *orientalis* LADL 07-285A and *F. noatunensis* subsp. *noatunensis* shared

more than 99% homology, but when comparing the *iglC* genes, the cod and the tilapia isolates only shared ~90% homology; making this gene a more specific target to differentiate between similar fish pathogens (Soto et al. 2009b). The differences found between the *iglC* sequences, make the present assay specific for the *F. noatunensis* subsp. *orientalis* isolates.

The sensitivity limit of the assay was found to be ~50 fg of DNA (equivalent to ~25 GE or CFU) of *F. noatunensis* subsp. *orientalis*. In this study we used different approaches to verify that our DNA extraction methodology and the real time PCR assay did not interfere with the results obtained in the assay. After suspending viable live bacteria in tilapia tissue homogenates and in PBS, performing CFU counts in CHAH, extracting the DNA under the same conditions, and running the assay we found that fish tissue did not negatively affect the real-time PCR detection or quantification of *F. noatunensis* subsp. *orientalis*.

When experimentally infected, tilapia were used to simulate wild epizootics, the real-time PCR assay enabled detection of the bacterium in all the dead, moribund and surviving fish 30 days post challenge, whereas it was possible to isolate the bacteria by conventional culturing on agar plates in only 58.8% (10 of 17) of dead and moribund fish, and in 38% (5 of 13) of the survivor fish after 30 days post challenge. The presence of secondary contaminants like *Aeromonas* spp. was greatly reduced by the use of selective media (CHAH with addition of polymixin B and ampicillin); when plating tissue from autolytic fish. The TaqMan probe real time PCR assay also allowed us to quantify the amount of GE of the bacterium in infected tilapia spleen, thus revealing a twofold higher amount of GE in dead tilapia spleen, than in survivors.

In conclusion, we have developed an *iglC* based TaqMan real-time PCR assay with high sensitivity and specificity for the detection and quantification of the emergent warm water fish pathogen *F. noatunensis* subsp. *orientalis*. The assay can be used not only as a rapid diagnostic test for francisellosis, but can also be used as a research tool for bacterial persistence, drug

therapy efficacy, epidemiological studies, screening of broodstock fish, and detection of

reservoirs for infection.

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CHAPTER 7

COMPARISON OF *IN VITRO* AND *IN VIVO* EFFICACY OF FLORFENICOL FOR TREATMENT OF *FRANCISELLA ASIATICA* INFECTION IN TILAPIA

INTRODUCTION

Members of the genus *Francisella* are small, pleomorphic, Gram-negative bacteria, belonging to the gamma group of the class *Proteobacteria* (Barker and Klose 2007; Clemens and Horwitz 2007; Sjostedt 2007). Many *Francisella* sp. are facultative intracellular pathogens, capable of replicating in macrophages and other various cell types in humans, rabbits, rodents, non-human primates, and fish. The bacteria may also exist as endosymbionts of amoebae and arthropods (Abd et al. 2003; Anthony et al. 1991, CLSI 2006; Schulert and Allen 2006; Soto et al. 2010; Vonkavaara et al. 2008). *Francisella asiatica* and *F. noatunensis* are two recently described members of the genus that cause piscine francisellosis in a wide variety of fish species (Mikalsen and Colquhoun 2009).

During the past five years *F. asiatica* has been implicated as the causative agent of mortality in tilapia (*Oreochromis* sp.) and other important warm water species cultured in the USA, Taiwan, Costa Rica, Latin America, Hawaii, and Japan (Hsieh et al. 2006; Kamaishi et al. 2005; Kay et al. 2006; Mauel et al. 2007; Mikalsen and Colquhoun, Soto et al. 2009; Vojtech et al. 2009). In tilapia the disease can present as an acute syndrome with few non-specific clinical signs and high mortality rates, or as a sub-acute to chronic syndrome with non-specific clinical signs like anorexia, exophthalmia, and anemia. The bacterium has a high infectivity rate in tilapia fingerlings. Low numbers (1-10 CFU) of the bacterium injected intra-peritoneally can cause significant damage to the head kidney and spleen, with a dose as low as 23 bacterium resulting in mortality (Soto et al. 2009). Macroscopic and microscopic examination often reveals enlarged internal organs containing widespread multifocal white nodules (Hsieh et al. 2006;

Kamaishi et al. 2005; Kay et al. 2006; Mauel et al. 2007; Mikalsen and Colquhoun, Soto et al. 2009; Vojtech et al. 2009). Moreover, *F. asiatica* has been found to be resistant to serum killing, and can penetrate, replicate and survive in tilapia head kidney derived macrophages (THKDM) (Soto et al. 2010).

Very limited data on fish pathogen susceptibility to antibiotics have been published. Only recently have guidelines been published for broth microdilution testing of fish pathogens (CLSI 2006), however, methods for fastidious organisms such as *Francisella asiatica* are not included in this publication. Clinical breakpoints are not available for this class of fish pathogens either. Currently, only three antibiotics have been approved by the United States Food and Drug Administration for use in United States aquaculture; oxytetracycline dehydrate (TERRAMYCIN® 200 for Fish; Phibro Animal Health, Fairfield, NJ), ormetoprim-sulfadimethoxine (ROMET-30 Type A medicated article; PHARMAQ AS, Oslo, Norway), and florfenicol (AQUAFLOR® Type A medicated article; Intervet/ Schering-Plough Animal Health, Roseland, NJ). Florfenicol is a fluorinated derivative of thiamphenicol that blocks the peptidyltransferase at the 50S ribosome subunit and acts against a wide variety of both gram-positive and gram-negative bacteria (Cannon et al. 1990). As a medicated feed, florfenicol (AQUAFLOR®) has been used to treat a wide variety of fish diseases in various warm and cold water cultured fish species, including *Vibrio anguillarum*, *Aeromonas salmonicida*, *Streptococcus iniae*, *Listonella anguillarum*, and *Edwardsiella ictaluri*, amongst others (Darwish et al. 2007; Gaunt et al. 2003; Samuelsen et al. 1998; Seljestokken et al. 2006).

Due to the emergent nature of francisellosis in fish, there is currently very little published data regarding antibiotic susceptibility of *F asiatica in vivo* or *in vitro*, and at present there are no known efficacious chemotherapeutants or vaccines available (Mauel et al. 2007; Ostland et al. 2006; Soto et al. 2009; Soto et al. 2010). Additionally, antimicrobial therapy in facultative

intracellular bacteria is more complex than in extracellular bacteria since the efficacy of the drug depends on its ability to penetrate and accumulate within the cell, cellular metabolism, subcellular disposition and bioavailability of the drug (Sandberg et al. 2009). For *F. noatunensis*, *in vitro* data was presented that indicated strain GM2212T was resistant to trimethoprim-sulfamethoxazole, penicillin, ampicillin, cefuroxime, and erythromycin, yet susceptible to ceftazidime, tetracycline, gentamicin and ciprofloxacin (Ottem et al. 2007).

The goal of the present study was to determine the ability of florfenicol medicated feed to control experimentally induced *F. asiatica* infection in tilapia. Additionally we evaluated the capacity of florfenicol to eliminate intracellular *F. asiatica* from THKDM *in vitro*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Francisella asiatica* strain LADL 07-285A, isolated from cultured tilapia (*Oreochromis* sp.), was described in previous work (Soto et al. 2009). *Francisella* sp. isolates demonstrating >99% identity to *F. asiatica* 16S rDNA were recovered from moribund hybrid striped bass (Ostland et al 2006) and tilapia (Kay et al. 2006) and kindly donated by Dr. John Hansen, Interdisciplinary Program in Pathology, University of Washington, Seattle, Washington, USA. Polymerase chain reaction (PCR) and sequence comparison of the *iglABCD* operon and 16S rDNA sequences, as well as phenotypic characteristics, temperature requirements, and host range analysis, demonstrated that isolated *Francisella* sp. #1, *Francisella* sp. #2, *Francisella* sp. #3 (Ostland et al. 2006) and *F. victoria* (Kay et al. 2006) are in fact members of the recently described species *F. asiatica* (Soto et al. 2009; Soto et al. 2010). *Francisella asiatica* isolates were grown on Cystine Heart Agar supplemented with bovine hemoglobin solution (CHAH) (Becton Dickenson (BD) BBL, Sparks, MD, USA) for 48 h at 28°C, or in Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL, Sparks, MD, USA) and 0.1% glucose (MMH) (Soto et al. 2009).

Broth cultures were grown overnight at 25°C in a shaker at 175 rpm, and bacteria were frozen at -80°C in the broth media containing 20% glycerol for later use. *Escherichia coli* 25922 used as a control organism for the MIC determinations was grown using Luria-Bertani broth or agar for 16 - 24 h at 28°C.

Antimicrobial Susceptibility Testing. The minimal inhibitory concentration (MIC) of florfenicol for *F. asiatica* isolates and *E. coli* were tested using Sensititre Just One® Strips (Trek Diagnostic Systems, West Sussex, UK), containing two fold dilutions of florfenicol (0.12-128 μg/ml), using slight modifications to the manufacturer's suggested protocol. Briefly, bacterial isolates were grown on agar plates as described previously, and 3-5 colonies were dislodged with a sterile cotton swab, suspended in 4 ml of MMH, and adjusted to a 0.5 McFarland. For *F. asiatica*, 100 μl of this suspension was transferred to 11 ml of MMH broth, mixed, and 50 μl added to each well of the sensititre plate containing florfenicol. For each plate, one well contained the bacterial inoculum without florfenicol (positive control) and one well contained the bacterial inoculum with an antibacterial agent to prevent bacterial growth (negative control). Test plates were covered with an adhesive seal, provided by the manufacturer, and incubated for 24–48 h at 28°C. Bacterial growth was checked visually after removing the adhesive seal at 24 and 48h post inoculation. The MIC value was defined as the lowest concentration exhibiting no visible growth. The MIC assay for the *E. coli* 25922 and *F. asiatica* isolates was replicated five times.

Fish. Tilapia fingerlings (16-27 g) were obtained from a source with no previous history of *Francisella* infection. A sub-sample of the population was confirmed negative for *F. asiatica* by complete clinical, bacteriological, and molecular analysis using previously published protocols to ensure they were negative for francisellosis (Soto et al. 2009). Fish were maintained at stocking densities of 10 fish per tank in 20 L flow through tanks at a temperature of 25°C. Fish were fed

2% fish body weight per day with a commercial tilapia feed (Burris Aquaculture Feeds, Franklinton, LA). Fish were acclimatized for at least four weeks prior to challenge.

Medicated Feed. The medicated feed was produced by mixing 660 mg of florfenicol (Intervet/Schering- Plough Animal Health, Roseland, NJ) with 880 g of tilapia feed (Burris Aquaculture Feeds, Franklinton, LA). This medicated ration, when fed at 2% of the fish body weight per day for a period of 10 days, corresponded to a daily drug dosage of 15 mg/kg fish. We utilized this dose since it has been suggested by preliminary studies that a dose of 15 mg drug for 10 d may be necessary to control infections of *Streptococcus* spp., another well know pathogen of cultured tilapia (Bowser et al. 2009). The commercial diet was ground to less than 600 μm in a Thomas-Wiley Laboratory Mill (Model 4) (Thomas Scientific, Swedesboro, NJ), and passed through a 600 μm sieve (USA Standard Testing Sieve, VWR Scientific Aquaculture supply). Florfenicol was added to the pulverized feed with 13 g of Carboxymethyl Cellulose (CMC) sodium salt (Sigma-Aldrich Corp., St. Louis, MO) and was thoroughly mixed in a Twin Shell Dry Blender (Patterson-Killey Co, Division of the Harsco Corporation, East Strasburg, PA) for 15 m. The dry ingredients were then placed in a commercial food mixer Model A- 200 (Hobart, Troy, OH) and an appropriate amount of distilled water was subsequently added until a uniform mixture was obtained. The moistened mixture was passed through a meat grinder equipped with a 3-mm die to obtain uniform pellets. Pelleted diets were air dried for 24 h under forced air in a temperature-controlled room at 23-25°C and kept dry in bags maintained at 4°C until used. High-performance liquid chromatography (HPLC) was performed by Eurofins Scientific (Memphis, Tennessee) to analyze florfenicol concentrations in the experimental diet. Medicated samples analyzed by HPLC revealed that the florfenicol dose administered was 12.9 mg florfenicol/kg fish/d (86% of target).

Infectivity Challenge. Six treatments were randomly assigned to 18 aquaria with three replicate tanks/group. Treatment groups were: three medicated, challenged treatments; a non-medicated, challenged treatment (positive control); a non-medicated, non-challenged treatment (negative control) and a medicated, non-challenged group to determine any detrimental effects the medication had on the fish. In all three medicated, challenged treatments, fish were fed medicated feed for a period of 10 days, starting at either one, three, or six days post challenge. Before and after the 10-d medication period, non-medicated feed was administered at 2% of body weight per day. Infectivity challenges in tilapia fingerlings were carried out according to a bath challenge model previously described (Soto et al. 2009). *Francisella asiatica* isolate LADL 07-285A recovered from moribund tilapia (Soto et al. 2009) was utilized in all infectivity trials. Feed was restricted for 24 h prior to challenge and the water supply was turned off immediately before the addition of bacteria. A challenge dose of 8×10^7 CFU/ml was obtained by adding 0.5 L of a bacterial suspension in 1x phosphate buffered saline (PBS; pH 7.3) to 10 L of tank water. Fish were maintained in the bath for 3 h, after which time the water supply was restored. Tanks were oxygenated continuously and water temperatures were maintained at 25°C for the duration of study. The negative controls were handled similarly but were not exposed to *F. asiatica.* Fish were observed twice daily during the 30 d of acclimation, and the 30 d post-challenge. Mortality in each tank was recorded twice daily, and when dead fish were removed from a tank, the quantity of feed administered to the tank was reduced proportionally to the decrease in tank biomass (based on mean fish weight).

Bacterial Load Determinations. Five fish from each medicated group and survivors from the non-medicated control group were sacrificed 30 d post-challenge, and spleens were harvested to determine approximate bacterial burdens. Organs were weighed, homogenized in 0.5 ml sterile PBS, plated in triplicate on CHAH and incubated at 25°C for three days prior to CFU

determinations. CFU were expressed as the mean ± SEM. Organs (head kidney, spleen and liver) from additional remaining survivors were used for histological examinations.

Intramacrophage Survival Assays. To determine internalization and intracellular growth of bacteria, THKDM were infected with *F. asiatica* following previously published protocols (Soto et al. 2010). The complete tilapia macrophage medium (CTMM) consisted of Roswell Park Memorial Institute (RPMI) medium 1640 (GIBCO, Invitrogen Corp., Carlsbad, CA) with 14mM Hepes Buffer (GIBCO, Invitrogen Corp.), 0.3% sodium bicarbonate (GIBCO, Invitrogen Corp.), 0.05 mM 2-beta mercaptoethanol (Sigma Chemical Co., St Louis, MO), and 5% heat-inactivated, pooled tilapia serum. Briefly, 96-well plates containing three day old cultures of THKDM at concentrations of 1-5 x 10^5 cells per well were used. *Francisella asiatica* was grown for 8 h in MMH at 25°C. Optical density (OD_{600}) of the culture was determined and the cells were adjusted to an estimated final concentration of 5 x 10^8 CFU/ml, based on an OD/CFU standard curve. One ml aliquots of the bacterial suspension were pelleted at 10,000 x g for 5 minutes in an Eppendorf 5415 D centrifuge (Eppendorf-Brinkman, Westbury, NY), and the pellet was resuspended in 1ml of normal autologous serum to opsonize the bacteria. Ten-fold serial dilutions were plated on CHAH plates after incubation to determine actual CFU/ml. Following a 30 min incubation at 25°C, the 96 well plate was inoculated with 5 μl of treated bacteria per well to achieve a multiplicity of infection (MOI) of 25 bacteria: 1 macrophage. The plates were centrifuged for 5 min at 400 x g to synchronize bacterial contact with macrophages. Following a two h incubation at 25°C with 5% CO_2, the cells were washed three times with warm CTMM (25°C), and further incubated with fresh CTMM containing 0 μg/ml, 1μg/ml, 10 μg/ml or 100 μg/ml of florfenicol for 0, 24, or 48 hours. Cells in five wells were lysed by the addition of 100 μl of 1% Saponin in PBS at each time point. The lysates were serially diluted and spread onto

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CHAH plates to determine viable counts. Experiments were performed in triplicate on a minimum of three separate occasions to affirm the reliability of the results.

Detection of *F. asiatica* Mediated Cytotoxicity. In order to monitor the THKDM cells during experiment, LDH (lactate dehydrogenase) cytotoxicity assay was performed using the colorimetric Cytotox 96 Kit (Promega, Madison, WI) according to the manufacturer's instructions. Cytotoxicity assays were performed on both infected and non-infected THKDM exposed to 0, 1, 10 and 100 µg/ml of florfenicol in CTMM and for 0, 24 and 48 h. The percentage of cytotoxicity was calculated as $100 \times$ [(experimental release – spontaneous release)] / [total release – spontaneous release)], where spontaneous release is the amount of LDH activity in the supernatant of uninfected cells and the total release is the activity in cell lysates (Soto et al. 2010).

Statistical Analysis. The Statistical Analysis System (SAS Institute, Inc. 2003) was used with the general linear models procedure (PROC GLM) to conduct analysis of variances (ANOVA) of factorial arrangements of treatment. When overall tests indicated significance, pairwise comparisons of main effects were calculated with Tukey's test. Interaction effects were examined by pairwise t-test comparisons of mean square means. For the mortality studies the percent mortalities were transformed with an arc-transformation to normalize the data. Colony forming units (CFU) recovered in the *in vitro* challenges were $\log_{10}$ transformed for statistical analysis. All comparisons were considered significant at ($P < 0.05$).

RESULTS

Susceptibility Testing (MIC Determination). Minimal inhibitory concentrations as determined using the Trek diagnostic sensititre plates containing florfenicol indicated that all *F. asiatica* isolates were susceptible to a concentration as low as 2 µg/ml of florfenicol (Table 7.1). Alternatively, the *E. coli* control consistently showed an MIC of 4 µg/ml which is within the

accepted quality control range for this organism in cation adjusted Mueller Hinton broth after 24-48 hr at 28°C (CSLI 2006).

Table 7.1 Minimum inhibitory concentration (MIC) of florfenicol against *Francisella asiatica* and *Escherichia coli* 25922 isolates obtained from culture fish.

Isolate	Source	Florfenicol MIC (µg/ml)
F. asiatica LADL 07-285A	Tilapia	2
F. asiatica LADL 07-285B	Tilapia	2
Francisella sp. #1	Hybrid Striped bass	2
Francisella sp. #2	Hybrid Striped bass	2
Francisella sp. #3	Hybrid Striped bass	2
F. victoria	Tilapia	2
E. coli 25922		4

***In-vivo* Efficacy.** The first mortalities of challenged fish occurred between three and five days post-challenge in non-medicated tanks (Non-medicated challenged group and fish given medicated feed 6 days post-immersion). In both groups mortalities rose rapidly to ~ 40% by day 15, and a final respective cumulative survival of 30 and 50% were recorded at day 30 when the experiment was terminated (Figure 7.1). In both groups, fish became anorexic beginning at four days post-challenge. Excess feed was removed from the tanks 30 m after feeding to avoid water quality problems. Conversely, groups of fish that received medicated feed one or three days post-challenge ate all medicated feed within a few minutes and presented significantly higher survivability than the non-medicated challenged group ($p<0.001$). No mortality events were observed in the group receiving medicated feed one day post-challenge, and only 13.3% of fish receiving medicated feed three days post-challenge died (Figure 7.1).

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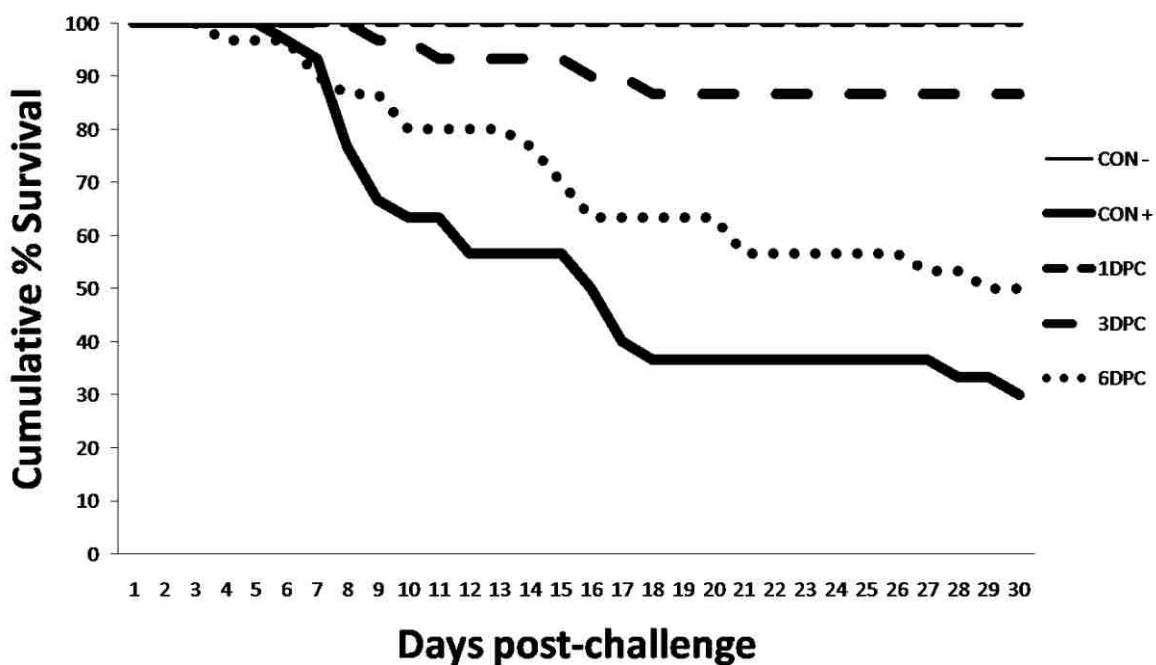
Figure 7.1 *In-vivo* infectivity trial. Cumulative percent survival of tilapia challenged by immersion exposure to *F. asiatica* and subsequently administered florfenicol-treated feed daily at 15 mg active ingredient/kg body weight for 10 d. The five treatments consisted of three medicated, challenged treatments, a non-medicated challenged treatment (CON+), and a non-medicated non-challenged treatment (CON-). The three medicated challenged treatments consisted of one group given medicated feed at day one post-challenge for a period of 10 days (1DPC). A second group was treated identically but medicated feed started at day three post-challenge (3DPC). The third medicated treatment started at day six post-challenge (6DPC). Each treatment group had 30 fish equally divided among three tanks.

In the fish fed medicated feed one day post-challenge, the spleen and head kidney contained multiple accumulations of melanomacrophages that surrounded or were adjacent to small, muscular splenic arterioles, and rare splenic arterioles contained luminal accumulations of melanomacrophages, as demonstrated by histological analysis performed in surviving fish 30 days post-challenge. In one fish, granulomatous inflammation was present in the spleen and kidneys with well delineated foci of necrosis and granuloma formation. No pathological changes were observed in any of the other fish in this group (Table 7.2).

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Table 7.2 Histopathological lesions in selected tissues from medicated and non-medicated tilapia 30 days post-challenge with *F. asiatica*. The five treatments consisted of three medicated, challenged treatments, a non-medicated challenged treatment (CON+), and a non-medicated non-challenged treatment (CON-). The three medicated challenged treatments consisted of one group given medicated feed at day one post-challenge for a period of 10 days (1DPC). A second group was treated identically but medicated feed started at day three post-challenge (3DPC). The third medicated treatment started at day six post-challenge (6DPC). Each treatment group had 30 fish equally divided among three tanks.

Mean values of granulomas in 10X microscopic field

Treatments	Spleen	Head kidney	Liver
1 DPC	None	None	None
3 DPC	Moderate	None	None
6 DPC	Severe	Severe	Moderate
Control +	Severe	Severe	Moderate
Control -	None	None	None

Legends:
None: X=0
Mild: X<7
Moderate: 7>X<20
Severe: X>20

Digests of harvested spleens from each group of animals challenged were plated on CHAH to determine CFU/mg of organ weight. Only one animal from the group presented with medicated feed one day post-challenge had detectable CFU in the spleen. The counts were significantly lower ($p<0.001$) than in any other treatments (Figure 7.2). The spleen from this animal looked normal and was not enlarged, suggesting that infection was not progressing. Bacterial counts in the spleens from fish medicated three days post-challenge (24.15 ± 7.4 CFU/mg of spleen), were significantly lower compared with the non-medicated challenged group (310 ± 77 CFU/mg of spleen) ($p<0.01$) (Figure 7.2). Histopathological analysis in the former group showed widespread granulomas and mixed inflammatory infiltrates composed mainly of

macrophages and lymphocytes in all the analyzed fish. The granulomas consisted of large, foamy, vacuolated macrophages encircled by thin fibrous capsules and small cuffs of lymphocytes with fewer neutrophils. The centers of the granulomas were often necrotic (Table 7.2). There were no significant differences between bacterial counts in the spleens of fish receiving medicated feed 6 days post-challenge (419±183 CFU/mg of spleen) and control (non-medicated and challenged) ($P<0.05$) (Figure 7.2). No mortality or lesions were observed in any of the medicated non-challenge controls or non-medicated non challenged controls.
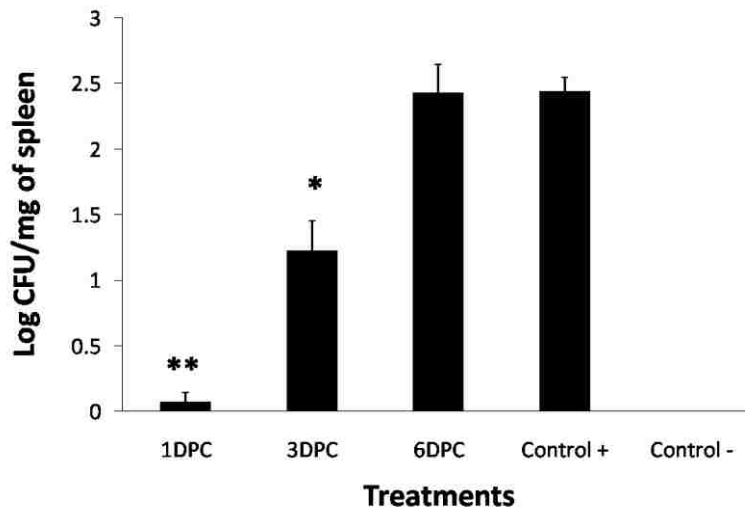
Figure 7.2 Reduced *Francisella asiatica* bacterial burden in the spleens of antibiotic fed tilapia. The five treatments consisted of three medicated challenged treatments, a non-medicated challenged treatment (CON+), and a non-medicated non-challenged treatment (CON-). The three medicated challenged treatments consisted of one group given medicated feed at day one post-challenge for a period of 10 days (1DPC). A second group was treated identically but medication feed started at day three post-challenge (3DPC) and in the third one medication started at day six post-challenge (6DPC). ** $P < 0.001$, *$P<0.05$. Errors bars represent mean ± SEM.

Efficacy of Florfenicol to Control Intracellular *Francisella asiatica In Vitro*. To evaluate the cytotoxic effects of florfenicol, THKDM were grown in 96-well plates in the presence of different concentrations of florfenicol (0, 1, 10, and 100 µg/ml) for 24 and 48 h. The LDH assay was used to assess the cytotoxicity of florfenicol in THKDM and there were no detectable

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cytotoxic effects of florfenicol at any concentration between 0 and 100 µg/ml. At 24 h, the

survival rates were 99.96 ± 0.004% in the 1 µg/ml, 99.95 ± 0.005% in the 10 µg/ml, and 99.95 ±

0.012% in the 100 µg/ml of florfenicol-treated macrophages compared with untreated

macrophages, which was set at 100%. At 48 h, the survival rates were 99.91 ± 0.02 in the 1

µg/ml, 99.9 ± 0.01 in the 10 µg/ml, and 99.9 ± 0.01 in the 100 µg/ml of florfenicol-treated

macrophages compared with untreated macrophages (0 µg/ml), which was set at 100%.

To investigate whether florfenicol affects *F. asiatica* survival within THKDM, cells were

infected at a MOI of 25 as described in materials and methods, and incubated for 0, 24, and 48 h

after bacterial infection in CTMM containing 0, 1, 10 or 100 µg/ml of florfenicol. The results

showed efficient intracellular replication of *F. asiatica* within THKDM cultured with 0 and

1µg/ml (Figure 7.3). At 24 and 48 h post-infection CFU counts of *F. asiatica* were greater

(p<0.001) than those at 0 h in both the control and the THKDM cultured with media containing

1µg/ml of florfenicol (Figure 7.3). On the other hand, infected macrophages that were cultured in

media with 10 and 100 µg/ml of florfenicol, showed no bacterial replication, and significantly

(p<0.001) reduced numbers of bacteria at 48 h post-infection (Figure 7.3).

The lactate dehydrogenase (LDH) cytotoxicity assay performed in infected cells

correlates with the results from the intracellular growth assays. The cytotoxicity observed in

THKDM cultured with media containing 10 and 100 µg/ml florfenicol was significantly lower

(p<0.001) than that observed in the control group (0 µg/ml) at both 24 and 48h (Figure 7.4).

Although the cytotoxicity observed in THKDM cultured with media containing 1µg/ml

florfenicol was significantly lower (p<0.001) than that observed in the control wells at 24h, by

48 h the cytotoxicity had increased in this group (Figure 7.4).
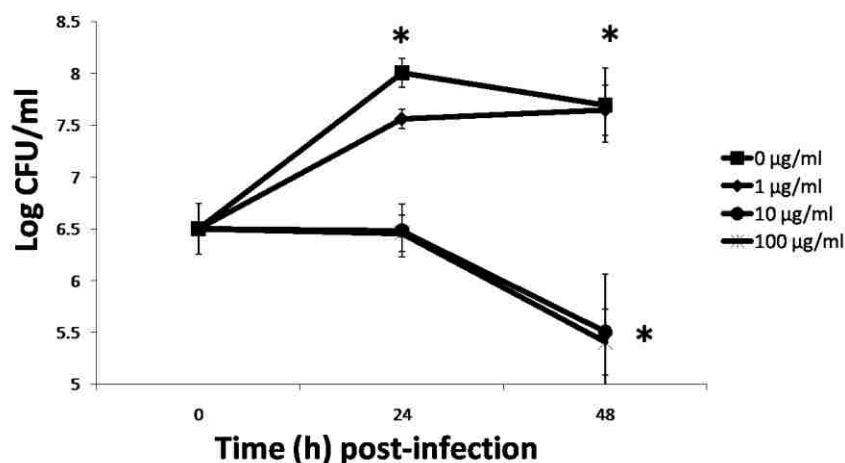
Figure 7.3 Florfenicol mediated killing of intracellular *F. asiatica* in infected tilapia head kidney derived macrophages. Bacteria were added at an MOI of 25:1 and incubated for 2 hours at 25°C with 5% CO2 followed by incubation with 0, 1, 10 or 100 µg/ml of florfenicol in complete tilapia macrophage media (CTMM). At 0, 24 and 48 h post-infection, cells were washed and lysed with 0.5% Saponin, followed by serial 10-fold dilutions plated on Cystein Heart Agar supplemented with hemoglobin (CHAH) plates and incubated at 25°C for 3 days for CFU determination. Experiment performed three times in triplicate. Error bars represent mean ± SEM. * Significantly different than time 0; P < 0.001.
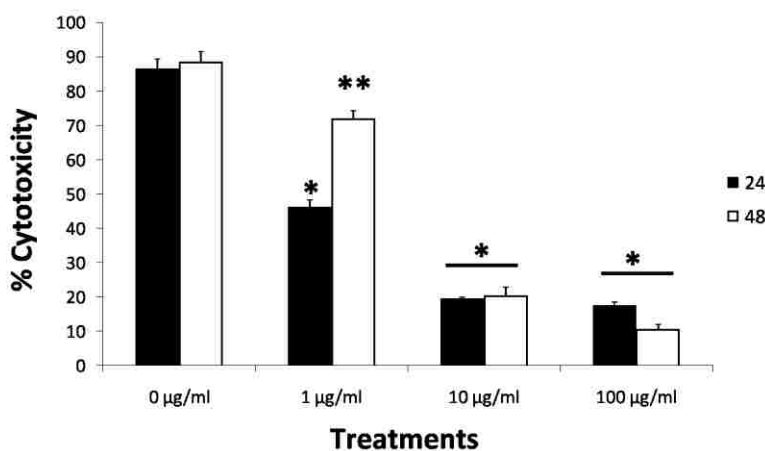
Figure 7.4 Cytotoxicity of *Francisella asiatica* in tilapia head kidney derived macrophages incubated in florfenicol. Bacteria were added to complete tilapia macrophage media (CTMM) at an MOI of 25:1 and incubated for 2 hours at 25°C with 5% CO_2 . Zero, one, 10 or 100 µg/ml of florfenicol was added to each culture. At 24 and 48 h cytotoxicity was assayed by the amount of lactate dehydrogenase released from infected cells. The error bars represent standard error of triplicate samples and results shown are representative of three independent experiments. Significant differences between treatments and the zero µg/ml of florfenicol are marked (*, $P<0.001$; **, P<0.05).

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DISCUSSION

The results obtained from the MIC determinations demonstrated that florfenicol is a suitable candidate for *in vivo* experimentation, since a low minimal inhibitory concentration (2µg/ml) was found. In mammalian pathogens like *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Mannheimia haemolytica*, and *Histophilus somni*, florfenicol has shown bactericidal activity at the respective MICs; whereas in others like *Staphylococccus aureus* MICs only have bacteriostatic activity (Pasmans et al. 2008). Therefore, maintaining concentrations in plasma above the MIC seem advisable to control *F. asiatica* in tilapia. Recent pharmacokinetic work has shown that serum, skin and muscle of tilapia medicated with florfenicol via a medicated ration at 15 mg florfenicol per kg fish body weight for 10 d, contain concentrations higher than the *in vitro* MIC (2µg/ml) for *F. asiatica* for the duration of the treatment (Bowser et al. 2009; Kosoff et al. 2009). In these studies, the mean T5 (midpoint of the 10-d treatment period) concentrations of florfenicol in serum (µg/mL) and florfenicol residue in muscle–skin (µg/g) where 7.14 ± 2.49 and 9.98 ± 2.23, respectively, in 100 g tilapia fed the medicated feed (Bowser et al. 2009).

The *in vivo* results showed that florfenicol treatment, initiated either one or three days post-challenge significantly decreased mortalities (p<0.001) to 0 and 13% (respectively), whereas 70% mortality was observed in the challenged non-medicated group. Treatment of francisellosis with florfenicol medicated feed prevented the development of an acute lethal form of disease, but was unable to provide complete clearance of the bacterial infection. By 30 days post-infection, bacteria were largely cleared from the spleen of fish feed at one or three days post-infection. Bacteriological analysis of splenic tissue of survivors demonstrated significantly (p<0.001) reduced bacterial numbers in spleen from medicated fish 30 days post-challenge, as compared with the control group. No significant differences were observed between the

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challenged non-medicated group and the fish that were given medicated feed 6 days post-challenge. We speculate this can be attributed to disease-induced anorexia, which began around four days post-challenge. By six days post-challenge, fish were not eating enough to achieve the desired dose, resulting in an ineffective treatment. This is in agreement with previously published work, where sunshine bass infected with *Streptococcus iniae* became anorexic two days post-infection. As a result, medicated feed administered after fish became anorexic was ineffective at treating the infection (Darwish 2007).

The pharmacokinetics of an antimicrobial in a given host species, at a given water temperature, relative to the *in vitro* MIC of the drug to the pathogen, theoretically should determine the outcome of therapeutic intervention, however in fish culture, other factors such as stressful environmental conditions may play a role. Also the ability of the bacteria to reside in privileged intracellular sites, should be considered as an important factor in determining effective treatments as these sites are not readily accessible to many antimicrobials. In *in vitro* experiments with THKDM, where macrophages were exposed to different concentrations of florfenicol for 48 h, a significant reduction ($p<0.001$) in the number of intracellular *F. asiatica* were obtained at florfenicol concentrations of 10 and 100 µg/ml in the extracellular environment (Figure. 7.3). Moreover, significant reduction ($p<0.001$) in cytopathogenesis was observed 24 and 48 h post-infection in THKDM that contained 10 or 100 µg/ml of florfenicol in the extracellular environment compared to the control group containing no florfenicol (Figure 7.4). Similar results were found in murine macrophages, where significant reductions of viable intracellular non-typhoid *Salmonella* were observed at extracellular chloramphenicol concentrations equal to or 10 times greater than the MIC (Chiu et al. 1999). Reduction of intracellular *Salmonella enterica* serovar *Typhimurium* PT99 was also observed in infected pigeon macrophages, although high concentrations of the antibiotic (>16 µg/ml) were required in

the extracellular environment (Pasmans et al. 2008). Various antibiotics like aminoglycosides, tetracyclines, fluoroquinolones, rifampin, and telithromycin have demonstrated bactericidal capacity and efficacy in killing intracellular *F. tularensis* in infected murine macrophage cells (Maurin et al. 2000). On the other hand penicillin G, amoxicillin, ceftriaxine, thiamphenicol, and erythromycin failed to display any significant activity against intracellular *F. tularensis* compared to drug-free controls (Maurin et al. 2000). No report has been made concerning the efficacy of florfenicol in the treatment of *F. tularensis* in mammals.

As previously described, tilapia receiving medicated feed at 15 mg/kg body weight, contained florfenicol concentrations greater than 10 µg/ml in tilapia serum, muscle and skin (Bowser et al. 2009, Kosoff et al. 2009), which is significantly greater than the 2 µg/ml MIC determined in this study. Moreover, in less than 24 h after a single oral dose of 10 mg/kg body weight of florfenicol, freshwater reared tilapia presented 5.21 µg/g, 5.27 µg/g, 4.59 µg/g and 5.50 µg/g concentrations of the antibiotic in the liver, gill, muscle and kidney, respectively (Feng et al. 2008). All these data suggests that florfenicol concentrations in medicated feed, as was used in this study, are sufficient to penetrate the intracellular environment and control infection in a dose dependent manner. Interestingly, concentrations as high as 100 µg/ml of florfenicol in the cultured media, did not affect survival of the THKDM, as demonstrated by the amount of LDH released in the medium (data not shown). Thus a higher concentration of antibiotic in the feed could potentially eliminate the persistent bacteria found in splenic tissue 30 days post-challenge. The effect of higher doses of drug in the feed on palatability is unknown.

In conclusion, florfenicol administered in medicated feed initiated at one day post-infection and fed daily for 10 days, significantly reduces mortalities in tilapia experimentally infected with *F. asiatica* and prevents dissemination of the bacterium to hematopoietic organs. No pathological changes occur and reduced numbers of bacteria remain in the spleen 30 days

post challenge. Conversely, administration of medicated feed for 10 days beginning 3-6 days post-infection led to the development of a chronic, non-lethal infection suggesting *F. asiatica* may have the propensity for latency. In these cases, the main reason for the ineffectiveness of the treatment seems to be associated with the anorexic condition that the diseases fish develop. The results from this study suggests that the infection could be contained or eliminated if early antibiotic treatment (<6 days post-infection) was initiated, preventing the bacterial load from reaching a lethal level in the host. Recently, an *iglC* based Taq-Man real-time PCR assay with high sensitivity and specificity for the detection and quantification *F. asiatica* has been developed (Soto et al. 2010). The assay can potentially by used as a rapid diagnostic test for francisellosis, with the great benefit of fast turnaround of results (hours), which can aid the producer and diagnostician in starting medicated feed protocols early; thus preventing the anorexic manifestation of sub-acute to chronic diseases fish.

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CHAPTER 8

CONCLUSIONS

Francisella asiatica is an emergent and important pathogen for fresh and marine aquaculture worldwide. This study provides methods for the culture, identification and initial description of the pathogenesis of *F. asiatica* in tilapia. Identification of homologous genes of the *F. tularensis* pathogenicity island in *F. asiatica* provided a basis for investigation of potential virulence genes. It also provides useful information on the development of a reliable real time PCR method for rapid diagnosis of the pathogen from infected tissue; and also demonstrates the use of an aquaculture approved drug for treatment of fish francisellosis. Finally, in this study, I demonstrated the potential use of an attenuated live vaccine for the prevention of *F. asiatica* infection in tilapia.

Members of the genus *Francisella* sp., are fastidious facultative bacteria that have been found to infect a great variety of animals (including humans), but very little is known regarding the virulence mechanisms and virulence factors of this genus (Barker and Klose 2007; Keim et al. 2007). *Francisella asiatica* was found to be one of the more virulent bacteria to affect the tilapia aquaculture industry. It was demonstrated that as few as 23 *F. asiatica* bacteria injected in the peritoneum are capable of causing mortalities in tilapia nilotica (*Oreochromis niloticus*), and that even fewer are enough to cause serious pathological lesions in important organs like the head kidney and spleen. This finding wasn't surprising; since a member of the same genus (*F. tularensis* subsp. *tularensis)* is extremely virulent in mammals and as few as 10 colony-forming units (CFU) are sufficient to cause mortalities in humans (McLendon et al. 2006).

In order to elucidate the pathogenicity of *F. asiatica* in tilapia, and to find a reliable and repetitive method for challenging fish with this pathogen, two different administration routes for challenging tilapia with *F. asiatica* were compared. The intra - peritoneal (IP) challenge was

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chosen since it was an easy and quick method to accurately administer suspended bacteria, but several problems developed when administering the bacteria by this method, including the lack of exposure of the bacteria to innate immune protection present in the skin, gills and other mucosa. As was expected, an acute onset of the disease was observed, with high mortalities and few clinical signs in the fish receiving the higher dosage. The low dose of bacteria, ~2 CFU injected into the peritoneum of the fingerlings, was able to cause mortalities. Even more surprising, was the amount and severity of lesions (granulomas) caused by a very low number of bacteria (~1 CFU/fish) in important hematopoietic and osmoregulatory organs like the spleen and the anterior kidney. Survivors of this treatment were observed with significant lesions in spleen, head kidney and liver, which will not only impair the fish's ability to osmoregulate, but will also result in immunosuppression by direct damage of their hematopoietic organs thus making them more susceptible to other important and common tilapia diseases seen in culture facilities such as streptococcosis, vibriosis and columnaris disease. The immersion challenge (IC) route was chosen because it more closely resembles a natural infection. This method, where the bacteria have to come into close contact with the innate immune system present in skin, gills, gastrointestinal mucosa, etc., mimics the way the disease progresses in nature. As expected, the amount of bacteria needed to cause mortality was higher than in the IP treatment, and the onset of the disease was more sub-acute to chronic, presenting anorexia, change in coloration, and pale gills. A dose of 2.3 x 10^2 CFU/ml of tank water was needed to cause mortality in the immersed fish, but when analyzing histopathological lesions of the survivors, it was evident that even a dose of 23 CFU/ml of tank water was able to cause significant lesions in the spleen and head kidney. The experiment was terminated 40 days after exposure to the bacterium, but it is suspected that the survivors of this trial may become carriers of the pathogen as seen in natural infections.

The capability of *F. tularensis* to multiply intracellularly is well documented in insects, as well as in a broad range of mammals, including rabbits, rodents, beavers, and man (Brotcke et al. 2006; Clemens and Horwitz 2007). In the case of *F. asiatica,* the ability to survive inside a wide variety of fish cells was hypothesized, but this conclusion was based only on histopathological analysis of infected tissue in natural cases (Ostland et al. 2006; Mauel et al. 2007). In the present study, *F. asiatica* was found to be capable of intracellular survival and replication within both 4h and 5d-old head kidney derived macrophages (HKDM) from tilapia. Effective internalization by both cell types was partially mediated by a heat-sensitive serum component, presumably complement, also, uptake of the bacterium by both HKDMs populations following opsonization with tilapia NS was significantly greater than uptake following pre-treatment with HINS, indicating involvement of complement and the complement receptor. It has been previously demonstrated that monocyte-derived macrophages (MDM) phagocytose more *F. tularensis* than monocytes, with a major contribution from the mannose receptor (MR) on MDM (Balagopal et al. 2006; Schulert and Allen 2006). Our results also indicated a substantial involvement of the MR in uptake of *F. asiatica* by HKDM. Other receptors demonstrated to be involved in the uptake of *F. tularensis* by mammalian macrophages, include Fc γ receptors, pulmonary collectins surfactant proteins, and type I and II class A scavangers (Balagopal et al. 2006; Schulert and Allen 2006). The difference between tilapia HKDM and the human blood-borne monocytes could be a result of the differential maturation of HKDM, but a clear involvement of both the CR and the MR was observed in both 4h- and 5D-HKDM. The combination of mannan pretreatment and opsonization with HINS reduced uptake of *F. asiatica* in 5D-HKDM by 99.7 % of uptake following NS treatment, indicating that the primary receptors involved are the CR and MR. This is in contrast to *F. tularensis*, where additional receptors were suspected for MDM (Balagopal et al. 2006; Schulert and Allen 2006). Differences between

the uptake of *F. asiatica* by 4h and 5d-HKDM indicates that the presence or absence of

receptors, like the MR, plays a role in the uptake of bacteria by fish phagocytes. In mammals,

uptake of *F. tularensis* by MDM was greater than by monocytes, presumably because the MR

are more abundant or more recently expressed on mature macrophages compared to monocytes.

More research is needed to elucidate the role of different bacterial receptors in fish mononuclear

cells. After uptake, regardless of whether the bacteria were pre-opsonized with normal serum,

heat inactivated serum, intracellular replication was equal in either HKDM population, similar to

the situation in *F. tularensis* (Nano et al. 2004; Nano and Schmerk 2007).

It is well described that several genes provide *F. tularensis* with properties for survival in

the extracellular compartment, and also for survival and multiplication inside of potent

phagocytes like neutrophils and macrophages (Baron and Nano 1998; Allen 2003; Nano et al.

2004). The *igl*ABCD operon was described by Nano et al. (2004) as part of the *F. tularensis*

pathogenicity island (FPI). The ability to survive inside macrophages and the presence of a Type

VI secretion system are two of the most important virulence factors described for this important

human pathogen present in the FPI. Several homologues to the *F. tularensis iglA, iglB, iglC*, and

iglD genes were found present in *F. asiatica* strain LADL 07-285A, isolated from diseased

tilapia. DNA sequence comparison between *F. asiatica* LADL 07-285A, *F. philomiragia subsp.*

philomiragia and *F. tularensis* subsp. *novicida* U112 *igl*ABCD operon, revealed > 83% identity

between the different *Francisella* sp. The functions of the conserved proteins corresponding to

the genes are elusive. Overall, Igl proteins appear to be essential for the ability of *F. tularensis*

to survive inside the macrophages and cause disease (Golovliov et al. 1997; Nano et al. 2004; Lai

et al. 2004; Lauriano et al. 2004; Santic et al. 2005; Brotcke et al. 2006; de Bruin et al. 2007).

Mutations of these four genes in *F. tularensis*, have shown decreased pathogenicity of the

bacterium both in-vivo and in-vitro in mammalian and insect tissues and cell lines (Lauriano et

al. 2003; Nano et al. 2004; de Bruin et al. 2007; Vonkavaara et al. 2008). It has been reported

that in *F. tularensis,* Δ*iglC* mutant strains are defective for survival and replication within

mammalian macrophages. Expression of *iglC* was induced during growth of *F. tularensis* in

macrophages, and was required for intracellular multiplication in macrophages and for virulence

in mice (Golovliov et al. 1997; Nano et al. 2004; Lai et al. 2004; Lauriano et al. 2004; Santic et

al. 2005; Brotcke et al. 2006; de Bruin et al. 2007). Inactivation of the *iglC* and *mglA* gene of *F.*

tularensis also abolishes its capacity to escape from the phagosome into the cytoplasm and to

multiply intracellularly in mouse peritoneal exudate macrophages (Lauriano et al. 2004; Santic et

al. 2005). After 24 h of infection with *F. tularensis*, the murine macrophage-like cell line

J774.A1 underwent apoptosis and pronounced cytopathogenesis. Further work by the same

group demonstrated that a *F. tularensis* Δ*iglC* mutant did not induce apoptosis in infected cells,

suggesting an involvement of IglC in the induction of apoptosis in *F. tularensis* infected

macrophages (Lai et al. 2001; Lai et al. 2004).

Insertional mutagenesis allowed us to select for a double recombination in the *F asiatica*

*igl*C gene, and as in *F. tularensis* subspecies, the *igl*C mutation significantly attenuates the

pathogen upon *in vivo* challenges, and increases the survival rates of the mutant infected fish

when compared with the wild type infected fish after both IP and IC challenges. Further work

demonstrated that *iglC* is required for intracellular survival and growth in tilapia HKDM. Similar

to what has been found in *F. tularensis* infected macrophages, significantly greater lactate

dehydrogenases (LDH) levels in supernatants of tilapia HKDM infected with the wild type and

the IglC complemented *Francisella* strains were found than in the Δ*iglC* mutant. The Δ*iglC*

mutant strain also induced significantly lower caspase 3/7 activity, similar levels as that of the

uninfected cells.

Since it was noted that the *F. asiatica* Δ*iglC* mutant strain wasn't causing any pathological changes in the tilapia *in vivo*; and that *in vitro* it persisted for several days inside HKDM; the efficacy of a Δ*iglC* mutant strain to protect tilapia against immersion challenge with the *F. asiatica* WT strain was evaluated. A live attenuated vaccine given to the fish by the immersion route, has the advantage of directly targeting the natural routes of attachment and penetration of the bacteria into the fish and hence inducing protective immunity at the primary site of infection. Results showed that an immersion vaccination with Δ*iglC* mutant significantly protects tilapia fingerlings against homologous *F. asiatica* immersion challenge. Our results show that the Δ*iglC* stimulated a systemic antibody response as demonstrated by the high antibody titers found in serum and skin mucus of vaccinated fish. Moreover, naive tilapia vaccinated by immersion with a suspension of the Δ*iglC* and subsequently challenged with wild-type (WT) *F. asiatica* were protected (90% survival) from the lethal challenges. *F. asiatica*-specific antibodies produced in response to immunization with the Δ*iglC* were subsequently found to protect naive tilapia against high-dose *F. asiatica* challenge in passive immunization experiments. Significant protection ($p<0.001$) was obtained when fish were passively immunized and challenged with 10^4 and 10^5 CFU/fish of WT *F. asiatica*; but not when challenged with 10^6 CFU/fish. Further work is necessary to elucidate the role of cell mediated immunity in the protection of tilapia upon infection with *F. asiatica*.

Due to the emergent nature of francisellosis in fish, there is currently very little published data regarding antibiotic susceptibility of *Francisella* spp. *in vivo* or *in vitro*, and at present there are no known efficacious chemotherapeutants available. Florfenicol is a fluorinated derivative of thiamphenicol that blocks the peptidyltransferase at the 50S ribosome subunit and acts against a wide variety of both gram-positive and gram-negative bacteria. As a medicated feed, florfenicol (AQUAFLOR®) has been used to treat a wide variety of fish diseases in various warm and cold

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water cultured fish species, including *Vibrio anguillarum*, *Aeromonas salmonicida*, *Streptococcus iniae*, *Listonella anguillarum*, and *Edwardsiella ictaluri*, amongst others. The use of florfenicol for treatment of *F. asiatica* infection in tilapia was also investigated during this work. The results obtained from the minimal inhibitory concentration (MIC) determinations demonstrated that florfenicol is a suitable candidate for *in vivo* experimentation, since a low minimal inhibitory concentration (2µg/ml) was found. In *in vitro* experiments with THKDM, where macrophages were exposed to different concentrations of florfenicol for 48 h, a significant reduction (p<0.001) in the number of intracellular *F. asiatica* were obtained at florfenicol concentrations of 10 and 100 µg/ml in the extracellular environment. Moreover, significant reduction (p<0.001) in cytopathogenesis was observed 24 and 48 h post-infection in THKDM that contained 10 or 100 µg/ml of florfenicol in the extracellular environment compared to the control group containing no florfenicol. *In vivo*, it was found that florfenicol administered in medicated feed initiated at 1 day post-infection and fed daily for 10 days, significantly reduced mortalities in tilapia experimentally infected with *F. asiatica* and prevents dissemination of the bacterium to hematopoietic organs. No pathological changes occur and reduced numbers of bacteria remain in the spleen 30 days post challenge. Conversely, administration of medicated feed for 10 days beginning 3-6 days post-infection led to the development of a chronic, non-lethal infection suggesting *F. asiatica* may have the propensity for latency. This suggests the infection could be contained or eliminated if very early antibiotic treatment (<6 days post-infection) was initiated, preventing the bacterial load from reaching a lethal level in the host.

The diagnosis of this highly virulent fish pathogen has many constraints including the fastidious nature of the bacterium, and the lack of biochemical, molecular, and serological tests specific for this aquatic animal pathogen; thus we developed a TaqMan real-time quantitative PCR assay for the rapid identification and quantification of this emergent fish pathogen. The

target region of the assay was the *F. tularensis iglC* gene homologue previously found in *F. asiatica*. Probe specificity was confirmed by the lack of signal and cross-reactivity with twelve common fish pathogens, two subspecies of *F. tularensis*, *F. noatunensis*, and tilapia tissue. The range of linearity was determined to be 50 fg to 1.4 mg, and the lower limit of detection was 50 fg of DNA (equivalent to ~25 genome equivalents (GE)) per reaction. A similar sensitivity was observed with DNA extracted from a mixture of *F.asiatica* and fish tissue. The assay was also able to detect and quantify *F. asiatica* from the spleens of experimentally infected tilapia. The development of this highly sensitive diagnostic method will enhance the diagnosis of this fastidious organism that could be present at low levels in fish tissue, may require specialized media to grow, and may possibly be overgrown by secondary contaminants following attempts at primary isolation. The assay can be used not only as a rapid diagnostic test for francisellosis, but can also be used as a research tool for bacterial persistence, drug therapy efficacy, epidemiological studies, screening of broodstock fish, and detection of reservoirs for infection.

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APPENDIX 1

JOURNAL OF FISH DISEASE PERMISSION

LSU

Journal of Fish Diseases permission
2 messages

Esteban Soto <esoto1@tigers.lsu.edu>   Fri, Mar 12, 2010 at 5:00 PM
To: rw3@stirling.ac.uk, heronpisces@btinternet.com

Dear Drs. Wooten and Roberts,

I hope everything is good. Our group published a manuscript in Journal of Fish Diseases (2009, Volume 32 Issue 8) with the title *Francisella* **sp., an emerging pathogen of tilapia,** *Oreochromis niloticus* **(L.), in Costa Rica (p 713-722).** This report was part of my phd dissertation research at Louisiana State University. In order for me to be able to include it in my dissertation I need a letter or email from the journal or copyright holder allowing me to add this data.

Could either of you please send me this letter. I greatly appreciate the help,

Esteban Soto

Rodney Wootten <rodney.wootten@stir.ac.uk>   Mon, Mar 15, 2010 at 4:37 AM
To: Esteban Soto <esoto1@tigers.lsu.edu>
Cc: RONALD ROBERTS <heronpisces@btinternet.com>

Dear Mr Soto

We are pleased to give permission for you to include data from your JFD paper (Vol 32, pp. 713 722) in your dissertation. I wish you the best of luck with your PhD.

Best wishes

Rod Wootten

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APPENDIX 2

JOURNAL OF AQUATIC ANIMAL HEALTH PERMISSION

Journal of Aquatic Animal Health Permission
2 messages

Esteban Soto <esoto1@tigers.lsu.edu>   Wed, Mar 17, 2010 at 11:23 AM
To: alerner@fisheries.org

Dear Mr. Lerner,

We published a paper with the title: "Attenuation of the fish pathogen Francisella sp. by mutation of the iglC* gene", in the Journal of Aquatic Animal Health 2009 Sep;21(3):140-9; and need a written permission from the Journal, which states that I can use the published material as part of my dissertation.

Could you please help me with this,

I greatly appreciate the help,

Esteban Soto

Aaron Lerner <alerner@fisheries.org>   Wed, Mar 17, 2010 at 11:37 AM
To: Esteban Soto <esoto1@tigers.lsu.edu>

Dear Mr. Soto,

The American Fisheries Society is pleased to grant you permission to use the below-referenced paper published in the Journal of Aquatic Animal Health 2009 21:3, 140-149, as part of your dissertation.
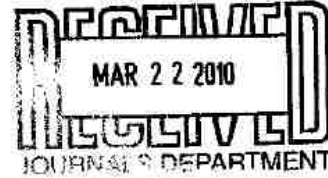

Aaron

APPENDIX 3

INFECTION AND IMMUNITY PERMISSION

Luckey, Adar

From:	Esteban Soto [esoto1@tigers.lsu.edu]
Sent:	Thursday, March 18, 2010 3:44 PM
To:	Luckey, Adar; Smith, Diane
Subject:	IAI Copyright permission
Follow Up Flag:	Follow up
Flag Status:	Red

To whom it may concern,

My name is Esteban Soto. We have a manuscript that was published online by IAI (IAI.01308-09) with the title Interaction of Francisella asiatica with Tilapia ....; and this project was part of my dissertation at LSU. The University needs a written permission by the Journal or copyright holder for me to include it in my dissertation. I already contacted the Copyright clearance center, and they told me that since the volume hasn't been published the permission has to come from the Journal. Dianne Smith told me to send you a email and see if you could send me this permission. With a email will be more than fine.

Thanks for the help,

Esteban Soto

PERMISSION GRANTED
CONTINGENT ON AUTHOR PERMISSION (which you must obtain)
AND APPROPRIATE CREDIT
American Society for Microbiology
Journals Department
Barbara M. Gillen Date 3/22/10

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APPENDIX 4

DISEASES OF AQUATIC ORGANISMS PERMISSION

Re: Diseases of Aquatic organisms permission, D 2204

1 message

Marianne Hiller <marianne@int-res.com>   Mon, Mar 15, 2010 at 10:33 AM
To: Esteban Soto <esoto1@tigers.lsu.edu>

Dear Dr. Soto,

We herewith give publisher permission for you to use the manuscript mentioned below in your PhD thesis. Please add the correct citation when the thesis is ready for printing.

Kind regards
Inter-Research
Marianne Hiller
Permission Department

==
Inter-Research Science Center
Nordbuente 23
D-21385 Oldendorf/Luhe
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Tel: (+49) (4132) 7127   Email: Marianne@int-res.com
Fax: (+49) (4132) 8883   http://www.int-res.com/
==

VITA

Esteban Soto was born in January, 1981, in San Jose, Costa Rica. His father Dr. Manuel E. Soto Quiros and mother Dr. Cecilia M. Martinez Sequeira raised Esteban in Heredia, Costa Rica. After graduation from Saint Paul's High School in 1997, he enrolled the School of Veterinary Medicine at the Universidad Nacional of Costa Rica in 1998. Esteban finished his veterinary education in 2005. That same year he left Costa Rica and moved to Mississippi, to pursue a master's degree at the College of Veterinary Medicine at the Mississippi State University under the guidance of Dr. Michael Mauel and Dr. Mark L. Lawrence studying the genetic and virulence diversity of the fish pathogen *Flavobacterium columnare*. Esteban finished his master's education on August 2007; and moved to Baton Rouge, Louisiana, to pursue his doctorate in The School of Veterinary Medical Sciences through the Department of Pathobiological Sciences under the guidance of Dr. John Hawke. In July 2008, Esteban married Tatiana Bonilla in Heredia, Costa Rica. Esteban finished his dissertation research and completed the requirements for the degree of Doctor of Philosophy on May 2010; and plans to graduate in August 2010.