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INCIDENCE AND PREVALENCE OF FRANCISELLA NOATUNENSIS SUBSP. ORIENTALIS (FNO) PATHOGENIC INFECTIONS IN FERAL HAWAIIAN TILAPIA

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INCIDENCE AND PREVALENCE OF *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS* (*FNO*) PATHOGENIC INFECTIONS IN FERAL HAWAIIAN TILAPIA

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TABLE OF CONTENTS

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ABSTRACT

Aquaculture has become a major food source for many countries and continues to grow each year. However, many of the fishes that are farmed are susceptible to pathogenic infections such as Francisellosis, a bacterial infection that contributes to disease and high mortality rate in many populations of farmed and wild fish around the world. In 1994, feral and farmed populations of tilapia (e.g. *Oreochromis mossambicus*) on Oahu, Hawaii were found to be infected with a novel Francisellosis strain, *Francisella noatunensis* subsp. *orientalis* (*Fno*). To prevent the spread of this pathogen, the Hawaii Department of Aquaculture (HDOA) initiated a ban on the exportation of tilapia to other Hawaiian Islands. To evaluate the effect of this ban and determine the prevalence of *Fno* throughout Hawaii, the goal of this study was to investigate the occurrence of *Fno* infections in wild tilapia on Oahu and the other islands using histological as well as molecular methods. Tissue samples from 222 tilapias collected on the islands of Oahu, Molokai, Kauai, Maui, and Molokai were screened via histology for evidence of granulomas and by real-time PCR for *Fno* bacterial DNA. Histological and molecular evidence of *Fno* infections were found in fish from Oahu, Kauai and Maui. This is the first report of *Fno* from the islands of Kauai and Maui suggesting that the quarantine efforts were not successful. However, we cannot rule out the possibility that infected fish were imported from outside of Hawaii. These findings have the potential to assist in the development of a disease management program for *Fno* that may result in an overall improvement in tilapia production throughout Hawaii.

LIST OF TABLES

Page

LIST OF FIGURES

Page

INTRODUCTION

Modern Economic Impacts of Aquaculture

In a world containing over seven billion people, food sources are vital to fulfill the nutritional requirements of the human population. As the population grows, the demand for sustainable food resources also increases (Kobayashi et al. 2015; Grealis et al. 2017). Food sources can come from hunting, raising livestock, and farming. In addition, fish are also an excellent nutritional source, as they provide high sources of protein, Omega-3-fatty acids, and vitamins essential to the human diet. According to the Food and Agriculture Organization (FAO), fisheries and aquaculture have been a major source of food, nutrition, income and livelihood for millions of humans around the world (2016). As fish have become an increasingly common food that has been incorporated into the human diet over the years, fish production for human consumption has increased significantly (*i.e.* 20%) in the last several decades (FAO 2016).

Aquaculture, the cultivation of aquatic plants and animals for human consumption, dates back to 2000 B.C. (Rabanal 1998) and today it has been estimated that nearly half of the world's seafood consumption comes from aquaculture (FAO 2018). In 2014, the global aquaculture industry produced an estimated 73.4 million tons of aquatic animals valued at 160.2 billion US dollars (FAO 2016). However, in 2016, aquaculture produced over 80 million tons of aquatic animals (232 billion dollars) (FAO 2018). According to the National Oceanic and Atmospheric Administration, the US produced 1.45 billion dollars in fish products (633 million pounds) in 2016 (NOAA 2019).

In past decades, Hawaii has shown growth in aquaculture, with eleven major aquaculture parks across the five major islands (Soto et al. 2013; State of Hawaii 2019). The development of

aquaculture in Hawaii allows Hawaiians to farm their own fish and generate income within the state, thereby creating jobs, which will help combat their reliance on imported seafood (Arita and Leung 2014; State of Hawaii: Animal Division 2017). Hawaiians are not the only people that consume fish as tourist visit Hawaii for high quality seafood that is fresh and delicious (State of Hawaii: Animal Division 2017). The total cost of seafood consumption between local Hawaiians and tourists was an estimated $664 million dollars in 2005 (State of Hawaii: Animal Division 2017).

Hawaii has many different environments that allows for the cultivation of many species of fish. Currently, Hawaii cultivates: Red and Japanese Abalone (*Haliotus refens* and *H. discus hanai* respectively), Broodstock and juvenile shrimp (*Litopenaeus vanamei, L. stylirostris* and *Penaeus monodon*), catfish (*Clarius fuscus*), prawn (*Macrobrachium rosenbergii*), kahala amberjack (*Seriola rivoliana*), milkfish (*Chanos chanos*), rainbow trout (*Oncorhynchus mykiss*), and several species of tilapia (State of Hawaii: Animal Division 2017). In 2011, Hawaii cultivated over 40 million US dollars worth of aquaculture products, 10 million US dollars more than the previous year (Soto et al. 2013; State of Hawaii: Animal Division 2017).

Tilapia Importance in Hawaii

Of the fishes Hawaii currently cultivates, tilapia plays an important role as a high selling product and is the second highest selling fish in global aquaculture production (Guyon et al. 2012; Klinger-Bowen et al. 2016). Tilapia are a group of freshwater fish within the family Cichlidae, which contain over 3000 different species found in both tropical and subtropical regions (Guyon et. al. 2012). The abundance of different tilapia species is one reason that makes tilapia so valuable to the aquaculture industry. Given their year round reproduction, ability to adapt to varying environmental conditions (temperature ranges of 12-43 °C, pH range of 6-10,

salt concentrations between 0-135 mg/L, and oxygen levels between 0.3-1.5 mg/mL) and resistance to a number of bacterial, viral, and parasitic infections, tilapia are an ideal species to cultivate (Guyon et al. 2012). Tilapia have increasingly been shown to replace cod and members of the cod family (e.g. hake) and have become a desirable product for many consumers (Costa-Pierce 2003).

Tilapia, such as the Mozambique tilapia (*Oreochromis mossambicus*), were first introduced into the Hawaiian Islands as a means of vegetation control and as bait for tuna fisheries in the 1950s and 1960s (Wu and Yang 2012). Later, additional tilapia species (e.g. Oreochromis species) were brought to Hawaii for research purposes from the United States mainland in the 1990s (Guyan et al. 2012; Wu and Yang 2012). Tilapia are used in various research programs that focus on their physiology, endocrinology, immunology, toxicology, and genetics (Guyon et al. 2012). Although, additional tilapia strains may have originally been brought to Hawaii for other purposes (e.g., ornamental pet trade), they have now become a vital part of the aquaculture and fisheries industry in Hawaii.

Aquaculture Biosecurity

Like every food source, fishes are susceptible to infectious pathogens that can have negative consequences for the entire aquaculture industry. There are many waterborne pathogens, such as Nodaviruses, *Amyloodinium ocellatum*, *Vibrio* species, *Mycobacterium* species, and *Moritella viscosa*., that can lead to health complications and mortality in many commonly cultured fish species (Lafferty et al. 2015). Aquaculture has suffered economic losses due to diseases in the past. For example, between 1992 and 1993 Asia lost approximately six billion US dollars to *Whispovirus* that infected Penaeid shrimp (Lafferty et al. 2015). The severity and pathogenicity of different pathogens vary, but one fish can infect the whole

aquaculture facility. As the aquaculture business continues to rise, the occurrence of waterborne pathogens that target fish such as tilapia, will rise accordingly, and has the potential to lead to production and economic losses.

Aquacultures must take preventative measures to decrease the presence of pathogens in their facilities. Bacteria, fungi and viruses are major pathogens that plague aquaculture facilities (Pridgeon and Klesius 2012). Biosecurity in aquaculture facilities depends on preventing the introduction of pathogens (bioexclusion) into aquaculture facilities (Caraguel et al. 2015). There are many vectors for pathogens to enter aquaculture facilities, such as fomites (e.g. equipment), food, employees, and the animals themselves (Caraguel et al. 2015). Having pathogen-free water is vital to aquacultures. In order to ensure facilities are healthy, measures such as feeding fish antimicrobial food, disinfection of water, and vaccinations are implemented (Pridgeon and Klesius 2012). In addition, ensuring fish that are used to restock populations are pathogen free are also aids in prevention of infection (Jeffery et al. 2010). However, these methods can have limitations and cannot guarantee the prevention of possible infections.

To detect the presence of pathogens, aquacultures rely on diagnostic tests more than clinical evidence as it is unrealistic and extremely difficult inspect every fish in a facility. These tests can include quarantine of potential infected fish (Caraguel et al. 2015). Unfortunately, diagnostic tests have the risk of inaccurately diagnosing the health status of fish (Caraguel et al. 2015). These inaccurate fish inspections can result in false positives as well as false negatives. A false negative may occur when a fish is infected, but asymptomatic. The dangers of having false negatives is that infected fishes may infect other fishes in the facility and the pathogen can quickly spread. Additionally, any new fish that are added to the stock can become infected as well. Furthermore, infected fishes can be transported to other stock or facilities, where the

infection will continue to spread. False positives can actually have more damaging impacts on aquaculture facilities than false negatives. Only aquacultures designated disease free are certified and are able to implement biosecurity barriers within their facilities (Caraguel et al. 2015). False positives can negatively impact a facility's certification, which makes it harder to implement biosecurity barriers in their facility (Caraguel et al. 2015).

Secondly, unknown or undiscovered pathogens can be hard to detect or implement/create biosecurity protocols for. For example, clinical symptoms associated with an infection could be common to a variety of pathogens. Regardless, new pathogens are very dangerous given the lack of knowledge on the infectious pathogen and the disease associated with it. Thus, the implementation of biosecurity plans is more difficult as there are no known protocols that are effective against the introduction of the pathogens, how the pathogens may have entered the facility or treatments/medicines that can be used to boost the fish population immunity to the future outbreaks.

Francisellosis in Tilapia

In 1994, Hawaii experienced an outbreak of a novel pathogen when wild and cultivated populations of tilapia in Oahu experienced high rates of mortality due to an unknown infection (Mauel et al. 2003; Klinger-Bowen et al. 2012). The pathogen was initially suspected to be *Piscirickettsia salmonis*, but serological testing was negative (Mauel et al. 2003). Unfortunately, isolation and complete characterization was not possible in 1994 due to limited knowledge. However, mortality events as well as clinical and histopathological findings were later found to be consistent with Francisellosis, a bacterial infection caused by the *Francisella* species, particularly *Francisella noatunensis* subsp. *noatunensis* and *Francisella noatunensis* subsp. *orientalis* (*Fno*) (Soto et al. 2013).

Francisella species are gram-negative, pleomorphic (ability to change shape and size in response to environmental conditions), non-motile, aerobic (obligate) facultative bacteria (Klinger-Bowen et. al. 2016). *Francisella* species, particularly *Fno*, causes high mortality rates in wild and cultured warm water fishes. In the past decade, *Fno* has contributed to mortality events and disease outbreaks in geographical locations such as Asia, Europe, North America and Central America, Latin America, the Caribbean Islands, and the Pacific Islands (Soto et al. 2010b; Soto et al. 2013). Francisellosis has been found in many animals including over 200 different mammals, crustaceans, birds, reptiles, and has been found in both water and soil samples (Soto et al. 2009a). In addition, *Francisella* species, including Fno, have been shown to persist in both fresh and salt-water, allowing transmission through currents (Duodu and Colquhoun 2010; Soto and Revan 2012).

Francisella species can infect their host by penetrating the skin or through cuts resulting from injury (Hsieh et al. 2006). Once infected, wild and farmed fishes may display few clinical signs, such as low blood count, abnormally low body weight, and bulging of the eye (Soto et al. 2010a). However, there have been cases where infected fishes are asymptomatic, but experience high mortality rates (Soto et al. 2010a). Fish affected with *Fno* appear to show abnormalities in the gills, heart, brain, spleen, liver, gastrointestinal tract, choroid glands, central nervous and kidneys, but these vary in severity (Soto et al. 2009a). Infected organs (i.e. kidneys and spleen) typically exhibits enlarged size and contain multifocal white nodules and multifocal granulomatous lesions (Hsieh et al. 2007; Soto et al. 2009a; Soto et al. 2010a)

In histopathological findings, the most severe change is the formation of granulomatous inflammation in almost all organs, with a large number of macrophages containing small pleomorphic coccobacilli. Moreover, in response to granulomas, the gills exhibit primary and

secondary lamellar fusion, which results from new growth of the epithelial cells (epithelial hyperplasia) (Soto et al. 2009a). Severely infected fish exhibit uncontrollable and erratic swimming for up to ten minutes and die (Soto et al. 2009a). This may be caused by the granulomatous inflammation in the central nervous system, which caused similar behaviors in the first outbreaks of Hawaii in the 1990s (Mauel et al. 2003; Soto et al. 2009a). Temperature and fish size have been shown to be important risk factors for *Fno* infection (Soto et al. 2013). Recent research has demonstrated that fish maintained at 25 °C are more likely to develop Francisellosis than those maintained at higher temperatures (Soto et al. 2013). Additionally, smaller fish (e.g. 15 grams or less) have been found to be more susceptible to developing Francisellosis than larger fish (Soto et al. 2013; Klinger-Bowen et al. 2016).

Diagnosing Francisellosis in Tilapia

In the past, diagnosing fishes infected with Francisellosis was time-consuming and expensive, given the lack of molecular and serological techniques (Soto et al. 2010a). Culturing the bacteria has proven to be very difficult. This could be due to *Francisella* species ability to enter a viable, but non-culturable state (VBNC) where the pathogen is in a starvation state (Colquhoun and Duodu 2011; Duodu and Colquhoun 2010; Soto and Revan 2012). This state is temperature dependent and it has been demonstrated that warmer temperatures (e.g. 30 °C) can cause the bacteria to enter this state more readily (Soto et al. 2012). In this state, the pathogen does not divide and remains dormant. Once *Fno* has entered the VBNC state, it has been proven difficult to resuscitate the cells when providing the necessary nutrients and incubations in the optimal temperatures (Forsman et al. 2000).

Currently, diagnosis has been improved with histopathology, polymerase chain reaction (PCR), culture assays with cysteine rich agars, and *in situ* hybridization. However, these methods

are not always reliable (Soto et al. 2010a). For example, secondary infections of other marine pathogens found in fishes infected with *Fno*, can cross react with primers specific to the *Francisella* species when diagnosing Francisellosis via PCR (Soto et al. 2010a). In addition, many of the methods, such as PCR and culturing, are very time consuming and laborious, making screening large sample size difficult. Given these limitations, it is often difficult for aquaculture facilities to identify fishes infected with francisellosis. In addition, the negative economic impact the pathogen can cripple aquaculture facilities severely. Although there has been research on the development of vaccines to prevent Francisellosis, there is not a commercial vaccine for the bacterial infection (Pridgeon and Klesius 2012; Brudal et al. 2015). It is imperative that there are methods that allow for the accurate and rapid identification of *Francisella* species.

Methods, such as real-time PCR, have been used to detect many pathogens that affect marine organisms in aquaculture. Real-time PCR has been used to detect many pathogens in aquaculture such as: Abdominal Segment Deformity Disease (ASDD) in *Penaeus vannamei* (Flegel 2009), Nervous Necrosis Virus (NNV) in grouper (Kuo et al. 2011), and Viral Haemorrhagic Septicaemia Virus (VHSV) in salmon (Matejusova et al. 2008). In addition, real-time PCR has been used for diagnosing other microbial pathogens (e.g. *Mycobacterium* species, *Francisella tularensis*, non-culturable microbes, and *Rickesttsia* species) in fishes and has been used for the quantification and identification of the *Francisella* species with a high degree of confidence (Soto et al. 2010a). Characterization of the gene 16S rRNA has shown that *Francisella* spp. have high levels of similarity, up to 99.9% (Forsman et al. 1994). However, the real-time PCR assay is specific to *Fno* representative isolates and does not amplify *Francisella*

noatunensis subsp. *noatunensis*, which is very closely related (Forsman et al. 1994; Lai et al. 2004; Soto et al. 2010a).

Soto and colleagues utilized a unique segment of DNA in for a real-time PCR target (Soto et al. 2010a). The intracellular growth genes have been identified in *Francisella* species as part of a 30kb pathogenicity island or the *Francisella* pathogenicity island (FPI) consisting of 16 to 19 genes and is duplicated in some strains (Nano and Schmerk 2007; Soto et al. 2010a). Simplistically, the open reading frame (ORF) of the FPI is effectively the same in all the *Francisella* species and subsp. (Nano and Schmerk 2007). The ORF is highly conserved and essentially the same among all species and subspecies indicating that the FPI are functional proteins, and not remnants of once functional proteins (Nano and Schmerk 2007). Additionally, the intracellular growth locus (IGL) proteins, found in the FPI, are essential for *Francisella* species to survive inside the host's macrophages and cause disease (Soto et al. 2009b).

These intracellular growth loci are also important for the virulence of *Francisella* species. There are four intracellular growth locus genes (iglA, iglB, iglC, and iglD). Research has shown iglC, along with other expressed genes, plays an important role in *Francisella* infection (Lai et al. 2004; Santic et al. 2005). Most intracellular pathogens that infect their host, rely on the ability to grow while inside their target. For example, *Salmonella enterica serovar Typhimurium* is an intracellular pathogen that depends on microbial growth inside the host for its pathogenetic characteristics (Lai et al. 2004). In *Francisella* species, the *igl*C protein is upregulated upon infection of the host macrophages or under stressful conditions and is necessary for the disruption of the macrophage and escape into the cytoplasm (Lai et al. 2004; Santic et al. 2005). An iglC mutant (lacked the expression of iglC) would not multiply upon infection of the host macrophages (Lai et al. 2004). Previous studies by Lai and colleagues showed that pathogens

also induced apoptosis in the host target cells after infection of the *Francisella* spp. (Lai et al. 2004). Moreover, igIC is suspected to play a role in decreasing the activation of NF-κβ and Mitogen-activating protein (MAP) kinase pathways, which are linked to inflammation, and apoptosis (Nano and Schmerk 2007).

The distribution of this *Fno* has reached many parts of the world in the last decade, including farmed and wild tilapia in Hawaii (Soto et al. 2010b; Soto et al. 2013; Klinger-Bowen et al. 2016). Aquaculture facilities can act as reservoirs for pathogens, such as *Fno,* and pose as a threat to wild tilapia as effluents of these facilities can transmit the infectious agent to wild populations (Lafferty et al. 2015). Francisellosis can be asymptomatic and very hard to detect, which poses a threat to aquacultures farming tilapia. The mortality rate of francisellosis is extremely high and poses a threat to both farmed and wild populations of tilapia. The suspension initiated by the Hawaii Department of Agriculture Plant Quarantine (HDOA PQ Policy 98-09, Section 150-A8, HRS) was put in place due to the 1994 outbreak of *Fno* in Hawaii and concern with high mortality rates in farmed and wild tilapia (Klinger-Bowen et al. 2016). In an effort to monitor the effects of this moratorium, it is imperative to assess the prevalence of *Fno* amongst the Hawaiian Islands.

Goals of the Study

Here, we aim to investigate the prevalence of *Francisella noatunensis* subsp. *orientalis* in wild tilapia throughout the Hawaiian Islands. Specifically, the objectives of this study are to: (1) collect tilapia from the islands of Hawaii, Oahu, Molokai, Kauai, and Maui, (2) necropsy the spleens of collected tilapia for histological evidence of granulomas indicative of *Fno* infection, (3) to validate and quantify the prevalence of *Fno* infection with molecular methods, and (4) compare histological evidence and molecular screenings.

Materials and Method

Sample Collection

Wild tilapia were collected via rod and reel and cast netting, from March 2017 to September 2018, across the islands of Oahu, Molokai, Maui, Kauai, and Hawaii for a total of 20 sites (Table 1). Water temperature, collection date, and fish length were recorded during specimen collection. Individuals were necropsied for the presence or absence of granulomas in their spleens at the University of Hawaii. Additionally, a portion of each spleen was stored in 95% ethanol and shipped to Georgia College in Milledgeville, Georgia for molecular screening. Once received, samples were stored at -20°C until further processing.

DNA Extraction

Total genomic DNA was extracted from spleen samples using DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol and resuspended in water. Successful extractions were confirmed via gel electrophoresis on a 1% agarose gel and viewed under a UV light. Lanes that contained fluorescent streaks indicated extracted DNA. Following extraction, ~10-70 ng of DNA was used as a template in *Francisella noatunensis* subsp. *orientalis* screening using both conventional and real-time PCR methods.

Conventional PCR

In order to amplify a ~1300 bp fragment of the *Francisella noatunensis* subsp. *orientalis* 16S rDNA gene, conventional PCR reactions were conducted in 25 µL volumes consisting of: deionized water, 10x PCR Buffer (2.5 µL), 200 mM, dNTPs, 0.4 µM F11 and F5 Primers (Table 2), and 1 U Taq Polymerase. Primers F11 and F5 were used as they have shown to provide specific and rapid identification of *Francisella* species without cross reaction to other pathogens (Forsman et al. 1994). To determine the optimal annealing temperature, a gradient PCR was

conducted at temperatures ranging from 60-70°C. PCR was conducted in a BIO-RAD T-100 thermocycler under the following conditions: an extension of 7 minutes at 95°C, followed by 31 cycles of 1 minute at 95°C, 1 minute at 65°C, 1 minute at 72°C and ending with a final extension of 10 minutes at 72°C. Five ng/µL of *Francisella noatunensis* subsp. *orientalis* DNA was used as a positive control.

In addition to the amplification of the 16S ribosomal gene, an ~630 bp fragment of the *Fno igl*C gene from the FPI, was amplified using conventional PCR. Reaction conditions were similar to those outlined above with the exception of primers; *igl*C forward and *igl*C reverse (Soto *et. al.* 2010a). These primers have been shown to be a more powerful option in screening for *Fno* as they are specific to *Fno* and do not amplify DNA from common marine pathogens or other *Francisella* species (Soto *et al.* 2010a). Again, a gradient PCR ranging from 60-70°C was conducted to find the optimal annealing temperature. Reaction conditions were as follows: an extension of 7 minutes at 95°C, followed by 31 cycles of 1 minute at 95°C, 1 minute at 62°C, 1 minute at 72°C and ending with a final extension of 10 minutes at 72°C. Again, *Fno* DNA (5 ng/µL) was used as the positive control.

Real-Time PCR

For real-time PCR, an ~630 bp fragment of the *Francisella noatunensis* subsp. *orientalis igl*C gene was amplified using the same *igl*C primers used in conventional PCR, as they were originally developed to screen *Fno* via real-time PCR (Soto et al. 2010a). Real-time PCR reactions were conducted in 25 µL volumes consisting of: 2X QuantiFast Probe Master Mix (12.5 µL), 2 µM *igl*C Forward and Reverse primer (Table 2), 0.4 µM *igl*C probe (Table 2) and RNase-free water (0.5 µL). To determine the optimal annealing temperature, a temperature gradient was run at temperatures ranging from 60-70°C. Reactions were conducted using a BIO-

RAD C1000 Thermal Cycler and CF96 Real-Time System under the following conditions: 2 minutes at 50°C, 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 62°C. The positive control was *Fno* DNA (5 ng/µL), while *Escherichia coli* (57.6 ng/µL) was chosen as the negative control. The sensitivity of the real-time PCR assay was estimated using serial dilutions of *Fno* DNA from 20 ng/µL to 0.0001 ng/µL. Similar to Soto et al. (2010a), a CT cutoff of 35 cycles was used to determine positive infections and positive samples were run twice for confirmation.

Species Identification

In a concurrent study by Weese et al. 2018, the tilapia specimens utilized in this study were identified to species level by amplification of the ~600 bp mitochondrial control region. Reactions were conducted in 25 µL volumes using the BIO-RAD T100 Thermal Cycler. The reaction mixtures were the same as for screening *Fno*, but with the ORMT-F and ORMT-R primers (Wu and Yang 2012) (Table 2). Wu and Yang demonstrated targeting the mitochondrial control region for DNA sequence comparison as a valid method for tilapia species identification of both wild and captive populations in Hawaii (2012). PCR conditions were as follows: an extension of 7 minutes at 95°C, followed by 31 cycles of 1 minute at 95°C, 1 minute at 50°C, 1 minute at 72°C and ending with a final extension of 10 minutes at 72°C.

Results

Sample Collection

Due to weather and accessibility, sample collection from ten of the twenty proposed sites were unsuccessful (Table 1; Figure 1). A total of 222 tilapia ranging in size from seven to 26 cm were successfully collected from ten sites across the islands of Oahu, Molokai, Maui, Kauai and

Hawaii. At these sites, water temperatures at the time of collection ranged from 23°C to 32°C (Table 3). From the total number of tilapias collected, 197 specimens were necropsied. The necropsy showed 63 specimens to possess histological evidence of granulomas: Two at Kuliouou Stream (Oahu), 17 at Kaunakakai Ditch (Molokai), 20 at Kahului Stream (Maui), four at Lahaina Stream (Maui), 17 at Puali Stream (Kauai), and three at Alenaio Stream (Hawaii). Specimens collected from Puali Stream (Kaui), Kaunakakai Ditch, (Molokai) and Kahului Stream (Maui) were found to have the highest presence of granulomas at 85%, 80%, and 80% respectively, whereas, Lahaina Stream (Maui), Alenaio Stream (Hawaii) and Kuliouou Stream (Oahu) had low presence of granulomas at 19%, 12.5%, and 9% respectively.

Conventional PCR

The results of the conventional PCR using 16S-rDNA primers were inconclusive as the products were not the expected sizes (~1300 bp) and not reproducible. Given this, we then attempted to amplify an ~630 bp fragment of the *igl*C gene. However, PCR amplification still proved to be inconsistent as band sizes were also different than the expected ~630 bp fragment. Unfortunately screening for *Francisella noatunensis* subsp. *orientalis* DNA via conventional amplification of the 16S-rDNA or *igl*C gene fragments was unsuccessful.

Real-time PCR

The real-time PCR assay was able to detect *Francisella noatunensis* subsp. *orientalis* infections as low as 0.001 ng/µL of bacterial DNA. The CT values of positive samples are shown in Table 5. In total, only 17 of the 197 tilapia specimens screened (~10%) from across three islands tested positive for *Fno* (Table 5). Molecular screenings showed infection rates of: 12 out of 20 specimens from Puali Stream (Kauai), three out of 25 specimens from Kahului Stream (Maui), one out of 20 specimens from Kawaihapai Reservoir (Oahu) and one out of 23

specimens from Kuliouou Stream (Oahu). Puali Stream had a high histological presence of granulomas (85%), which correlated with the high percentage of *Fno* DNA (60%) found via molecular analysis. However, Kahuli Stream and Kaunakakai Ditch both possessed a high presence of granulomas (80% of samples for both) but had low to no evidence of *Fno* infection (12% and 0% respectively) according to the real-time PCR assay. Sites testing positive for the presence of *Fno* had recorded water temperatures ranging from 23°C to 26.7°C.

Tilapia Species

Because of the high hybridization with many tilapia species, a concurrent study by Weese et al. (2018), the tilapia specimens screened for *Francisella noatunensis* subsp. *orientalis* were sequenced to determine the species. Several species of tilapia were identified, including *Oreochromis. niloticus, O. mossambicus*, *O. aureus*, *Coptodon* spp., *O. mossambicus* X *O. niloticus*, and *O. niloticus* X *O. aureus* hybrids, and *Sarotherodon melanotheron*, which accounted for approximately 50% of the tilapia species collected in this study (Figure 1). Species found to be infected with Fno were *S. melanotheron*, *O. mossambicus* and *O. niloticus* hybrid, and *Coptodon* spp. (Figure 2). *Coptodon* spp. had the highest infection at 25%, followed by *S. melanotheron* at 16.7%. *O. mossambicus* and *O. niloticus* hybrid had infection rate of 3.2%.

Discussion

Conventional PCR vs Real-time PCR

In this experiment, screening for *Fno* infections via conventional PCR was unsuccessful. Amplification of the intended ~1300 bp 16S rDNA or ~630 bp *igl*C gene were not consistent or reliable as PCR products were smaller or larger than the desired size and successful amplifications were not reproducible. This suggests that amplified PCR products possibly

represent false positives. Conventional PCR has worked in previous studies with the ability to differentiate between *Francisella* species. (Forsman et al. 1994) However, this study had several limitations. For example, screening of *Fno* via conventional PCR proved to be time consuming, with no success in optimizing the method. Soto et al. stated that conventional PCR has the potential to produce false positives when screening for *Fno* infections and that more reliable molecular screening methods are needed (2010a). There are other methods for *Fno* screening, however this pathogen is very difficult to detect and culture. For example, the ability of *Fno* to enter a dormant state (VBNC) makes it very laborious and difficult to culture (Duodu and Colquhoun 2010; Colquhoun and Duodu 2011; Soto et al. 2012). In this study we did not culture *Fno,* as culturing would require additional methods of screening (e.g. *in situ* hybridization) in order to detect *Fno* if it were to have entered the VBNC state, which would have been beyond the means of this project.

Many studies that screen for pathogenic infection have suffered from the limitations of Conventional PCR. For example, both wild and farmed grouper in Taiwan have suffered from outbreaks of Nervous Necrosis Virus (NNV). The detection of the virus was relied on by both histology and conventional PCR screening, however there were limitations in detecting small amounts of viral DNA in tissue (Kuo et al. 2011). White Spot Syndrome Virus (WSSV) in cultured shrimp has made a serious impact in aquaculture and is typically detected by conventional PCR (Jang et al. 2009). However, conventional PCR was only able to determine the presence or absence of WSSV in shrimp and not the degree infection (Jang et al. 2009). Real-time PCR has been a more common method for screening both NNV and WSSV, as well as many other marine pathogens that impact aquaculture due to its high sensitivity and ability to

detect low amounts of DNA in tissue samples. Real-time PCR allows for the detection pathogenic infection in low level infected organisms.

In this study, Real-time PCR was more sensitive and reliable than conventional PCR in screening for *Fno* infections as it provided consistent and repeatable results. Additionally, this method was able to detect small concentrations of *Fno* DNA (as low as 0.001 ng/µL). The significance is that histology cannot confirm *Fno* infection as granulomas are clinical symptoms for a variety of bacterial infections. Molecular screening must be done in tandem with histology to confirm that the observed granulomas are caused by *Fno*. Unfortunately, despite the efficiency, real-time PCR is much more expensive than conventional PCR. The kits and equipment are more costly and may be outside the means of many labs. However, recently real-time PCR instruments and kits have become more affordable and accessible for molecular screening. In addition, real-time PCR is less laborious than conventional PCR and culturing bacteria, which may make it a better alternative.

Histological Evidence and Real-time PCR Findings

Puali Stream, Kaunakakai Ditch, and Kahului Stream exhibited high numbers of granulomas present in the spleens of the tilapia sampled. At least 80% of the spleens necropsied at these sites contained granulomas, with Puali Stream (Kaui) having the most. However, this may not confirm *Fno* as the causative agent. Spleen granulomas are a clinical sign of *Fno* infection; however, they can also be linked to a number of other bacterial infections such as *Mycobacterium* (Heckert 2001). At Kaunakakai Ditch the majority of specimens exhibited histological evidence granulomas. However, none of these specimens contained *Fno* DNA suggesting that the granulomas are potentially caused by another pathogen such as *Piscirickettsia salmonis* which was thought to be the original culprit of the 1994 Oahu outbreak (Mauel 2003;

Soto et al. 2009a). Eliminating other possible bacterial infections indicative of granulomas would require screening for additional pathogens.

Real-time PCR confirmed *Fno* infection in less than ten percent of collected specimens, at sites Puali Stream, Kahuli Stream, Kawaihapai Reservoir, and Kuliouou Stream, with Puali Stream showing the highest infection rate. Three of the 17 infected specimens confirmed via real-time PCR (two from Puali Stream and one from Kawaihapai Reservoir) had no histological evidence of granulomas. However, *Fno* DNA was still present in the spleens of these tilapia demonstrating that histology may miss asymptomatic or freshly infected specimens. These findings study suggest that both histological analysis and real-time PCR screening is necessary for accurately determining *Fno* infection rates. Real-time PCR results confirmed *Fno* infection in less than 30% of specimens that contained granulomas. This suggest that histological screening of spleens alone can misrepresent infection rates of *Fno* because granulomas are clinical symptoms that can be potentially caused by multiple pathogens (Heckert 2001). The significance of both molecular screening and histology allows for accurate diagnosis of the correct pathogen. Although other bacterial infections may produce similar clinical symptoms, treatments may differ. Identification of the known pathogen, in this case *Fno*, may help develop treatments and preventative protocols for francisellosis. Molecular screening via real-time PCR allows for the detection of bacterial infections before clinical symptoms appear. This can allow for quicker treatments to be implemented, as *Fno* can be detected without the need for clinical symptoms to be displayed from potentially infected fish.

Hawaiian Island Infection

In previous studies, screening for *Fno* has been limited to wild and cultured populations of tilapia on the island of Oahu, where the outbreak first occurred (Mauel et al. 2003; Soto et al.

2009a; Soto and Revan 2012; Klinger-Bowen et al. 2016). Given the history of *Fno* on Oahu, the HDOA suspended exportation of tilapia from Oahu to the other islands in 1994 (HDOA PQ Policy 98-09, Section 150-A8, HRS). Unfortunately, despite this quarantine, this study has found evidence of *Fno* infections on the islands of Kauai and Maui in addition to Oahu (discussed above). This may suggest that isolation attempts of *Fno* were unsuccessful. However, we cannot rule out the possibility that infected fish were imported into Hawaii. Wild and cultivated fishes have a high chance of interacting with one another which could spread the infection of *Fno* throughout the island of Oahu or to other islands. *Fno* has shown to be prevalent in the wild of other regions. Wild Cod caught in Sweden showed a prevalence of 20% and approximately 8% in Norway (Colquhoun and Duodu 2011). There has been evidence of high *Fno* prevalence in Hawaiian farms in Oahu (Soto et al. 2013), which may suggest high prevalence of *Fno* in the wild. With the quarantine in effect from 1994 by the HDOA, it was suggested the spread of *Fno* would stay on Oahu. Thus, many studies aimed to continue assessing the prevalence of infection and the extent of the pathogen solely on Oahu (Mauel et al. 2003; Soto et al. 2009a; Klinger-Bowen et al. 2016). However, the infection of *Fno* reaching Kauai and Maui demonstrates the need for a more extensive screenings across the Hawaiian Islands. Additionally, infection of *Fno* is not tilapia specific, suggesting that other fishes could potentially be infected. This would suggest that *Fno* found on Kauai and Maui may have come from a different aquatic species. In addition, it is suggested the lack of *Fno* screenings on other islands in the last two decades may have allowed the pathogen to spread undetected. In addition, this study only screened a total of ten sites from Oahu, Kauai, Molokai, Maui and Hawaii. It is probable that *Fno* infection has reached both Molokai and Hawaii, similar to Kauai and Maui, but it has not yet been confirmed.

More screening across the islands is needed to monitor the spread of *Fno* throughout Hawaii, which can help to develop better biosecurity.

Water Temperature and Fish Size

At the time of collection, water temperatures of infected sites ranged from 23.0°C to 26.7°C. Sites that contained granuloma presence in sampled spleens, but no evidence of *Fno* infection had water temperatures of at least 29.0°C or higher. These findings suggest that water temperature may not have been optimal for *Fno* to thrive, which correlates with findings of previous studies (Soto et al. 2012; Soto et al. 2013; Klinger-Bowen 2016). Water temperature has been shown to impact tilapia susceptibility to francisellosis (Soto et al. 2013; Klinger-Bowen 2016) and it has been demonstrated that tilapia at 25°C were more likely to be infected with *Fno*. (Soto et al. 2013). The sites with positive *Fno* infections support previous studies demonstrating tilapia raised at cooler water temperatures (i.e. 25.0°C $\mp$ 2°C) are more likely to be infected (Soto et al. 2013).

Like temperature, fish size has also been found to be correlated with infection rates (Soto et al. 2009b; Soto et al. 2013; Klinger-Bowen 2016). In previous studies, farmed fishes from five to 15 grams in weight, one year or less in age (i.e. fingerlings), and of the same species or genus were used to demonstrate how size influences infectivity of *Fno* (Klinger-Bowen et al. 2016, Soto and Revan 2012; Soto et al. 2012; Soto et al. 2009b). However, the fishes collected in this study were from wild populations and unfortunately age could not be determined. Additionally, our sampling included seven different species of tilapia, belonging to three different genera. Given this, we were unable to determine how fish size, weight or length, correlated with *Fno* infection rates.

Tilapia Species and Infection Rate

In a concurrent study, by Weese et al. 2018, the tilapia specimens screened for *Fno* infections were also sequenced to determine species identity. Several species of tilapia were identified, including *Oreochromis niloticus*, *O. mossambicus*, *O. aureus*, *Sarotherodon melanotheron*, and *Coptodon* spp. as well as several potential hybrids such as *O. niloticus X O. mossambicus* hybrids and *O. niloticus X O. aureus* hybrids. Of the specimens collected, 46.8% were identified as being *S. melanotheron* (Figure 1). *Sarotherodon melanotheron* also accounted for 82% of fish found to be infected with *Fno* (Figure 1). Both *S. melanotheron* and *O. mossambicus* have been shown in the past to be highly susceptible to *Fno* infection and were the first species to be identified with francisellosis in Hawaii (Mauel et al. 2003; Klinger-Bowen et al. 2016; Soto et al. 2013). Additionally, *O. mossambicus* has been demonstrated to be more susceptible to francisellosis compared to other *Oreochromis* species such as *O. aureus* and *O. niloticus*. (Soto et al. 2013; Klinger-Bowen et al. 2016). Although, *O. mossambicus* was not found to be infected with *Fno* in this study, *O. niloticus X O. mossambicus* hybrids were found to be infected. Infection of *Fno* has been reported in *O. niloticus* in Costa Rica (Soto et al. 2009a), suggesting that *O. niloticus X O. mossambicus* hybrids, along with *O. mossambicus* and *S. melanotheron*, are potential carriers for *Fno*. These species have shown high susceptibility to *Fno* in the last two decades and may contribute to widespread infections throughout the Hawaiian Islands. This knowledge can aid aquaculture and biosecurity efforts to label these species as being at high risk and potential *Fno* reservoirs. In addition, aquaculture facilities can choose to cultivate tilapia species that may be more resistant to *Fno* infection.

Conclusion

In this study, we investigated the prevalence of *Francisella noatunensis* subsp. *orientalis* across the Hawaiian Islands. Histological screening found evidence of granulomas indicative of a pathogenic infection on the islands of Oahu, Molokai, Kauai, Maui, and Hawaii. However, molecular analysis only confirmed *Fno* infection on the islands of Oahu, Kauai and Maui. Prior to this study, *Fno* had only been reported from the island of Oahu, but here we found evidence of infection on the islands of Kauai and Maui. These results suggest that, despite the quarantine initiated by the HDOA, *Fno* has reached other islands as well. This suggests that *Fno* has failed to be contained and there needs to be more rigorous screening protocols put in place.

Molecular techniques, such as conventional PCR, have been developed to detect *Fno* without the need to culture the pathogen (Forsman et al. 1994). However, our results using conventional PCR showed unreliability by inconsistencies in amplification and band sizes that were different from the intended fragment size. Therefore, we suggest using both histology and real-time PCR *Fno* screening is a better method for screening *Fno* infections in tilapia. These methods allow for rapid results, and confirmation of the suspected pathogen. Assays utilizing real-time PCR are able to detect smaller concentrations of bacterial DNA than conventional PCR and the primers developed by Soto et al. (2010a). are specific for *Fno*, without fear of cross reacting with other common marine pathogens. For aquaculture, these methods would make preventative protocols or developing treatment methods much easier.

For future research, additional sampling across the Hawaiian Islands is warranted. While we were able to detect *Fno* infections on the islands of Oahu, Kauai, and Maui, our sampling was limited. Islands with very few collection sites, such as Hawaii and Molokai, did not provide enough data to determine *Fno* infection. Additional sampling could aid in determining overall prevalence of *Fno* in Hawaii. Additionally, future sampling should target areas close to

aquaculture facilities in order to detect *Fno* before entering the facilities. Lastly, continued screening would help the aquaculture industry to develop biosecurity protocols for fish species commonly found in Hawaii.

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List of Tables

Table 1: Locations of collection sites across five Hawaiian Islands. * denotes successful collection sites.

Island	Site	GPS Coordinates
Oahu	Kahuku Pond*	N: 21.70632; W: -157.96550
	Kuliouou Stream*	N: 21.2843; W: -157.7214
	Kawaihapai Reservoir*	N: 21.580849; -158.185329
	Ahuimanu	N: 21.70632; W: -157.96550
	Anahuli	N: 21.597774; W: -158.11026
Molokai	Kaunakakai Ditch*	N: 21.090651; W: -157.02071
	Kawela Stream*	N: 21.064640; W: -156.94886
Maui	Kahului Stream*	N: 20.897526; W: -156.44699
	Lahaina Stream*	N: 20.869900; W: -156.67550
Kauai	Puali Stream*	N: 21.951975; W: -159.36198
	Anahola*	N: 22.145622; W: -159.30251
	Kumukumu	N: 22.106996; W:-159.29927
	Kapaa	N: 22.096961; W: -159.30939
	Wailua 1	N: 22.052896; W: -159.34763
	Wailua 2	N: 22.053374; W: -159.34222
Hawaii	Alenaio Stream*	N:19.431964; W: -155.05008
	Southern Hamakua Coast	N: 19.989233; W: -155.23749
	Nuilii	N: 20.224399; W: -155.75026
	Puwaiole Gulch	N: 20.220755; W: -155.75876
	Kohala	N: 20.206812; W: -155.79118

Table 2: Primers and probe used for amplification of the 16Sr-RNA and *iglC Francisella noatunensis* subsp. *orientalis* genes.

Primers used	**Sequence**	**Reference**
F11	5'-TACCAGTTGGAAACGACTGT-3'	Forsman et al. 1994
F5	5'-CCTTTTTGAGTTTCGCTCC-3'	Forsman et al. 1994
*igl*C Forward	5'-GGGCGTATCTAAGGATGGTATGAG-3'	Soto et al. 2010a
*igl*C Reverse	5'-AGCACAGCATACAGGCAAGCTA-3'	Soto et al. 2010a
Probes Used	**Sequence**	**Reference**
iglC Probe	FAM-ATCTATTGATGGGCTCACAACTTCACAA-BHQ-1	Soto et al. 2010a

Table 3: Number of individuals collected (n), collection date, water temperature, average fish weight and length from each site sampled across Hawaii islands.

Island	Site	*n*	Date Collected	Water Temp. (C)	Avg. Weight (g)	Avg. Length (cm)
Oahu	Kahuku Pond	30	11/22/17	23.5°C	35.2 ∓ 16.4	12.3 ∓ 1.7
	Kuliouou Stream	23	2/27/18	24.5°C	13.3 ∓ 25.7	7.3 ∓ 3.1
	Kawaihapai Reservoir	20	3/5-6/18	23°C	7.7 ∓ 3.5	7.5 ∓ 1.2
Molokai	Kaunakakai Ditch	21	5/31/18	32°C	305.2 ∓ 99.8	26.3 ∓ 3.8
	Kawela Stream	13	5/31/18	24°C	100.6 ∓ 117.2	16.9 ∓ 5.5
Maui	Kahului Stream	25	6/26/18	26.7°C	90.8 ∓ 43.7	16.9 ∓ 2.4
	Lahaina Stream	21	6/26/18	30°C	99.5 ∓ 55.4	17.4 ∓ 3.7
Kauai	Puali Stream	20	7/10/18	25°C	237.5 ∓ 101.2	21.7 ∓ 3.8
	Anahola	25	8/14/18	29°C	9.5 ∓ 4.7	7.7 ∓ 1.4
Hawaii	Alenaio Stream	24	9/18/18	24°C	318.3 ∓ 204.8	25.4 ∓ 6.7

Table 4: Real-time PCR CT values for samples testing positive for *Fno*. Starred samples indicate failure to amplify the second time screened and were not included for further data analysis. The first and second CT values are the first and second qPCR reaction respectively.

Island	Site	Sample	CT Value
Oahu	Kuliouou Stream	B3	30.58, 32.26
	Kawaihapai Reservoir	B14	32.82
Maui	Kahului Stream	B4	32.09, 33.66
		B14*	32.02
		B15	32.65, 32.90
		B19	8.53, 31.14
		B20*	29.38
Kauai	Puali Stream	B1	34.81, 35.88
		B4	30.54, 30.13
		B6	32.55, 32.42
		B7	34.77, 35.39
		B9	33.54, 34.27
		B11	31.28, 31.58
		B12	34.83, 33.93
		B14	34.63, 32.86
		B16	31.69, 31.70
		B17	33.47, 33.93
		B18	33.45, 33.44
		B20	28.67, 28.89

Table 5: Histological evidence of granuloma presence and percent infected based on real-time PCR.

Island	Site	Granuloma Presence	Infected %
Oahu	Kahuku Pond	0%	0%
	Kuliouou Stream	9%	4%
	Kawaihapai Reservoir	0%	6%
Molokai	Kaunakakai Ditch	80%	0%
	Kawela Stream	0%	0%
Maui	Kahului Stream	80%	12%
	Lahaina Stream	19%	0%
Kauai	Puali Stream	85%	60%
	Anahola	Not tested	0%
Hawaii	Alenaio Stream	12.5%	0%
Total		32%	7.7%

List of Figures

Figure 1: Geographic map of collection sites. Oahu (1. Kahuku, 2. Kuliouou Stream, and 3. Kawaihapai Reservoir), Molokai (4. Kaunakakai Ditch and 5. Kawela Stream), Maui (5. Kahului Stream and 6. Lahaina Stream), Kauai (8. Puali Stream and 9. Anahola) and Hawaii (10. Alenaio Stream).

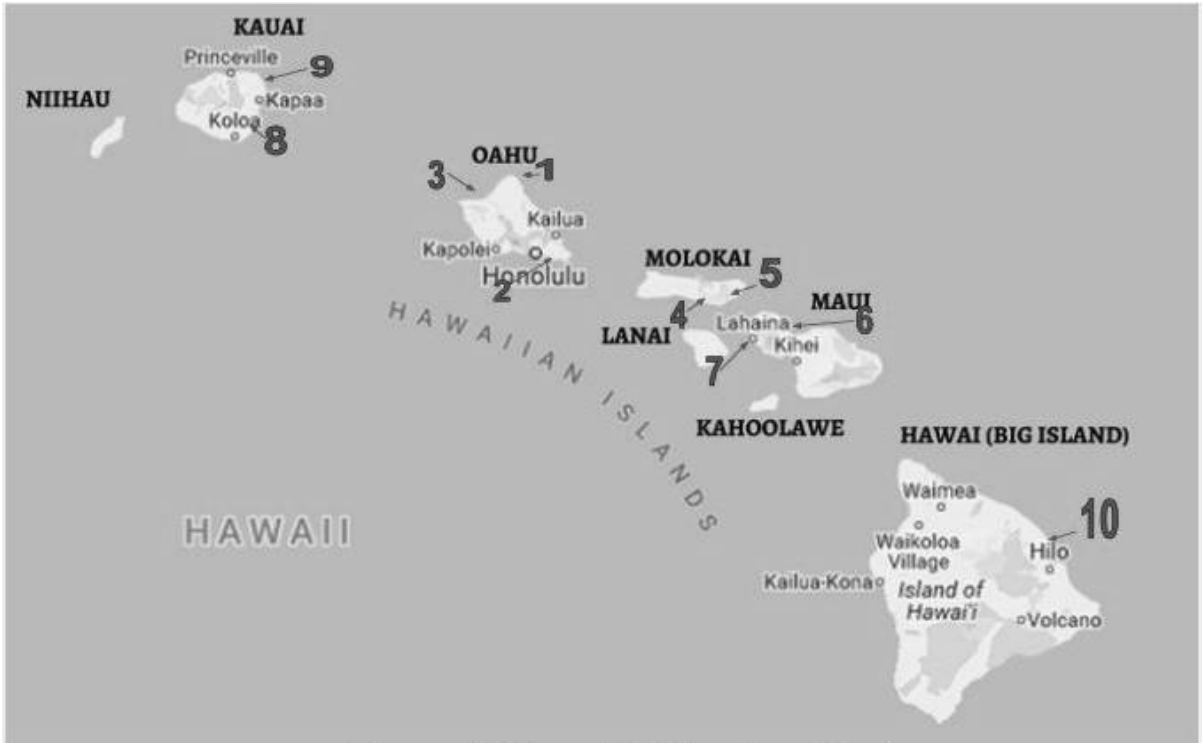

Figure 2: Number of individual tilapia species and number of tilapia species infected.