FELIPE LUIZ PEREIRA

Omics approaches provide new insights into the epidemiology and pathogenicity of the emerging fish-pathogen *Francisella noatunensis* subsp. *orientalis*

Belo Horizonte/MG
June of 2019

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA INTERUNIDADES DE POS-GRADUAÇÃO EM BIOINFORMÁTICA

TESE DE DOUTORADO

Omics approaches provide new insights into the epidemiology and pathogenicity of the emerging fish-pathogen *Francisella noatunensis* subsp. *orientalis*

Tese de Doutorado apresentada ao Curso de Pós-Graduação em Bioinformática da Universidade Federal de Minas Gerais como requisito para obtenção do título de Doutor em Bioinformática.

Discente: Felipe Luiz Pereira, M.Sc.

Orientador: Prof. Henrique César Pereira Figueiredo, DMV, PhD.

Co-orientador: Prof. Vasco Ariston de Carvalho Azevedo, DMV, PhD.

Belo Horizonte/MG
Junho de 2019

043 Pereira, Felipe Luiz.

Omics approaches provide new insights into the epidemiology and pathogenicity of the emerging fish-pathogen Francisella noatunensis subsp. orientalis [manuscrito] /Felipe Luiz Pereira. – 2019.

69 f. : il. ; 29,5 cm.

Orientador: Prof. Henrique César Pereira Figueiredo. Co-Orientador: Prof. Vasco Ariston de Carvalho Azevedo.

Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa Interunidades de Pós-Graduação em Bioinformática.

1. Bioinformática - Teses. 2. Genômica. 3. Tilápia do Nilo. 4. Transcriptômica. I. Figueiredo, Henrique César Pereira. II. Azevedo, Vasco Ariston de Carvalho. III. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. IV. Título.

CDU: 573:004

Ficha elaborada por Sônia M. S. Moraes CRB 6/1357 - ICB/UFMG

Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
Programa Interunidades de Pós-Graduação em Bioinformática da UFMG

ATA DA DEFESA DE TESE

110/2019
entrada
1°/2016
CPF:
053.072.499-50

Felipe Luiz Pereira

Às quatorze horas do dia **06 de setembro de 2019**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Omics approaches provide new insights in the epidemiology and pathogenicity of the emerging fish-pathogen Francisella noatunensis subsp. orientalis"**, requisito para obtenção do grau de Doutor em **Bioinformática.** Abrindo a sessão, o Presidente da Comissão, **Dr. Henrique Cesar Pereira Figueiredo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	CPF	Indicação
Dr. Henrique Cesar Pereira Figueiredo	UFMG	952.717.716-04	APROVADO
Dr. Vasco Ariston de Carvalho Azevedo	UFMG	273.141.225-49	APROVADO
Dr. Rafael Diego da Rosa	UFSC	036.423.889-57	Aprovado
Dr. Frederico Augusto de Alcântara Costa	UFU	07224495697	APROVADO
Dr. Aristóteles Góes Neto	UFMG	54432882520	APROVADO
Dr. Francisco Lobato	UFMG	2247166x71	APROVADO
Dr. João Luís Reis Cunha	UFMG	015.105.936-05	Aprovado

Pelas indicações, o candidato foi considerado: APROVADO
O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.
Belo Horizonte, 06 de setembro de 2019.

Dr. Henrique Cesar Pereira Figueiredo - Orientador _____
Dr. Vasco Ariston de Carvalho Azevedo - Coorientador _____
Dr. Rafael Diego da Rosa _____
Dr. Frederico Augusto de Alcântara Costa _____
Dr. Aristóteles Góes Neto _____
Dr. Francisco Lobato _____

Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
Programa Interunidades de Pós-Graduação em Bioinformática da UFMG

Dr. João Luís Reis Cunha _____

Acknowledgements

To my wife, Paula Mateus Pereira, my partner of this discovery journey. Sometimes I think that it is a dream come true for both of us.

To my daughter, Valentina, just for existing and making the world more beautiful.

To my parents, Fernando and Márcia, for all-life support.

To my advisor, Henrique Figueiredo, for his patience, for always believing in me and to lapidate my way through this project.

To my academic friends, Tarcisio and Thiago, for hours of studies, theories and friendship. To the other colleagues, collaborators and Professors of the Bioinformatics Pos-Graduate Program.

To my AQUACEN coworkers and friends for the talkings and works.

Resumo

Francisella noatunensis subsp. *orientalis* (FNO) é um patógeno emergente com grande disseminação nas fazendas produtoras de tilápia do Nilo, em diferentes partes do mundo. No Brasil, perdas economicamente expressivas são associadas a surtos de franciselose desde o ano de 2012. Normalmente o desenvolvimento da doença e a mortalidade de peixes ocorrem quando a temperatura da água está abaixo de 26ºC. Por tratar-se de um patógeno recentemente idetificado, informações quanto ao seu repertório genético e expressão dos seus genes virulência são pouco conhecidas. O objetivo deste trabalho foi realizar uma comparação filogenética desses isolados com linhagens de outros países, utilizando genomas de isolados obtidos de diferentes surtos reportados no Brasil e caracterizar o perfil expressão de todos os genes presentes no genoma nas temperaturas de 22ºC e 28ºC. As sequências dos genomas dos isolados (n = 16) permitiu confirmar que a espécie está sob forte decaimento gênico (número elevado de pseudogenes e genoma reduzido, quando comparado com espécies de vida livre do mesmo gênero), mas não descartou a existência de outras linhagens em outros países. No Brasil, existe apenas uma linhagem circulante, com mutações pontuais, que apresentam uma deriva genética desde os primeiros casos reportados, o que permite concluir que a doença teve uma única inserção no país que ocorreu antes do ano de 2012. Em termos de quantificação da virulência do patógeno, um desafio de infecção experimental identificou-se que é necessária uma dose média inferior (*i.e.*, < 3 logs) para mortalidadedos hospedeiros a 22ºC em comparação com os hospedeiros mantidos a 28ºC. Entretanto, no experimento *in vitro,* os genes relacionados à virulência do patógeno não sofreram alteração na expressão quando avaliados em diferentes temperaturas. Isso demonstra que há uma evolução divergente da FNO em relação a outras do mesmo gênero, que apresentam uma alteração significativa da expressão de genes de virulência quando são submetidas a temperaturas similares a de seus hospedeiros. Essa alta expressão dos genes de virulência pode ser uma adaptação para determinado tipo de hospedeiro, uma vez que os peixes são animais heterotérmicos, com variações substanciais na temperatura corporal como resultado da alteração na temperatura do ambiente em que se encontra. Por outro lado, genes relacionados a metabolismo em FNO foram sub- e sobre-regulados na comparação entre as temperaturas, o que pode representar uma adaptação no processo de replicação na temperatura em que a doença clínica se desenvolve.

PALAVRAS-CHAVE: franciselose, tilápia do Nilo, genômica, transcriptômica.

Abstract

Francisella noatunensis subsp. *orientalis* (FNO) is an emerging pathogen with large dissemination on Nile tilapia fish farms worldwide. In Brazil, extensive economic losses were associated to francisellosis outbreaks since 2012. Normally the disease cases and the fish mortalities occur when the water temperature is bellow 26ºC. It is an emerging pathogen and thus information about its genetic repertoire and virulence gene expression profiles are still poorly understood. The aims of this work were to compare phylogenetically the Brazilian isolates of each outbreak with sequenced genomes from other countries and to obtain the whole-genome profile expression at the temperatures of 22ºC and 28ºC. The sequenced genome sequences (n = 16) allows the confirmation that this species in under a strong genome decay (high number of pseudogenes and small genome, when compared with free-living species of the same genus), but it does not discard the existence of other strain in other countries. In Brazil, there is only one strain, with slight mutation events and with genetic diversions in the first reported cases, which demonstrates that this bacterium has only one insertion into the country before 2012. Regarding the quantification of the pathogen's virulence, the observed median lethal doses were smaller (*i.e.*, < 3 logs) when the hosts were maintained at 22ºC in comparison with those maintained at 28ºC. However, in *in-vitro* assays, the pathogen genes related to virulence did not change the expression when submitted to different temperatures. These results show a divergent evolution of this species as compared with other species of the same genus, which shows a significant regulation of these genes when submitted to similar temperatures as their hosts. This high expression of virulence genes migth be a host niche adaptive strategy. Since fishes are poikilothermic, substantial variations of their body temperature occur as a result of water temperature changes around them. On the other side, metabolism genes of FNO were up- and down-regulated between tested temperatures, which might represent an improved host fitness trait for replication at lower temperatures.

KEYWORDS: francisellosis, Nile tilapia, genomics, transcriptomics.

Figure list

Abbreviations list

16S rDNA	16S gene of Ribosomal DNA
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
FNO	*Francisella noatunensis* subsp. *orientalis*
FPI	*Francisella* Pathogenicity Island
MLSA	Multilocus sequence analysis
MLVA	Multiple-locus variable-number of tandem repeat analysis
NVI	National Veterinary Institute
PCR	Polymerase Chain Reaction
PTR	Protein Tandem Repeats
qPCR	Real time quantitative PCR
REP-PCR	Repetitive extragenic palindromic-PCR
RT-qPCR	Reverse transcription qPCR
SRA	Single read archive
WGS	Whole-genome shotgun

Structure of the thesis

This thesis is structured in 3 chapters:

1. Introduction: literature revision of the pathogen *Francisella noatunensis* subsp. *orientalis* (FNO), the *Francisella* genus pathogenesis, and the genomic and transcriptomic approaches applied to aquaculture fish pathogens; hypothesis; and objectives;

2. Paper published at Genomics of Elsevier in November, 2018: "**Complete genome sequencing of sixteen *Francisella noatunensis* subsp. *orientalis* isolates: A genomic approach for molecular characterization and spread dynamics of this clonal population**". The study aim was to provide the whole-genome sequence of a comprehensive number of Brazilian FNO-outbreak isolates, and to compare their genetic population structure and evolution and their population spread dynamics. Each Brazilian isolate could be identified as a single node of a clonal population, presenting slight genetic differences associated to mutational events. The common ancestry node suggests a single entry into the country before 2012.

3. Paper published at Microbial Pathogenesis in May, 2019: "**Effects of the temperature changes in the transcriptional profile of the emerging fish pathogen *Francisella noatunensis* subsp. *orientalis***". This paper aim was to evaluate the pathogenicity of a FNO isolate in Nile tilapia experimentally infected and maintained under temperatures of 28 ºC and 22 ºC, and to evaluate the effect *in-vitro* of the temperature change on the global transcription of FNO genes. Shift of virulence was observed in *in-vivo* assay with a reduction of 3 logs on mean lethal doses required, however, classical expression changes of virulence gene was not observed in microarray assay.

After these chapters, it will be presented the Discussion, Conclusion and Final Considerations.

Summary

Chapter 1. Introduction

Aquaculture and infectious diseases

Aquaculture, which is the rearing of aquatic animals for food, is the main approach to meet the world rising demand of fish consumption (FAO, 2018; Kubitza, 2015). The population growth had an annual increase of 1.6%, while the fish consumption increase was about 3.2% (data available since 1961); nowadays the per capita consumption is about 20.5 kg, and it seems to be increasing (FAO, 2018). Besides, it is expanding around the world: during the 1960s almost half percent of processed fish products were concentrated in Europe, Japan and in the United States of America and today, this amount is around only 20% (FAO, 2018). In the last four decades, capture fisheries production has remained stable (~90 million tons $year^{-1}$), whereas aquaculture production has risen continuously, reaching 48% of the total production (~80 million tons $year^{-1}$) in 2016 (FAO, 2018).

The first FAO statistical data of Brazilian aquaculture production dates from the late 60's, however, after the mid of 90', with the permission for the use of public waters (dams and lakes) for aquaculture purpouses, production was expanded (Barroso, *et al.*, 2018). Nowadays, fisheries and aquaculture have a comparatible market share within the Aquaculture industry (Kubitza, 2015). Among most produced species in Brazil, such as Tambaqui, Tambacu, Tambatinga and Carp, the Nile tilapia is the most farmed fish with a production ~283 thousand tons in 2017, representing ~58 % of all Brazilian aquaculture production (IBGE, 2017). The Nile tilapia fish farms are concentrated in Northeast (tropical climate), Southeast (tropical/subtropical climate) and South (subtropical climate) states regions. The states with the highest production are, respectively, Paraná, São Paulo, Minas Gerais, and Santa Catarina with ~91, ~42, ~28, and ~24 mil tons in 2017 (IBGE, 2017).

Due to intensive fish husbandry, the occurrence of infectious disease outbreaks has become one of the main obstacles to the expansion of tilapia farming (Leal, *et al.*, 2014). In last decades *Lactococcus garvieae*, *Streptococcus agalactiae*, *Streptococcus iniae*, *Streptococcus dysgalactiae* and *Francisella noatunensis* subsp. *orientalis* (FNO) have emerged as tilapia pathogens, causing infectious diseases with high economic losses (Fukushima, *et al.*, 2017; Barony, *et al.*, 2017; Agnew & Barnes, 2007; Soto, *et al.*, 2009).

FNO is one of the most important bacteria associated with disease outbreaks in farm-raised Nile tilapia in Brazil (Leal, *et al.*, 2014; Sebastião, *et al.*, 2017), mainly in the

subtropical climate region where a seasonal occurrence of francisellosis was established (Delphino, *et al.*, 2019). The first description of FNO in the country occurred in different commercial farms located in the state of Minas Gerais in 2012 (Leal, *et al.*, 2014). After this description, other Brazilian states such as São Paulo (2013), Paraná (2015) and Santa Catarina (2015) also reported mortalities of tilapia due to FNO infections (Sebastião, *et al.*, 2017).

FNO is a species from the *Francisella* genus, which includes the *Francisella tulerensis* species, a highly infective human pathogen, with designation by the Centers for Disease Control and Prevention (CDC) of the United States of America (USA), as potential bioterrorism agents (Rowe & Huntley, 2015). Thus, *F. tularensis* is a highly studied pathogen, with many aspects of infection and dissemination patters being very well described. Based on these facts, the biology of the *Francisella* genus will be described below.

Francisella genus

In 1911, McCoy and Chapin isolated the agent of a plague-like disease of squirrel in Tulare County, California/USA. Years later, in 1922, Edward Francis after knowledge refinement over time had established the agent of several disease cases in humans, nominating the disease as "Tularemia" (Kingry & Petersen, 2014). Thus, the genus *Francisella* refers to Edward Francis, and the first isolate of the Tulare County was named *F. tularensis* (Kingry & Petersen, 2014; Foley & Nieto, 2010).

In 1955, Larson and collaborators isolated a bacterium from salt water in Utah/USA that morphologically resembled *F. tularensis*. However, this bacterium had some differences in sucrose fermentation and reaction with serum inoculated with *F. tularensis*, and then it was nominated as *Francisella novicida* (Kingry & Petersen, 2014). The taxonomical classification as a new species was further substantiated in 1964 by Owen and collaborators (Owen, *et al.*, 1964). Other studies from 1980s to 2010 (Hollis, *et al.*, 1989; Huber, *et al.*, 2010; Johansson, *et al.*, 2010) showed divergence of opinions by the authors regarding the reclassification of this species as a subspecies, *Francisella tularensis* subsp. *novicida*, or else to maintain as a species solely. Nowadays, this controversy still remains, with *F. novicida* being recognized on the Approved List of Bacterial Names (Skerman, *et al.*, 1980) and *F. tularensis* subsp. *novicida* being considered as a valid published species (Huber, *et al.*, 2010).

Three subspecies of *F. tularensis*, regardless of the subsp. *novicida*, were cited: the subsp. *tularensis*, an extremely virulent pathogen being geographically distributed in North

America, the subsp. *holarctica,* highly virulent in the Old World and being sporadically distributed in North America, and the subsp. *mediasiatica* also highly virulent and being distributed in Central Asia (Keim, *et al.*, 2007).

Still in the 1980s, Hollis and his team proposed another reclassification of the species, as *Francisella philomiragia* that it was previously denominated *Yersinia philomiragia*, described as the causative agent of the granulomatous disease, with DNA-relatedness characteristics of *F. tularensis* and *F. novicida* (Hollis, *et al.*, 1989). In the same study, Hollis inferred that the infection in humans occured after saltwater exposure and that the vector or reservoirs could be voles or water rats.

In 2007, researchers of the National Veterinary Institute (NVI) from of Norway (Mikalsen, *et al.*, 2007) isolated the bacteria from Atlantic cod (*Gadus morhua* L.) characterizing as a subsp. of *F. philomiragia*, which is a pathogen responsible for disease outbreaks along the Norwegian coast that was proposed as *Francisella philomiragia* subsp. *noatunensis*. This new subspecies was proposed considering its differences in growth requirements (such as the optimal temperature growth of 22 ºC), DNA-DNA reassociation values, and phylogenetic and genetic distances of conserved genes (like as *groEL*, *atpA*, *pgm*, *shdA*, *rpoB* and *rpoA*).

Also in 2007, the Bergen University group proposed the creation of a new species, the *Francisella piscicida*, considering analyses performed with *F. philomiragia* that was isolated also from Atlantic cod by the same group a year earlier (Ottem, *et al.*, 2007). At this time, the proposition was made considering the 16S rDNA sequence variations and the ecological niches (host and temperature optima) between the *F. philomiragia* and the new species.

Two years later, in 2009, the Ottem's group, considering an extensively taxonomic characterization based on sequenced 16S rRNA and several house-keeping genes, and biochemical and phenotypic properties, proposed the elevation of *F. philomiragia* subsp. *noatunensis* to the species *Francisella noatunensis*, with *F. piscicida* being considered as a heterotypic synonym. Also, this group proposed the creation of the subsp. *Francisella noatunensis* subsp. *orientalis*, characterized by the strain *Francisella* sp. Ehime-1, isolated from three-line grunt (*Parapristipoma trilineatum*) in Japan in 2001 (Ottem, *et al.*, 2009).

Besides these organisms, there are other species being recognized in the literature: the *Francisella hispaniensis,* a reclassification from *F. novicida* (Huber, *et al.*, 2010), the *Francisella halioticida,* a causative pathogen in giant abalone (*Haliotis gigantea*) (Brevik, *et*

al., 2011), and *Francisella persica,* a reclassification from *Wolbachia persica* (Larson, *et al.*, 2016). Furthermore, there is a citation of an unpublished data in the literature of the fish pathogen *Francisella victoria* (Kay, *et al.*, 2006) which it might be a syn. of FNO. Other species with a brief participation in the genus is the *Francisella guangzhouensis,* a strain that was isolated from an air-conditioning system in Guangzhou city, China, in 2013 (Qu, *et al.*, 2013), and was placed in the *Allofrancisella* genus in 2016 (Qu, *et al.*, 2016). Recently, in 2018, Soto and collaborators described a new species, *Francisella marina*, isolated from spotted rose snapper fish in Central America.

Currently the *Francisella* genus is comprised by eight species: *F. tularensis* (with subspp. *tularensis*, *holarctica* and *novicida*), *F. novicida*, *F. philomiragia*, *F. noatunensis* (with subspp. *noatunensis* and *orientalis*), *F. marina*, *F. hispaniensis*, *F. halioticida*, and *F. persica*. Figure 1 shows the phylogenetic relations of the *Francisella* genus (Soto, *et al.*, 2018). The genus is composed by Gram-negative bacillus, with 0.1-1.5 × 0.1-3 μm, non-motile, aerobic and non-sporulated (Foley & Nieto, 2010; Gonçalves, *et al.*, 2016; Huber, *et al.*, 2010). Optimal growth temperatures are 37 ºC for *F. tularensis*, *F. philomiragia*, and *F. hispaniensis* (Foley & Nieto, 2010; Huber, *et al.*, 2010; Mikalsen, *et al.*, 2007), 22 ºC for *F. noatunensis* (Ottem, *et al.*, 2007), and 20 ºC for *F. halioticida* (Brevik, *et al.*, 2011).

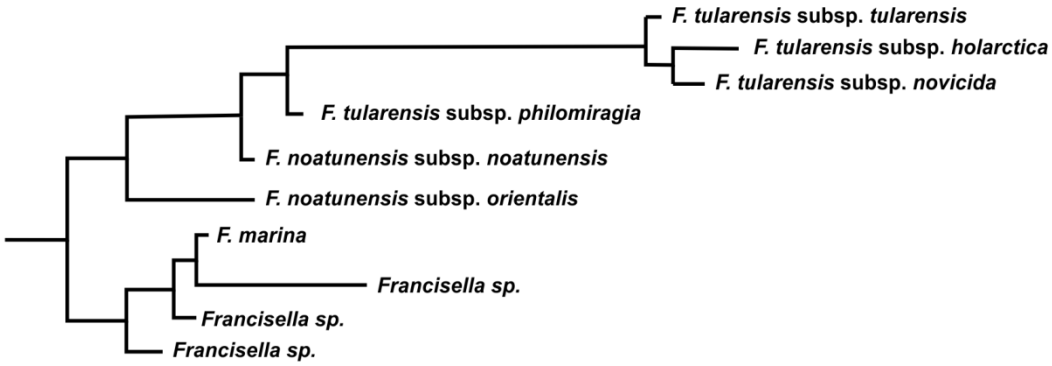

Figure 1. Phylogenetic tree based on Bayesian inference on 1,357bp of the 16S gene sequence adapted from Soto and collaborators (Soto, *et al.*, 2018).

In summary, *F. tularensis* is a human-pathogen, while *Francisella noatunensis* is a pathogen of aquatic hosts, and *F. novicida, F. hispaniensis* and *F. philomiragia* are rarely identified as pathogens, only in cases of immunocompromised humans, and are normally classified as free-living organisms.

Francisella virulence

Based on studies, the virulence of *F. tularensis* was quantified to 1 CFU for mice, guinea pig and rabbit using subcutaneous or intradermal route (Kingry & Petersen, 2014). However, studies with *F. novicida* using the same animals and infection routes reviewed that the infectious doses necessary for disease development ranged, from 10 to 10^7, 10 to 10^5 and $>10^8$ CFU, respectively (Kingry & Petersen, 2014). Pulpipat *et al.* reported FNO median lethal doses of 9.06×10^3 CFU/fish by intraperitoneal routes, at 25ºC (Pulpipat, *et al.*, 2019), whereas Soto and collaborators reported lethal doses up to 23 CFU/fish in an experimental assay (Soto, *et al.*, 2009).

Although not fully understood, the cycle of life and infection routes of *Francisella* spp. have already been highly discussed in the literature. Asare and Kwaik in a review, described the *F. tularensis* intracellular trafficking within macrophages (Figure 2) (Asare & Kwaik, 2011). They reported that *F. tularensis* enters into host cells through binding to surface receptors, generating an uptake inside as a phagocytosis. After 30-60 min, the bacterium performs the acidification of the phagosome to dissolve it and escape to the cytosol. Once in the cytosol, *F. tularensis* promote the delay of cell death for its survival and replication. And, in the end of the infectious cycle, the pathogen induces cell apoptosis allowing the release of the bacteria, starting a new infectious cycle. Modulation of phagosome biogenesis and escape into the cytosol is encoded by the genes of type VI secretion system (T6SS) (Asare & Kwaik, 2011).

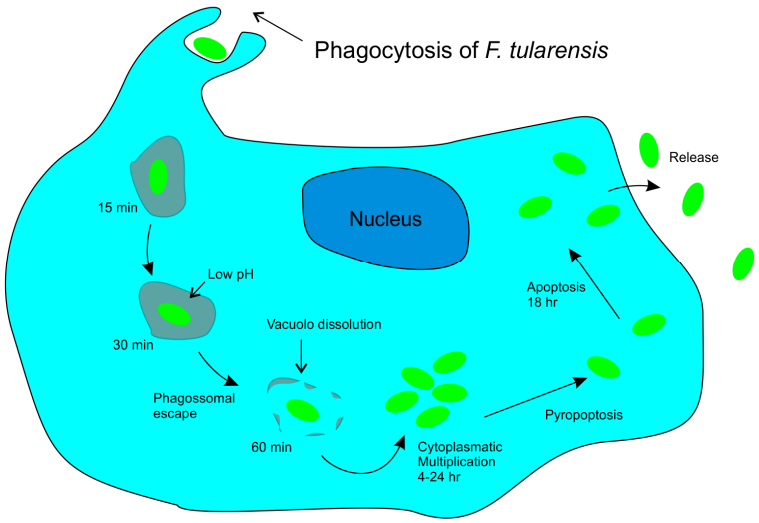

Figure 2. Intracellular trafficking of *Francisella tularensis* within macrophages, adapted from Asare and Kwaik (Asare & Kwaik, 2011).

Several genes of T6SS in *Francisella* spp. are clustered in a genome island (Figure 3), referred as *Francisella* Pathogenicity Island (FPI) (Nano & Schmerk, 2007). The FPI is a remarkable feature of *Francisella* genus (Eshraghi, *et al.*, 2016), and there are studies discussing the pathogen virulence mechanisms, such as phagosomal escape, intracellular replication, and evasion of host immune responses (Hare & Hueffer, 2014) that are related to these genes. In *F. tularensis*, *iglC* gene is required for the phagosomal escape (Barker, *et al.*, 2010). The *iglA*, *iglB*, *iglD*, and *pdpA* genes were showed to be required for intramacrophage growth (Nano & Schmerk, 2007). While *dotU* and *icmF* showed to have a role on secretion of VgrG (Zheng & Leung, 2007). The role of VgrG has been poorly understood. It is essential to *Edwardsiella tarda* virulence to fish (Zheng & Leung, 2007). Furthermore, transcription of several FPI genes is under the positive control of the regulators MglA and MglB located outside FPI (Lauriano, *et al.*, 2004).

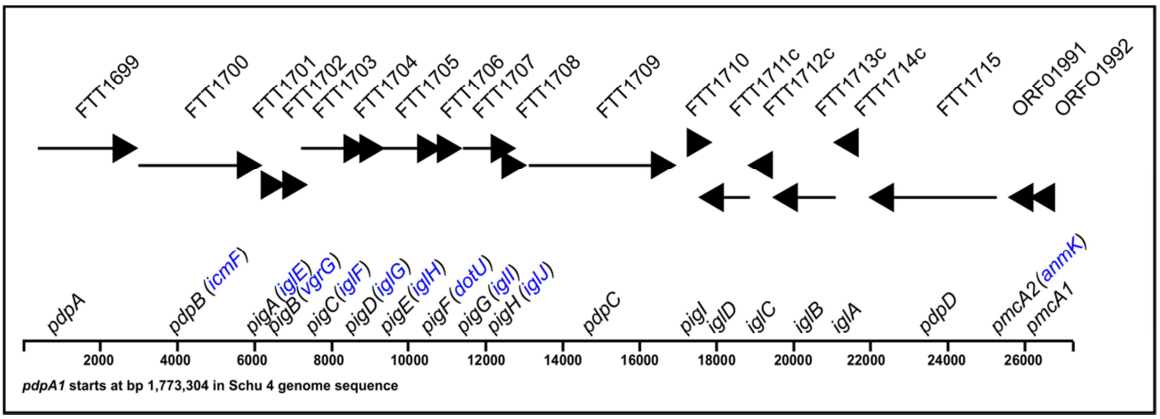

Figure 3. Schematic representation of the *Francisella* pathogenicity island (FPI) with gene names on genome sequence of *F. tularensis* Schu4 strain. Adapted from (Nano & Schmerk, 2007) and (de Bruin, *et al.*, 2007). Gene names in blue as cited by de Bruin et al.

Furthermore, *Francisella* genus codify genes related with type IV pili (T4p) (*i.e.*, responsible for adhesion, aggregation and DNA uptake) (Rowe & Huntley, 2015). Differenly from FPI, the genes related to T4p are not comprised on a genomic island.

T4p biogenesis is a process whereby a major pilin (*pilA*), is processed and translocated across the inner membrane to form a dynamic multimeric filament (Figure 4) (Salomonsson, *et al.*, 2011). PilD is responsible to translocate the major pilin across the inner membrane to form a multimeric pilus fimber, and afterwards is secreted across the outer membrane by PilQ (Salomonsson, *et al.*, 2011). Pili assembly is promoted by PilB (Turner, *et al.*, 1993), whereas

PilT promotes disassembly and retraction (Salomonsson, *et al.*, 2011). Eventhough PilC is essential for pili formation, its main functions are still unknown (Turner, *et al.*, 1993).

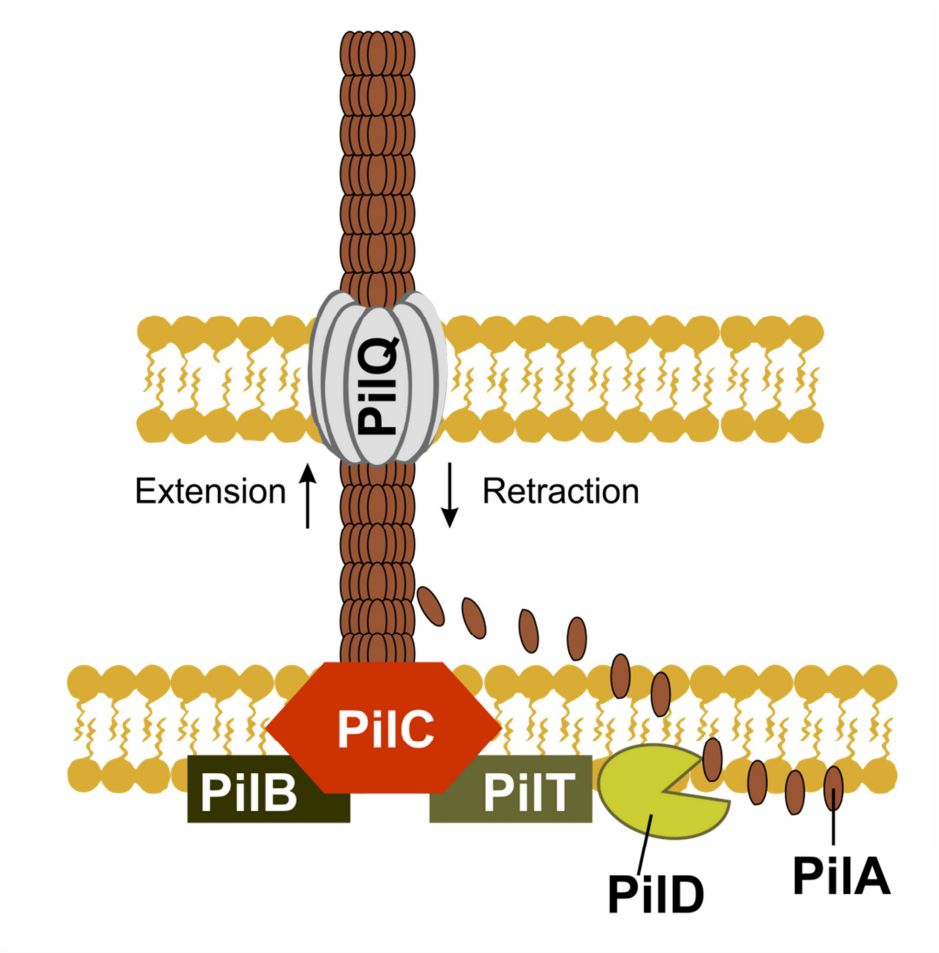

Figure 4. Schematic representation of the T4p machinery of *Francisella*. Adapted from (Salomonsson, *et al.*, 2011)

Furthermore, the TolC, a protein involved in the type I secretion system, which exports several bacterial virulence factors, plays an important role in *Francisella* pathogenicity, modulating their macrophage apoptotic and proinflammatory responses (Kopping, *et al.*, 2019). Proteins involved in siderophore biosynthesis, controlled by ferric uptake Fur, also were involved in virulence in the macrophage-like cells (Pérard, *et al.*, 2018). Also, a recent study showed that the outside pathogenicity island A (OpiA), a substrate of FPI, acts on phagosome escape (Ledvina, *et al.*, 2018). These virulence factors demonstrate the modulation of host immune response and different features of infection route-dependent impact on tularemia disease.

The pathogen *F. tularensis* has a broad host range with ~250 species of mammals, birds, reptiles, fishes, and invertebrates (Foley & Nieto, 2010) and its ability to multiply intracellularly is well documented (Soto, *et al.*, 2010). *F. tularensis* readily infects macrophages and reticulo-endothelial cells in *in-vivo* animal models, and also can be responsible for obliteration of hepatocytes (Foley & Nieto, 2010). The intracellular pathogen resides preferentially within different cell types, according to its nutritional requirement, for example, *F. tularensis* has its primary target the macrophages (Ray, *et al.*, 2009; Fortier, *et al.*, 1994).

FNO

Initiating in the 1990s, a granulomatous disease was identified in cultured tilapia in Taiwan (Chern & Chao, 1994), which was believed to be caused by a *Rickettsia*-like organism, due to the presence of intracellular organisms. Then, in 1994 and 1998, similar cases were verified in the USA, in tilapia and white seabass (*Atractoscion nobilis*), respectively, which were diagnosed as piscirickettsiosis-like (Mauel, *et al.*, 2003; Chen, *et al.*, 2000). It should be remembered that, the genus *Francisella* is part of the family *Francisellaceae*, order *Thiotrichales*, that also includes the family *Piscirickettsiaceae*, which contains the important fish pathogen, *Piscirickettsia salmonis* (Birkbeck, *et al.*, 2011).

Finally, in 2001, mortalities in cultured three-line grunt (*Parapristipoma trilineatum*) were observed in Japan, and from this outbreak, bacterial colonies were isolated and submitted to molecular analysis (*i.e.*, 16S rDNA gene sequencing), resulting in the identification of a *Francisella* sp. as the infectious agent (Kamaishi, *et al.*, 2005). In the same year, tilapia with suggestive clinical signs of *Rickettsia*-like organisms (Chern & Chao, 1994) from Taiwan was re-evaluated, and based on phylogenetic analysis, the isolates were identified as *Francisella* sp. (Hsieha, *et al.*, 2006).

Since then, other cases of francisellosis in tilapia have been reported in other countries, such as Indonesia (2004) (Ottem, *et al.*, 2009), Central America (2004) (Mauel, *et al.*, 2007), Costa Rica (2007) (Soto, *et al.*, 2009), Colombia (2009) (Iregui, *et al.*, 2011), the Midwest of the United States (2010) (Soto, *et al.*, 2011), the UK (2010) (Jeffery, *et al.*, 2010), Northern Europe (2012) (Ramírez-Paredes, *et al.*, 2017), Mexico (2012) (Ortega, *et al.*, 2016), Brazil (2012) (Leal, *et al.*, 2014), Thailand (2013) (Nguyen, *et al.*, 2016) and China (2013)

(Lin, *et al.*, 2016), as well as cases in ornamental fish from the USA (2013) (Soto, *et al.*, 2014) and from Austria (2014) (Lewisch, *et al.*, 2014).

Nowadays, FNO is one of the most important bacteria associated with disease outbreaks in farm-raised Nile tilapia in Brazil (Leal, *et al.*, 2014; Sebastião, *et al.*, 2017). The first description of FNO in the country occurred in different commercial farms located in the state of Minas Gerais in 2012 (Leal, *et al.*, 2014). After this description, other Brazilian states such as São Paulo (2013), Paraná (2015) and Santa Catarina (2015) also reported mortality of tilapia due to FNO infection (Sebastião, *et al.*, 2017).

Pathogenesis

The disease caused by FNO, known as francisellosis, primarily affects the fry and fingerling stages of fish, with sizes ranging between 6.5-9.0 cm (Leal, *et al.*, 2014), but it might also affect adults (Soto, *et al.*, 2010), presenting granulomatosis with multifocal granulomas in liver, spleen, and kidney (Soto, *et al.*, 2009). The affected fish may show an acute syndrome and high mortalities or a subacute to a chronic syndrome (Soto, *et al.*, 2012) with nonspecific clinical signs, such as anorexia, melanosis, exophthalmia, erratic swimming, skin ulcers, and gill pallor (Figure 5) (Mauel, *et al.*, 2007; Soto, *et al.*, 2009; Leal, *et al.*, 2014). In microscopic examinations, internal organs appear enlarged with white multifocal nodules, and in histological examinations, the presence of multifocal granulomatous lesions containing numerous small coccobacilli was found (Figure 6) (Soto, *et al.*, 2009; Soto, *et al.*, 2013).

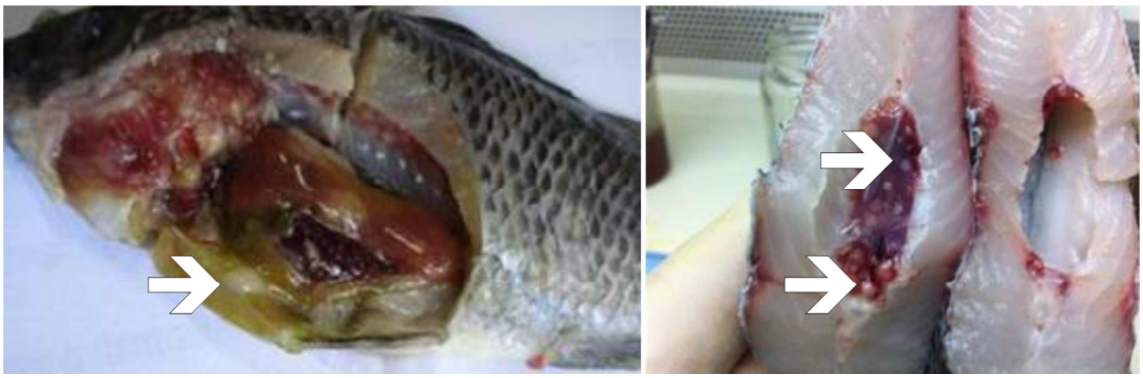

Figure 5. Multifocal nodules (arrows) found in clinical exams at National Reference Laboratory of Aquatic Animal Diseases – AQUACEN.

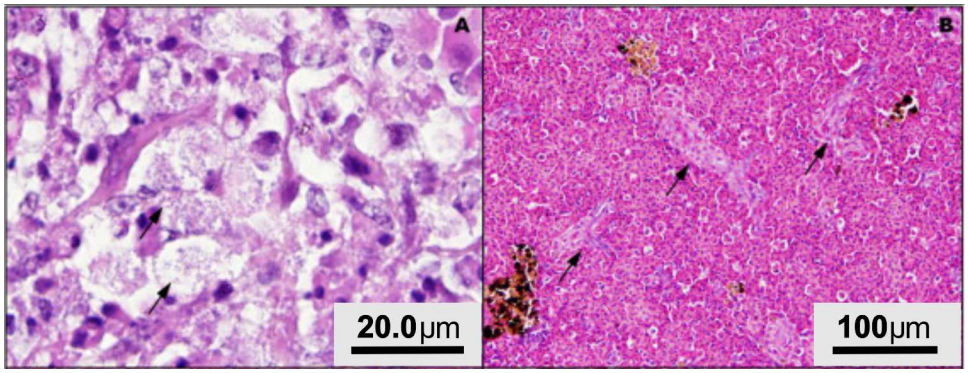

Figure 6. Sequential development of experimentally induced FNO granulomas in tilapia spleen. (A) Typical granuloma composed of macrophages with large vacuoles containing small coccoid bacteria (arrows). (B) Normal tilapia spleen illustrating ellipsoids or terminal arterioles sheathed by macrophages (arrows) adapted from (Soto, *et al.*, 2013).

Differently from *F. tularensis*, FNO species are restricted to hosts of water environments and that have the ability to survive in a wide range of cell types based on histopathological analysis of infected fish tissues (Soto, *et al.*, 2010). An additional study of Bakkemo *et al.* showed the mechanisms of invasion, survival and replication of FNO in Atlantic cod macrophage and epithelial-like cells (Bakkemo, *et al.*, 2016).

In fish, macrophages have an important role in the innate and acquired defense mechanisms (Norum, *et al.*, 2005). This cell type has been shown to be capable of producing reactive oxygen and nitrogen intermediates, and phagolysosome fusion, and thus, being a potent antimicrobial host defense (Rieger & Barreda, 2011). Fish macrophages are abundant in kidney, spleen and peritoneal cavity (Norum, *et al.*, 2005), which were the organs that have been reported to have multifocal granulomatous lesions (Soto, *et al.*, 2009; Leal, *et al.*, 2014).

Regarding *F. asiatica* (syn of FNO), a previously work (Soto, *et al.*, 2010) described the participation of the heat-sensitive serum component and mannose receptors for the efficient uptake of the microorganism, although the authors pointed to the fact that this may not be the only factors. This previous work and other reports of the same group (Soto, *et al.*, 2009) also indicate that the *iglC* gene (from FPI) is indispensable for survival, replication and cytotoxicity by promoting host cell apoptosis in Nile tilapia. Other gene of the *igl** operon, the *iglD*, plays an important role to guarantee the escape of the pathogen from the vacuole trap in *F. tularensis* (Bönquist, *et al.*, 2008), however its function is still unknown for FNO.

Regarding the environment, the literature shows that the FNO-outbreaks occurs when water temperature is between 21 and 24 ºC (Mauel, *et al.*, 2007; Soto, *et al.*, 2009; Iregui, *et*

al., 2011; Jeffery, *et al.*, 2010; Ortega, *et al.*, 2016; Nguyen, *et al.*, 2016). Soto and collaborators performed an experimental infection using two assays: 25 and 30 ºC and the mortalities and clinical signals were only identified at 25 ºC. Interestingly, it was also observed the disappearance of typical clinical signals of infected animals when the water temperature was gradually heated to 30 ºC (Soto, *et al.*, 2012). Likewise, the FNO cases reported in Brazil often occurred during the winter season, when water temperatures were less than or equal to 26 ºC (Leal, *et al.*, 2014), and without a FNO detection in warm-season, as demonstrated in a longitudinal study (Delphino, *et al.*, 2019). Using a water microcosm assay, the survival and viability of this bacterium was shown to decrease rapidly (Soto & Revan, 2012).

Host spectra and experimental infections routes

FNO and Rickettsia-like organisms are responsible for disease outbreaks in several aquatic hosts according to the literature: tilapia (*Oreochronuis* spp.) (Chern & Chao, 1994), atlantic salmon (*Salmo salar*) (Cvitanich, *et al.*, 1995), blue-eyed plecostomus (*Panaque suttoni*) (Khoo, *et al.*, 1995), sea bass (*Dicentrarchus labrax*) (Comps, *et al.*, 1996), one-blotch grouper (syn. black-spot grouper, blackspot honeycomb gouper, mero espaldaron; *Epinephelus melanostigma*) (Chen, *et al.*, 2000), three-line grunt (*Parapristipoma trilineatum*) (Fukuda, *et al.*, 2000), Norwegian cod (*Gadus morhua*) (Nylund, *et al.*, 2006), hybrid striped bass (*Morone chrysops* x *Morone saxitalis*) (Ostland, *et al.*, 2006), giant abalone (*Haliotis gigantea*) (Kamaishi, *et al.*, 2010), and various Malawi cichlids (*Nimbochromis venustus*, *Nimbochromis linni*, *Aulonocara stuartgranti*, *Placidochromis* sp., *Protomelas* sp., *Naevochromis chryosogaster*, *Copadichromis mloto*, *Otopharynx tetrastigma*) (Lewisch, *et al.*, 2014). Natural co-infections were found in Nile and Red tilapia (Assis, *et al.*, 2016; Pradeep, *et al.*, 2016). In addition to the above mentioned species, experimental infections have also been conducted: spotted wolffish (*Anarhichas minor* Olafsen) macrophages (Norum, *et al.*, 2005), zebrafish model (*Danio rerio*) (Vojtech, *et al.*, 2009), and sunfish (*Lepomis gibbosus*) (Lewisch, *et al.*, 2016). Also, there is a study that reports a natural vertical transmission of FNO in Red tilapia (Pradeep, *et al.*, 2017).

In *in-vivo* experimental infections, three pathways of infection have been largely described: intraperitoneal injections (Soto, *et al.*, 2009; Soto, *et al.*, 2009; Soto & Revan, 2012; Leal, *et al.*, 2014), gill spraying (Soto, *et al.*, 2009) and water submersion (Soto, *et al.*, 2009; Soto, *et al.*, 2013). Intraperitoneal injections showed the least median lethal doses, in

which clinical signals were observed with less than 23 CFU and at 23 CFU it can be observed acute infections, and eventually deaths (Soto, *et al.*, 2009).

Diagnosis

Methods to detect FNO in diseased fish include: bacteriological culture (nowadays, considered as the reference gold standard), electron microscopy, histopathology, PCR, real time quantitative PCR (qPCR) and *in situ* hybridization (Hsieh, *et al.*, 2007; Soto, *et al.*, 2010; Colquhoun & Duodu, 2011; Mauel, *et al.*, 2007; Ostland, *et al.*, 2006; Ottem, *et al.*, 2008; Kulkarni, *et al.*, 2011; Dong, *et al.*, 2016; Shahin, *et al.*, 2018). Real time quantitative PCR assays with high analytical sensitivities and specificities were developed (Soto, *et al.*, 2010; Duodu, *et al.*, 2012). In a previous work (Assis, *et al.*, 2017), it was calculated the sensitivities and specificities of bacteriological cultures and qPCR. It was determined that direct qPCR, using fresh or ethanol-fixed tissues was the best method to infer the FNO as the causative agent of francisellosis in Nile tilapia.

Genomics and Transcriptomics of fish pathogens

Genomics

Genomics is a multi-disciplinary field to study the whole-genome in large scale (McKusick & Ruddle, 1987; Venter, *et al.*, 2001). Once the read of the genetic sequence code was solved, the origins of all biological life could be better investigated and thus a revolution of biological studies has began (Zhang, *et al.*, 2011). Since the “original” sequencing methodology, the Sanger chemistry (Sanger, *et al.*, 1977; Sanger, 1975), several studies were performed to identify the whole-genomic contents of microorganisms, such as the first published *Haemophilus influenzae* Rd (Fleischmann, *et al.*, 1995).

Sanger’s technology allows sequencing of 600-1200 bp and in order to sequence longer DNA sections, new technologies were developed, such as the Next-Generation Sequencing (Venter, *et al.*, 2003) that is used for sequencing large genomes, like the human genome. Microbiology studies were shifted to the genomic era, which allows acquiring a big wave of data to be analyzed (Metzker, 2005; Forde & O’Toole, 2013). Whole-genomes and microbiomes can be sequenced in a few hours or days (Rothberg, *et al.*, 2011; Bentley, *et al.*, 2008), and thus at this time another scientific revolution has been created in different science

fields including molecular medicine, microbial physiology, agriculture and other fields (Tritt, *et al.*, 2012).

Among the NGS platforms, Roche/454 and Illumina/Solexa dominated the global market for the first generation sequencing. Basically, these technologies use a camera to identify the color of marked oligonucleotides during the synthesis of a complementary DNA (Bentley, *et al.*, 2008; Zhang, *et al.*, 2011). Afterwards, the second generation, Ion Torrent from Life Technologies was introduced and it uses a semiconductor to detect ion releases during the same complementary-DNA synthesis (Rothberg, *et al.*, 2011). Finally, the third and fourth generation were introduced, respectively, the PacBio (Schadt, *et al.*, 2010; Rhoads & Au, 2015) with the sequencing of a single cell and the MinION with a revolutionary reading process without polymerases, using a transmembrane reactive protein to identify the nucleotide sequence (Mikheyev & Tin, 2014). Likewise, the bioinformatics evolution field provides new applications to operate each feature of the NGS platforms (Nagarajan & Pop, 2009).

Starting with sequencing, sequence comparisons may be considered the most important method to identify microbial and animal diversity (Woese, *et al.*, 1990), and it is also the method that more benefited from the NGS generated data (Galperin & Kolker, 2006). Nowadays, sequence comparisons can be applied to all genes from a specific genome group (Maiden, *et al.*, 2013).

Comparison of sequences have initiated the field of comparative genomics, which is composed of tools to infer correlations and divergences of genome sequences, such as: composition of genome sequences of pathogenic and non-pathogenic strains (Arif, *et al.*, 2015; Prasanna & Mehra, 2013; Załuga, *et al.*, 2014), identification of common genes in a pathogen lineage to be used as vaccine targets (Seib, *et al.*, 2012), and genomic epidemiology to infer phylogenetic relations of isolates from different outbreaks (Ranjbar, *et al.*, 2014).

FNO genomics

Currently, there are available at the GenBank: eight complete genomes, one whole-genome shotgun (WGS) project and two single read archive (SRA) projects of FNO (Benson, *et al.*, 2013). The first released complete genome is the Toba04 isolate from Lake Toba of Indonesia (Sridhar, *et al.*, 2012). The second was LADL--07-285A strain from Costa Rica (GenBank accession number: CP006875.1). Four other complete genomes, the FNO01,

FNO12, FNO24 and FNO190 isolates, were submitted by our group and have described their genome sequences (Figueiredo, *et al.*, 2016; Gonçalves, *et al.*, 2016). One other complete genome comes from Brazil, which is the F1 isolate (Genbank accession number: CP018051.1). The WGS and SRA projects are from isolates from the United Kingdom, Costa Rica and Japan, respectively. The WGS project had a genome announcement published by Ramirez-Paredes *et al.* (Ramírez-Paredes, *et al.*, 2017).

The FNO genome is comprised in one chromosome with mean length size of 1,862,099±10,705 bp and a G+C content of 32.30±0.05%. The predicted gene varies from 1886 to 1917 (Gonçalves, *et al.*, 2016). After comparing with *F. philomiragia* subsp. *philomiragia* and *F. tularensis* subsp. *novicida,* which are two species with isolates of free-living lifestyle, the FNO fish parasite genome was found to be more compact (*i.e.*, genome of 1.8Mbp comparing with 2.05-2.20Mbp) and with a greater number of pseudogenes (*i.e.*, mean of ~260 comparing with 3-48) (Sridhar, *et al.*, 2012). And, in comparison with the mammalian parasite, *F. tularensis*, the FNO genome has a lower number of FPI (*i.e.*, $n = 1$ comparing with $n = 2$) and lower numbers of IS elements (*i.e.*, $n = 0$ comparing with $8>n>116$) (Sridhar, *et al.*, 2012; Gonçalves, *et al.*, 2016).

A recent study suggested that the FNO species is undergoing genome decay (Sjödin, *et al.*, 2012). The features of this reductive evolution are: the compact genome length and the high number of pseudogenes. This process can be associated with a niche selection or host specialization (Williams, *et al.*, 2009), or also associated with increase of virulence with loss of important metabolism genes (Kenn, 2012; Georgiades & Raoult, 2011).

Comparative genomic and typing applied to *Francisella*

Molecular genetics followed by molecular genomics methods were updated several times in last 20 years, increasing in resolution or cost-effectiveness (MacCannell, 2013). These advances generated a plethora of methods, that can even now be considered obsolete (Keim, *et al.*, 2007). Nowadays, for *Francisella* genus there are methods available for differentiation from the species level and between isolates, as shown in Figure 7 from Keim and collaborators (Keim, *et al.*, 2007). From these methods, the 16S rRNA is the most used method to identify species and subspecies (Soto, *et al.*, 2009; Kamaishi, *et al.*, 2010; Birkbeck, *et al.*, 2011), whereas whole-genome multilocus sequence typing is used to discriminate even isolates.

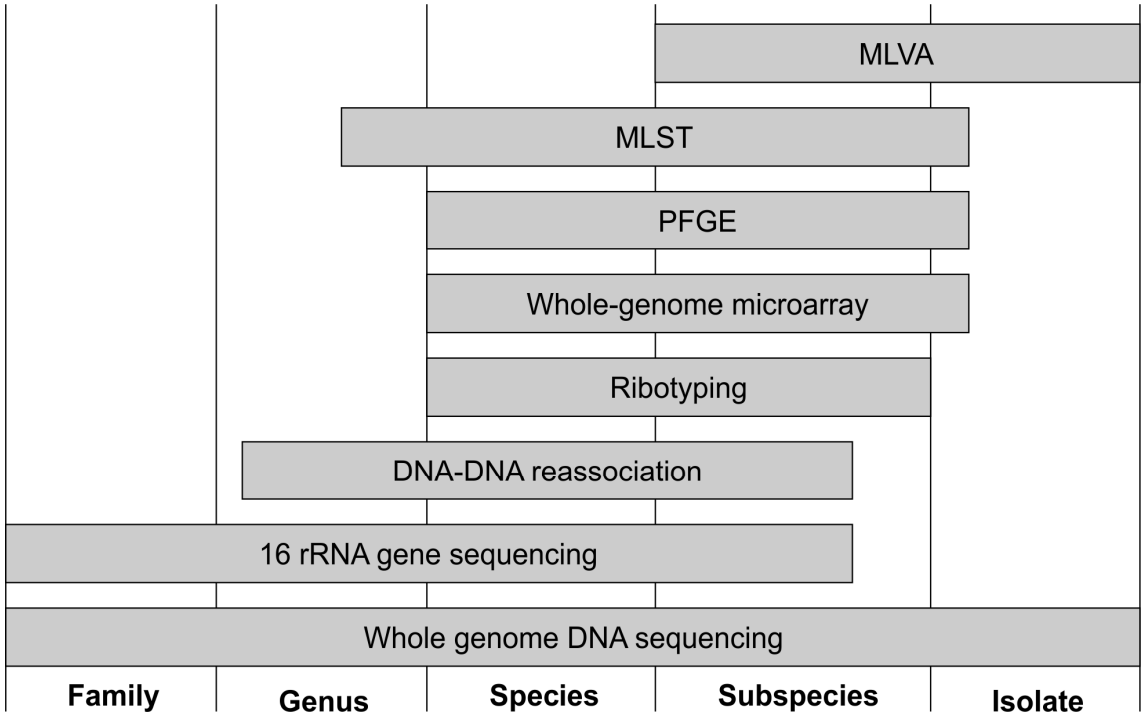

Figure 7. *Francisella* spp. resolution typing methods adapted from Keim *et al.* 2007

In the study of FNO elevation species from *F. philomiragia* subsp. *noatunensis* performed by the Bergen University group, the molecular typing methods applied were DNA-DNA hybridization, 16S rRNA gene sequencing and several other housekeeping genes (*i.e.*, a multilocus sequence analysis - MLSA) (Ottem, *et al.*, 2009). In another study, it was demonstrated the *Francisella* subspecies discrimination using a multiple-locus variable-number of tandem repeat analysis (MLVA). Although the results showed the discrimination of FNO from other *Francisella* spp., the number of used isolates were very low ($n = 2$), and the discrimination of the isolates could not be achieved (Duodu, *et al.*, 2013).

A previous work by our group showed the molecular relation of FNO isolates from different fish farms in Brazil using repetitive extragenic palindromic-PCR (REP-PCR) (Leal, *et al.*, 2014). In this study, it was found the first indication that the Brazilian isolate belonged to a clonal population. In another study by our group (Gonçalves, *et al.*, 2016), three isolates were compared using genomic techniques, such as blast nucleotide identity and synteny analyses. Once again, considering the high similarities of the genome sequences of the isolates, a clonally relation was suggested (Figure 8).

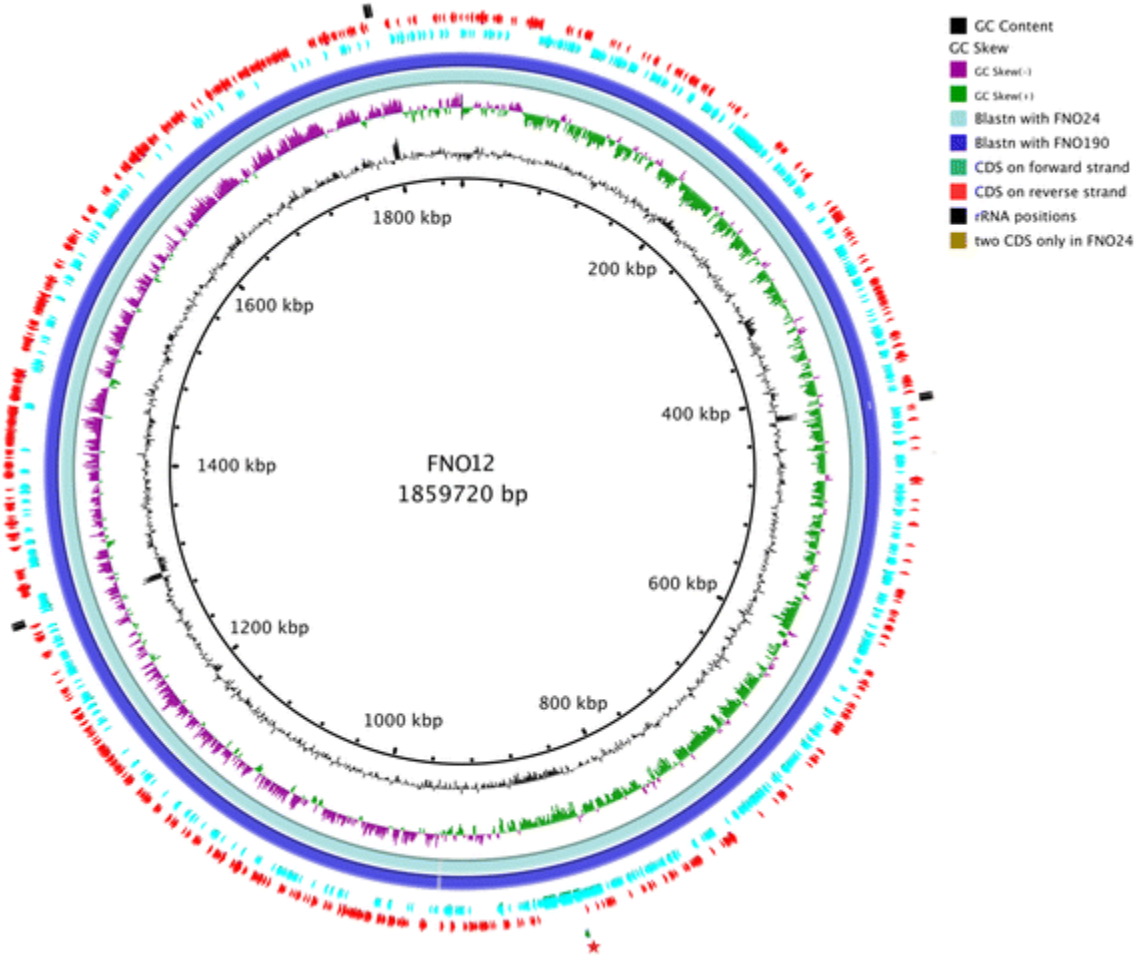

Figure 8. Graphical circular map of *Francisella noatunensis* subsp. *orientalis* strain FNO12 in comparison with FNO24 and FNO190, reproduced from (Gonçalves, *et al.*, 2016).

Transcriptomics

Transcriptomic is an "omic" field to study the whole repertoire of mRNA transcripts (*i.e.*, transcriptome) in a cell or organism in a specific developmental stage or physiological condition (Wang, *et al.*, 2009). The mRNAs is the first step for protein synthesis, responsible for phenotypical and morphological features of cells, and, hence, the mRNAs may denote a sensitive response to external environmental triggers (Lockhart & Winzeler, 2000).

The study of gene level expression dates from 1977 using northern-blots. Since then, RNase-protection assays, differential plaque-filter hybridization, Sanger sequencing of cDNA, EST libraries and qPCR techniques were applied to discover the existence and abundance of mRNAs (Kozian & Kirschbaum, 1999). With the NGS advent, a large scale of gene sequences and new techniques were created. The first technique and still used nowadays in large scale was the DNA microarray, consisting in directly synthesis of known DNA

sequences on a glass slide, followed by hybridization of the complementary cDNA and photolithographic technology (Koizumi, 2004).

Also following NGS technologies, a technique called RNA-Seq was described (Bainbridge, *et al.*, 2006), consisting in using the NGS platforms to sequence whole cDNA of a cell/microorganism. This method allowed RNA sequencing without the knowledge of the genome, hence, some authors recognize as a genome-wide unbiased method (Mäder, *et al.*, 2011). Another feature of RNA-Seq that needs to be considered as a technological upgrade is the increase of sensibility by sequencing the mRNAs with minor abundances. This is quite important in eukaryote cells, which contains mRNAs with more hundred folds than other organisms (Wang, *et al.*, 2009).

During the pathogen infective process, a different combination of gene expression levels reveals distinct patterns and behaviors of the parasitic microorganism, such as the environments' metabolites, pathogen life cycle, and/or host immune scape necessity (Koizumi, 2004). Likewise, pathogenic bacteria were also studied by using transcriptomic assays, such as: *S. agalactiae* isolates from human and fish hosts at different incubation temperatures (Mereghetti, *et al.*, 2008; Tavares, *et al.*, 2018), *Escherichia coli* upon glucose repression (Borirak, *et al.*, 2015), heat shock stress of differential expression in *Corynebacterium pseudotuberculosis* (Gomide, *et al.*, 2018) and temperature influences on gene expression of *Lactococcus garvieae* (Castro, *et al.*, 2017; Aguado-Urda, *et al.*, 2013).

FNO transcriptomics

For FNO, there are only two studies evaluating gene expression. Brudal *et al.* used Reverse Trancription qPCR (RT-qPCR) to determine between eight reference gene candidates, the most suitable for normalization in transcriptomic studies (Brudal, *et al.*, 2013).

The second study, more recently, Lewis and Soto evaluated the expression of T6SS genes, present on FPI, on different temperature and oxidative and acid stress conditions (Lewis & Soto, 2019). This work indicates that the T6SS putative genes expression change at low temperatures and oxidative stress suggesting a role in pathogenicity (Lewis & Soto, 2019).

FNO emergency

The FNO is still an emerging pathogen and being spread continuosly worldwide, with high economic losses to the Nile tilapia fish-farms, mainly for farms located in regions which have at least one cold season during the year. With this in mind, Brazil that has Nile tilapia as its principal commodity and has the major production farms in subtropical regions (IBGE, 2017). FNO infections are a major hindrance to the devevelopment of the nacional fish production.

Despite its emergency, FNO genomics are still poorly studied. Clonality inferences were discussed in the literacture using pre-genomic tools and average nucleotide identity. However, there are no studies using high resolution whole-genome based approaches to allow better knowledge about the relations of the genome and epidemiogenomics of this species.

Furthermore, the pathogen's genomic regulation and the disease epidemiology remain widely unknown. Further studies should be conducted to determine the seasonal effects of disease outbreaks especially including at temperatures below 26ºC. There had been only a few studies using a small part of the whole-genome (*i.e.*, <10 genes). A screening of the whole-genome regulatory mechanisms might provide a better understanding about host-pathogen interactions and their environment.

Hypothesis

The FNO fish pathogen has one single entry into Brazil in 2012, potentially related to acquisition of broodsotck fish to produce fingerlings, and modulates their gene profile expression in response to temperature.

Objectives

General objective

The aims of this study were to evaluate the phylogenomic relationship of FNO isolates from Brazilian outbreak between 2012 and 2016, and to caraterize the virulence of this pathogen associated to temperature changes.

Specific objectives

- To sequency, to assembly and to annotate one isolate from each farm FNO outbreak;
- To perform analysis using high-resolution genomic approach to characterize the FNO Brazilian population and compare with isolate from other countries;
- To perform recombination analysis in order to identify multi-strain occurence;
- To quantify the median lethal doses (LD_{50}) of this bacteria in cultive temperature of 22º and 28ºC, using an *in-vivo* experiment assay with one Brazilian isolate;
- To evaluate whole-genome transcriptomic expression to identify changes in adaptation of this pathogen under different temperatures.

Chapter 2. Paper "Complete genome sequencing of sixteen *Francisella noatunensis* subsp. *orientalis* isolates: A genomic approach for molecular characterization and spread dynamics of this clonal population"

Chapter 3. Paper "Effects of temperature changes in the transcriptional profile of the emerging fish pathogen *Francisella noatunensis* subsp. *orientalis*"

General Discussion

The franciselosis affects mainly the fry and fingerlings, and the major productive cost of fish-farming is the fattening stage, then, theoretically, the losses not entail significant investment losses. However, the planning of productive chain is constantly broken, with losses of almost whole lots, and a new starts of production cicle is required affecting the final weight of the animals on slaughter industry. Nowadays, the Brazilian aquaculture sector considers that, the FNO shares with *Streptococcus agalactiae* the main challenge in infection diseases of cultured tilapia. The data produced in this thesis not only contributed with literacture knowlegment about the pathogen and the relation of its host, but starts discussions of how the tilapia farmers can improve the management to mitigate the losses with franciselosis disease.

A molecular approach with genome resolution was applied to study isolates from different Brazilian outbreaks and showed to be effective to depict an epidemiological status of this pathogen. The genetic drift of isolates found with high-resolution genomic analysis allowed the access of each Brazilian outbreak from South and Central-south state regions. However, there is no evidence of transit of animals between fish farms. The spread of the pathogen might be more linked with animal acquisition from hatcheries, which produce firgerlings to several grow-out fish farms. To decrease and/or to avoid the dissemination of this pathogen is necessary that the Government applies rigorous controls in animal transit, mainly of the fry and fingerling stages from markets sale.

Regarding to the genomes sequence, data corroborated that FNO species is in a reductive evolution with a small genome sequence (when compared with free-living species of the genus) and with a high number of pseudogenes (Sjödin, *et al.*, 2012; Sridhar, *et al.*, 2012). The proteogenomic assay used in the present study is the first comprehensive omics approach to show a confirmation of this genomic indication. This reductive evolution seems to affect the complete genome, once the pseudogenes were found equally distributed by entire genome sequence.

The generation of genomic data was perfomed using different approaches and technologies. Ion Torrent Personal Genome Machine and Illumina MiSeq were used to the sequencing. An optical map of one isolate was acquired, from OpGen Map, to orient the *ab initio* assembly. Furthermore, after the assembly and scaffolds generations, gaps were found

in repetitive regions and in protein tandem repeats (PTR) loci. Repetitive regions are filled using bioinformatics techniques and PTR loci were supported by prime design and Sanger sequencing. On the final of assembly process, the reads were mapped against the final chromosome and a mean of 1.2±1% of reads were discarded; this mean is normally found in assembly process and is composed of sequencing artifacts. The final genome chromosome sequence presented a GC content of 33.4±0%, which is common between bacterium of the *Francisella* genus.

In order to compare the generated FNO genome sequences of this work with the genomes of FNO strains from other countries, the genomes data were downloaded from GenBank. Among then, the whole-genome sequence of FNO Toba04 isolate from Idonesia. This was the first genome of FNO sequenced, in 2012, by Sridhar *et al*. (Sridhar, *et al.*, 2012). They used the 454-pyrosequencing to sequencing 56,522,682 bp, its means a vertical coverage of 30×. In the analyses on this work, the most divergent genome between Brazilian and worldwide isolates was the Toba04 strain. Although considered in all analyses, this isolate may inputed a bias on the results, which was pondered in the published papers. A resequencing of this isolate, using the new state of the art in sequencing technologies, can improve the knowledge about this pathogen.

Likewise genomic information, FNO pathogenesis information still reduced in the literature. Fields' information of the seasonal characteristic of FNO-pathogenesis found in Brazilian fish-farms and in longitudinal studies was confirmed. The Chapter 3 is the first peer reviewed study to demonstrante the LD50 at 22ºC and to show a comparison of LD50 required in two different temperatures. This data is also relevant to assure the results generated by *in-vitro* experiments of gene expression. The expression of the putative virulence genes did not change it's regulation in tested temperatures, although the same temperature change was a significant effect in the infecting doses of the *in-vivo* experiment. The metabolism can be associated with an improved fitness of replication and, maybe, promoting the disease clinical signals and host mortalities. Further studies need to be conducted to explain the relationship between metabolic associated genes and the virulence of this pathogen. Genes related to virulence maintain the high-expression (in comparison with other genes) in both temperatures. Taken together this demonstrates a different adaptation of FNO, a host niche adaptation, since the fish is a poikilothermic animal, where substantial variations of body temperature occur as a result of water temperature changes.

Conclusion

Similar genomic contents of Brazilian isolates accessed by pre-genomic tools and dates of the first cases suggest a single entry of the FNO pathogen into Brazil in 2012. In order to confirm these sugestions, the experiments of Chapter 2 were conducted. Recombination analysis showed that isolates belongs to a clonal population, and high-resolution genomic analysis reveal slight mutational events from each isolate. The Brazilian lineage forms a paraphyletic clade with strains isolated in other countries, which did not enabled to suggest the origin of this lineage, but allows the inference about single entry into the country. The mutational events identified at the first cases displayed that the entrance of pathogen in the country occurred before this year in a not accessed case.

Regarding the virulence shifted by temperature, in the cases of *F. tularensis*, an important human-pathogen from the same genus, the expression of several genes, including the virulence related, are modulated by temperature (*i.e.*, increasing the expression when stay in the temperature of the host and facilitating the infection). Franciselosis outbreaks in fish-farms is associated with water temperature < 25ºC. In order to confirm the similar adaptation of the FNO to temperature, experiments of Chapter 3 were conducted. LD_{50} assay showed that temperature plays an important role in infectivity at 22ºC (*i.e.*, temperature with field evidences of disease) when compred with 28ºC (*i.e.*, temperature without or undetectable disease). However, *in vitro* assay at the same temperatures, genes related with metabolism and oxidative stress had significative expression modulation.

References

Agnew, W., Barnes, A. C. (2007) Streptococcus iniae: An aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Veterinary Microbiology.* **122**(1-2), 1-15.

Aguado-Urda, M., Gibello, A., Blanco, M. M., Fernández-Garayzábal, J. F., López-Alonso, V., López-Campos, G. H. (2013) Global transcriptome analysis of Lactococcus garvieae strains in response to temperature. *PLoS One.* **8**(11), e79692; doi:10.1371/journal.pone.0079692.

Arif, M., Zaidi, N. W., Haq, Q. M., Singh, Y. P., Taj, G., Kar, C. S., Singh, U. S. (2015) Morphological and comparative genomic analyses of pathogenic and non-pathogenic Fusarium solani isolated from Dalbergia sissoo. *Mol Biol Rep.*. **42**(6), 1107-22; doi:10.1007/s11033-014-3849-3..

Asare, R., Kwaik, Y. A. (2011) Exploitation of host cell biology and evasion of immunity by Francisella tularensis. *Front. Microbiol.*. **1**, 145; doi:10.3389/fmicb.2010.00145 .

Assis, G. B. N., de Oliveira, T. F., Gardner, I. A., Figueiredo, H. C. P., Leal, C. A. G. (2017) Sensitivity and specificity of real-time PCR and bacteriological culture for francisellosis in farm-raised Nile tilapia (Oreochromis niloticus L.). *J Fish Dis.*. **40**(6), 785-795.

Assis, G. B. N., Tavares, G. C., Pereira, F. L., Figueiredo, H. C. P., Leal, C. A. G. (2016) Natural coinfection by Streptococcus agalactiae and Francisella noatunensis subsp. orientalis in farmed Nile tilapia (Oreochromis niloticus L.). *Journal of Fish Diseases.* **40**(1), 51-63; doi:10.1111/jfd.12493.

Bainbridge, M. N., Warren, R. L., Hirst, M., Romanuik, T., Zeng, T., Go, A., Delaney, A., Griffith, M., Hickenbotham, M., Magrini, V., Mardis, E. R., Sadar, M. D., Siddiqui, A. D., Marra, M. A., Jones, S. J. M. (2006) Analysis of the prostate cancer cell line LNCaP transcriptome using a sequencing-by-synthesis approach. *BMC Genomics.* **7**, 246; doi:10.1186/1471-2164-7-246.

Bakkemo, K. R., Mikkelsen, H., Johansen, A., Robertsen, B., Seppola, M. (2016) Francisella noatunensis subsp. noatunensis invades, survives and replicates in Atlantic cod cells. *Dis Aquat Org.* **121**, 149–159.

Barker, J. R., Chong, A., Wehrly, T. D., Yu, J.-J., Rodriguez, S. A., Liu, J., Celli, J., Arulanandam, B. P., Klose, K. E. (2010) The Francisella tularensis Pathogenicity Island Encodes a Secretion System that is required for Phagosome Escape and Virulence. *Mol Microbiol.*. **74**(6), 1459–1470.

Barony, G. M., Tavares, G. C., Pereira, F. L., Carvalho, A. F., Dorella, F. A., Leal, C. A. G. F. H. C. P. (2017) Large-scale genomic analyses reveal the population structure and evolutionary trends of Streptococcus agalactiae strains in Brazilian fish farms. *Sci Rep.*. **7**, 13538; doi:10.1038/s41598-017-13228-z.

Barroso, R. M., Muñoz, A. E. P., Tahin, E. F., Webber, D. C., Albuquerque-Filho, A. C., Pedrosa-Filho, M. X. (2018) Diagnóstico da cadeia de valor da tilapicultura no Brasil. *EMBRAPA.*

Benson, D., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D., Ostell, J., Sayers, E. (2013) GenBank. *Nucleic Acids Res.*. **41**(Database issue), D36-42; doi:10.1093/nar/gks1195.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G. e. (2008) Accurate whole human genome sequencing using reversible terminator chemistry. *Nature.* **456**, 53-59; doi:10.1038/nature07517.

Birkbeck, T. H., Feist, S. W., Verner-Jeffreys, D. W. (2011) Francisella infections in fish and shellfish. *Journal Fish Diseases.* **34**(3), 173-87.

Bönquist, L., Lindgren, H., Golovliov, I., Guina, T., Sjöstedt, A. (2008) MglA and Igl proteins contribute to the modulation of Francisella tularensis live vaccine strain-containing phagosomes in murine macrophages. *Infect Immun.*. **76**(8), 3502-10.

Borirak, O., Rolfe, M. D., de Koning, L. J., Hoefsloot, H. C., Bekker, M., Dekker, H. L., Roseboom, W., Green, J., de Koster, C. G., Hellingwerf, K. J. (2015) Time-series analysis of the transcriptome and proteome of Escherichia coli upon glucose repression. *Biochim Biophys Acta.*. **1854**(10 Pt A), 1269-79; doi:10.1016/j.bbapap.2015.05.017.

Brevik, Ø. J., Ottem, K. F., Kamaishi, T., Watanabe, K., Nylund, A. (2011) Francisella halioticida sp. nov., a pathogen of farmed giant abalone (Haliotis gigantea) in Japan. *Journal of Applied Microbiology.* **111**, 1044-1056.

Brudal, E., Winther-Larsen, H. C., Colquhoun, D. J., Duodu, S. (2013) Evaluation of reference genes for reverse transcription quantitative PCR analyses of fish-pathogenic Francisella strains exposed to different growth conditions. *BMC Research Notes.* **6**, 76.

Castro, R., Reguera-Brito, M., López-Campos, G. H., Blanco, M. M., Aguado-Urda, M., Fernández-Garayzábal, J. F., Gibello, A. (2017) How does temperature influences the development of lactococcosis? Transcriptomic and immunoproteomic in vitro approaches. *J Fish Dis.*. **40**(10), 1285-1297; doi:10.1111/jfd.12601.

Chen, M. F., Yun, S., Marty, G. D., McDowell, T. S., House, M. L., Appersen, J. A., Guenther, T. A., Arkush, K. D., Hedrick, R. P. (2000) A Piscirickettsia salmonis-like bacterium associated with mortality of white seabass Atractoscion nobilis. *Dis. Aquat. Organ.*. **43**(2), 117-26.

Chen, S. C., Wang, P. C., Tung, M. C., Thompson, K. D., Adams, A. (2000) A Piscirickettsia salmonis-like organism in grouper, Epinephalus melanostigma, in Taiwan. *Journal of Fish Diseases.* **23**, 415-418.

Chern, R. S., Chao, C. B. (1994) Outbreaks of a disease caused by Rickettsia-like organism in cultured Tilapias in Taiwan. *Fish Pathology.* **29**, 61-71.

Colquhoun, D. J., Duodu, S. (2011) Francisella infections in farmed and wild aquatic organisms. *Vet Res.*. **42**, 47.

Comps, M., Raymond, M. J., Plasiart, G. N. (1996) Rickettsia-like organism infecting juvenile sea bass (Dicentrarchus labrax). *Bulletin of the European Association of Fish Pathologists.* **16**, 30–33.

Cvitanich, J. D., Garate, N. O., Silva, P. C., Andrave, V. M., Figueroa, P., Smith, C. E. (1995) Isolation of a new rickettsia-like organism from Atlantic salmon in Chile. *American Fisheries Society Fish Health Section Newsletter.* **23**, 1-3.

de Bruin, O. M., Ludu, J. S., Nano, F. E. (2007) The Francisella pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth. *BMC Microb.*. **7**, 1; doi:10.1186/1471-2180-7-1.

Delphino, M. K. V. C., Leal, C. A. G., Gardned, Y., Assis, G. B. N., Roriz, G. D., Ferreira, F., Figueiredo, H. C. P., Gonçalves, V. S. P. (2019) Seasonal dynamics of bacterial pathogens of Nile tilapia farmed in a Brazilian reservoir. *Aquaculture.* **498**, 100-108; doi:10.1016/j.aquaculture.2018.08.023.

Dong, H. T., Gangnonngiw, W., Phiwsaiya, K., Charoensapsri, W., Nguyen, V. V., Nilsen, P., Pradeep, P. J., Withyachumnarnkul, B., Senapin, S., Rodkhum, C. (2016) Duplex PCR assay and in situ hybridization for detection of Francisella spp. and Francisellanoatunensis subsp. orientalis in red tilapia. *Dis Aquat Organ.*. **120**(1), 39-47; doi:10.3354/dao03021.

Duodu, S., Larsson, P., Sjodin, A., Forsman, M., Colquhoun, D. J. (2012) The distribution of Francisella-like bacteria associated with coastal water in Norway. *Microbial Ecology.* **64**, 370-377.

Duodu, S., Wan, X., Tandstad, N. M., Larsson, P., Myrtennäs, K., Sjödin, A., Forsman, M., Colquhoun, D. J. (2013) An improved multiple-locus variable-number of tandem repeat analysis (MLVA) for the fish pathogen Francisella noatunensis using capillary electrophoresis. *BMC Vet Res.*. **9**, 252; doi:10.1186/1746-6148-9-252.

Eshraghi, A., Kim, J., Walls, A. C., Ledvina, H. E., Miller, C. N., Ramsey, K. M., Whitney, J. C., Radey, M. C., Peterson, S. B., Ruhland, B. R., Tran, B. Q., Goo, Y. A., Goodlett, D. R., Dove, S. L., Celli, J., Veesler, D., Mougous, J. D. (2016) Secreted Effectors Encoded within and outside of the Francisella Pathogenicity Island Promote Intramacrophage Growth. *Cell Host & Microbe.* **20**(5), 573-583; doi:10.1016/j.chom.2016.10.008.

FAO, (2018) The State of World Fisheries and Aquaculture (SOFIA). Available at: http://www.fao.org/3/CA0191EN/CA0191EN.pdf; Accessed in June, 26, 2019..

Figueiredo, H. C. P., Leal, C. A. G., Pereira, F. L., Soares, S. C., Gongalves, L. A., Dorella, F. A., Carvalho, A. F., Azevedo, V. A. C. (2016) Whole-Genome Sequence of Francisella noatunensis subsp. orientalis Strain FNO01 Isolated from Diseased Nile Tilapia in Brazil. *Genome Announc.*. **4**(1), e01603-15; doi:10.1128/genomeA.01603-15.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M., al., e. (1995) Whole-Genome Random Sequencing and Assembly of Haemophilus influenzae Rd. *Science.* **269**(5223), 496-512.

Foley, J. E., Nieto, N. C. (2010) Tularemia. *Veterinary Microbiology.* **140**(3-4), 332-338.

Forde, B. M., O'Toole, P. W. (2013) Next-generation sequencing technologies and their impact on microbial genomics. *Briefings in Functional Genomics.* **12**(5), 440-53; doi:10.1093/bfgp/els062.

Fortier, A. H., Green, S. J., Polsinelli, T., Jones, T. R., Crawford, R. M., Leiby, D. A., Elkins, K. L., Meltzer, M. S., Nacy, C. A. (1994) Life and death of an intracellular pathogen: Francisella tularensis and the macrophage. *Immunol Ser.*. **60**, 349–361.

Fukuda, Y., Okamura, A., Nishiyama, M., Kawakami, H., Kamaishi, T., Yoshinga, T. (2000) Granulomatosis of cultured three-line grunt Parapristipoma trilineatum caused by an intracellular bacterium. *Fish Pathology.* **37**, 119-124.

Fukushima, H. C. S., Leal, C. A. G., Cavalcante, R. B., Figueiredo, H. C. P., Arijo, S., Moriñigo, M. A., Ishikawa, M., Borra, R. C., Ranzani-Paiva, M. J. T. (2017) Lactococcus garvieae outbreaks in Brazilian farms Lactococcosis in Pseudoplatystoma sp. – development of an autogenous vaccine as a control strategy.. *J Fish Dis.* **40**, 263–272.

Galperin, M. Y., Kolker, E. (2006) New metrics for comparative genomics. *Current Opinion in Biotechnology.* **17**(5), 440-447; doi:10.1016/j.copbio.2006.08.007.

Georgiades, K., Raoult, D. (2011) Defining pathogenic bacterial species in the genomic era. *Front. Microbiol.*. **1**, 151; doi:10.3389/fmicb.2010.00151.

Gomide, A. C. P., de Sa, P. G., Cavalcantes, A. L. Q., Souza, T. J., Gomes, L. G. R., Ramos, R. T. J., Azevedo, V., Silva, A., Folador, A. R. C. (2018) Heat shock stress: Profile of differential expression in Corynebacterium pseudotuberculosis biovar Equi. *Gene.* **645**, 124-130; doi:10.1016/j.gene.2017.12.015.

Gonçalves, L. A., Soares, S. C., Pereira, F. L., Dorella, F. A., Carvalho, A. F., Almeida, G. M. F., Leal, C. A. G., Azevedo, V., Figueiredo, H. C. P. (2016) Complete genome sequences of Francisella noatunensis subsp. orientalis strains FNO12, FNO24 and FNO190: a fish pathogen with genomic clonal behavior. *Standards in Genomic Sciences.* doi:10.1186/s40793-016-0151-0.

Hare, R. F., Hueffer, K. (2014) Francisella novicida Pathogenicity Island Encoded Proteins Were Secreted during Infection of Macrophage-Like Cells. *PLoS One.* **9**(8), e105773; doi:10.1371/journal.pone.0105773.

Hollis, D. G., Weaver, R. E., Steigerwalt, A. G., Wenger, J. D., Moss, C. W., Brenner, D. J. (1989) Francisella philomiragia comb. nov. (formerly Yersinia philomiragia) and Francisella tularensis biogroup novicida (formerly Francisella novicida) associated with human disease. *J Clin Microbiol.* . **27**(7), 1601-8.

Hsieha, C. Y., Tunga, M. C., Tub, C., Changa, C. D., Tsaia, S. S. (2006) Enzootics of visceral granulomas associated with Francisella-like organism infection in tilapia (Oreochromis spp.). *Aquaculture.* **254**(1-4), 129–138.

Hsieh, C. Y., Wu, Z. B., Tung, M. C., Tsai, S. S. (2007) PCR and in situ hybridization for the detection and localization of a new pathogen Francisella-like bacterium (FLB) in ornamental cichlids. *Diseases of Aquatic Organisms.* **75**, 29-36.

Huber, B., Escudero, R., Busse, H. J., Seibold, E., Scholz, H. C., Anda, P., Kämpfer, P., Splettstoesser, W. D. (2010) Description of Francisella hispaniensis sp. nov., isolated from human blood, reclassification of Francisella novicida (Larson et al. 1955) Olsufiev et al. 1959 as Francisella tularensis subsp. novicida comb. nov. and emended description of the genus Franc. *Int J Syst Evol Microbiol.*. **60**(Pt 8), 1887-96.

IBGE, (2017) Produção da Pecuária Municipal. *Instituto Brasileiro de Geografia e Estatística.* **45**, 1-8, Rio de Janeiro..

Iregui, C. A., Vasquez, G. M., Rey, A. L., Verjan, N. (2011) Piscirickettsia-Like Organisms as a Cause of Acute Necrotic Lesions in Colombian Tilapia Larvae. *Journal of Veterinary Diagnostic Investigation.* **23**(1), 147-151.

Jeffery, K. R., Stone, D., Feist, S. W., Verner-Jeffreys, D. W. (2010) An outbreak of disease caused by Francisella sp. in Nile tilapia Oreochromis niloticus at a recirculation fish farm in the UK. *Dis Aquat Organ.*. **91**, 161-5; doi:10.3354/dao02260.

Johansson, A., Celli, J., Conlan, W., Elkins, K. L., Forsman, M., Keim, P. S., Larsson, P., Manoil, C., Nano, F. E., Petersen, J. M., Sjöstedt, A. (2010) Objections to the transfer of Francisella novicida to the subspecies rank of Francisella tularensis. *Int J Syst Evol Microbiol.*. **60**(Pt 8), 1717-8.

Kamaishi, T., Fukuda, Y., Nishiyama, M., Kawakami, H., Matsuyama, T., Yoshinaga, T., Oseko, N. (2005) Identification and Pathogenicity of Intracellular Francisella Bacterium in Three-line Grunt Parapristipoma trilineatum. *Fish Pathology.* **40**(2), 67-71.

Kamaishi, T., Miwa, S., Goto, E., Matsuyama, T., Oseko, N. (2010) Mass mortality of giant abalone Haliotis gigantea caused by a Francisella sp. bacterium. *Diseases of Aquatic Organisms.* **89**, 145-154.

Kay, W., Petersen, B. O., Duus, J. Ø., Perry, M. B., Vinogradov, E. (2006) Characterization of the lipopolysaccaride and β-glucan of the fish pathogen Francisella victoria. *FEBS Journal.* **273**, 3002–3013.

Keim, P., Johansson, A., Wagner, D. M. (2007) Molecular Epidemiology, Evolution, and Ecology of Francisella. *Annals of the New York Academy of Sciences.* **1105**, 30-66.

Kenn, E. C., (2012) Paradigms of pathogenesis: targeting the mobile genetic elements of disease. *Front Cell Infect Microbiol.*. **2**, 161; doi:10.3389/fcimb.2012.00161.

Khoo, L., Dennis, P. M., Lewbart, G. (1995) Rickettsia-like organisms in the blue-eyed plecostomus, Panaque suttoni (Eigenmann and Eigenmann). *Journal of Fish Diseases.* **18**, 157–164.

Kingry, L. C., Petersen, J. M. (2014) Comparative review of Francisella tularensis and Francisella novicida. *Front Cell Infect Microbiol.*. **4**, 35.

Koizumi, S. (2004) Application of DNA Microarray in Occupational Health Researct. *J Occup Health.*. **46**, 20-25.

Kopping, E. J., Doyle, C. R., Sampath, V., Thanassi, D. G. (2019) Contributions of TolC Orthologs to Francisella tularensis Schu S4 Multidrug Resistance, Modulation of Host Cell Responses, and Virulence. *Infect Immun.*. **87**(4), e00823-18; doi:10.1128/IAI.00823-18.

Kozian, D. H., Kirschbaum, B. J. (1999) Comparative gene-expression analysis. *Trends in Biotechnology.* **17**(2), 73-78.

Kubitza, F. (2015) Aquicultura no Brasil: principais espécies, áreas de cultivo, rações, fatores limitantes e desafios. *Panorama da Aquicultura.* **25**(150), 10-23.

Kulkarni, A., Caipang, C. M., Korsnes, K., Brinchmann, M. F., Kiron, V. (2011) Molecular diagnosis of francisellosis, a systemic granulomatous inflammatory disease in Atlantic cod, Gadus morhua L. *Vet Res Commun.*. **35**(2), 67-77; doi:10.1007/s11259-010-9451-9.

Larson, M. A., Nalbantoglu, U., Sayood, K., Zentz, E. B., Cer, R. Z., Iwen, P. C., Francesconi, S. C., Bishop-Lilly, K. A., Mokashi, V. P., Sjostedt, A., Hinrichs, S. H. (2016) Reclassification of Wolbachia persica as Francisella persica comb. nov. and emended description of the family Francisellaceae. *Int J Syst Evol Microbiol.*. **66**, 1200-1205.

Lauriano, C. M., Barker, J. R., Yoon, S. S., Nano, F. E., Arulanandam, B. P., Hassett, D. J., Klose, K. E. (2004) MglA regulates transcription of virulence factors necessary for Francisella tularensis intraamoebae and intramacrophage survival. *Proc Natl Acad Sci U S A.* **101**(12), 4246-9.

Leal, C. A. G., Tavares, G. C., Figueiredo, H. C. P. (2014) Outbreaks and genetic diversity of Francisella noatunensis subsp orientalis isolated from farm-raised Nile tilapia (Oreochromis niloticus) in Brazil. *Genet. Mol. Res.*. **13**(3), 5704-12; doi:10.4238/2014.July.25.26.

Ledvina, H. E., Kelly, K. A., Eshraghi, A., Plemel, R. L., Peterson, S. B., Lee, B., Steele, S., Adler, M., Kawula, T. H., Merz, A. J., Skerrett, S. J., Celli, J., Mougous, J. D. (2018) A Phosphatidylinositol 3-Kinase Effector Alters Phagosomal Maturation to Promote Intracellular Growth of Francisella. *Cell Host Microbe.*. **24**(2), 285-295.e8; doi:10.1016/j.chom.2018.07.003.

Lewisch, E., Dressler, A., Menanteau-Ledouble, S., Saleh, M., El-Matbouli, M. (2014) Francisellosis in ornamental African cichlids in Austria. *Bull. Eur. Ass. Fish Pathol.*. **34**(2), 65.

Lewisch, E., Menanteau-Ledouble, S., Tichy, A., El-Matbouli, M. (2016) Susceptibility of common carp and sunfish to a strain of Francisella noatunensis subsp. orientalis in a challenge experiment. *Dis Aquat Organ.*. **121**(2), 161-166.

Lewis, J., Soto, E. (2019) Gene expression of putative type VI secretion system (T6SS) genes in the emergent fish pathogen Francisella noatunensis subsp. orientalis in different physiochemical conditions. *BMC Microbiology.* **19**, 21; doi:10.1186/s12866-019-1389-7.

Lin, Q., Li, N., Fu, X., Hu, Q., Chang, O., Liu, L., Zhang, D., Wang, G., San, G., Wu, S. (2016) An outbreak of granulomatous inflammation associated with Francisella noatunensis subsp. orientalis in farmed tilapia (Oreochromis niloticus × O. aureus) in China. *Chinese Journal of Oceanology and Limnology.* **34**(3), 460–466.

Lockhart, D. J., Winzeler, E. A. (2000) Genomics, gene expression and DNA arrays. *Nature.* **405**, 827-836; doi:10.1038/35015701.

MacCannell, D. (2013) Bacterial Strain Typing. *Clinics in Laboratory Medicine.* **33**, 629-650; doi:10.1016/j.cll.2013.03.005.

Mäder, U., Nicolas, P., Richard, H., Bessières, P., Aymerich, S. (2011) Comprehensive identification and quantification of microbial transcriptomes by genome-wide unbiased methods. *Current Opinion in Biotechnology.* **22**(1), 32-41.

Maiden, M. C., Jansen van Rensburg, M. J., Bray, J. E., Earle, S. G., Ford, S. A., Jolley, K. A., McCarthy, N. D. (2013) MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat Rev Microbiol.*. **11**(10), 728-36; doi:10.1038/nrmicro3093.

Mauel, M. J., Miller, D. L., Frazier, K., Liggett, A. D., Styer, L., Montgomery-Brock, D., Brock, J. (2003) Characterization of a Piscirickettsiosis-Like Disease in Hawaiian Tilapia. *Dis. Aquat. Organ.*. **53**(3), 249-255.

Mauel, M. J., Soto, E., Moralis, J. A., Hawke, J. (2007) A piscirickettsiosis-like syndrome in cultured Nile tilapia in Latin America with Francisella spp. as the pathogenic agent. *Journal of Aquatic Animal Health.* **19**(1), 27-34.

McKusick, V. A., Ruddle, F. H. (1987) Toward a complete map of the human genome. *Genomics.* **1**(2), 103-106.

Mereghetti, L., Sitkiewicz, L., Green, N. M., Musser, J. M. (2008) Remodeling of the Streptococcus agalactiae Transcriptome in Response to Growth Temperature. *PLoS One.* **3**(7), e2785; doi:10.1371/journal.pone.0002785.

Metzker, M. L., (2005) Emerging technologies in DNA sequencing. *Genome Research.* **15**(12), 1767-1776; doi:10.1101/gr.3770505.

Mikalsen, J., Olsen, A. B., Tengs, T., Colquhoun, D. J. (2007) Francisella philomiragia subsp. noatunensis subsp. nov., isolated from farmed Atlantic cod (Gadus morhua L.).. *Int J Syst Evol Microbiol.*. **57**(Pt 9), 1960-5.

Mikheyev, A. S., Tin, M. M. Y. (2014) A first look at the Oxford Nanopore MinION sequencer. *Molecular Ecology Resources.* **14**(6), 1097-1102.

Nagarajan, N., Pop, M. (2009) Parametric Complexity of Sequence Assembly: Theory and Applications to Next Generation Sequencing. *Journal of Computational Biology.* **16**(7), 897-908; doi:10.1089/cmb.2009.0005.

Nano, F. E., Schmerk, C. (2007) The Francisella Pathogenicity Island. *Annals of the New York Academy of Sciences.* **1105**, 122-137; doi:10.1196/annals.1409.000.

Nguyen, V. V., Dong, H. T., Senapin, S., Pirarat, N., Rodkhum, C. (2016) Francisella noatunensis subsp. orientalis, an emerging bacterial pathogen affecting cultured red tilapia (Oreochromis sp.) in Thailand. *Aquaculture Research.* **47**(11), 3697–3702.

Norum, M., Bøgwald, J., Dalmo, R. A. (2005) Isolation and characterisation of spotted wolffish (Anarhichas minor Olafsen) macrophages. *Fish & Shellfish Immunology.* **18**(5), 381-391.

Nylund, A., Ottem, K. F., Watanabe, K., Karlsbakk, E., Krossøy, B. (2006) Francisella sp (Family Francisellaceae) causing mortality in Norwegian cod (Gadus morhua) farming. *Archives of Microbiology.* **185**, 383-392.

Ortega, C., Mancera, G., Enríquez, R., Vargas, A., Martínez, S., Fajardo, R., Avendaño-Herrera, R., Navarrete, M. J., Romero, A. (2016) First identification of Francisella noatunensis subsp. orientalis causing mortality in Mexican tilapia Oreochromis spp.. *Diseases of Aquatic Organisms.* **120**, 205-215; doi:10.3354/dao02999.

Ostland, V. E., Stannard, J. A., Creek, J. J., Hedrick, R. P., Ferguson, H. W., Carlberg, J. M., Westerman, M. E. (2006) Aquatic Francisella-like bacterium associated with mortality of intensively-cultured hybrid striped bass Morone chrysops x M. saxitalis. *Disease of Aquatic Organisms.* **72**, 135-145.

Ottem, K. F., Nylund, A., Isaksen, T. E., Karlsbakk, E., Bergh, Ø. (2008) Occurrence of Francisella piscicida in farmed and wild Atlantic cod, Gadus morhua L., in Norway. *Journal of Fish Diseases.* **31**, 525-534.

Ottem, K. F., Nylund, A., Karlsbakk, E., Friis-Møller, A., Krossøy, B., Knappskog, D. (2007) New species in the genus Francisella (Gammaproteobacteria; Francisellaceae); Francisella piscicida sp. nov. isolated from cod (Gadus morhua). *Arch Microbiol.* **188**, 547.

Ottem, K. F., Nylund, A., Karlsbakk, E., Friis-Møller, A., Kamaishi, T. (2009) Elevation of Francisella philomiragia subsp. noatunensis† to Francisella noatunensis comb. nov. [syn. Francisella piscicida Ottem et al. (2008) syn. nov.] and characterization of Francisella noatunensis subsp. orientalis subsp. nov., two important fish pa. *Journal of Applied Microbiology.* **106**, 1231-1243; doi:10.1111/j.1365-2672.2008.04092.x.

Owen, C. R. B. E. O., Jellison, W. L., Lackman, D. B., F, B. J. (1964) Comparative studies of Francisella tularensis and Francisella novicida. *J Bacteriol.*. **87**, 676-83.

Pérard, J., Nader, S., Levert, M., Arnaud, L., Carpentier, P., Siebert, C., Blanquet, F., Cavazza, C., Renesto, P., Schneider, D., Maurin, M., Coves, J., Crouzy, S., Michaud-Soret, I. (2018) Structural and functional studies of the metalloregulator Fur identify a promoter-binding mechanism and its role in Francisella tularensis virulence. *Commun Biol.*. **1**, 93; doi:10.1038/s42003-018-0095-6.

Pradeep, P. J., Suebsing, R., Sirithammajak, S., Kampeera, J., Turner, W., Jeffs, A., Kiatpathomchai, W., B, W. (2016) Vertical transmission and concurrent infection of multiple bacterial pathogens in naturally infected red tilapia (Oreochromis spp.). *Aquaculture Research.* **48**(6), 2706-2717.

Pradeep, P. J., Suebsing, R., Sirithammajak, S., Kampeera, J., Turner, W., Jeffs, A., Kiatpathomchai, W., Withyachumanarnkul, B. (2017) Vertical transmission and concurrent infection of multiple bacterial pathogens in naturally infected red tilapia (Oreochromis spp.). *Aquac Res.*. **48**, 2706-2717.

Prasanna, A. N., Mehra, S. (2013) Comparative phylogenomics of pathogenic and non-pathogenic mycobacterium. *PLoS One.* **8**(8), e71248; doi:10.1371/journal.pone.0071248.

Pulpipat, T., Lin, K., Chen, Y., Wang, P., Chen, S. (2019) Molecular characterization and pathogenicity of Francisella noatunensis subsp. orientalis isolated from cultured tilapia (Oreochromis sp.) in Taiwan. *J. of Fish Dis.*. **42**(5), 643-655.

Qu, P. H., Chen, S. Y., Scholz, H. C., Busse, H. J., Gu, Q., Kämpfer, P., Foster, J. T., Glaeser, S. P., Chen, C., Yang, Z. C. (2013) Francisella guangzhouensis sp. nov., isolated from air-conditioning systems. *Int J Syst Evol Microbiol.*. **63**(Pt 10), 3628-35.

Qu, P. H., Li, Y., Salam, N., Chen, S. Y., Liu, L., Gu, Q., Fang, B. Z., Xiao, M., Li, M., Chen, C., Li, W. J. (2016) Allofrancisella inopinata gen. nov., sp. nov. and Allofrancisella frigidaquae sp. nov., isolated from water-cooling systems, and transfer of Francisella guangzhouensis Qu et al. 2013 to the new genus as Allofrancisella guangzhouensis comb. nov.. *Int J Syst Evol Microbiol.*. **66**(11), 4832-4838.

Ramírez-Paredes, J. G., Larsson, P., Wehner, S., Bekaert, M., Öhrman, C., Metselaar, M., Thompson, K. D., Richards, R. H., Penman, D. J., Adams, A. (2017) Draft Genome Sequence of Francisella noatunensis subsp. orientalis STIR-GUS-F2f7, a Highly Virulent Strain Recovered from Diseased Red Nile Tilapia Farmed in Europe. *Genome Announc.*. **11**, e01555-16; doi:10.1128/genomeA.01555-16.

Ranjbar, R., Karami, A., Farshad, S., Giammanco, G. M., Mammina, C. (2014) Typing methods used in the molecular epidemiology of microbial pathogens: a how-to guide. *New Microbiol.*. **37**(1), 1-15.

Ray, K., Marteyn, B., Sansonetti, P. J., Tang, C. M. (2009) Life on the inside: the intracellular lifestyle of cytosolic bacteria. *Nature Reviews Microbiology.* **7**, 333–340.

Rhoads, A., Au, K. F. (2015) PacBio Sequencing and Its Applications. *Genomics, Proteomics & Bioinformatics.* **13**(5), 278-289.

Rieger, A. M., Barreda, D. R. (2011) Antimicrobial mechanisms of fish leukocytes. *Developmental & Comparative Immunology.* **35**(12), 1238-1245.

Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., et.al., (2011) An integrated semiconductor device enabling non-optical genome sequencing. *Nature.* **475**, 348-352; doi:10.1038/nature10242.

Rowe, H. M., Huntley, J. F. (2015) From the Outside-In: The Francisella tularensis Envelope and Virulence. *Front Cell Infect Microbiol.*. **5**, 94: doi:10.3389/fcimb.2015.00094.

Salomonsson, E. N., Forslund, A.-L., Forsberg, Å. (2011) Type IV pili in Francisella – a virulence trait in an intracellular pathogen. *Frontiers in Microbiology.* **2**, 29; doi:10.3389/fmicb.2011.00029.

Sanger, F. (1975) The Croonian Lecture, 1975. Nucleotide sequences in DNA. *Proc R Soc Lond B Biol Sci.*. **191**(1104), 317-33.

Sanger, F., Nicklen, S., Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci.*. **74**, 5463-5467.

Schadt, E., Turner, S., Kasarskis, A. (2010) A window into third-generation sequencing. *Human Molecular Genetics.* **19**(R2), R227-R240.

Sebastião, F. A., Pilarski, F., Kearney, M. T., E, S. (2017) Molecular detection of Francisella noatunensis subsp. orientalis in cultured Nile tilapia (Oreochromis niloticus L.) in three Brazilian states. *J. Fish Dis.*. doi:10.1111/jfd.12636.

Seib, K. L., Zhao, X., Rappuoli, R. (2012) Developing vaccines in the era of genomics: a decade of reverse vaccinology. *Clin Microbiol Infect.*. **5**, 109-16; doi:10.1111/j.1469-0691.2012.03939.x..

Shahin, K., Ramirez-Paredes, J. G., Harold, G., Lopez-Jimena, B., Adams, A., Weidmann, M. (2018) Development of a recombinase polymerase amplification assay for rapid detection of Francisellanoatunensis subsp. orientalis. *PLoS One.* **13**(2), e0192979; doi:10.1371/journal.pone.0192979.

Sjödin, A., Svensson, K., Öhrman, C., Ahlinder, J., Lindgren, P., Duodu, S., Johansson, A., Colquhoun, D. J., Larsson, P., Forsman, M. (2012) Genome characterisation of the genus Francisella reveals insight into similar evolutionary paths in pathogens of mammals and fish. *BMC Genomics.* **13**, 268; doi:10.1186/1471-2164-13-268.

Skerman, V. B. D., Mcgowan, V., Sneath, P. H. A. (1980) Approved Lists of Bacterial Names. *Int J Syst Bacteriol.* **30:225-420**.

Soto, E., Abrams, S. B., Revan, F. (2012) Effects of temperature and salt concentration on Francisella noatunensis subsp. orientalis infections in Nile tilapia Oreochrimis niloticus. *Dis Aquat Organ.*. **101**(3), 217-23.

Soto, E., Baumgartner, W., Wiles, J., Hawke, J. P. (2011) Francisella asiatica as the causative agent of piscine francisellosis in cultured tilapia (Oreochromis sp.) in the United States. *Journal of Veterinary Diagnostic Investigation.* **23**(4), 821–825.

Soto, E., Bowles, K., Fernandez, D., Hawke, J. P. (2010) Development of a real-time PCR assay for identification and quantification of the fish pathogen Francisella noatunensis subsp. orientalis. *Diseases of Aquatic Organisms.* **89**, 199-207.

Soto, E., Fernandez, D., Hawke, J. P. (2009) Attenuation of the Fish Pathogen Francisella sp. by Mutation of the iglC* Gene. *Journal of Aquatic Animal Health.* **21**(3), 140-1449; doi:10.1577/H08-056.1.

Soto, E., Fernandez, D., Thune, R., Hawke, J. P. (2010) Interaction of Francisella asiatica with Tilapia (Oreochromis niloticus) Innate Immunity. *Infect. Immun.*. **78**(5), 2070-2078; doi:10.1128/IAI.01308-09.

Soto, E., Griffin, M. J., Morales, J. A., Calvo, E. B., Sebastião, F. A., Porras, A. L., Viques-Rodriguez, X., Reichley, S. R., Rosser, T. G., Ware, C., Byrne, B. A., Garcia, J. C., LaFrentz, B. R., Camus, A. C. (2018) Francisella marina sp. nov., Etiologic Agent of Systemic Disease

in Cultured Spotted Rose Snapper (Lutjanus guttatus) in Central America. *Applied and Environmental Microbiology.* **84**(16), e00144-18; doi:10.1128/AEM.00144-18.

Soto, E., Hawke, J. P., Fernandez, D., Morales, J. A. (2009) Francisella sp., an emerging pathogen of tilapia, Oreochromis niloticus (L.), in Costa Rica. *Journal of Fish Diseases.* **32**(8), 713-722; doi:10.1111/j.1365-2761.2009.01070.x.

Soto, E., Illanes, O., Hilchie, D., Morales, J. A., Sunyakumthorm, P., Hawke, J. P., Goodwin, A. E., Riggs, A., Yanong, R. P., Pouder, D. B., Francis-Floyd, R., Arauz, M., Bogdanovic, L., Castillo-Alcala, (2012) Molecular and immunohistochemical diagnosis of Francisella noatunensis subsp. orientalis from formalin-fixed, paraffin-embedded tissues. *J. Vet. Diagn. Invest.*. **24**(5), 840-5.

Soto, E., Kidd, S., Mendez, S., Marancik, D., Revan, F., Hiltchie, D., Camus, A. (2013) Francisella noatunensis subsp. orientalis pathogenesis analyzed by experimental immersion challenge in Nile tilapia, Oreochromis niloticus (L.). *Vet Microbiol.*. **164**(1-2), 77-84.

Soto, E., Primus, A. E., Pouder, D. B., George, R. H., Gerlach, T. J., Cassle, S. E., Johnson, T., Boyd, S., Handsel, T., Yanong, R. P. E. (2014) Identification of Francisella noatunensis in novel host species French grunt (Haemulon flavolineatum) and Caesar grunt (Haemulon carbonarium). *Journal of Zoo and Wildlife Medicine.* **45**(3), 727-731.

Soto, E., Revan, F. (2012) Culturability and persistence of Francisella noatunensis subsp. orientalis (syn. Franciesella asiática) in sea-and freshwater microcosms. *Micro Ecol.*. **63**(2), 398-404; doi:10.1007/s00248-011-9932-6.

Sridhar, S., Sharma, A., Kongshaug, H., Nilsen, F., Jonassen, I. (2012) Whole genome sequencing of the fish pathogen Francisella noatunensis subsp. orientalis Toba04 gives novel insights into Francisella evolution and pathogenecity. *BMC Genomics.* **13**, 598; doi:10.1186/1471-2164-13-598.

Tavares, G. C., Carvalho, A. F., Pereira, F. L., Rezende, C. P., Vasco, V. A. C., Leal, C. A. G., Figueiredo, H. C. P. (2018) Transcriptome and Proteome of Fish-Pathogenic Streptococcus agalactiae Are Modulated by Temperature. *Frontiers in Microbiology.* **9**, 2639; doi:10.3389/fmicb.2018.02639.

Tritt, A., Eisen, J. A., Facciotti, M. T., Darling, A. E. (2012) An Integrated Pipeline for de Novo Assembly of Microbial Genomes. *Plos One.* **7**(0), e42304; doi:10.1371/journal.pone.0042304.

Turner, L. R., Lara, J. C., Nunn, D. N., Lory, S. (1993) Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in Pseudomonas aeruginosa. *J Bacteriol.*. **175**(16), 4962-9.

Turner, L. R., Lara, J. C., Nunn, D. N., Lory, S. (1993) Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in Pseudomonas aeruginosa. *J Bacteriol.*. **175**(16), 4962-9.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W. M. R. J., Sutton, G. G., al, e. (2001) The Sequence of the Human Genome. *Science.* **291**(5507), 1304-1351; doi:10.1126/science.1058040.

Venter, J. C., Levy, S., Stockwell, T., Remington, K., Halpern, A. (2003) Massive parallelism, randomness and genomic advances. *Nat. Genet.*. **33**, 219-227.

Vojtech, L. N., Sanders, G. E., Conway, C., Ostland, V., Hansen, J. D. (2009) Host immune response and acute disease in a zebrafish model of Francisella pathogenesis. *Infection and Immunity.* **77**, 914-925.

Wang, Z., Gerstein, M., Snyder, M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics.* **10**, 57-63; doi:10.1038/nrg2484.

Williams, D. L., Slayden, A., Amin, A., Martinez, A. N., Pittman, T. L., Mira, A., Mitra, A., Nagaraja, V., Morrison, N. E., Moraes, N., Gillis, T. P. (2009) Implications of high level pseudogene transcription in Mycobacterium leprae. *BMC Genomics.* **10**, 397; doi:10.1186/1471-2164-10-397.

Woese, C. R., Kandler, O., Wheelis, M. L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *PNAS.* **87**, 4576-4579.

Załuga, J., Stragier, P., Baeyen, S., Haegeman, A., Van-Vaerenbergh, J., Maes, M., De Vos, P. (2014) Comparative genome analysis of pathogenic and non-pathogenic Clavibacter strains reveals adaptations to their lifestyle. *BMC Genomics.* **15**, 392; doi: 10.1186/1471-2164-15-392.

Zhang, J., Chiodinic, R., Badr, A., Zhang, G. (2011) The impact of next-generation sequencing on genomics. *Journal of Genetics and Genomics.* **38**(3), 95-109.

Zheng, J., Leung, K. Y. (2007) Dissection of a type VI secretion system in Edwardsiella tarda. *Mol Microbiol.*. **66**(5), 1192-206.