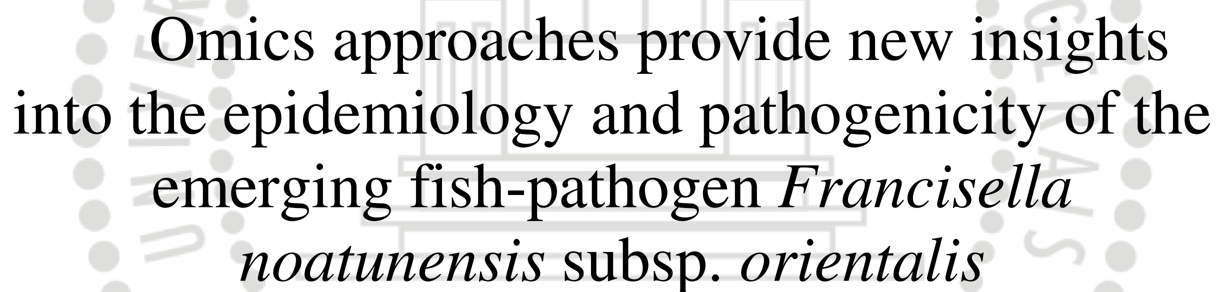
**FELIPE LUIZ PEREIRA**



# 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**

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**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*

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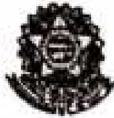

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# Felipe Luiz Pereira

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 | 95271171/6-04 | 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 | 54434188520 | APROVADO |
| Dr. Francisco Lobato | UFMG | 22471662671 | APROVADO |
| Dr. João Luís Reis Cunha | UFMG | 095.105.936-05 | 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 ____________

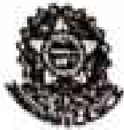
Dr. Vasco Ariston de Carvalho Azevedo - Coorientador ____________

Dr. Rafael Diego da Rosa ____________

Dr. Frederico Augusto de Alcântara Costa ____________

Dr. Aristóteles Góes Neto ____________

Dr. Francisco Lobato ____________



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Dr. João Luís Reis Cunha ______________________

CONFERE COM O ORIGINAL
Em: 24 / 09 / 19
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# 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.

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# 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

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# 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).

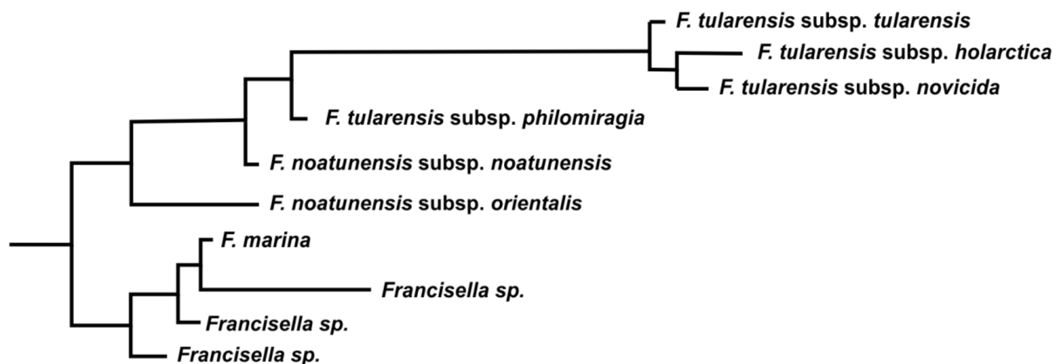


**Figure 1. Phylogenetic tree based on Bayesian inference on 1,357bp of the 16S gene sequence adapted from Soto and collaborators (Soto, *et al.*, 2018).**

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 $9.06 \times 10^3$ 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).

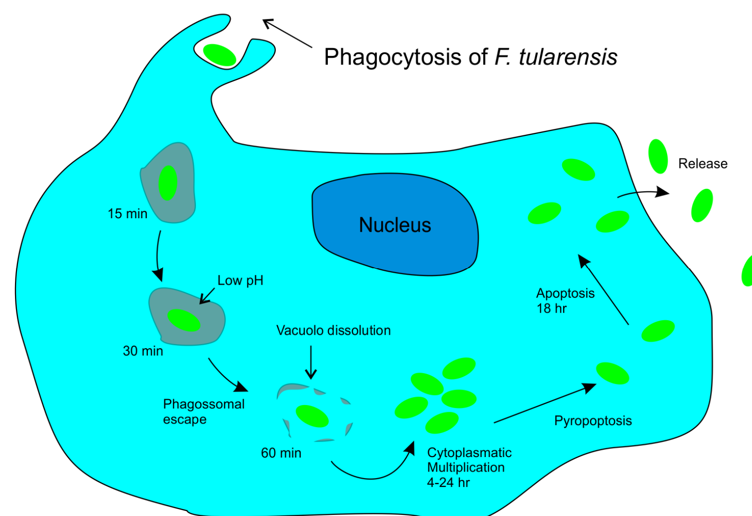


**Figure 2. Intracellular trafficking of *Francisella tularensis* within macrophages, adapted from Asare and Kwaik (Asare & Kwaik, 2011).**

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).

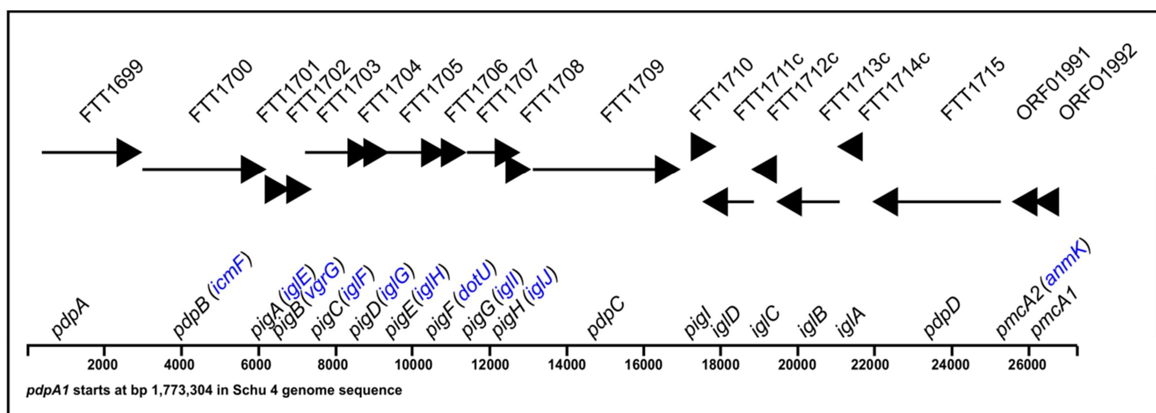


**Figure 3. Schematic representation of the *Francisella* pathogenicity island (FPI) with gene names on genome sequence of *F. tularensis* Schu4 strain. Adapted from (Nano & Schmerk, 2007) and (de Bruin, *et al.*, 2007). Gene names in blue as cited by de Bruin et al.**

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).

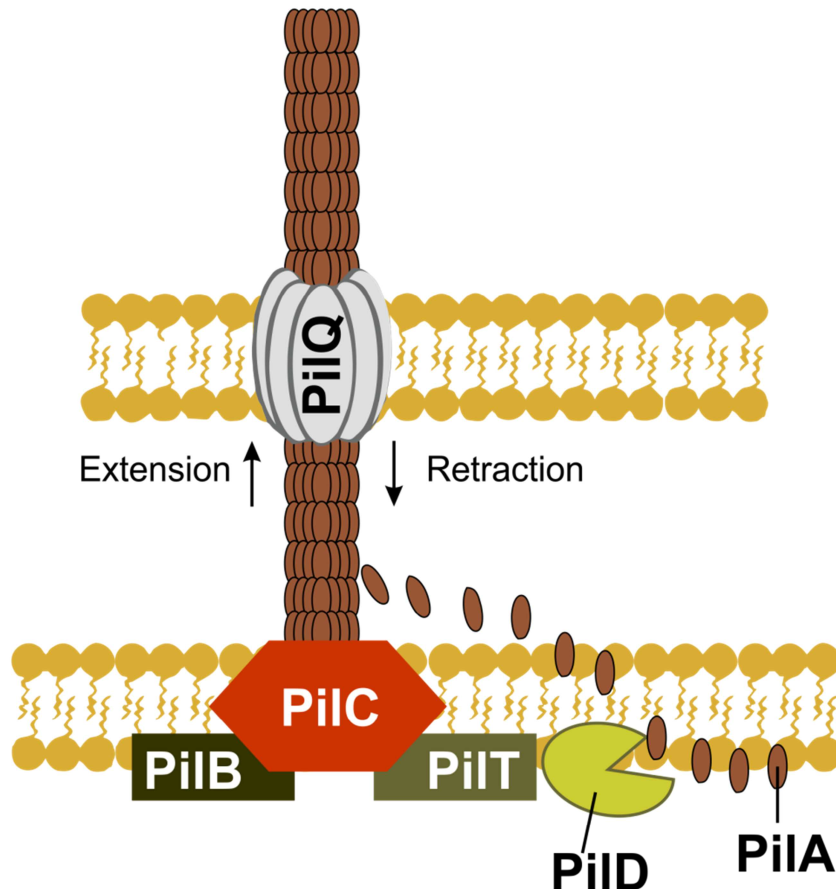


**Figure 4. Schematic representation of the T4p machinery of *Francisella*. Adapted from (Salomonsson, *et al.*, 2011)**

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.**

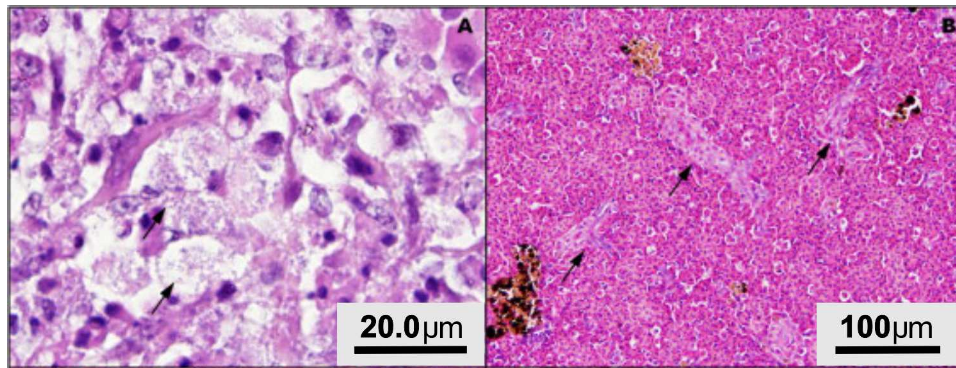


**Figure 6. Sequential development of experimentally induced FNO granulomas in tilapia spleen. (A) Typical granuloma composed of macrophages with large vacuoles containing small coccoid bacteria (arrows). (B) Normal tilapia spleen illustrating ellipsoids or terminal arterioles sheathed by macrophages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

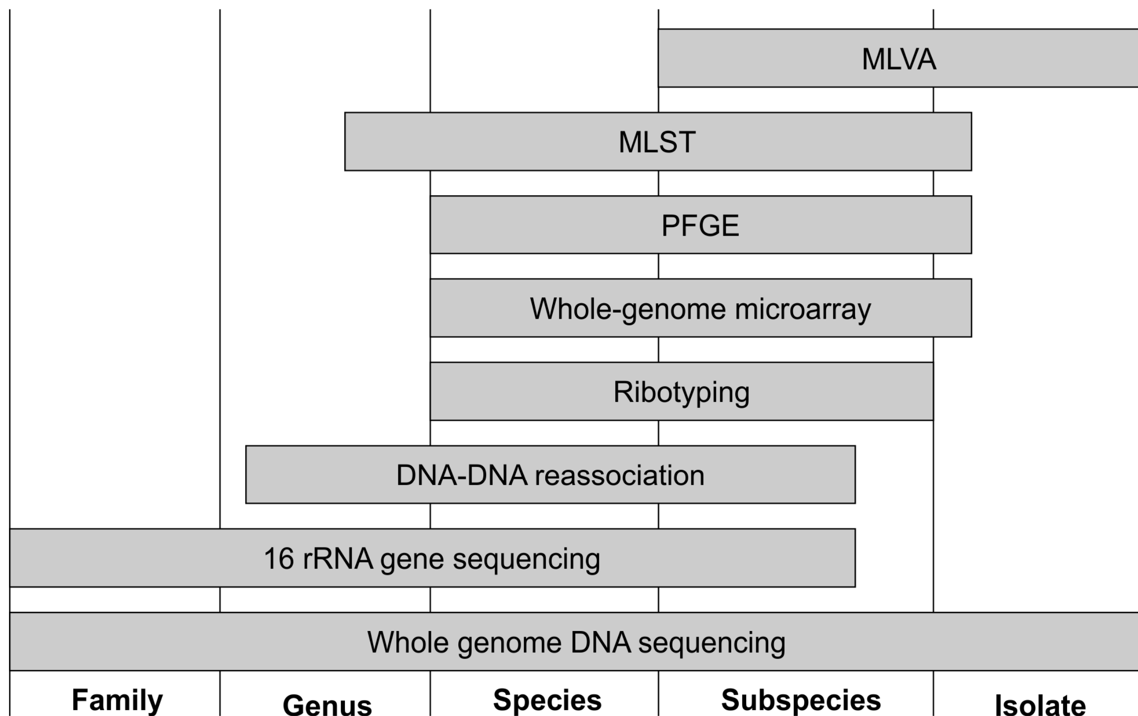


**Figure 7. *Francisella* spp. resolution typing methods adapted from Keim *et al.* 2007**

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).

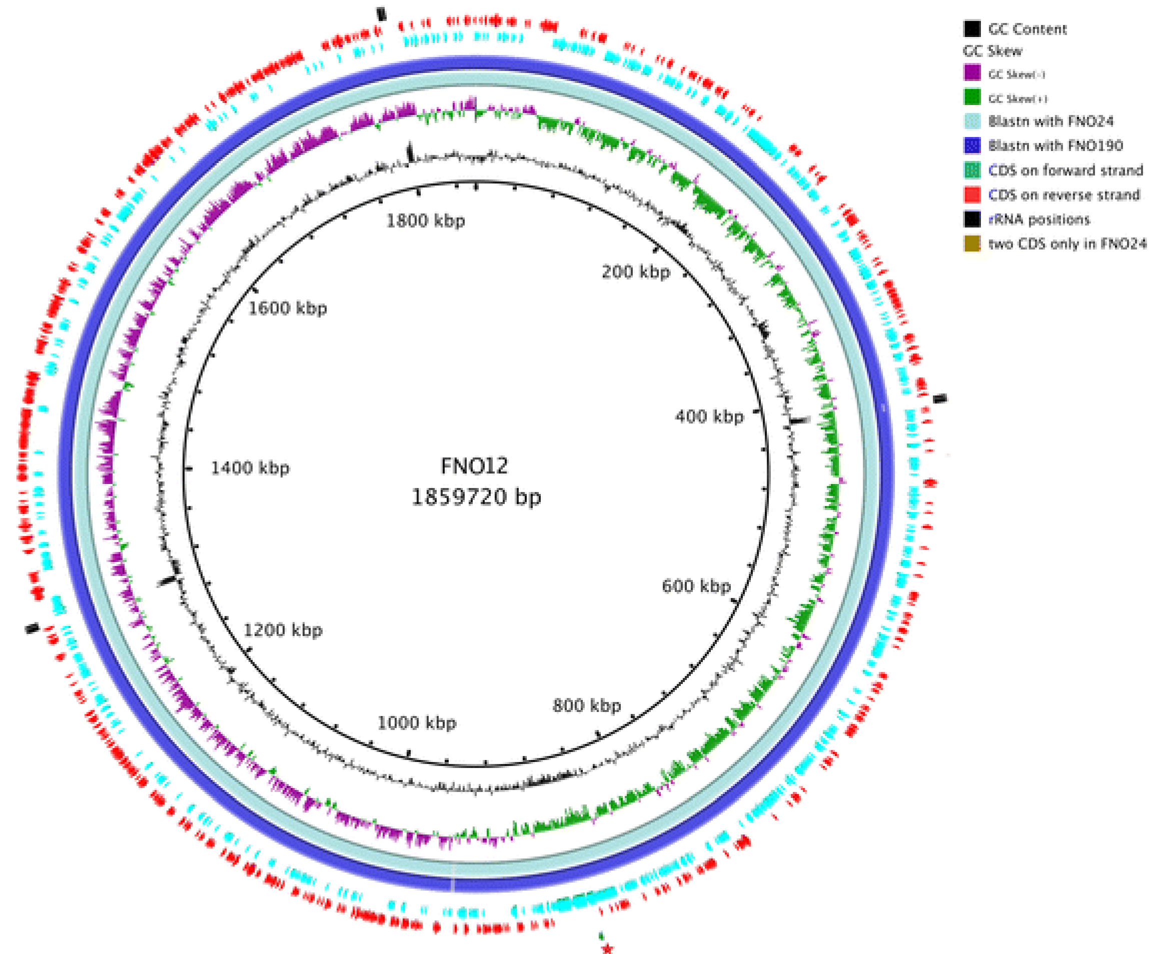


**Figure 8. Graphical circular map of *Francisella noatunensis* subsp. *orientalis* strain FNO12 in comparison with FNO24 and FNO190, reproduced from (Gonçalves, *et al.*, 2016).**

## 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 literature 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"

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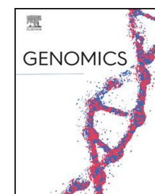
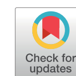


# Complete genome sequencing of sixteen *Francisella noatunensis* subsp. *orientalis* isolates: A genomic approach for molecular characterization and spread dynamics of this clonal population



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Nile tilapia

ABSTRACT

*Francisella noatunensis* subsp. *orientalis* (FNO) is an important emerging pathogen associated with disease outbreaks in farm-raised Nile tilapia. FNO genetic diversity using PCR-based typing, no intra-species discrimination was achieved among isolates/strains from different countries, thus demonstrating a clonal behaviour pattern. In this study, we aimed to evaluate the population structure of FNO isolates by comparing whole-genome sequencing data. The analysis of recombination showed that Brazilian isolates group formed a clonal population; whereas other lineages are also supported by this analysis for isolates from foreign countries. The whole-genome multilocus sequence typing (wgMLST) analysis showed varying numbers of dissimilar alleles, suggesting that the Brazilian clonal population are in expansion. Each Brazilian isolate could be identified as a single node by high-resolution gene-by-gene approach, presenting slight genetic differences associated to mutational events. The common ancestry node suggests a single entry into the country before 2012, and the rapid dissemination of this infectious agent may be linked to market sales of infected fingerlings.

## 1. Introduction

*Francisella noatunensis* subsp. *orientalis* (FNO) has emerged as an important fish pathogen, mainly for Nile tilapia (*Oreochromis niloticus*), in Japan, Thailand, China, Indonesia, Northern Europe, the United Kingdom, Costa Rica, Colombia, the Midwest of the United States, Mexico, Central America, and Brazil [1–11]. FNO is a Gram-negative bacterium from *Francisella* genus, with highly infectivity indices [3–5,11] and commonly associated to outbreaks when water temperature is lower than 24 °C [11,12]. This genus belongs to the *Francisellceae* family and have been detected in environment samples (e.g., free living), from mammals (e.g., human, rabbits) and aquatic hosts (e.g., giant abalone, Nile tilapia), with species classified as virulent and non-virulent [13]. Recent reports have shown serious losses in FNO-outbreak in farm-raised tilapia [5–11]. Tilapia has become the second most produced fish species worldwide, and its production has been expanded to Asia, South America and Africa to serve domestic and international markets [14]. Nile tilapia is the most farmed fish in Brazil, and in 2015, its production reached ~220 thousand tons, representing ~45% of all Brazilian aquaculture production [15].

Prior to the use of genomics, several tools were applied to determine the epidemiological relationships of agents, such as pulse-field gel electrophoresis, capsular serotype, and multilocus sequence typing (MLST), and in the genomics era, techniques such as k-mer-based

*Abbreviations:* FNO, Francisella noatunensis subsp. orientalis; MLST, multilocus sequence typing; wgMLST, whole-genome multilocus sequence typing; AQUACEN, National Reference Laboratory for Aquatic Animal Diseases; PGM, Personal Genome Machine; CLC-gw, CLC Genomics Workbench; CDS, coding DNA sequences; r/m, recombination ratio by mutation; BIGSdb, Bacterial Isolate Genome Sequence Database

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approaches, nucleotide blast of complete genomes, variant calling and whole-genome MLST (wgMLST) are being widely used [16]. In spite of its importance, molecular epidemiological data of FNO from different outbreaks in Brazil and from other countries remain scarce and most of the case reports could not determine the pathogen origin. ERIC- and BOX-PCR were used to identify the genomic fingerprints of ten FNO isolates from USA, Costa Rica and Central America. As a result, these isolates were found to share the same genotype based on both technologies [17]. Regarding the Brazilian FNO isolates, a previous work by our group [11] using REP-PCR-based genotyping of 62 isolates from Minas Gerais state, has shown a homogenous population pattern among these isolates. Based on another study by our group [18], it was suggested a clonal behaviour among three different isolates, considering the average of normalized BLAST score values (i.e., 100 ± 0 with threshold of 30%) and co-localization of all genetic loci (i.e., all synteny blocks equally showed by the isolates). Currently, the advent of cost-effective and rapid generation of the complete genome sequences of prokaryotes has revolutionized many scientific fields including taxonomy, phylogeny and epidemiology [19]. Moreover, the identification of subpopulations of closely related bacterial pathogens by modern genotyping methods is an appealing opportunity to study the genomic background according to their geographic distribution or transmission patterns [20]. Nevertheless, little is known about the genomic features of FNO, since few whole-genome sequences are available in public databases.

The aim of this study was to provide the whole-genome sequence of a comprehensive number of Brazilian FNO-outbreak isolates, among different farms and from different states, to compare their genetic population structure and evolution and to study their population spread dynamics, using a high-resolution type tools with gene-by-gene approach.

## 2. Materials and methods

### 2.1. Bacterial strains

A total of 20 isolates of FNO from diseased Nile tilapia, each of them representing a Brazilian FNO outbreak, were selected from the National Reference Laboratory for Aquatic Animal Diseases (AQUACEN) culture collection (Table 1). The FNO01, FNO12, FNO24 and FNO190 isolates had been sequenced in previous work of our group [18,21]. The remaining isolates ($n = 16$) were from routine diagnosis by AQUACEN, which are stored at −80 °C in Mueller-Hinton cation-adjusted broth supplemented with 2% VX supplement (Laborclin, Brazil), 0.1% glucose, and 15% glycerol. All isolates were obtained from outbreaks at 17 fish farms located in four states (i.e., Minas Gerais, São Paulo, Espírito Santo and Santa Catarina) between 2012 and 2015. These isolates were previously identified as FNO by qPCR assay according to Soto, et al. [22]. Also, the genome sequences of FNO isolates/strains reported in other works ($n = 6$), which are the all FNO genome sequence available, from Indonesia, Costa Rica, Brazil (Paraná state), Japan and the United Kingdom (Accession numbers: CP003402.1, CP006875.1, CP018051.1, LTDO01, SRX147950, SRX147952), were included in this study.

### 2.2. Sequencing, assembly and annotation

Isolates were thawed and streaked onto cysteine heart agar supplemented with 2% bovine haemoglobin (CHAH; BD Biosciences, USA) and incubated at 28 °C for 48 h. Colonies were collected and subjected to DNA extraction using the Maxwell 16 MDx Research Instrument (Promega, USA) following the manufacturer's instructions. Genomic DNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Thermo Scientific, USA). The isolates sequencing libraries were constructed using 100 ng of genomic DNA (gDNA). The gDNA was sheared with the Ion Shear™ Plus Reagents Kit and barcoded using the Ion Xpress Fragment Library kit and Ion Xpress™ Barcode Adapters (Life Technologies, USA). Size selection of ~400 bp was performed with 2% *E*-Gel® SizeSelect™ Agarose Gels (Invitrogen, USA). The libraries were amplified with the OneTouch Template 400 kit on the Ion One Touch™ 2 (Life Technologies) and enriched on the Ion OneTouch™ ES (Life Technologies). The annealing of the sequencing primers was made binding the Ion PI Sequencing Polymerase and loading the Ion 318v2 Chip. The gDNA were sequenced on an Ion Torrent Personal Genome Machine™ (PGM) (Life Technologies) with the Ion PGM Sequencing 400 bp Kit with required 1100 flows. All steps were conducted following the manufacturers' recommendations.

The quality of all raw sequenced data was analysed using FastQC 0.11.121, and an *in-house* script (https://www.github.com/aquacen/fast_sample) was used to exclude adaptor sequences. Genomes were then de novo assembled using SPAdes v3.9.1 [23], and scaffolds were generated using CONTIGuator 2.0 [24], with FNO12 as a reference strain. Both programs were run with the default parameters. The FNO12 isolate was chosen as reference because it is the first assembled genome among the FNO Brazilian isolates and had the support of optical mapping data to generate the final complete genome [18]. Gap filling was performed using CLC Genomics Workbench 7.0 (CLC-gw) (Qiagen, USA), mapping the reads to the genomes and extending the flanks of the gaps and including the contigs that were not used in scaffold process (due to the small length or more than one similar hit location with the reference). All contigs of each isolate assembly process were included in final whole-genome. Annotations were performed using the software Prokka version 1.11 [25] using nested databases in the following order: coding DNA sequences (CDSs) from FNO12 and all proteins from RefSeq. The annotated genome visualization and the manual correction of putative frameshifts were performed using Artemis [26] and CLC-gw, respectively. The sequences of the SRA projects of isolate/strain from Costa Rica and Japan were assembled as described above and were used as draft genomes. A search for potential insertion sequence (IS) elements was performed using the IS Finder [27] web tool. The fasta files containing complete or draft genomes were used as queries for BlastN search. The following parameters were used: word size = 11, e-value $10e^{-3}$, gap open existence = 5 and gap open extension = 2.

### 2.3. Nucleotide blast identity percentage and gene synteny analyses

The strain/isolates sequences ($n = 26$) were compared using Gegenees software version 2.0 [28] to obtain a matrix of the percentage of identical fragments. A heatmap was built with this matrix. The parameters set in Gegenees were a fragmentation length of 100 nucleotides and a threshold of 0%.

The Mauve program was used to determine the gene synteny between the genomes of the studied isolates/strains. This program performs orthology comparisons between the genomes to predict syntenic blocks, which reveal rearrangement events between the genomes [29]. The progressiveMauve algorithm was conducted using the standard parameters. Two rounds were performed: the first with all Brazilian isolates and the second with one Brazilian representative isolate (FNO12) together with the foreign strains. For the second round, the FNO12 isolate was chosen considering its high syntenic conservation with other Brazilian isolates in the first round. Before run the progressiveMauve algorithm, the contigs of the draft genomes were ordered according.

to genome synteny with the FNO12 sequence for better visualization, using "Move contig" step of Mauve software. Block regions that were not present in all genomes were validated using BLASTN algorithm.

### 2.4. Recombination analyses

In order to evaluate the recombination events, two alignments were performed using ClonalFrameML software version 1.11 [30] with

**Table 1**
Isolate information. Information about the sources of the isolates, weights of the fishes, and city, state, season and year of FNO outbreak of the 20 isolates from the AQUACEN culture collection, and other 6 isolates available at GenBank. All isolates are from Nile tilapia.

| Strain | Farm #[a] | Farm city/state[b] | Organ of isolation | Year of isolation | Accession |
|---|---|---|---|---|---|
| FNO01 | Farm 1 | Areado/MG | Kidney | 2012 | CP012153.1 |
| FNO12 | Farm 1 | Areado/MG | Kidney | 2012 | CP011921.1 |
| FNO24 | Farm 2 | Alterosa/MG | Spleen | 2012 | CP011922.1 |
| FNO39 | Farm 3 | Capim Branco/MG | Kidney | 2012 | CP022938, this work |
| FNO44 | Farm 4 | Alfenas/MG | Spleen | 2013 | CP022939, this work |
| FNO61 | Farm 5 | Ribeirão da Neves/MG | Kidney | 2013 | CP022940, this work |
| FNO75 | Farm 5 | Ribeirão da Neves/MG | Kidney | 2013 | CP022941, this work |
| FNO93 | Farm 6 | Fama/MG | Kidney | 2013 | CP022942, this work |
| FNO95 | Farm 7 | Passos/MG | Kidney | 2013 | CP022943, this work |
| FNO111 | Farm 8 | Varginha/MG | Kidney | 2013 | CP022944, this work |
| FNO117 | Farm 9 | Guapé/MG | Kidney | 2013 | CP022945, this work |
| FNO135 | Farm 10 | Alfenas/MG | Kidney | 2013 | CP022946, this work |
| FNO137 | Farm 11 | Santa Fé do Sul/SP | Kidney | 2013 | CP022947, this work |
| FNO190 | Farm 12 | Santa Fé do Sul/SP | Spleen | 2013 | CP011923.1 |
| FNO191 | Farm 13 | Três Marias/MG | Kidney | 2013 | CP022948, this work |
| FNO205 | Farm 14 | Carmo do Rio Claro/MG | Kidney | 2014 | CP022949, this work |
| FNO215 | Farm 15 | Linhares/ES | Kidney | 2014 | CP022950, this work |
| FNO222 | Farm 16 | Linhares/ES | Kidney | 2014 | CP022951, this work |
| FNO364 | Farm 17 | Joinville/SC | Kidney | 2015 | CP022952, this work |
| FNO371 | Farm 17 | Joinville/SC | Kidney | 2015 | CP022953, this work |
| F1 | | Londrina/PR | Kidney | 2015 | CP018051.1 |
| Toba04 | | Indonesia | Spleen | 2004 | CP003402.1 |
| LADL–07-285A | | Costa Rica | n/i[e] | < 2013 | CP006875.1 |
| FSC770 | | Costa Rica | n/i[e] | < 2012 | SRX147950[c] |
| FSC771 | | Japan | n/i[e] | 2001 | SRX147952[c] |
| STIR-GUS-F2f7 | | The United Kingdom | n/i[e] | 2012 | LTDO01[d] |

[a] Only for isolates from AQUACEN culture collection.
[b] MG: Minas Gerais; SP: São Paulo; ES: Espírito Santo; SC: Santa Catarina; PR: Paraná.
[c] SRA project.
[d] WGS project.
[e] n/i: Not informed.

default parameters, in two independents runs. The first dataset was composed of all the complete genomes available ($n = 23$; 3 draft genome sequences were not used), and the second one contained the Brazilian isolates ($n = 21$). Alignments were generated with MAFFT v7.302b [31] with whole-genome sequences and the "–auto" parameter, and then they were converted to PHYLIP format using CLC-gw. The starting maximum-likelihood tree phylogeny was inferred using RAxML version 8.0.0 [32] under the General Time-Reversible model with gamma correction together with invariant sites.

The ClonalFrameML software calculates the parameters $r/\Theta$ (i.e., per-site rate of initiation of recombination relative to mutation over mean length of DNA imported by homologous recombination), $\delta$ (i.e., mean of the exponentially distributed lengths of segments involved in recombination), and $\nu$ (i.e., probability with which each site is substituted). Thus, the recombination ratio by mutation ($r/m$) (i.e., where $r$ is the relative recombination events and $m$ is the relative mutation events), is obtained with the equation $(r/\Theta) \times \delta \times \nu$ [30].

Also, a pairwise homoplasy index (PHI) test was conducted using homology genes present in all genomes sequences (identified on wgMLST analysis – see below), concatenated in a fasta file format, using the "PHI test for recombination" option of the SplitsTree 4.0 software [33] that implements the algorithm of Bruen et al. [34].

### 2.5. wgMLST analysis

In order to improve the discrimination of isolates and to allow the tracking of their origin, a high-resolution tool, which uses a gene-by-gene approach, was evaluated. The CDSs of the FNO12 isolate ($n = 1917$), including pseudogenes, were imported into Bacterial Isolate Genome Sequence Database (BIGSdb) version 1.15.4 [35] on a local server installation. All genome sequences of FNO, including those available at GenBank (see Bacterial strains section), were imported as isolates. All loci were compared between isolates using a gene-by-gene approach [36] with the GenomeComparator plugin with a minimum of 90% identity and alignment. A distance matrix with the relative genomic divergences between all the isolates was obtained and used to construct a phylogenomic NeighborNet network using SplitsTree 4.0 [33].

## 3. Results

### 3.1. Whole-genome sequences

To perform genomic comparisons of the Brazilian isolates with the available foreign genomes, the sequences of the 16 isolates selected in this work were sequenced and assembled in a mean of 22 ± 9 contigs (Table 2). After gap filling, each isolate comprised one chromosome with mean length of 1,862,299 ± 112 bp. The expected coverage varied from 101- to 344-fold. The number of CDSs, including pseudogenes, was ~1916. The numbers of ribosomal RNA genes, transfer RNA genes, and transfer-messenger RNA genes were 10, 39 and 1, respectively, for all sequenced isolates. The sequences of whole-genome were submitted to GenBank and the accession numbers of the isolates are CP022938 to CP022953. Two SRA projects with strains/isolates from Costa Rica and from Japan were assembled in two draft genomes with 29 and 88 contigs, respectively. The 16S rRNA sequences from these assemblies were used as search queries on blast nr database and they showed ~99.8% of identity with FNO species. Only one IS element was found on genome sequence. The IS element ISFtu1 was identified on "gi|1,030,122,488|gb|LTDO01000004.1" contig of STIR-GUS-F2f7. This contig was used as a query on BlastN nt database and a 99% of identity and 100% of query coverage with *F. tularensis* strain 12T0050_FLI were found, in the region of an IS630 family transposase.

**Table 2**
Isolate sequence features. Isolate genome sequence information about assembly (length of the whole genome, number of contigs generated by the assembly software and coverage depth of sequencing) and annotation features (number of CDSs, pseudogenes, tRNA, rRNA, and tmRNA).

| Isolate | Length | #CDS | #Pseudo | #tRNA | #rRNA | #tmRNA | #contigs | ~ coverage |
|---|---|---|---|---|---|---|---|---|
| FNO01[a] | 1,862,440 | 1918 | 363 | 39 | 10 | 1 | 14 | 381 |
| FNO12[a] | 1,862,215 | 1917 | 363 | 39 | 10 | 1 | 15 | 1382 |
| FNO24[a] | 1,862,323 | 1917 | 365 | 39 | 10 | 1 | 57 | 79 |
| FNO190[a] | 1,862,208 | 1917 | 361 | 39 | 10 | 1 | 16 | 203 |
| FNO39 | 1,862,552 | 1916 | 363 | 39 | 10 | 1 | 22 | 150 |
| FNO44 | 1,862,369 | 1918 | 363 | 39 | 10 | 1 | 26 | 168 |
| FNO61 | 1,862,353 | 1916 | 364 | 39 | 10 | 1 | 18 | 102 |
| FNO75 | 1,862,202 | 1916 | 366 | 39 | 10 | 1 | 53 | 127 |
| FNO93 | 1,862,329 | 1916 | 361 | 39 | 10 | 1 | 19 | 160 |
| FNO95 | 1,862,342 | 1916 | 361 | 39 | 10 | 1 | 18 | 212 |
| FNO111 | 1,862,278 | 1916 | 361 | 39 | 10 | 1 | 16 | 220 |
| FNO117 | 1,862,139 | 1916 | 364 | 39 | 10 | 1 | 32 | 272 |
| FNO135 | 1,862,148 | 1916 | 360 | 39 | 10 | 1 | 18 | 197 |
| FNO137 | 1,862,215 | 1916 | 361 | 39 | 10 | 1 | 16 | 179 |
| FNO191 | 1,862,129 | 1916 | 362 | 39 | 10 | 1 | 25 | 106 |
| FNO205 | 1,862,338 | 1916 | 361 | 39 | 10 | 1 | 19 | 345 |
| FNO215 | 1,862,430 | 1916 | 361 | 39 | 10 | 1 | 18 | 159 |
| FNO222 | 1,862,403 | 1916 | 361 | 39 | 10 | 1 | 27 | 298 |
| FNO364 | 1,862,310 | 1917 | 362 | 39 | 10 | 1 | 18 | 162 |
| FNO371 | 1,862,249 | 1916 | 361 | 39 | 10 | 1 | 16 | 177 |

[a] Genomics feature obtained from previous works.

### 3.2. Similarity between genomes and synteny analysis

To compare the sequence similarities, the complete or draft whole genomes were fragmented and blasted all-against-all. The isolates of this work, including the previously described sequences of FNO01, FNO12, FNO24, and FNO190, achieved ~100 in average of normalized BLAST score values of fragments from the genomes. The score slightly decreased, to ~99.8, after the isolate from Brazilian Paraná state (FNO F1) was included in the analysis. Furthermore, when the other strains from Indonesia (Toba04), Costa Rica (LADL–07-285A and FSC770), Japan (FSC771) and the United Kingdom (STIR-GUS-F2f7) were included, the minimum score of fragments was ~99.7.

In order to evaluate the genome rearrangements that allow the development of different traits a co-localization of loci analysis was performed. The Brazilian isolates from this work, together with FNO01, FNO12, FNO24, FNO190 and F1, showed a high degree of synteny, with four blocks flanked by repeated regions (). When the strains from other countries were analysed in comparison with the representative Brazilian isolate, the number of blocks increased to ten, with some translocation and inversion in the Toba04 strain (Fig. 1). Furthermore, one block was present in only Toba04, FSC770 and FSC771. This block comprises the ribosomal RNA operon and it was used as a query search in BLASTN algorithm. The block was present in all genomes (data not shown) and it was included as a false-positive new block in Mauve results.

### 3.3. Recombination events

Considering the high similarities and synteny found, we decided to investigate the evidence of a clonal population among isolates using recombination events analysis. These analysis were conducted with all available FNO complete genomes ($n = 23$) resulted in a ratio of homologous $r/m$ (see $r/m$ description on Material and Methods section) across the overall phylogeny of 0.759, with $r/\Theta = 64.9285$, $\delta = 0.0335074$, and $\nu = 0.348705$. In contrast, analysis with the complete genomes of the Brazilian isolates ($n = 21$; isolates from this study together with FNO01, FNO12, FNO24, FNO190 and F1) resulted in an $r/m = 0.005$, with $r/\Theta = 0.464445$, $\delta = 0.0340392$, and $\nu = 0.318853$. The main divergent parameter in these two groups was the $r/\Theta$ value. These results suggest that recombination events in Brazilian isolates cannot be completely discarded, however this occurred in much higher degree when all available genome sequences were analysed. Statistically, the PHI test showed significant evidence ($p = 5.07^{-3}$) of recombination using foreign genomes.

### 3.4. Phylogenomic analysis based on wgMLST

Considering that Mauve and Gegenees results were not able to systematically differentiate these closely related Brazilian isolates in a high-resolution discriminatory way, a survey for a gene-by-gene approach was conducted. A wgMLST analysis showed that 1901 of 1917 genes (~99.16%) were present in all FNO sequenced genomes. Among these, there are 1657 homologous genes (~86%) with the identical sequences in the Brazilian isolates ($n = 21$), whereas 1383 genes (~72%) remained identical when the sequenced foreign strains were included in the analysis (). Considering solely the group of Brazilian isolates, only seven genes were not shared by all of them, whereas when these isolates were compared with the strains from other countries, thirteen genes were not shared by all genome sequences. Then, the number of homologous genes with alternative alleles ranged from 8 to 335, with 8 between the FNO12 and FNO371 isolates and 335 between the FNO01 isolate and Toba04 strain.

The NeighborNet phylogenomic network (Fig. 2A) showed a distantly related strain and a close group: the Toba04 strain and a group containing all other strains/isolates. In the group composed by the other strains/isolates (Fig. 2B), LADL–07-285A, FSC770, FSC771, and STIR-GUS-F2f7 formed a monophyletic subgroup that was found before a single node that expanded in a radial network comprising all the Brazilian isolates. Isolates from the outbreaks of 2012 and 2013 in Minas Gerais state (Fig. 2B, green and yellow leafs) were distributed in paraphyletic subgroups inside the Brazilian node. In 2013, the isolates from two outbreaks reported in São Paulo state belonged to a monophyletic subgroup with isolates from Minas Gerais state. Cases of 2014 were comprised in two subgroups, the first one from Paraná state (isolate F1) and the second from Santa Catarina. Both subgroups also possessed isolates from Minas Gerais state. Fig. 3 shows the geographical distribution of the Brazilian outbreaks.

## 4. Discussion

Currently, FNO outbreaks represent an increasing threat for the development of tilapia culture in several countries [3,6,7,37]. In Brazil,

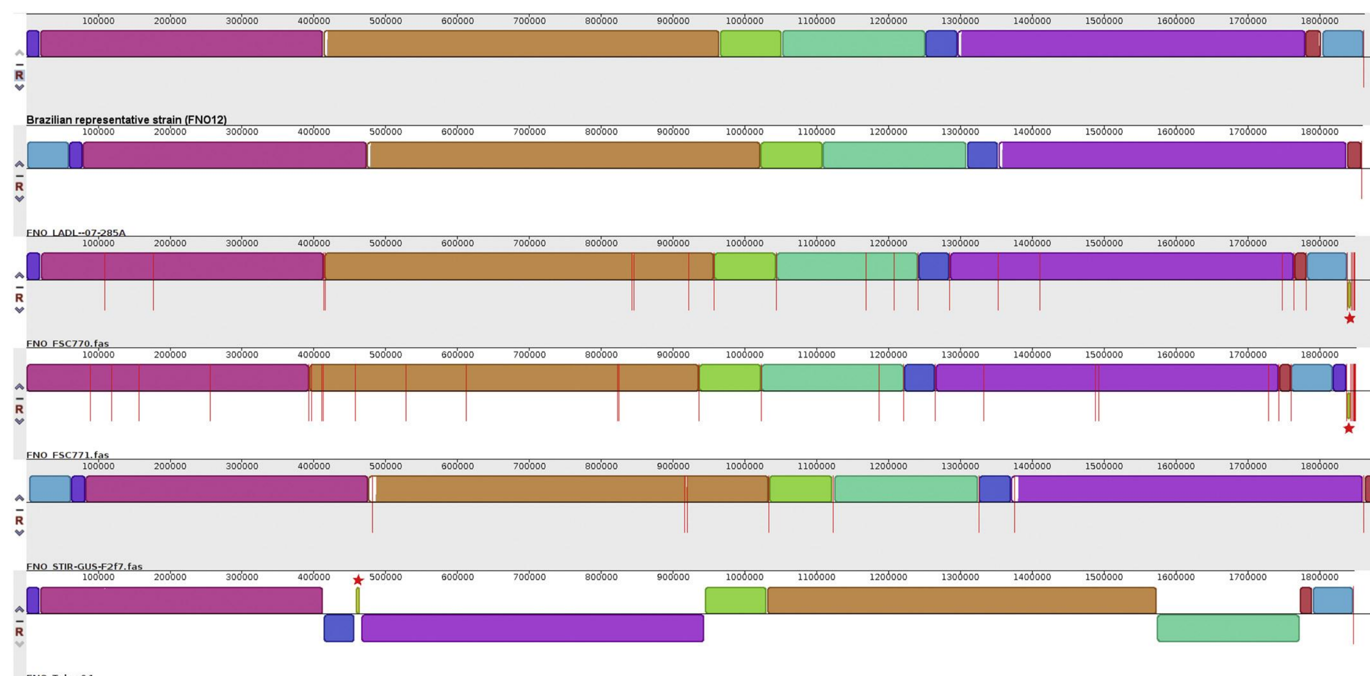


**Fig. 1.** Synteny block analysis. From top to bottom: the Brazilian representative isolate (FNO12), LADL–07-285A, FSC770, FSC771, STIR-CD-F2f7, and Toba04. Ten distinct blocks were found. Blocks with same colour represent large regions conserved between the genomes, while the white spots inside the blocks are regions of low similarity. Red vertical lines delimit the contigs of the draft genomes. Red stars are close to the block present only in Toba04, FSC770 and FSC771. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

after the first outbreaks that occurred in the states of Minas Gerais in 2012 [11] and São Paulo in 2013 [18], successive cases were reported in different farms from former states and other three states (i.e., Minas Gerais in 2013 and 2014; Espírito Santo in 2014; São Paulo in 2015; Paraná in 2015; and Santa Catarina in 2015; Fig. 3) that did not share the same water source. Thus, to provide a comprehensive number of genome sequences for the analyses, isolates from each outbreak in Brazil ($n = 16$) were sequenced and assembled in complete genomes.

To evaluate the molecular epidemiology and perform source tracking of the FNO isolates in Brazil, comparative genomic analyses of sequence identity and synteny were conducted. FNO strains have shown to have large backbones of intra-subspecies common genes (~100 normalized score of sequence similarity). Furthermore, synteny block analysis showed a high congruence between the sequenced isolates (). The progressiveMauve algorithm fragments the blocks between repeated regions, otherwise, in the result with only Brazilian isolates would be a single block containing the complete genomes. These results suggest a high probability of clonality among all the large number of sequenced genomes, corroborating the results of a previous study performed by our group [18]. Only Toba04 isolate had shown rearrangement events (translocation and inversion). This suggests that the Toba04 might be a different lineage of the Brazilian isolates (Fig. 1). Although these events can be artefact results from the 454-pyrosequencing single reads and the assembler software performed for FNO Toba04, these biological features of rearrangements have also been reported in other *Francisella* species [38]. The other foreign isolates/strains did not show rearrangements but they are draft genomes (i.e., not comprised on unique chromosome) and this analysis cannot provide complete evidence of these biological events in these isolates.

The FNO molecular epidemiology are still scarce and although the clonality of FNO isolates was largely hypothesized in the literature [11,13,18,39], there is no peer reviewed study using whole-genome to demonstrate if FNO is indeed a clonal population. According to Shapiro et al., clonal population is an isolates population that its genome is in complete linkage and in which selective sweeps will affect the entire genome that never or rarely undergoes recombination events [40]. An $r/m$ value of 0.005 was found for the Brazilian FNO isolates, demonstrating that mutations are the leading force of evolution with recombination being rarely found and thus all isolates belong to a clonal population. Regarding the isolates from other countries, the existence of different genetic lineages is supported by the complete linkage ($r/m = 0.759$) and the PHI test ($p = 5.07^{-3}$) analyses, besides the presence of a transposase in the genome sequence of the STIR-GUS-F2f7 strain. Other works also showed possible indications of a clonal population, including the study demonstrating that the FNO strains are undergoing genome decay [13] and a previous study that reported the absence of IS elements [39]. Nevertheless, both studies were performed with a limited number of genomes and our results further corroborates by using Brazilian and foreign isolates. Clonal behaviour has been reported in other pathogens, such as *Vibrio cholerae* [41], associated with niche persistence, and *Staphylococcus aureus* [42], associated with host specialization.

Considering the evidence of a clonal population pattern of the Brazilian FNO isolates and to allow epidemiological tracking and evolution analysis, the wgMLST analysis was conducted. A previous study that proposed wgMLST showed the potential of gene-by-gene analysis to discriminate very closely related strains, using core and accessory genome in a single analysis. This study, even demonstrated the presence of two populations of *Staphylococcus aureus* in a unique health care system, one from patients and the second from workers [36]. For *F. tularensis*, the wgMLST was essential to understand the micro-evolutionary events and for typing this clonal species [20]. In our work, the wgMLST analysis showed varying numbers of homologous genes with alternative alleles. The Toba04 strain, isolated from Indonesia, appeared to be the most divergent among all the tested isolates, followed by LADL–07-285A, from Costa Rica. Each Brazilian isolate could be distinguished as a single individual from the clonal population. The single node that intercepted all Brazilian isolates showed a genetic drift since of the first isolates in 2012 (variable loci varying from 30 to 86), and considering the knowledge that there has been no import authorization for live Nile tilapia to Brazil since 2005 to 2016 [Personal Communication with Cunha, 2017] [43], we might suggest that the

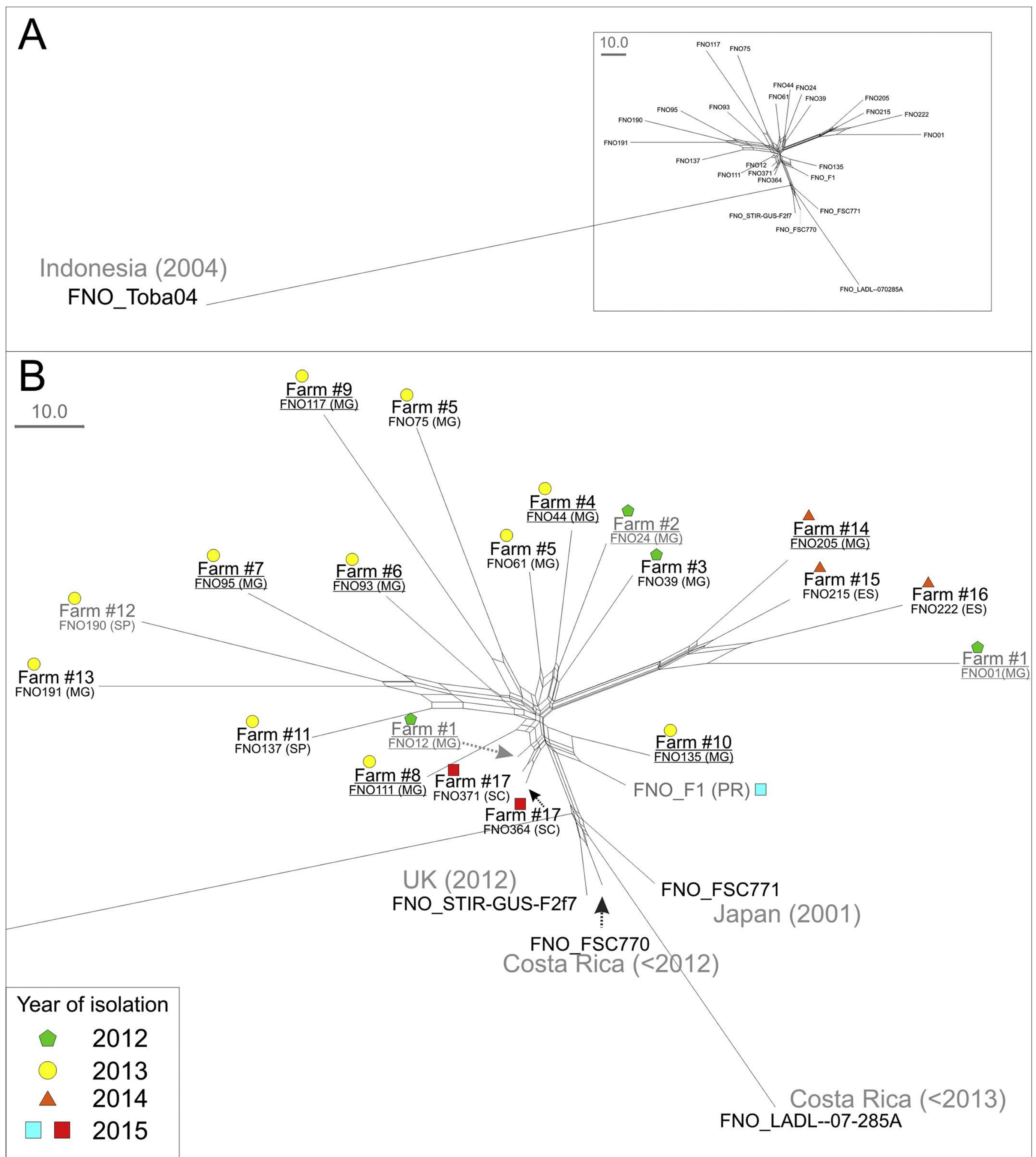


**Fig. 2.** Phylogenomic Neighbornet network of wgMLST data. Brazilian isolates and years of isolation are identified by polygons (2012), circles (2013), triangles (2014) and square (2015). Strains from other countries are indicated by the name of the origin country and the year of isolation. The years of isolation of the FSC770 and LADL–07-285A strains are untraceable; in these cases, the years of creation of the NCBI BioSamples were used. Isolates from south region of Minas Gerais state are underlined. (A) All isolates, showing a distantly related strain and a close group, the Toba04 strain and group with all other isolates; (B) the group composed by the other isolates.

pathogen had a single entry into the country previous to the 2012 outbreaks. Furthermore, all monophyletic subgroups had at least one isolate of the southern region of Minas Gerais state, including those in which there was the presence of isolates from outbreaks reported in the states of São Paulo, Espírito Santo, Paraná and Santa Catarina, which are geographically distant with unconnected water sources. This molecular discriminate view is consistent with the information about intensive animals transfer between farms (data not shown), which

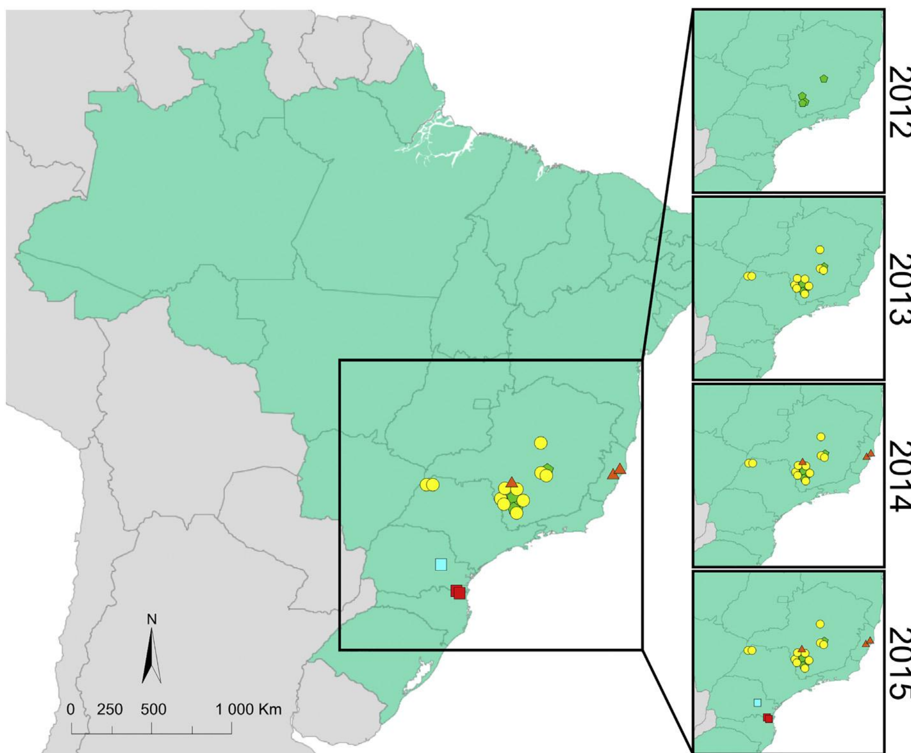


**Fig. 3.** Map distribution of *Francisella noatunensis* subsp. *orientalis* isolations between 2012 and 2015. The symbols are over the city of isolation, except the light blue (the isolation city of the F1 strain is untraceable). Green polygons are from 2012; yellow circles are from 2013; orange triangles are from 2014; and red squares and light blue square are from 2015. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

indicates that the rapid dissemination of the infectious agent might be linked to other common sources, such as market sale of infected fingerlings. Further studies of FNO may apply the wgMLST to study the dynamics of pathogen transmission between different fish farms and from different regions.

## 5. Conclusion

Taken together, these analyses demonstrate that the FNO isolates from Brazil, from previous and from this study, belong to a clonal population, indicating that this pathogen had a single entry into the country with pathogen spread probably being associated with animal transfer between farms. Additionally, through wgMLST analyses, it was shown that slight genetic differences have accumulated between FNO isolates from different farms in recent years, and that these differences are associated with mutation events, showing an expansion of this clonal population.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2018.10.011.

## Conflict of interests

The authors declare that they have no conflicts of interest.

## Authors' contributions

F.L.P. and H.C.P.F.: wrote the manuscript. F.L.P., L.A.G., G.C.T., and S.C.S.: performed the bioinformatics analyses. F.A.D., A.F.C., and M.P.L.: performed the culture growth and sequencing of isolates. H.C.P.F., L.A.G.L., and V.A.C.A: conceived and designed the experiments. H.C.P.F.: Coordinated all analyses of the project. All authors read and approved the final manuscript.

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

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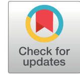
# Effects of temperature changes in the transcriptional profile of the emerging fish pathogen *Francisella noatunensis* subsp. *orientalis*



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A B S T R A C T

One of the major challenges in Nile tilapia (*Oreochromis niloticus* L.) farming is the occurrence of bacterial infections, and the *Francisella noatunensis* subsp. *orientalis* (FNO) is an important pathogen that has emerged in last decades. Francisellosis outbreaks have been reported in the literature as occurring seasonally when water temperature is below 24 °C. The aim of this study was to quantify the median lethal doses ($LD_{50}$) of FNO in experimental challenges at 28 °C and 22 °C, and to investigate the impact of temperature changes in whole genome expression using microarray technology. The $LD_{50}$ for Nile tilapia at 28 °C was $\sim 10^{5.7}$, whereas at 22 °C, the $LD_{50}$ was $\sim 10^{2.2}$, showing that the decrease in temperature enhanced disease outcome. Out of 1917 genes screened, a total of 31 and 19 genes were down- and up-regulated at 22 °C, respectively. These genes were grouped by orthology into functional categories of: amino acid, inorganic ion, and carbohydrate transport and metabolism; transcription; and posttranslational modification, protein turnover, and chaperones. Expression of genes related to metabolism, oxidative stress, and thermal shock were regulated by temperature changes, reflecting an ability of FNO to adapt to the environment. Expression of virulence genes usually required for the *Francisella* genus was not changed between tested temperatures, including that of genes located on the *Francisella* Pathogenicity Island.

## 1. Introduction

*Francisella noatunensis* subsp. *orientalis* (FNO) is a worldwide important causative agent of outbreaks in fish farms of Nile tilapia (*Oreochromis niloticus* L.), a major aquaculture commodity [1–5]. This bacterium is a Gram-negative, aerobic, pleomorphic, non-motile, and intracellular facultative agent of francisellosis in fish [6]. Fry and fingerling stages of Nile tilapia are most commonly affected by francisellosis, exhibiting, in acute cases, systemic granulomatosis with multifocal granulomas in internal organs, including the liver, spleen, and kidneys and, in subacute or chronic cases, anorexia, exophthalmia, and anemia [4,7].

In last decades, francisellosis outbreaks have been reported in several countries as a seasonal disease, with water temperature below 26 °C triggering onset [4,8–11]. A study evaluated the effects of temperature on FNO infection in Nile tilapia, showing the presence of clinical signal and mortality in controlled infection at 25 °C but not at 30 °C [12]. In Brazilian fish farms, mortality has been shown to be substantially increased in the cold seasons, when the water temperature is under 24 °C [3], and the infections were not detected in the warm season [13]. However, no further studies to quantify the pathogenicity of Brazilian FNO circulating strain in Nile tilapia at different temperatures have been conducted.

The FNO is still an emerging pathogen and much remains unknown about the pathogenesis of this species. However, *Francisella* spp. contain a known cluster of 16–19 genes located in a genome island, referred as the *Francisella* Pathogenicity Island (FPI) [14], which has been well characterized. The FPI is a remarkable feature of the *Francisella* genus and contains several genes of the bacterial type VI secretion system (T6SS) [14,15], related to characteristics of virulence, such as

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phagosomal escape, intracellular replication, and evasion of host immune responses [16]. A recent study indicated that the expression level of putative FNO T6SS genes were changed when the bacterium is exposed to low temperatures (25 °C) and oxidative stress [17]. Previous studies with FNO have shown an attenuation in virulence of a Δ*iglC* FPI gene mutant isolate in experimental infection of Nile tilapia, due to a decrease in intramacrophage growth/survival [18,19]. Furthermore, the *Francisella* spp. presents other virulence genes, such as Type IV pili (Tfp) genes that encode adhesion, aggregation, and twitching motility [20], which have not been investigated in FNO species.

Evaluation of the whole transcriptional profile, which might explain the effects of temperature change on FNO infection, is not currently available. The evaluation of gene expression level is one of the most important applications of the microarray approach, allowing monitoring of the production of mRNA (i.e., the first step in the protein synthesis) responsible for morphological and phenotypic differences, and indicative of response to environmental changes [21]. Among the methods used to measure gene expression, the microarray detects all genes of a microbial genome simultaneously, allowing analysis of physiologic adaptation under various conditions [22] such as temperature [23], or by different species/genotypes [24,25].

The aim of this study is to evaluate the pathogenicity of an FNO isolate in Nile tilapia experimentally infected and maintained under temperatures of 28 °C and 22 °C, as well as to evaluate the effect *in vitro* of the temperature change on the global transcription of FNO genes.

## 2. Materials and methods

### 2.1. Bacterial strain

The FNO12 isolate was selected from culture collection of the National Reference Laboratory for Aquatic Animals Diseases (AQUACEN). This isolate was previously characterized by our group [3] and the whole-genome sequence was published [26]. The genome had 1917 predicted genes, hereafter referred to only as 'genes', including 363 pseudogenes. This pathogen was isolated from a diseased Nile tilapia from Aerado city on Minas Gerais state, during the winter season of 2012 (the outbreak occurred when water temperature was ≤22 °C).

### 2.2. Experimental infection and $LD_{50}$ to Nile tilapia

The protocols for our *in vivo* experiments were approved by the Ethics Committee in Animal Experimentation of Federal University of Minas Gerais (Protocol number: CEUA 96/2013). A total of 140 Nile tilapia fingerlings with an average weight of 45 g from a commercial hatchery were acquired. Fourteen experimental groups with ten fish were created (seven groups for each temperature condition). The groups were maintained in separate 57 L tanks supplied with flow-through dechlorinated tap water ($0.5\,L\,h^{-1}$) at a water temperature of 28 °C or 22 °C. Ten fish for each temperature were randomly selected for bacteriological analysis and FNO-specific qPCR [27] to ensure that they were FNO-free infection. Fish were fed to satiation four times a day with commercially available feed containing 32% crude protein (Presence, Paulinia, Brazil).

For the groups at 28 °C, the FNO12 isolate was streaked onto cysteine heart agar supplemented with 2% bovine hemoglobin (CHAH; BD Biosciences, San Jose, USA), and incubated at 28 °C for 96 h. One colony was inoculated in Mueller-Hinton cation-adjusted broth supplement with 2% VX supplement (Laborclin, Sao Paulo, Brazil) and 0.1% glucose (MMH) and incubated at 28 °C for approximately 29 h. The bacterial suspension was then adjusted to an optical density ($OD_{600}$) of 0.150 corresponding to $\sim 10^8\,CFU\,mL^{-1}$. The fish were anesthetized by immersion in a bath containing $100\,mg\,L^{-1}$ benzocaine, and a 0.1 mL intraperitoneal injection of bacterial inoculum was administered. The inoculum dilutions ranged from $10^3$ to $10^8\,CFU\,mL^{-1}$ (one dilution per group). The control group was injected intraperitoneally with 0.1 mL of sterile MMH. After inoculation, the fish were maintained in water at a temperature of 28 °C.

For the groups at 22 °C, the FNO12 isolate was streaked on a CHAH plate, and incubated at 22 °C for 120 h. MMH was inoculated with one colony, and incubated at 22 °C for approximately 36 h. The bacterial suspension, the process of fish anesthesia and the intraperitoneal injection were performed as described above. The fish were maintained in water at a temperature of 22 °C.

In both groups, the fish were monitored for 15 days post-inoculation. All surviving fish at the end of the observation period were euthanized in a bath containing a dose of benzocaine ($300\,mg\,L^{-1}$). Tissue samples were collected from the kidneys and spleen of both the dead and euthanized fish and subjected to bacteriological analysis, followed by identification of bacterial isolates by FNO-specific qPCR [27].

The $LD_{50}$ value was determined based on a logistic regression model using the *dose. p* function in the MASS program of the R statistical software [28].

### 2.3. Bacterial growth condition for microarray analysis

In order to compare the differential gene expression between the four biological replicates of the FNO12 isolate grown under each temperature, 28 °C and 22 °C, a transcriptomic gene expression using microarray was performed. This isolate was streaked onto a CHAH plate and incubated at 28 °C for 96 h. Then, one colony was inoculated into quadruplicate cultures of MMH, and incubated at 28 °C or 22 °C for approximately 29 h and 120 h, respectively, under constant agitation (100 rpm). The bacterial suspension from each culture ($n = 8$) was collected for RNA extraction once reaching an $OD_{600}$ of 0.150.

### 2.4. RNA extraction and sample preparation

For RNA extraction, a culture volume of 5 mL of each biological quadruplicate was immediately mixed in 10 mL of RNAlater (Life Technologies, Carlsbad, USA) and incubated at room temperature for 5 min (according to the manufacturer's instructions). The mixture was centrifuged at 10,000 rpm for 10 min at 4 °C and the pellets were stored at −80 °C until use. Total RNA was extracted using TRIzol RNA Isolation Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The extracted RNA of each replicate was treated with Turbo DNA-free kit (Ambion, Carlsbad, USA) and 1 μL was submitted to FNO-specific qPCR [27] to determine the absence of genomic DNA. The extracted RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA) and its quality and integrity was evaluated using TapeStation 2200 (Agilent Technologies, Santa Clara, USA). All RNA samples used in this study had a high integrity [29] confirmed by the RNA integrity number value ≥ 9.5 (Supplementary Fig. 1).

Sample preparation was performed in three steps, according to the Agilent One-Color Microarray-Based Expression Analysis protocol (Agilent Technologies), using the Agilent Quick Amp Labeling kit (Agilent Technologies). First, RNA spike-in controls (Agilent Technologies) were combined with 100 ng of total RNA to allow the normalization of expression levels among replicates during data analysis. The replicates were labeled by the addition of 1 μL WT primer and incubation at 65 °C for 10 min, followed by a 5 min incubation on ice. The second step consisted of cDNA synthesis using a cDNA Master Mix (Agilent Technologies). The reaction was performed in a final volume of 10 μL containing 2 μL of 5X First Strand Buffer, 1 μL of 0.1 M DTT, 0.5 μL of 10 mM dNTP mix, 1.2 μL of Affinity Script RNase Block mix, and 5.3 μL of labeled RNA. The mixture was incubated at 40 °C for 2 h, followed by incubation at 70 °C for 15 min, and subsequently a 5 min incubation on ice. The third step consisted of a transcription reaction using Transcription Master Mix (Agilent Technologies). The reactions were performed in final volumes of 16 μL each, containing 0.75 μL of nuclease-free water, 3.2 μL of 5X Transcription Buffer, 0.6 μL of 0.1 M

DTT, 1 μL of NTP mix, 0.21 μL of T7 RNA Polymerase Blend, 0.24 μL of Cyanine 3-CTP, and 10 μL of cDNA. The mixture was incubated at 40 °C for 2 h in order to obtain cRNA. Then, the cRNA of each quadruplicate was purified using the RNAeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The concentration and specific activities of each cRNA were measured using a NanoDrop spectrophotometer (Thermo Scientific).

### 2.5. Hybridization analysis

The cRNA fragmentation and hybridization were performed using Agilent Gene Expression Hybridization kit (Agilent Technologies), according to the manufacturer's instructions. The fragmentation reactions consisted of 600 ng cRNA, 5 μL of 10X Blocking agent, and nuclease-free water to reach a final volume of 24 μL. Then, 1 μL of 25X Fragmentation Buffer was added to each replicate, incubated on ice for 1 min, and briefly centrifuged. After centrifugation, 25 μL of 2X GEx Hybridization Buffer were added in each replicate, which were centrifuged at 14,000 rpm for 2 min. Next, 45 μL of hybridization solution were dispensed into a custom-made Agilent slide (8 × 60 K) formulated based on the whole-genome sequence of the FNO12 isolate (Agilent.SingleColor.62611) for microarray-based gene expression analysis. This slide contains probes controls (e.g., negative control, spike-in) and 5751 probes (3 probes for each of the 1917 genes). The slide was incubated at 65 °C for 18 h at 10 rpm in a hybridization oven (Agilent Technologies). Then, the slide was washed in two buffers (Gene Expression Wash Buffer 1 and 2, both Agilent Technologies) and scanned using an Agilent DNA Microarray Scanner (Agilent Technologies). The data obtained from the array image were extracted using Agilent Features Extraction software v11.5 (Agilent Technologies).

### 2.6. qRT-PCR validation analysis

Three differentially expressed genes (*gprE*, *groS*, and *gyrA*) were randomly selected together with *ftsZ* gene for further validation by real-time polymerase chain reaction of reverse transcriptase of RNA (qRT-PCR). The primers were designed using Primer Express 3.0 software (Life Technologies) and synthetized by Integrated DNA Technologies (IDT, Coralville, USA).

The qRT-PCR reactions were performed using a GoTaq qPCR Master Mix (Promega, USA) intercalating dye kit in a final volume of 20 μL consisting of 10 μL 1x Master Mix, 0.5 μM of primers, 0.2 μL of CXR reference dye, and 50 ng of RNA template. The qRT-PCR assay was performed using a ViiA 7 Real-Time PCR System (Life Technologies) with the following cycle protocol: an initial step at 50 °C for 20 min followed by 1 cycle of 95 °C for 10 min and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The relative mRNA expressions of genes evaluated were normalized with the *ftsZ* gene, which has been considered a reference gene for *Francisella noatunensis* ssp. expression experiments [30]. Data acquisition and analysis were performed using the ViiA 7 software v.1.2.3 (Life Technologies). The correlation between $\Delta\Delta Ct$ from qRT-PCR and mean log2 fold-change from the microarray analysis was calculated using R Software [28], using the Pearson method.

### 2.7. Bioinformatics analyses

Microarray raw data were evaluated using the *limma* package of R statistical software. Background corrections were performed with the "normexp" method. Normalization between arrays was done using the "cyclicloess" method. Average reps of probes were linked by ProbeName. Down- and up-regulated genes were selected when at least 2 out of the 3 probes with *p*-adjusted-values ≤ 0.05, and log2 fold-change ≤ −1 and ≥1. The *p*-adjusted-values were obtained using a moderated *t*-test with Benjamin-Hochberg multiple testing corrections. Volcano and pseudogene expression plots were done using *ggplot2* graph library and *limma* package's results. To evaluate the hierarchical expression of genes inside each condition, a dynamic range (e.g., genes ordered by expression abundance) comparison was done with an *in-house* application (available at: https://github.com/aquacen/dynamic_compare). Dynamic range was divided into four quartiles groups: (1) high intensities > 3/4 of expressed genes; (2) medium/high intensities > 2/4 of expressed genes; (3) medium/low intensities > 1/4; and (4) low intensities, the least quarter of expressed genes. Moreover, the 50 genes highly expressed in each condition (Hi50) were obtained from the dynamic range. In order to predict orthologous group by functional category, genes were analyzed using the Cluster of Orthologous Genes (COG) version 2014 db [31]. The COG database search was performed using an *in-house* script (available at: https://github.com/aquacen/blast_cog). The COG categories were considered enriched when the percentage of expressed genes in the category was 1% greater than was expected (i.e., percentage of genes in category considering all genes) for the category. To predict the genes related with virulence factors, all genes were used as query in the Virulence Factors Database (VFDb) [32] using blastp algorithm; result was filtered by pident ≥ 70%. Also, the search by FPI was done using the *Francisella tularensis* subsp. *tularensis* SCHU S4 strain (GenBank accession number: NC_006570.2) genes as query on the BLASTn algorithm. The prediction of vaccine candidates in the Dynamic Vaxign analysis was performed with the Vaxign webserver [33] using all predicted code sequences (CDS). Parameters of Gram-negative bacterium and similarity to host proteins of humans were set. Only proteins with adhesion probabilities ≥0.51 and no-similar human protein were considered. Finally, genes predicted in the FNO12 isolate without gene names were searched in the Uniprot database [34] to identify homologous genes and complete this information in this work.

### 2.8. Validation of FNO12 pseudogene expression through proteomic analysis

Due to the detection of mRNA expression for several protein CDS annotated as pseudogenes in the results of the microarray experiment, a shotgun proteomic approach was applied to verify actual protein translation for each pseudogene in the genome of FNO12. For protein extraction, a volume of 50 mL of a culture cell was prepared as described in section 2.3, then immediately centrifuged at 16,100 × *g* for 20 min at 4 °C and washed three times with 50 mM Tris-HCl pH 7.5. Then, the pellets were resuspended in 1 mL of lysis buffer (42% (w/v) urea, 15% thiourea, 4% sodium deoxycholate (SDC), 12.5 mM Tris-HCl pH 7.5, and 1.5% dithiothreitol) containing 1% of a protease inhibitor mix (GE Healthcare, Pittsburgh, USA). Subsequently, the samples were incubated on ice for 15 min, then sonicated on ice using a cell ultrasonic disruptor (Unique, Indaiatuba, Brazil) for 20 min, in a cycle of 1 min at maximum power (495 W) and 1 min off. The lysates were centrifugated at 21,900 × *g* for 40 min at 4 °C. The supernatant was collected, loaded into a Vivaspin 500 column with a threshold of 10 kDa (GE HealthCare), concentrated, and washed five times with 50 mM $NH_4HCO_3$. After washing, the concentrated samples were collected and quantified on a Qubit 2.0 fluorometer (Invitrogen, Oregon, USA) using a Qubit protein assay kit (Molecular Probes, Oregon, USA).

Tryptic digestion was performed as described in previous work of our group [35] with a step for the removal of SDC using two-phase solvent extraction with ethyl acetate (Sigma Aldrich) (2:1) followed by the addition of 0.5% TFA, and centrifuged at 15,000 × *g* for 5 min at 20 °C. After centrifugation, the aqueous phase was collected and desalted using C18 MacroSpin Columns (Harvard Apparatus, Holliston, USA), according to the manufacturer's instructions. The sample were dried under vacuum in a Vacufuge Concentrator (Eppendorf, Hamburg, Germany), resuspended in 100 μL of 20 mM ammonium formate (Sigma Aldrich), transferred to Waters Total Recovery vial (Waters), and stored at −80 °C until use.

The vial was analyzed by LC-MS using a nanoACQUITY ultra-

performance liquid chromatography (nanoUPLC) system coupled to a Synapt G2-Si HDMS mass spectrometer (Waters) in three technical replicates. Bidimensional nanoUPLC tandem nano electrospray high definition mass spectrometry (nanoESI-HDMS$^E$) experiments, using multiplexed data-independent acquisition (DIA), was conducted using both: (1) a 1 h reverse-phase gradient from 7% to 40% (v/v) acetonitrile (0.1% v/v formic acid) and a 500 nL min$^{-1}$ nanoACQUITY UPLC 2D Technology system. Typical on-column sample loads were 500 ng of total protein digests for each of the five fractions (500 ng per fraction/load).

For each measurement, the mass spectrometer was operated in resolution mode with a typical *m/z* resolving power of at least 25,000 full width at half maximum (FWHM), an ion mobility cell that was filled with helium gas, and a cross-section resolving power of at least 40 Ω/Δ Ω. Analyses were performed using nano-electrospray ionization in the positive ion mode nanoESI(+) and a NanoLock-Spray ionization source (both from Waters). Mass spectrometric analysis of tryptic peptides was performed using a mass spectrometer equipped with a T-Wave-IMS device in MSE and HDMS$^E$ modes as previously described [36]. The radio frequency offset (MS profile) was adjusted such that the nanoESI-HDMS$^E$ data were effectively acquired from *m/z* 400 to 2000 by the MassLynx v.4.1 software (Waters), ensuring that any masses that were observed in the high energy spectra of less than *m/z* 400 arose from dissociations in the collision cell.

Using the database management tool of the ProteinLynx Global Server (PLGS), the sequence of each CDS together with all pseudogene open read frames (ORF) regions (i.e., ORF ≥ 10 amino acids in pseudogene region in all six frames) of the FNO12 isolate genome sequence were reversed during the database queries and appended to the original database to assess the false positivity rate during identification. Then, protein identification was performed using PLGS as described in previous work of our group [35]. The correlation between technical replicates was calculated using R Software [28], using the Pearson method. Pseudogenes with at least one ORF identified in the proteomics assay were considered as "putative translated".

## 3. Results

### 3.1. $LD_{50}$ analysis

Major clinical signs of infection (e.g., anorexia, lethargy, melanosis, exophthalmia, and ascites) were identified in both temperatures. Moreover, the first signs also appeared at 6 h post-inoculation (hpi) also in both temperatures. The first mortality was recorded at 20 hpi in groups at 22 °C and at 48 hpi in groups at 28 °C. The final mortalities observed in 28 °C and 22 °C occurred on 11- and 8-days post-inoculation, respectively. The calculated $LD_{50}$ for the fish maintained at 28 °C was $1.64 \times 10^{5.74}$ CFU, and the $LD_{50}$ for the fish maintained at 22 °C was $2.4 \times 10^{2.21}$ CFU. In the analysis of the fish that died during the observation period, necropsy examination revealed splenomegaly, hepatomegaly, renal congestion, and the presence of multifocal white nodules in the spleen, kidney, and liver. All isolates obtained from bacteriological assays presented positive results in FNO-specific qPCR.

### 3.2. General microarray quality parameters

In order to identify genes that might be involved in the impact of temperature changes on $LD_{50}$ experimental analysis, the differential expression of genes was evaluated in FNO12 cultivated at 22 °C and 28 °C. The reproducibility of the biological replicates can be observed in the PCA analysis (Fig. 1). Samples of each condition had been grouped in principal component 1 (95.23%) in different quadrants. Samples of the same condition differ at the most in ~2.8% (i.e., in principal component 2). The spike-in results were obtained following the manufacturer's instructions, with the logarithmic relative concentration of abundance agreement for the ten probes (data not shown). The microarray raw results were deposited in NCBI's Gene Expression Omnibus (GEO) database [37], with the accession number GSE125115.

### 3.3. Validation of microarray analysis by qRT-PCR

In order to validate the microarray results, a set of three genes related to cold- and heat-shock response were randomly selected (*gprE*, *groS*, and *gyrA*) together with the *ftsZ* gene. The Pearson correlation between qRT-PCR and microarray data was 0.993. The *gprE* gene showed significantly up-regulated expression ($p < 0.05$) at 22 °C in the microarray analysis and qRT-PCR. While, the *gyrA* gene was relatively stable in both conditions, and besides the statically significantly results, the *groS* gene was expressed in the same regulatory direction as the microarray results.

### 3.4. Differential gene expression at 28 °C and 22 °C

For a total of 1917 genes evaluated from the FNO12 genome, 31 (~1.6%) and 19 (~1.0%) genes were down- and up-regulated at 22 °C, respectively (Fig. 2). Hypothetical proteins comprised ~22% of the down-regulated genes and ~52% of the up-regulated genes. Fifteen of the 24 COG categories had genes down-regulated and eleven had up-regulated genes (Fig. 3). The most abundant category of down-regulated genes was amino acid transport and metabolism (*n* = 11); followed by inorganic ion transport and metabolism (*n* = 6); and carbohydrate transport and metabolism (*n* = 4). On the other hand, the most abundant categories of up-regulated genes were posttranslational modification, protein turnover and chaperones (*n* = 4); and transcription (*n* = 3). Enriched COG categories in the down-regulated group were: inorganic ion transport and metabolism; carbohydrate transport and metabolism; and amino acid transport and metabolism. Enriched COG categories in up-regulated group were: transcription; and post-translational modification, protein turnover, and chaperones. All up- and down-regulated genes are summarized in Table 1. Regarding virulence genes, for both those found in FPI and those predicted in VFDb analysis, no up- or down-regulation of expression was observed.

### 3.5. Qualitative gene expression

Considering the small percentage of down- and up-regulated genes, a qualitative search was performed to classify genes identified in the transcriptomic assay to better understand the hierarchical distribution of gene expression in each condition. From the 1917 genes, a total of 1776 (~93%) were found as expressed genes in microarray analysis, with 1765 genes being in at least two replicates at 28 °C and 1770 genes in at least two replicates on 22 °C. The dynamic range between the least and most abundantly expressed genes was ~2.3 logs for both tested temperatures.

Among the Hi50 from each condition (i.e., the 50 genes with greater expression level), 42 (84%) were common for both experimental temperatures. For the Hi50 at 28 °C and 22 °C, 14 COG categories were identified: Translation, ribosomal structure, and biogenesis (*n* = 31); posttranslational modification, protein turnover, and chaperones (*n* = 5); energy production and conversion (*n* = 4); general function prediction only (*n* = 3); secondary metabolites biosynthesis, transport, and catabolism (*n* = 3); lipid transport and metabolism (*n* = 3), intracellular trafficking, secretion, and vesicular transport (*n* = 2); and coenzyme transport and metabolism (*n* = 2). Other categories with one representative gene were: cell cycle control, cell division, chromosome partitioning, amino acid transport and metabolism, transcription, replication, recombination and repair, cell wall/membrane/envelope biogenesis, and defense mechanisms. Additionally, five genes from FPI and those that had homology with genes found in VFDb were detected in the Hi50 group: intracellular growth locus protein C (*iglC*; FNO12_1367), intracellular growth locus protein B (*iglB*; FNO12_1368), pathogenicity determinant protein D (*pdpD*; FNO12_1370), 60 kDa

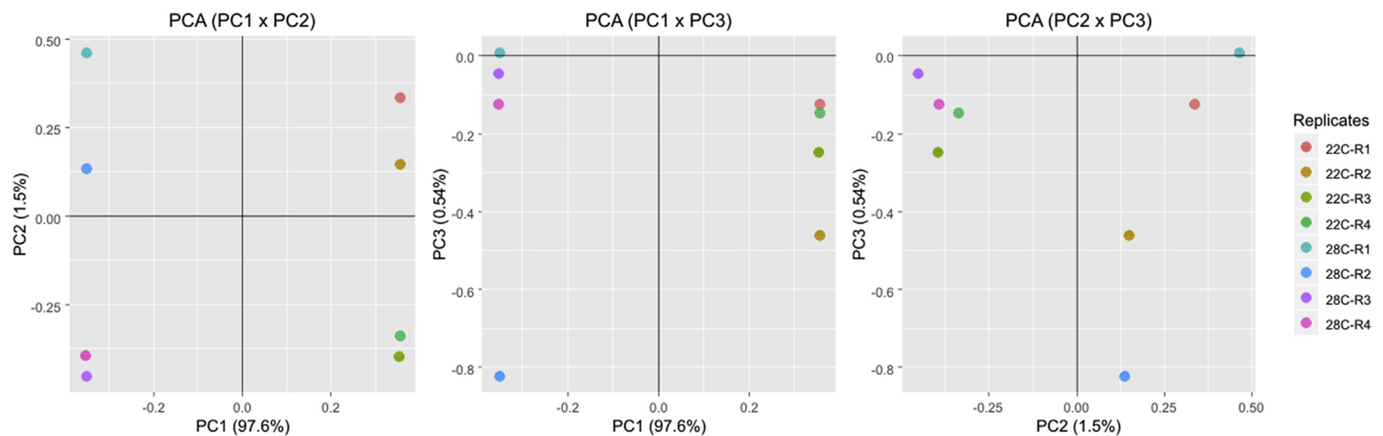


**Fig. 1. Principal component analysis of biological FNO12 quadruplicates maintained at 22 and 28°C.** The biological replicates from each temperature are represented as R1, R2, R3, and R4. Left plot represents the PC1 and PC2 components, central plot represents the P1 and PC3 components, and right plot represents the PC2 and PC3 components.

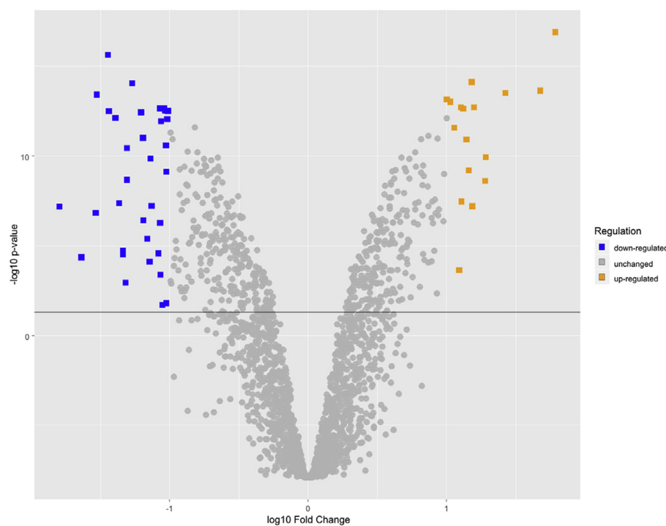


**Fig. 2. Volcano plot of down- and up-regulated genes.** Blue squares are down-regulated genes (≤1 log10-fold), while orange squares are up-regulated genes (≥1 log10-fold) and grey circles are unchanged (> −1 and < 1 log10-fold) or not statically significant (p ≥ 0.05). Black horizontal line represents –log10 of 0.05 *p*-adjusted-values confidence values.

chaperonin (*groL*; FNO12_1627), and elongation factor Tu (*tuf*; FNO12_1691).

### 3.6. FPI expression and virulence genes

In the FNO12 isolate the FPI genes were found between locus tag FNO12_1356 and FNO12_1371, homologous of *pdpA*, *pdpB* (syn. *icmF*), *pigA* (syn. *iglE*), *pigB* (syn. *vgrG*), *pigC* (syn. *iglF*), *pigD* (syn. *iglG*), *pigE* (syn. *iglH*), *pigF* (syn. *dotU*), *pigG* (syn. *iglI*), *pigH* (syn. *iglJ*), *iglD*, *iglC*, *iglB*, *iglA*, *pdpD*, and *pmcA* (syn. *anmK*) of *F. tularensis* subsp. *tularensis* SCHU S4 FPI described by Nano and Schmerk [14], and synonymous gene names from de Bruin et al. [38]. Compared with that of *F. tularensis* subsp. *tularensis* SCHU S4 strain genes, only *pdpC* and *pigI* do not have homologous in FNO12. There were no genes of FPI that had altered expression levels (up- or down-regulation) between conditions of 28 °C and 22 °C, and all genes had been listed in high ($n = 7$), medium/high ($n = 8$) and medium/low ($n = 1$) intensities groups (Fig. 4) from the dynamic ranges.

Beyond the sixteen genes present in the FPI, six other genes were found in search of a homolog of VFDb. Two of these genes were: *groL* and *tuf*, that belong to the Hi50 group described above. Additionally, a superoxide dismutase (*sodC*, FNO12_0402) and a hypothetical protein (FNO12_0442) were found at high and medium/high intensities groups, respectively, in both temperatures. Moreover, two Tfp genes (*pilE5*, FNO12_0046; and *pilD*, FNO12_0791) were found at medium/high intensity group. In addition to *pilE5* and *pilD*, another sixteen homologous genes of Tfp were predicted in the FNO12 isolate, and among them, thirteen were predicted as pseudogenes. None of these genes were found in the up- or down-regulated group of genes, and, Tfp gene and pseudogene expression levels were distributed between all intensity groups (Fig. 5). Genes from FPI, Tfp, VFDb analysis, and literature search of virulence genes associated with *Francisella* genus are summarized in Table 1.

### 3.7. Transcription and translation of FNO12 pseudogenes

The FNO12 isolate presented a high number of pseudogenes ($n = 363$) on the annotated whole-genome sequence, approximately 20% of its genes. A total of 344 predicted pseudogenes were expressed in our microarray analysis. Additionally, the intensity of expression was significantly different between functional genes and predicted pseudogenes ($p < 0.05$) in both conditions, as showed in Fig. 6.

In order to validate the annotation of the pseudogenes, a proteomics analysis was performed. From 1554 predicted CDS sequences, a total of 856 proteins (~55%) were identified, being 852, 860, and 845 on replicate 1, 2, and 3, respectively. The Pearson correlation between proteomic technical replicates ranged from 0.929 to 0.945. A total of 1334 ORFs were predicted in pseudogene regions, considering all six frames (see material and methods section). From these ORFs, only 13 were identified in at least two replicates, which were classified as putative translated. The results are summarized in Supplementary Table 1. There were no pseudogenes with more than one ORF identified in the proteomics approach. The MS proteomics data are available at the ProteomeXchange Consortium via the PRIDE [39] partner repository under the identifier PXD012449.

## 4. Discussion

The clinical outcome of francisellosis in tilapia has a clear relationship with water temperature. A recent study revealed a seasonal dynamic of FNO infection in a longitudinal study in Brazilian fish farms, where the clinical signals and mortalities caused by this pathogen were registered only when the water temperatures decreased to 24-26 °C [13]. Previous work has demonstrated that the impact of the temperature of 30 °C (without mortalities) and 25 °C (with high effects) in experimental infections [12] and in Brazilian fish farms in cold seasons

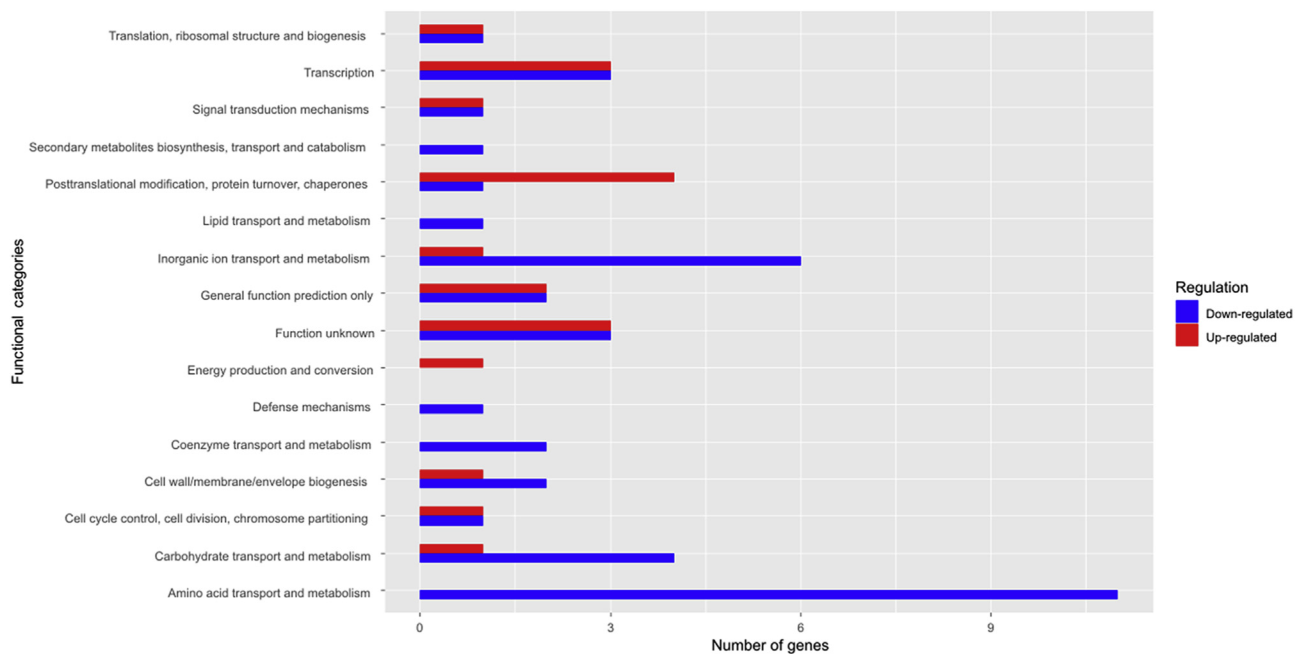


**Fig. 3. COG categories of down- and up-regulated expressed genes at 22°C.** The Y-axis is the COG categories. Blue (down-regulated) and red (up-regulated) bars represent the number of genes classified in each COG category.

[3], may or may not be associated with coinfection with another pathogen [40]. In this study, we used the FNO12 isolate, that is a representative of the a clonal population from Brazilian francisellosis outbreaks, with small genetic dissimilarities between isolates from other countries [41], which suggest that it can be considered a good representative of how this pathogen behaves worldwide. In our work the $LD_{50}$ of FNO infection at water temperatures of 28 °C and 22 °C were determined. A decrease in water temperature of 6 °C resulted in a three-log reduction in $LD_{50}$ for the Brazilian FNO12 isolate, which demonstrated a high level of virulence ($LD_{50}$ $2.4 \times 10^{2.21}$ CFU) to Nile tilapia at 22 °C. This shows that the temperature change plays an important role in FNO12 infecting dose.

The $OD_{600}$ corresponding to $10^8$ CFU was reached in abs 0.15 for the analyzed strain FNO12 (data not shown). This is similar to found by Ramirez-Paredes et al. [42], where $1.0 \times 10^9$ CFU was found at $OD_{600}$ 0.4, using FNO strain STIR-GUS-F2f7, from the United Kingdom. By other hand, this $OD_{600}$ showed a divergence with Soto et al. [4] where $\sim 10^7$ CFU was reached in $OD_{600}$ 0.48, using LADL07-285A strain from Costa Rica. This slight divergence between our work and the cited study could be due to differences in the initial bacterial load on culture media and/or some differences of growth kinetics at strain level.

Microarray technology is largely used to assess the gene expression levels under different conditions. Critical differences relative to pathogenesis of *F. tularensis* and *Francisella novicida* for humans [43] have been showed using this technology. Several pathogens have been evaluated over different temperatures with the same technologies, including *Escherichia coli* [44], *Streptococcus pyogenes* [45], *Streptococcus agalactiae* [46,47], and *F. tularensis* [23]. Roughly 93% of the genes were found in our microarray experimental assay, showing that the whole-genome had been monitored by the technology, and had provided a comprehensive view about the predicted genes. Additionally, the PCA analysis showed a high similarity between each condition (divergence ≤ 2.8%), and qRT-PCR validation showed a similar pattern of gene expression levels (Pearson correlation = 0.807). Taking together, this microarray raw data was acceptable to perform comparisons.

Although the aim of this study was not to evaluate genome decay, some features were observed in our microarray data. The FNO12 isolate had 363 predicted pseudogenes [26]. However, predicted pseudogenes were identified by homology with gene databases, such as Uniprot or Genbank, and the expression of these putative pseudogenes can represent genetic events (e.g., alternative start codons, alternative stop codons, gene fusions) with still active function or mutation that can be silenced by other translational "silencing" mechanisms, as shown in a previous study with *Mycobacterium leprae* [48]. Variation of the strength of ribosome-binding site (data not shown) might change the level of transcription of these pseudogenes (Fig. 6). Proteomics analysis showed that only 13 of the 363 pseudogenes were classified as putative translated. Therefore, the transcripts from annotated pseudogenes, but not those classified as putative translated, were discarded in the functional/comparative analysis.

The metabolism gene expression reduction was shown by an enrichment of several metabolism/transport COG categories in down-regulated gene groups. The lower metabolic activity at low temperatures has previously been suggested by Soto and Revan, considering the duration time of the culturability, in experiments with FNO at 20 °C [49]. However, two hypothetical proteins related to metabolisms and energy production/conversion on COG were up-regulated. Furthermore, genes from ribosomal activities and metabolism (e.g., fatty acid synthesis) represent ~50% of the Hi50. Also in this way, the microorganism monitors and adapts, changing the expression level of genes related to several functions, including stress tolerance (e.g., cold- and heat-shock proteins [CSP and HSP] and oxidative stress regulators) [50,51] that might improve the fitness and enlarge the growth in the temperature changes. In our work, *cspC*, a global stress response regulator, associated also with reduction in the stability of *groL/S* transcripts [52,53] was down-regulated. The *groL/S* is responsible for the refolding of misfolded proteins [54]. Additionally, three other genes were up-regulated: *hsp15*, which is involved in the recycling of free RNA 50S subunits [55], *grpE* and *cbpA*, which are both associated with *dnaK* regulation [56,57], and *DnaK*, which is responsible for ATPase activity [56]. Altogether, these finding turns inconclusive with regard to the suggestion of a metabolism reduction of this pathogen in the tested temperature. Future work, evaluating the total bacterial load in host cells, needs to be undertaken to better understand the replication time/growth *in vivo*.

In relation to the oxidative stress, one (i.e., acid phosphatase/phosphotransferase - *acp*) and four (i.e., *lysR1*, *lysR2*, *msrB*, and *tspO*)

**Table 1**
Down- and up-regulated genes at 22 °C and other genes related with virulence and stress response.

| Locus tag | Gene name | Product/function | Function described in literature | Regulation or Group[1] |
|---|---|---|---|---|
| **Thermal stress** | | | | |
| FNO12_0817 | *cspC* | cold-shock protein | regulation of RpoS protein, a global stress response regulator of OsmY, Dps, and UspA [52]; negative regulation of CspA [53]; reduce the stability of *groL* and *groS* transcripts [54] | **Down-regulated** |
| FNO12_0474 | *cspA* | cold-shock protein CspA | anti-freezing protein; *E. coli* [72] | Hi50 |
| FNO12_0246 | *htpG* | heat-shock protein | folding, signal transduction, cell proliferation; involved in pathogenicity of *Edwardsiella tarda* [73] | Hi50 |
| FNO12_0385 | *htpX* | heat-shock protein | unknown function with no particular phenotype in inactivated mutant [74] | Hi50 |
| FNO12_1328 | *grpE* | heat-shock protein | regulation of *dnaK* [56] | **Up-regulated** |
| FNO12_0808 | *hsp15* | heat-shock protein 15 | involved in the recycling of free RNA 50S subunits [55] | **Up-regulated** |
| FNO12_1627 | *groL* | 60 kDa chaperonin | refolding of misfolded proteins [54] | Hi50 |
| FNO12_1628 | *groS* | 10 kDa chaperonin | refolding of misfolded proteins [54] | Hi50 |
| FNO12_1326 | *dnaJ* | chaperone protein | regulation of *dnaK* [56] | Hi50 |
| FNO12_1327 | *dnaK* | chaperone protein | ATPase activity [56] | Hi50 |
| FNO12_1859 | *clpB* | chaperone | degradation of proteins, peptides and glycopeptides [75] | High |
| FNO12_1606 | *cbpA* | DnaJ-class molecular chaperone CbpA | *dnaJ* homolog [57] | **Up-regulated** |
| FNO12_0099 | *secB* | protein export chaperone | facilitate translocase of secretory proteins [76] | High |
| **Oxidative stress** | | | | |
| FNO12_1067 | *oxyR* | Oxidative stress transcriptional regulator | regulation of several proteins in response of oxidative stress [77]; absence of LysR family on *Francisella tularensis* subsp. *mediasiatica* is associated to virulence attenuation [59]. | Medium/low and Low |
| FNO12_0026 | *lysR1* | LysR family transcriptional regulator | | **Up-regulated** |
| FNO12_0071 | *lysR2* | LysR family transcriptional regulator | | **Up-regulated** |
| FNO12_0078_p | *lysR3* | LysR family transcriptional regulator | | Medium/low and Low |
| FNO12_0581_p | *lysR4* | LysR family transcriptional regulator | | Medium/low and Low |
| FNO12_1207 | *lysR5* | LysR family transcriptional regulator | | High and Medium/high |
| FNO12_1739_p | *lysR6* | LysR family transcriptional regulator | | Medium/low and Low |
| FNO12_0401 | *msrB* | Methionine sulfoxide reductase B | antioxidant enzyme that converts oxidized methionine into methionine [61] | **Up-regulated** |
| FNO12_0630 | *katG* | Catalase-peroxidase | catalase-peroxidase activity with $H_2O_2$ scavenging function, protecting cells from $H_2O_2$ toxicity [78] | High |
| FNO12_0841 | *acp* | Acid phosphatase/phosphotransferase | inhibition of the oxidative burst inside the phagosome [43] | **Down-regulated** |
| FNO12_1046_p | *tspO* | Tryptophan-rich sensory protein | fine-regulation of oxidative stress response [62] | **Up-regulated** |
| FNO12_1079 | *ahpC* | Alkyl hydroperoxide reductase | reduction of organic peroxides [79]; induction and maintenance of the viable but non-culturable state in *Vibrio parahaemolyticus* [65] | Hi50 |
| **Virulence** | | | | |
| FNO12_1334 | *mglA* | Macrophage growth locus A | FPI regulation [80] | High |
| FNO12_1335 | *mglB* | Macrophage growth locus B | | Medium/high |
| FNO12_1691 | *tuf* | Elongation factor Tu | adhesion and entry process in host tissues for *F. tularensis* [81] | Hi50 |
| FNO12_1368 | *iglB* | Intracellular growth locus protein B | FPI [14] | Hi50 |
| FNO12_1367 | *iglC* | Intracellular growth locus protein C | intra macrophage growth; phagosome scape; FPI [14] | Hi50 |
| FNO12_1370 | *pdpD* | Pathogenicity determinant protein D | intra macrophage growth; FPI [14] | Hi50 |
| FNO12_1366 | *iglD* | Intracellular growth locus protein D | FPI [14] | High |
| FNO12_1369 | *iglA* | Intracellular growth locus protein A | intra macrophage growth; FPI [14] | High |
| FNO12_1364 | *pigG* | Hypothetical protein | FPI [14] | High |
| FNO12_1356 | *pdpA* | Pathogenicity determinant protein A | intra macrophage growth; induce the proapoptotic caspase-1 and access to the host cell cytoplasm; FPI [14] | High |
| FNO12_1357 | *pdpB* | Pathogenicity determinant protein B | entry into and growth in macrophage; FPI [14] | Medium/high |
| FNO12_1358 | *pigA* | Hypothetical protein | FPI [14] | Medium/high |
| FNO12_1371 | *pmcA* | Anhydro-*N*-acetylmuramic acid kinase | FPI [14] | Medium/high |
| FNO12_1359 | *pigB* \| *vgrG* | Hypothetical protein | FPI [14] | Medium/high |
| FNO12_1365 | *pigH* | Hypothetical protein | FPI [14] | Medium/high |
| FNO12_1363 | *pigF* \| *dotU* | Hypothetical protein | FPI [14] | Medium/high |
| FNO12_1360 | *pigC* | Hypothetical protein | FPI [14] | Medium/high |
| FNO12_1362 | *pigE* | Hypothetical protein | FPI [14] | Medium/high |
| FNO12_1361 | *pigD* | Hypothetical protein | FPI [14] | Medium/low |
| FNO12_0411 | *pilA* | Type IV pilus assembly protein | Tfp; prepilin [66,67] | High |
| FNO12_0669_p | *pilT1* | Type IV pili fiber building block protein | Tfp [66,67] | High |
| FNO12_1708 | | Type IV pili, pilus assembly protein | Tfp [66,67] | Medium/high |
| FNO12_0791 | *pilD* | Type 4 prepilin-like proteins leader | Tfp [66,67] | Medium/high |
| FNO12_0410_p | *pilB1* | Type IV pilus biogenesis protein | Tfp; inner membrane associated with elongation [66,67] | Medium/high |
| FNO12_0046 | *pilE5* | Type IV pili | Tfp; prepilin [66,67] | Medium/high |
| FNO12_1024_p | *pilF* | Type IV pili, pilus assembly protein | Tfp; inner membrane associated with elongation [66,67] | Medium/high |
| FNO12_0384_p | *pilE4* | Type IV pili, pilus assembly protein | Tfp; prepilin [66,67] | Medium/high |
| FNO12_1133_p | *pilQ* | Type IV pili secretin component | Tfp; outer membrane secretin [66,67] | Medium/high |
| FNO12_1137_p | *pilM* | Type IV pili | Tfp [66,67] | Medium/low |
| FNO12_1136_p | *pilN* | Type IV pili associated protein | Tfp [66,67] | Medium/low |
| FNO12_1135_p | *pilO* | Type IV pili glycosylation protein | Tfp [66,67] | Medium/low |
| FNO12_0705_p | *pilB2* | Type 4 fimbrial assembly protein PilB | Tfp; inner membrane associated with elongation [66,67] | Medium/low |

(*continued on next page*)

**Table 1** (*continued*)

| Locus tag | Gene name | Product/function | Function described in literature | Regulation or Group[1] |
|---|---|---|---|---|
| FNO12_1733_p | *pilT2* | Type IV pili nucleotide-binding protein | Tfp [66,67] | Medium/low |
| FNO12_0409_p | *pilC1* | Type IV pilin assembly protein PilC | Tfp; inner membrane associated with structure [66,67] | Low |
| FNO12_1134_p | *pilP* | Type IV pili lipoprotein | Tfp [66,67] | Low |
| FNO12_0429_p | *pilO* | Type IV pili glycosylation protein | Tfp [66,67] | Low |
| FNO12_0704_p | *pilC2* | Type IV pili polytopic inner membrane protein | Tfp; inner membrane associated with structure [66,67] | Low |
| **Metabolism** | | | | |
| FNO12_0141 | *ftsZ* | Cell division protein | prokaryotic cytoskeletal protein [30] | Hi50 |
| FNO12_1397 | *panB* | 3-methyl-2-oxobutanoate hydroxymethyltransferase | pantothenate production and allow PurF-independent thiamine synthesis on glucose medium [82] | Hi50 |
| FNO12_1384 | *fabG* | 3-oxoacyl-(Acyl-carrier-protein) | Lipid transport and metabolism [31] | Hi50 |
| FNO12_1386 | | 3-oxoacyl-(Acyl-carrier-protein) | Lipid transport and metabolism [31] | Hi50 |
| FNO12_1563 | *lpxA* | 3-oxoacyl-(Acyl-carrier-protein) | Lipid transport and metabolism [31] | Hi50 |
| FNO12_1266 | *fabL* | Enoyl-[acyl-carrier-protein] reductase | Lipid transport and metabolism [31] | High |
| **Others** | | | | |
| FNO12_0201 | | Hypothetical protein | Transcription [31] | **Up-regulated** |
| FNO12_0269 | | Hypothetical protein | | **Up-regulated** |
| FNO12_0270 | | Hypothetical protein | | **Up-regulated** |
| FNO12_0436 | | Hypothetical protein | Cell cycle control, cell division, chromosome partitioning [31] | **Up-regulated** |
| FNO12_0796 | | ATP-dependent protease peptidase subunit | Posttranslational modification, protein turnover, chaperones [31] | **Up-regulated** |
| FNO12_0834_p | | Hypothetical protein | | **Up-regulated** |
| FNO12_1260 | *kdsD* | Arabinose 5-phosphate isomerase | isomerization of *d*-dribulose 5-phosphate to D-arabinose 5-phosphate in lipopolysaccharide biosynthetic pathway [83] | **Up-regulated** |
| FNO12_1352_p | | Hypothetical protein | | **Up-regulated** |
| FNO12_1453_p | | Hypothetical protein | | **Up-regulated** |
| FNO12_1531 | | Hypothetical protein | Inorganic ion transport and metabolism [31] | **Up-regulated** |
| FNO12_1867 | | Hypothetical protein | | **Up-regulated** |
| FNO12_1873 | | Hypothetical protein | Energy production and conversion [31] | **Up-regulated** |
| FNO12_0107_p | | Betaine–homocysteine *S*-methyltransferase | Amino acid transport and metabolism [31] | **Down-regulated** |
| FNO12_0139 | *ftsQ* | Cell division protein FtsQ | Cell cycle control, cell division, chromosome partitioning [31] | **Down-regulated** |
| FNO12_0161 | | Permease of ABC transporter | Inorganic ion transport and metabolism [31] | **Down-regulated** |
| FNO12_0162 | | ABC transporter | Inorganic ion transport and metabolism [31] | **Down-regulated** |
| FNO12_0164 | | Major facilitator transporter | Inorganic ion transport and metabolism [31] | **Down-regulated** |
| FNO12_0245_p | | Hypothetical protein | Inorganic ion transport and metabolism [31] | **Down-regulated** |
| FNO12_0264 | *murJ* | Probable lipid II flippase MurJ | Cell wall/membrane/envelope biogenesis [31] | **Down-regulated** |
| FNO12_0281 | | Amino acid transporter | Amino acid transport and metabolism [31] | **Down-regulated** |
| FNO12_0343_p | | Ribosomal protein S12 methylthiotransferase | Translation, ribosomal structure, and biogenesis [31] | **Down-regulated** |
| FNO12_0452 | | 2-octaprenylphenol hydroxylase | Coenzyme transport and metabolism [31] | **Down-regulated** |
| FNO12_0628 | | Metabolite:H+ symporter (MHS) family protein | Amino acid, carbohydrate and inorganic ion transport and metabolism [31] | **Down-regulated** |
| FNO12_0738 | | Hypothetical protein | Secondary metabolites biosynthesis, transport, and catabolism [31] | **Down-regulated** |
| FNO12_0749 | | HflK | Posttranslational modification, protein turnover, chaperones [31] | **Down-regulated** |
| FNO12_0810 | | hypothetical protein | | **Down-regulated** |
| FNO12_0811 | | HlyD family secretion protein | Defense mechanisms [31] | **Down-regulated** |
| FNO12_0934 | | Alkaline phosphatase | Amino acid transport and metabolism [31] | **Down-regulated** |
| FNO12_0954 | | Transcriptional regulator | Transcription | **Down-regulated** |
| FNO12_1167 | | Hypothetical protein | | **Down-regulated** |
| FNO12_1217 | | Hypothetical protein | | **Down-regulated** |
| FNO12_1320 | | Drug:H+ antiporter-1 (DHA2) | Amino acid, carbohydrate and inorganic ion transport and metabolism [31] | **Down-regulated** |
| FNO12_1462 | | Transcription termination factor Rho | Transcription [31] | **Down-regulated** |
| FNO12_1754_p | | Hypothetical protein | Carbohydrate transport and metabolism [31] | **Down-regulated** |
| FNO12_1842_p | | Aspartate aminotransferase | Amino acid transport and metabolism [31] | **Down-regulated** |
| FNO12_1845_p | | 4-hydroxy-tetrahydrodipicolinate synthase | Amino acid transport and metabolism [31] | **Down-regulated** |
| FNO12_1847_p | | Aspartokinase | | **Down-regulated** |
| FNO12_1853 | | Hypothetical protein | Carbohydrate transport and metabolism [31] | **Down-regulated** |
| FNO12_1854 | | Chloride channel protein | Amino acid transport and metabolism [31] | **Down-regulated** |
| FNO12_1896_p | | Anthranilate phosphoribosyltransferase | Amino acid transport and metabolism [31] | **Down-regulated** |
| FNO12_1898_p | | Anthranilate synthase component I | Amino acid transport and metabolism [31] | **Down-regulated** |

1 – Group of intensity (i.e., divide in quartiles: High, Medium/high, Medium/low, Low – and the top 50 from both tested temperature: Hi50) for 22 °C and 28 °C microarray expression experiments. If the intensity was in the same group, it was cited once.

genes were down- and up-regulated, respectively. The *acp* gene encodes a virulence factor described in *F. tularensis*, which is required for survival and intra-macrophage growth [58]. The other three acid phosphatase genes were observed in our study at medium/high expression levels under both conditions. For the *lysR* family, in *F. tularensis* subsp. *mediasiatica*, it's absence was associated with attenuation of virulence [59,60] and regulation of several genes. The *msrB*, a methionine sulfoxide reductase, an antioxidant enzyme that converts oxidized methionine into methionine, is linked with an important role in the intracellular replication of *F. tularensis* in macrophages and infections in mice [61]. Finally, *tspO* is a fine-regulator of oxidative stress response in eukaryotic cells, contributing to protection against reactive oxygen species with alterations in tetrapyrrole homeostasis [62]. Although these genes have not yet had their function defined in FNO, comparison with that of *Francisella* spp. homologous genes suggests that they could facilitate bacterial survival inside the host cell and could be a factor contributing to the pathogenicity shift modulated by temperature.

The pathogenesis of *Francisella* spp. depends on adherence, entering,

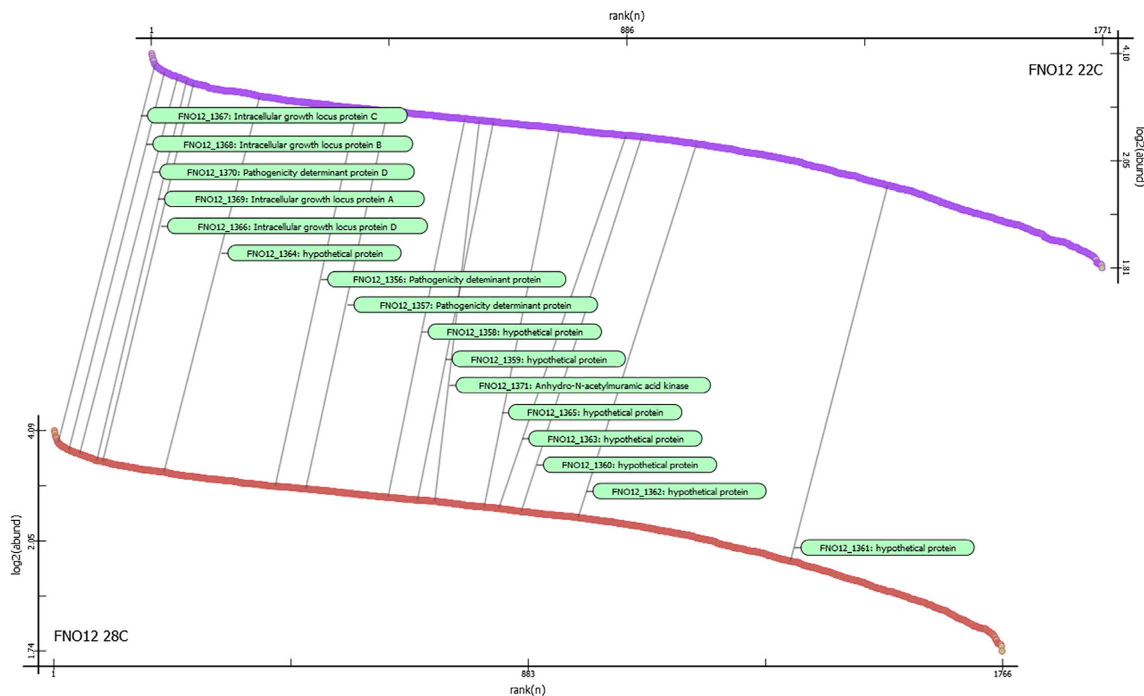


**Fig. 4. Comparison of expression levels of FPI genes according to the dynamic range.** Top: dynamic range (purple) of FNO12 isolate at 22 °C. Bottom: dynamic range (red) of FNO12 isolate at 28 °C. Grey lines show the position of each gene of the *Francisella* Pathogenic Island under both conditions.

vacuole escape, and growth inside the host cell. The pathogenic members of this genus have a well characterized genomic pathogenicity island [14] that encodes genes related to the type VI secretion system [63], the FPI, and vacuole escape and growth inside the host cell. The genes comprised in the island and *mlgA/B*, which are known to regulate the gene island, did not shown any regulatory changes between tested temperature conditions. These genes were found in very similarly high abundance in both conditions, demonstrating that the FPI genes are expressed actively. Interestingly, this result shows a different evolution of regulation of FPI system in FNO when compared to *F. tularensis* in human-infections, which showed an up-regulation of this island of genes at host temperature [23,64]. Also, it suggests a host niche adaptation of FNO, since the fish is a poikilothermic animal, where substantial variations of body temperature can occur as a result of water temperature changes.

The *iglB* and *pdpB* genes, of FNO FPI, have remained in unchanged level expression in our study. Whereas in the study conducted by Lewis & Soto [17], they have shown a different pattern. In this work, during

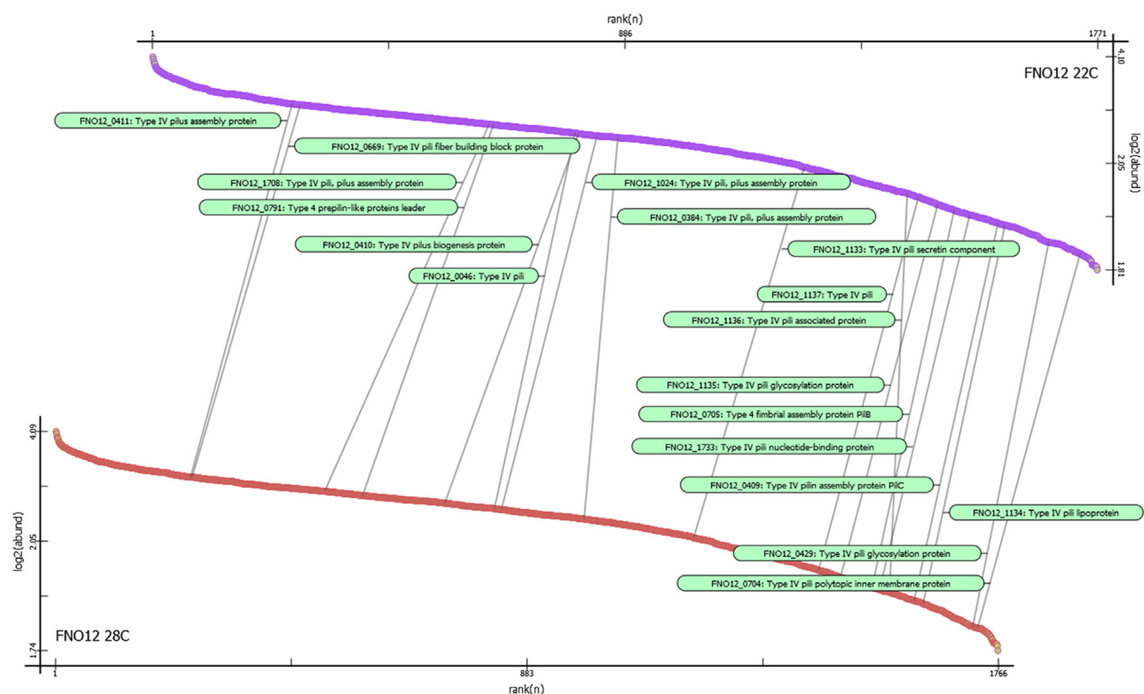


**Fig. 5. Comparison of expression levels of Tfp genes according to the dynamic range.** Top: dynamic range (purple) of FNO12 isolate at 22 °C. Bottom: dynamic range (red) of FNO12 isolate at 28 °C. Grey lines show the position of each gene related with Type IV secretion under both conditions.

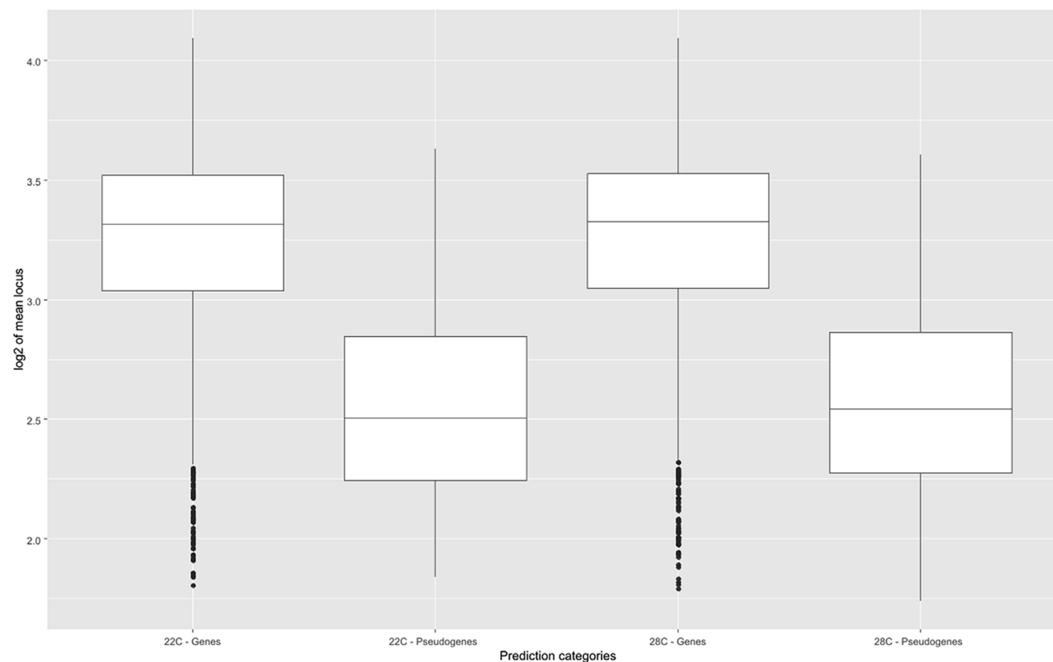


**Fig. 6. Mean expression levels of gene and pseudogene at 28°C and 22°C.** The differential expression between genes and pseudogenes were statistically significant ($p < 0.05$) for both tested temperatures.

the exponential phase at 25 °C compared to 30 °C, the *iglB* was observed in up-regulation, while the *pdpB* was observed in down-regulation. This difference in the results can be because of the slight difference on temperature used in the experiments, or due to the genetic diversity observed [41] between FNO12 isolate of our work and the LADL–07-285A strain, isolated in Costa Rica, used by the Lewis & Soto.

The *iglB*, together with *dotU* and *vgrG* showed an up-regulation when the bacterium was exposed an oxidative stress by Lewis & Soto [17]. In our study, these genes have remained in stable level of expression. Although our study did not exposed the *in-vitro* cultures to an oxidative stress, genes related specifically to oxidative stress have shown an up-regulation when temperature changed from 28 °C to 22 °C. Further studies need to be done in order to evaluate how the composition of theses triggers (*i.e.*, oxidative stress and temperature) could influence to the FNO genes expression.

In previous work analyzing the survival of FNO in the water, the pathogenic properties of FNO decreased after 24 h, becoming non-infective after two days without a fish host, and its survival rate was inversely proportional to the temperature of microcosm experiment [49]. In other work, FNO infections were detected in consecutive winter seasons in the same Brazilian fish farm, without the introduction of infected live fish and after a period without identification of FNO [13]. It is understood that FNO does not survive in the environment for long periods of time without a host, which leads us to believe that during the period with subclinical tilapia, the survival of the FNO inside the host may stay linked with high and unchanged expression of the FPI virulence genes. Still in this way, entering and recovering from a viable but non-culturable (VBNC) state of the pathogen is a theory that cannot be discarded, which was also suggested by Delphino and collaborators, based on epidemiological data [13]. The expression of these genes may allow infection, might in VBNC state, without disease development at higher studied temperature. Another gene that has an unchanged regulation expression, identified in the Hi50 group in our analysis, and can corroborate with this suggestion, is the *ahpC*. Although this function is not confirmed for FNO, the *ahpC* is related with induction and maintenance of a VBNC state in *Vibrio parahaemolyticus* [65].

Other secretion system very studied for *F. tularensis* is the Type IV pili machinery [66]. Pre-pilin genes, *pilA*, *pilE,* and *pilD,* from this machinery, have been found to be indispensable to virulence of *F. tularensis,* and *pilE* is also required for the intradermal route of infection by *F. novicida* [67]. The *pilA* was confirmed to be exported to the bacterial surface by immunogold electron microscopy analysis [68]. Several genes from Tfp were annotated as pseudogene in FNO and their translation was not confirmed in our proteomics assay, suggesting that the Tfp as fimbrial machinery is not functional. Otherwise, *pilA*, *pilE* and *pilD* were expressed in high and medium/high groups, possibly presenting another relevant role for virulence like a non-fimbrial adhesins. These genes can also be involved in virulence maintenance of this pathogen in both tested temperatures, and, considering the adhesion probabilities of *pilA* and *pilE* on Vaxign analysis (data not shown), they are good candidate targets for vaccine development against francisellosis.

It is known that others triggers are able to induce expression of virulence genes; in *F. tularensis*, the genes from FPI were up-regulated in iron and oxygen restriction, or in oxidative and acid stress [64,69]. In our work these triggers could not be accessed by the limitation of the experimental design in the *in vitro* assay. Further studies are required to complement these features to FNO pathogen and to evaluate the host immune response and susceptibility for this pathogen in different temperatures. The Nile tilapia usually displays progressive stress in temperatures outside the optimal growth temperature range of 28–30 °C [70,71], and this, associated with the unchanged FNO virulence activity found of this work, might explain the development of the francisellosis disease in cold seasons and maintenance, but the absence of FNO disease in host fish during the hot water seasons.

## 5. Conclusion

In conclusion, FNO12 isolate demonstrated an increase in infectivity in our *in vivo* assay at 22 °C compared with that observed at 28 °C. The temperature changes modulate the gene level expression of oxidative stress and thermal shock genes, and genes related to metabolism, which may indicate an ability of FNO to adapt to the aquatic environment or the fish host at both temperatures. However, expression levels of several genes related to virulence remained unchanged and were identified in high abundance under both temperature conditions. The expected

increase of virulence factor expression at the lower temperature, which might help to explain the virulence shift, was not observed.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2019.103548.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Authors' contributions

FLP: wrote the manuscript and performed the bioinformatics analyses. GCT, AFC: performed the transcriptomic experiments. JCCR and CPR: performed the proteomics experiments. FLP, GCT, HCPF: contributed to data interpretation and to revisions of the manuscript. CAGL, HCPF: conceived and designed the experiments. HCPF: coordinated all analyses of the project. All authors read and approved the final manuscript.

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# 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.

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