GMR
The Original by FUNPEC-RP

# Complete genome sequence of *Francisella noatunensis* subsp. *orientalis* strain F1 and prediction of vaccine candidates against warm and cold-water fish francisellosis

**C.T. Facimoto [1], R.T. Chideroli[1], G.W. Di Santis[1], D.D. Gonçalves[2], A.O. do Carmo[3], E. Kalapothakis[3], A.F. Oliveira Junior[3], V.A.C. Azevedo[3], L.J. Benevides[3,5], S.S. Castro[4], N.M.L. Barrero[1,6], A.G. de Oliveira[7] and U.P. Pereira[1]**

[1] Laboratório de Bacteriologia em Peixes, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Londrina, PR, Brasil
[2] Laboratório de Medicina Veterinária Preventiva e Saúde Pública, Universidade Paranaense, Umuarama, PR, Brasil
[3] Departamento de Biologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[4] Departamento de Imunologia, Microbiologia e Parasitologia, Instituto de Ciências Biológicas e Ciências Naturais, Universidade Federal do Triângulo Mineiro, Uberaba, MG, Brasil
[5] Commensal and Probiotics-Host Interactions Laboratory, Micalis Institute, Institut National de la Recherche Agronomique, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France
[6] Centro de Pesquisa em Aquicultura e Genética, Departamento de Zootecnia, Universidade Estadual de Londrina, Londrina, PR, Brasil
[7] Laboratório de Biotecnologia Microbiana, Departamento de Microbiologia, Universidade Estadual de Londrina, Londrina, PR, Brasil

Corresponding author: U.P. Pereira
E-mail: upaduapereira@uel.br

Genet. Mol. Res. 18 (2): gmr18302
Received March 14, 2019
Accepted May 21, 2019
Published May 28, 2019
DOI http://dx.doi.org/10.4238/gmr18302

**ABSTRACT.** *Francisella noatunensis* subsp. *orientalis* (Fno) is a Gram-negative bacterium that causes granulomatous infections in fish reared at low water temperatures; it has been responsible for a large number of deaths in tilapia fish farms. Fno has been reported in many countries in the last decade. Studies on phylogenomic

 

relationships among isolates are needed because of the increasing importance of this disease. Here, we present the complete genome of the Fno F1 strain isolated in 2015 from a tilapia farm in São Paulo state, Brazil. The genome is a circular chromosome with a length of 1,854,333 bp, containing 32.26% G+C DNA content, 1,448 protein-coding genes and 393 pseudogenes. In addition, a prediction of conserved vaccine targets was made between the two subspecies of *F. noatunensis* that cause disease in tropical and cold-water fish species. Five proteins conserved between Fno and *Francisella noatunensis* subsp. *noatunensis* were predicted to be good vaccine candidates for the development of a recombinant vaccine against francisellosis. This genome also provides useful data to help understand the pathogen evolution and epidemiology of this disease in Brazil.

**Key words:** Infectious diseases; Vaccine; Fish pathogen; Genome sequencing; Phylogeny

## INTRODUCTION

Aquaculture has become increasingly intensified and diversified worldwide over the years; this has resulted in the identification of "new" infectious agents, previously considered unknown diseases (Colquhoun and Duodu, 2011). Bacteria of the genus *Francisella*, are part of the *Francisellaceae* family and the *Thiotrichales* order. In recent years, bacteria of the *Francisella* genus, *Francisella noatunensis* subsp. *noatunensis* (Fnn) (Ottem et al., 2009; Oren and Garrity, 2016), and *Francisella noatunensis* subsp. *orientalis* (Fno), have been associated with bacteriosis in fish species from cold and warm waters, respectively (Soto et al., 2009; Vestvik et al., 2013; Nguyen et al., 2016a). *Francisella* spp. infections are characterized as an acute (with few clinical signs and high mortality), a subacute, or a chronic (non-specific clinical signs with varying degrees of mortality) syndrome (Colquhoun and Duodu, 2011). In Brazil, francisellosis losses in Nile Tilapia (*Oreochromis niloticus*) cultivated in cage tanks have been reported since 2012 and mortality rates can reach up to 60%. The most common clinical signs associated with infections are: anorexia, melanosis, exophthalmia, erratic swimming, skin ulcers (primarily at the base of the fins) and gill pallor (Leal et al., 2014).

A short time ago, the *Francisella* genus consisted of only two species: *F. tularensis* subsp. *tularensis* and *F. philomiragia* subsp. *philomiragia*. However, new species and strains have been isolated, including *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* (Ottem et al., 2009), being recognized as among the most important pathogens of cultivated tilapia (Soto et al., 2012a). This pathogen affects mainly fry, fingerlings, and young adult fish, exhibiting multifocal granulomas in internal organs, mostly in the liver, spleen, and kidney (Ottem et al., 2009; Nguyen et al., 2016b). Cases of Fno in farm-raised tilapia have been reported in Taiwan (Chen et

 

al., 1994), Costa Rica (Soto et al., 2009), Indonesia (Ottem et al., 2009), the UK (Jeffery et al., 2010), the USA (Soto et al., 2012b), and Brazil (Jatobá et al., 2016).

Research on *Francisella* phylogenomic relationships and diversity is in constant development as whole genomes have been made publicly available. However, although many microbiological features and biochemical and genomic characterizations are available for the *Francisella* genus, the studies focus on subspecies of *F. tularensis* because of their relevance for human health and biosafety (Oyston et al., 2004). On the other hand, there are few studies about other species, such as *F. noatunensis* subsp. *orientalis*, pathogens of veterinary and economic importance (Soto et al., 2012a). No commercial vaccine against fish francisellosis is currently available; however, attempts using attenuated mutant strains of *F. noatunensis* subsp. *orientalis* have provided promising results (Soto et al., 2011).

Due to its importance for aquaculture, *F. noatunensis* has gained more attention in recent years; however, the genomic data of this pathogen has been poorly explored. In this paper, we describe the whole genome features and potential vaccine candidates (conserved between Fnn and Fno) for the F1 strain isolated from a fish farm outbreak in the state of São Paulo, Brazil in 2015.

## MATERIAL AND METHODS

### Isolation and sequencing

The F1 strain was obtained from Southeast Brazil in the 2015 winter season from an outbreak in a fish farm with water temperature below 21º C. Swabs of kidney tissue were sampled, streaked onto cysteine heart agar supplemented with 1% bovine hemoglobin (CHAH) (HIMEDIA, India) and incubated at 28°C for 4–7 days. Colonies were tested with catalase and cytochrome oxidase assays. Further, the isolate was submitted to PCR genus confirmation using primers described by Forsman et al. (1994).

Genomic DNA was obtained with a phenol-chloroform-isoamylic alcohol extraction protocol (Bollet et al., 1991). Genome sequencing of the F1 strain was performed with the MiSEQ platform (Illumina®, USA), using a 300-bp paired-end-library. Sequencing and partial assembly were performed at the Laboratory of Cellular and Molecular Genetics (LGCM), Minas Gerais, Brazil.

### Assembly of F. noatunensis subsp orientalis F1

Reads were uploaded in FASTQ format to CLC Genomics Workbench 8 (Qiagen, USA) software, for trimming and assembly steps. Reads with average phred scores below 30 and presenting any ambiguities were discarded. The last 10 nucleotides of each 3' end and reads smaller than 50 bp were also discarded. Filtered reads were submitted to *ab initio* assembly to generate contigs.

In order to build the scaffold draft of the genome, contigs were ordered through CONTIGuator 2.7 software (Galardini et al., 2011), utilizing the FNO01 (CP012153.1) strain as a reference. Remaining gaps were processed through CLC Genomics

Workbench 8. During this step, the reads were mapped multiple times in the scaffold sequence (mainly to observe gap flanking regions) in order to complete the missing nucleotides of the genome. All the raw sequencing data was mapped in the final genome sequence and absence of contamination with other genomes was confirmed by the coverage and the low number of unmapped reads (less than 0.1%). Automatic structural and functional annotation (to predict genes, rRNAs and tRNAs) of the genome was performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016).

## Phylogeny and Conserved Vaccine Candidate Prediction

Twelve whole genome strains in the *Francisella* genus were submitted to Gegenees V2.2.1 (Ågren et al., 2012) analysis with high accuracy, generating a heatmap displaying the similarity among whole genome *Francisella* strains. Accession numbers and hosts are displayed in Table 1. A phylogenetic tree was drawn using SplitsTree4 set to EqualAngleRoot split and UPGMA methods in order to estimate the phylogenetic distance of our strain from other species in the genus (Huson, 1998).

**Table 1.** *Francisella* genome sequences used in comparison with strain F1.

| Species | Strain | Accession | Size (Mb) | GC% | Genes | Protein | Host | Country |
|---|---|---|---|---|---|---|---|---|
| *F. noatunensis* subsp. *noatunensis* | FSC772 | CP022207.1 | 1.93382 | 32.7 | 1891 | 1754 | Atlantic Salmon | Chile |
| *F. noatunensis* subsp. *orientalis* | F1 | CP018051.1 | 1.85433 | 32.3 | 1889 | 1448 | Tilapia | Brazil |
| *F. noatunensis* subsp. *orientalis* | FNO01 | CP012153.2 | 1.86244 | 32.3 | 1900 | 1452 | Tilapia | Brazil |
| *F. noatunensis* subsp. *orientalis* | FNO12 | CP011921.2 | 1.86221 | 32.3 | 1899 | 1446 | Tilapia | Brazil |
| *F. noatunensis* subsp. *orientalis* | FNO190 | CP011923.2 | 1.86221 | 32.3 | 1900 | 1451 | Tilapia | Brazil |
| *F. noatunensis* subsp. *orientalis* | FNO24 | CP011922.2 | 1.86232 | 32.3 | 1899 | 1446 | Tilapia | Brazil |
| *F. noatunensis* subsp. *orientalis* | LADL--07-285A | CP006875.1 | 1.85899 | 32.3 | 1896 | 1422 | Tilapia | Costa Rica |
| *F. noatunensis* subsp. *orientalis* | Toba 04 | CP003402.1 | 1.8472 | 32.2 | 1886 | 1418 | Tilapia | Indonesia |
| *F. halioticida* | DSM_23729 | CP022132.1 | 2.19743 | 31.2 | 2351 | 2096 | Giant Abalone | Japan |
| *F. tularensis* subsp. *tularensis* | Schu_S4 | NC_006570 | 1.89278 | 32.3 | 1834 | 1556 | Human | United States |
| *F. persica* | FSC845 | CP012505.1 | 1.51668 | 31.4 | 1485 | 1067 | Tick | Egypt |
| *F. philomiragia* | O_319_029 | CP009343.1 | 2.04493 | 32.6 | 1986 | 1876 | Water | United States |

In order to predict vaccine targets, the protein sequences of genome of the F1 strain were submitted to MEDpipe workflow (Santos et al., 2013), which classifies subcellular localization of proteins as cytoplasmatic (CYT), secreted (SEC), potentially surface exposed (PSE) and membrane proteins (MEM). Results were analyzed and proteins with the highest immunogenicity potential (score MED, mature epitope density), were chosen (Barinov et al., 2009). Only SEC and PSE proteins were selected for the next steps, due to the close relationship with host immunity (Rappuoli, 2000). One hundred of the highest MED score protein candidates were checked for protein conservation and presence of premature stop codons. Using BLASTp, protein sequences were compared with other proteins belonging to Fno and Fnn, aiming to find proteins that were conserved intra and inter-subspecies. The degree of conservation of protein targets between Fno and Fnn was measured using BLASTp software (https://blast.ncbi.nlm.nih.gov). Only proteins with query coverage and identity above

 

90% were considered conserved between Fnn and Fno and consequently good vaccine candidates. A search in Uniprot (https://www.uniprot.org) and Interproscan (https://www.ebi.ac.uk) was used to determine candidate functional role/annotation. Vaxign software was used to verify proteins with adhesion probability larger than 0.51 and MHC I and MHC II binding properties (He et al., 2010).

Proteins were also checked for similarity with *Oreochromis* spp. proteins to avoid autoimmune reactions in the host; none of them presented considerable coverage or identity with host proteins (more than 50%). Also, the same analysis was performed using GenBank genome data of the genus *Gadus* (since Fnn is a pathogen of some fish belonging to that genus); no close proteins were found (*Gadus*- taxid: 8048).

## RESULTS

### Isolation and Assembly

The bacteria presented growth in the CHAH plates after 96h of incubation; the colonies were gray, smooth, and convex. Microscopy showed Gram-negative coccobacilli shapes. The bacteria were catalase positive and cytochrome oxidase negative. They were also PCR positive for the *Francisella* genus.

Sequencing generated 2,524,734 raw reads that resulted in 2,492,893 reads after trimming. The F1 strain *ab initio* assembly produced 12 contigs, with an N50 value of 304,012. The maximum contig length was 478,741 bp and the minimum 1,782 bp. Eleven gaps were filled by iterative reads mapping against gap flanking regions of the scaffold sequence. The complete genome of F1 strain comprises a single circular chromosome 1,854,333 bp in length, 32.26% G+C content, 7 rRNA operons, 37 tRNA genes and 393 pseudogenes. The genome project is housed in the Genomes OnLine Database (GOLD) (Liolios et al., 2010) project ID Go0336213; and complete genome sequence and annotation data are available in the DDBJ/EMBL/GenBank under accession number CP018051.1.

### Histopathological characterization of lesions

Specific signs of the disease were observed internally, including a large number of white nodules on the gills, spleen, kidney, gonads, liver and heart. Microscopically, we observed a large accumulation of inflammatory cells promoting an increase in nodular volume between the choroid and the sclera (Figure 1A), with areas of well vascularized stroma, and marked monophyletic and polymorphonuclear infiltrates (Figure 1B), alternating with some that were clearly granulomatous (Figure 1C), composed of epithelioid cells and macrophages containing intracytoplasmic coccoid bacteria (Figure 1D). The same type of lesion was also seen in other histological sections, extending discreetly to the retrobulbar periocular tissues. Granulomatous lesions were also observed on the spleen and cranial kidney.

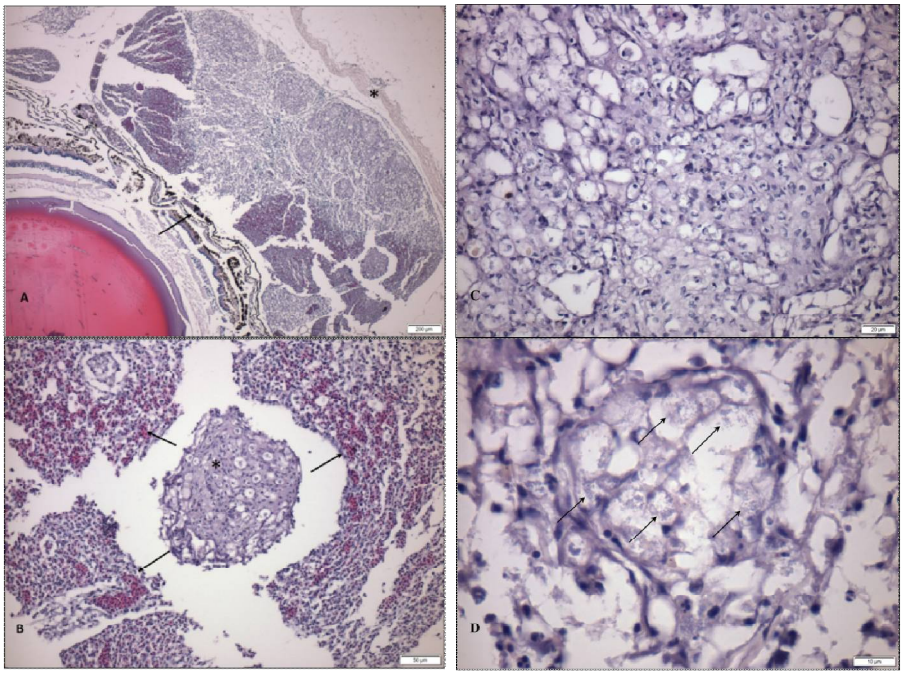


**Figure 1.** Ocular globe of Nile tilapia (*Oreochromis niloticus*) infected by *Francisella noatunensis* subsp. *orientalis* strain F1. A) Presence of marked nodular inflammatory reaction between choroid (arrow) and sclera (*). Hematoxylin and Eosin. B) Accentuated accumulation of polymorphonuclear and mononuclear inflammatory cells in well-vascularized stroma (arrows) with granulomatous formation (*). Hematoxylin and Eosin. C) Granulomatous area composed of vacuolated macrophages and epithelioid cells. Hematoxylin and Eosin. D) Granulomatous portion evidencing intracytoplasmic coccoid bacteria (arrows). Hematoxylin and Eosin.

## *In silico* Prediction of Vaccine Candidates

In total, 188 proteins were assigned as MEM, 1053 CYT, 49 SEC and 158 PSE. Only 8 SEC and 40 PSE proteins attended the criteria of our study. The highest and lowest MED scores picked were 55.27 and 11.05 respectively. Among the 48 proteins submitted, only 5 had a binding score higher than 0.51 and no similarity with human, mouse and pig. The highest adhesivity scores were displayed by proteins classified as SEC (WP_014715657.1, locus_tag BMT43_RS09195 and WP_014714512.1, locus_tag BMT43_RS02215); the remaining proteins were classified as PSE (Table 2).

**Table 2.** Protein vaccine candidates against francisellosis.

| Protein Accession | Protein Note | Locus_tag | Adhesin Probability | Location |
|---|---|---|---|---|
| WP_014715657.1 | DUF3568 | BMT43_RS09195 | 0.701 | SECRETED |
| WP_014714512.1 | DUF3573 | BMT43_RS02215 | 0.664 | SECRETED |
| WP_012280666.1 | MULTISPECIES: hypothetical protein | BMT43_RS07125 | 0.577 | PSE |
| WP_014714564.1 | MCE family protein | BMT43_RS06520 | 0.565 | PSE |
| WP_014714557.1 | HlyD family secretion protein | BMT43_RS06560 | 0.551 | PSE |

DUF – Domain of Unknown Function; PSE – Potentially Surface Exposed

# DISCUSSION

## Phylogeny

We evaluated the evolutionary relationships of *Francisella* spp. complete genomes deposited in GenBank. The *Francisella* spp. genus also contains species that infect humans, such as *Francisella tularensis* (the agent responsible for tularemia) (Sjöstedt, 2007), and *Francisella philomiragia* (Relich et al., 2015), both associated with chronic and necrotizing granulomas. Although the available genomes of species from the genus *Francisella* have relatively similar features, their genomes are highly rearranged (Rohmer et al., 2007).

In the heatmap (Figure 2) all Fno strains formed a cluster with high similarity, while the only genome of Fnn (strain FSC772) evaluated, displayed a relatively low similarity with the Fno cluster, similar to what was found in another study of the *Francisella* genus (Gonçalves et al., 2016). Also, *F. philomiragia* O_319_029 strain presented a high similarity with the Fnn strain, which shows a close relationship between these species. The phylogenetic tree generated by SplitsTree (Figure 3) gives an overview of the phylogenetic distances within the *Francisella* genus. In agreement with other studies using whole genome analysis (Sjödin et al., 2012; Sridhar et al., 2012), species that infect fish, are grouped in a different cluster from species that infect mammals, suggesting an independent evolutionary path through adaptation to these host species.

| Organism | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1: *F. halioticida* DSM 23729 | 100.0 | 3.95 | 3.31 | 4.46 | 4.47 | 4.27 | 4.27 | 4.27 | 4.27 | 4.27 | 4.27 | 4.27 |
| 2: *F. tularensis* subsp *tularensis* SCHU S4 | 4.6 | 100.0 | 25.68 | 14.04 | 13.23 | 11.2 | 11.21 | 11.21 | 11.21 | 11.21 | 11.21 | 11.2 |
| 3: *F. persica* FSC845 | 4.55 | 31.74 | 100.0 | 6.54 | 6.4 | 6.05 | 6.05 | 6.05 | 6.05 | 6.05 | 6.05 | 6.04 |
| 4: *F. philomiragia* O_319_029 | 4.82 | 12.54 | 5.06 | 100.0 | 80.08 | 59.87 | 59.86 | 59.88 | 59.88 | 59.88 | 59.87 | 59.8 |
| 5: *F. noatunensis* subsp *noatunensis* FSC772 | 5.03 | 12.95 | 5.23 | 84.47 | 100.0 | 63.41 | 63.4 | 63.4 | 63.41 | 63.41 | 63.41 | 63.35 |
| 6: *F. noatunensis* subsp *orientalis* FNO24 | 4.98 | 11.36 | 5.09 | 65.41 | 65.78 | 100.0 | 99.96 | 99.96 | 99.98 | 99.84 | 99.85 | 99.86 |
| 7: *F. noatunensis* subsp *orientalis* FNO190 | 4.93 | 11.46 | 5.13 | 65.52 | 65.76 | 99.96 | 100.0 | 99.96 | 99.98 | 99.85 | 99.86 | 99.86 |
| 8: *F. noatunensis* subsp *orientalis* FNO01 | 4.94 | 11.38 | 5.12 | 65.36 | 65.73 | 99.96 | 99.95 | 100.0 | 99.97 | 99.84 | 99.85 | 99.86 |
| 9: *F. noatunensis* subsp *orientalis* FNO12 | 4.91 | 11.39 | 5.1 | 65.39 | 65.78 | 99.98 | 99.98 | 99.97 | 100.0 | 99.87 | 99.87 | 99.88 |
| 10: *F. noatunensis* subsp *orientalis* LADL-07-285A | 4.86 | 11.33 | 5.07 | 65.5 | 65.83 | 99.96 | 99.95 | 99.95 | 99.97 | 100.0 | 99.98 | 99.87 |
| 11: *F. noatunensis* subsp *orientalis* F1 | 4.69 | 11.23 | 4.93 | 65.42 | 65.72 | 99.97 | 99.97 | 99.97 | 99.98 | 99.97 | 100.0 | 99.89 |
| 12: *F. noatunensis* subsp *orientalis* Toba04 | 4.48 | 10.96 | 4.68 | 65.3 | 65.67 | 99.89 | 99.89 | 99.88 | 99.91 | 99.79 | 99.79 | 100.0 |

**Figure 2.** Heatmap of similarity among non-core regions of *Francisella* spp. genomes using Gegenees all-against-all fragmented comparison with high accuracy.

Noteworthy, *F. halioticida* DSM_23729, a Giant Abalone strain, was closer to the phylogenetic tree root, reinforcing the hypothesis that *Francisella* species originated from a marine habitat (Sjödin et al., 2012). Interestingly, *F. halioticida* DSM_23729, showed a closer ancestral relationship with human strains instead of marine species. An enlarged view of the Fno cluster (Figure 3B) shows a clonal relation of Latin American strains and also a close similarity with Toba_04 strain isolated from Indonesia. This cluster branch displays Toba_04 slightly out of the group, indicating that this strain is not directly related to the strains in Latin America; however, Toba_04 could have originated outbreaks in the American continent.

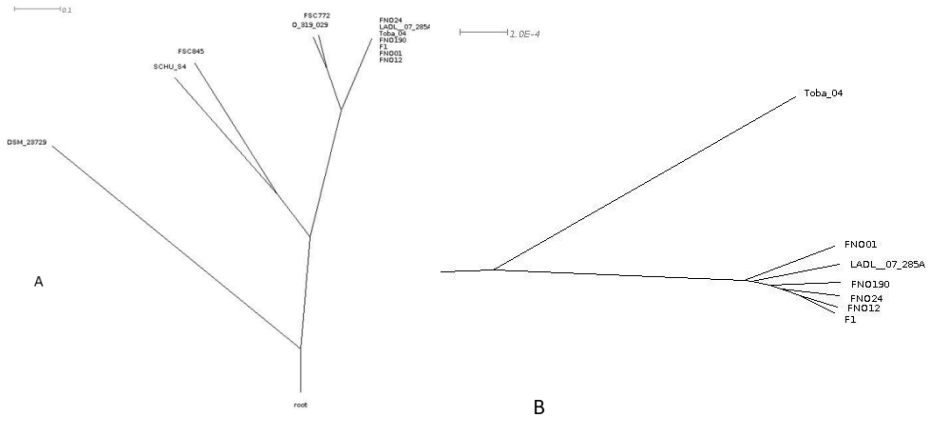


**Figure 3.** Phylogenetic tree based on whole genomes of *Francisella* spp. strains using EqualAngleRoot and UPGMA methods. A) Phylogenetic tree comparing different species in the *Francisella* genus, showing the distance of strains. B) A zoomed view of the Fno cluster.

## In silico Prediction of Vaccine Candidates

Only WP_014714564.1 (locus_tag BMT43_RS06520) and WP_014714557.1 (locus_tag BMT43_RS06560) were assigned to a protein function domain through Interproscan analysis, which classified these proteins as MCE family protein and HlyD family secretion protein, respectively. These proteins may be considered a good option for universal vaccine candidates because they are exclusive to the bacteria as they do not show homology with the host (*Oreochromis niloticus*) or other species of fish (*Gadus morhua*).

The MCE family proteins are related to survival in macrophages and are reported in live vaccines of attenuated mutant strains, resulting in reduction of virulence in guinea pigs to *Mycobacterium tuberculosis* (Obregón-Henao et al., 2011). HlyD, along with other membrane proteins, form a transport apparatus for hemolysin secretion. Other studies argue that this system is suitable for the development of heterologous vaccine antigens (Gentschev et al., 2002).

## CONCLUSIONS

Here we present the complete genome of an Fno F1 strain isolated from a francisellosis outbreak in the state of Sao Paulo, Brazil. The genome resembles those of other strains isolated in Brazil, due to a similar count of RNA genes and coding sequences, especially the large number of pseudogenes, which suggests genome decay in this species. The phylogenetic analysus showed high similarity among species in the Fno cluster. Interestingly, the Fno Toba_04 strain was placed slightly distant from American Fno isolates, suggesting that the Indonesian strain was not directly "introduced" into Brazil or the American continent. The Fnn strain was somewhat outside of this cluster, closer to a *F. philomiragia* isolate. Nevertheless, five potential vaccine proteins were selected in the vaccine candidate prediction; these have the potential to induce an immune response against both subspecies of *F. noatunensis* without negatively affecting the host. These results

highlight the usefulness of reverse vaccinology for vaccine development against francisellosis in fish (in both tropical and cold regions).

## ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and to the Researcher Support Office of the Universidade Estadual de Londrina (PROPPG/UEL).

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## REFERENCES

Ågren J, Sundström A, Håfström T and Segerman B (2012). Gegenees: Fragmented Alignment of Multiple Genomes for Determining Phylogenomic Distances and Genetic Signatures Unique for Specified Target Groups. *PLoS One*. 7: e39107. doi: 10.1371/journal.pone.0039107

Barinov A, Loux V, Hammani A, Nicolas P, et al. (2009). Prediction of surface exposed proteins in *Streptococcus pyogenes*, with a potential application to other Gram-positive bacteria. *Proteomics*. 9: 61–73. doi: 10.1002/pmic.200800195

Bollet C, Gevaudan MJ, de Lamballerie X, Zandotti C, et al. (1991). A simple method for the isolation of chromosomal DNA from gram positive or acid-fast bacteria. *Nucleic Acids Res*. 19: 1955. doi: 10.1093/nar/19.8.1955

Chen SC, Tung MC, Chen SP, Tsai JF, et al. (1994). Systematic granulomas caused by a rickettsia-like organism in Nile tilapia, *Oreochronuis niloticus* (L.), from southern Taiwan. *J. Fish Dis.* 17: 591–599. doi: 10.1111/j.1365-2761.1994.tb00257.x

Colquhoun DJ and Duodu S (2011) *Francisella* infections in farmed and wild aquatic organisms. *Vet. Res.* 42: 47. doi: 10.1186/1297-9716-42-47

Forsman M, Sandstrom G and Sjostedt A (1994). Analysis of 16S Ribosomal DNA Sequences of *Francisella* Strains and Utilization for Determination of the Phylogeny of the Genus and for Identification of Strains by PCR. *Int. J. Syst. Bacteriol.* 44: 38–46. doi: 10.1099/00207713-44-1-38

Galardini M, Biondi EG, Bazzicalupo M and Mengoni A (2011). CONTIGuator: a bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol. Med*. 6: 11. doi: 10.1186/1751-0473-6-11

Gentschev I, Dietrich G and Goebel W (2002.) The *E. coli* α-hemolysin secretion system and its use in vaccine development. *Trends Microbiol.* 10: 39–45. doi: 10.1016/S0966-842X(01)02259-4

Gonçalves LA, Soares S de C, Pereira FL, Dorella FA, et al. (2016). Complete genome sequences of *Francisella noatunensis* subsp. *orientalis* strains FNO12, FNO24 and FNO190: A fish pathogen with genomic clonal behavior. *Stand Genomic Sci*. 11: 1–8. doi: 10.1186/s40793-016-0151-0

He Y, Xiang Z and Mobley HLT (2010). Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. *J. Biomed. Biotechnol.* 2010: 297505. doi: 10.1155/2010/297505

Huson DH (1998). SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics*. 14: 68–73.

Jatobá A, Klipp SP and Hoppe R (2016). Primeiro relato de *Francisella noatunensis* subespécie *orientalis* no sul do brasil - relato de caso. *Acta Vet. Bras.* 10: 172–176. doi: 10.21708/avb.2016.10.2.5512

Jeffery K, Stone D, Feist S and Verner-Jeffreys D (2010). An outbreak of disease caused by *Francisella* sp. in Nile tilapia *Oreochromis niloticus* at a recirculation fish farm in the UK. *Dis. Aquat. Organ.* 91: 161–165. doi: 10.3354/dao02260

Leal CAG, Tavares GC and Figueiredo HCP (2014). Outbreaks and genetic diversity of *Francisella noatunensis* subsp *orientalis* isolated from farm-raised Nile tilapia (Oreochromis niloticus) in Brazil. *Genet. Mol. Res.* 13: 5704–5712. doi: 10.4238/2014.July.25.26

Liolios K, Chen I-MA, Mavromatis K, Tavernarakis N, et al. (2010). The Genomes On Line Database (GOLD) in 2009: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Res*. 38: D346-54. doi: 10.1093/nar/gkp848

Nguyen VV, Dong HT, Senapin S, Pirarat N, et al. (2016a). *Francisella noatunensis* subsp. *orientalis* , an emerging bacterial pathogen affecting cultured red tilapia ( *Oreochromis* sp.) in Thailand. *Aquac. Res.* 47: 3697–3702. doi: 10.1111/are.12802

Obregón-Henao A, Shanley C, Bianco MV, Cataldi AA, et al. (2011). Vaccination of guinea pigs using mce operon mutants of *Mycobacterium tuberculosis*. *Vaccine*. 29: 4302–7. doi: 10.1016/j.vaccine.2011.04.027

Oren A and Garrity GM (2016). List of new names and new combinations previously effectively, but not validly, published. *Int. J. Syst. Evol. Microbiol.* 66: 4299–4305. doi: 10.1099/ijsem.0.001585

Ottem KF, Nylund A, Karlsbakk E, Friis-Møller A, et al. (2009). Elevation of *Francisella philomiragia* subsp. *noatunensis* Mikalsen et al., (2007) to *Francisella noatunensis* comb. nov. [syn. *Francisella piscicida* Ottem et al., (2008) syn. nov.] and characterization of *Francisella noatunensis* subsp. *orientalis* subsp. nov., two important fish pathogens. *J. Appl. Microbiol.* 106: 1231–1243. doi: 10.1111/j.1365-2672.2008.04092.x

Oyston PCF, Sjostedt A and Titball RW (2004). Tularaemia: Bioterrorism defence renews interest in *Francisella tularensis*. *Nat. Rev. Microbiol.* 2: 967–978. doi: 10.1038/nrmicro1045

Rappuoli R (2000). Reverse vaccinology. *Curr. Opin. Microbiol.* 3: 445–50.

Relich RF, Humphries RM, Mattison HR, Miles JE et al. (2015). *Francisella philomiragia* Bacteremia in a Patient with Acute Respiratory Insufficiency and Acute-on-Chronic Kidney Disease. *J. Clin. Microbiol.* 53: 3947–50. doi: 10.1128/JCM.01762-15

Rohmer L, Fong C, Abmayr S, Wasnick M, et al. (2007). Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the emergence of human pathogenic strains. *Genome Biol.* 8: R102. doi: 10.1186/gb-2007-8-6-r102

Santos AR, Pereira VB, Barbosa E, Baumbach J et al. (2013). Mature Epitope Density--a strategy for target selection based on immunoinformatics and exported prokaryotic proteins. *BMC Genomics*. 14 Suppl 6: S4. doi: 10.1186/1471-2164-14-S6-S4

Sjödin A, Svensson K, Ohrman C, Ahlinder J, et al. (2012). Genome characterisation of the genus *Francisella* reveals insight into similar evolutionary paths in pathogens of mammals and fish. *BMC Genomics*. 13: 268. doi: 10.1186/1471-2164-13-268

Sjöstedt A (2007). Tularemia: History, epidemiology, pathogen physiology, and clinical manifestations. *Ann. N. Y. Acad. Sci.* 1105: 1–29. doi: 10.1196/annals.1409.009

Soto E, Abrams SB and Revan F (2012a). Effects of temperature and salt concentration on *Francisella noatunensis* subsp. *orientalis* infections in Nile tilapia *Oreochromis niloticus*. *Dis. Aquat. Organ.* 101: 217–223. doi: 10.3354/dao02533

Soto E, Hawke JP, Fernandez D and Morales JA (2009). *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica. *J. Fish Dis.* 32: 713–722. doi: 10.1111/j.1365-2761.2009.01070.x

Soto E, Illanes O, Hilchie D, Morales JA et al. (2012b). Molecular and immunohistochemical diagnosis of *Francisella noatunensis* subsp. *orientalis* from formalin-fixed, paraffin-embedded tissues. *J. Vet. Diagnostic Investig.* 24: 840–845. doi: 10.1177/1040638712452108

Soto EE, Wiles JJ, Elzer PP, Macaluso KK, et al. (2011). Attenuated *Francisella asiatica* iglC mutant induces protective immunity to francisellosis in tilapia. *Vaccine*. 29: 593–598. doi: 10.1016/j.vaccine.2010.06.040

Sridhar S, Sharma A, Kongshaug H, Nilsen F et al. (2012). Whole genome sequencing of the fish pathogen *Francisella noatunensis* subsp. *orientalis* Toba04 gives novel insights into *Francisella* evolution and pathogenecity. *BMC Genomics*. 13: 1. doi: 10.1186/1471-2164-13-598

Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, et al. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* 44: 6614–24. doi: 10.1093/nar/gkw569

Vestvik N, Rønneseth A, Kalgraff CAK, Winther-Larsen HC, et al. (2013). *Francisella noatunensis* subsp. *noatunensis* replicates within Atlantic cod (*Gadus morhua* L.) leucocytes and inhibits respiratory burst activity. *Fish Shellfish Immunol.* 35: 725–33. doi: 10.1016/j.fsi.2013.06.002