The fish pathogen *Francisella orientalis*: characterisation and vaccine development

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By

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Declaration

This thesis has been composed in its entirety by me, the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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To my ever supportive parents

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Abstract

Piscine francisellosis in an infectious emerging bacterial disease that affects several marine and fresh water fish species worldwide, including farmed salmon, wild and farmed cod, farmed tilapia and several ornamental species, for which no commercial treatment or vaccine exists. During 2011 and the first semester of 2012, chronic episodes of moderate to high levels of mortality with nonspecific clinical signs, and widespread multifocal white nodules as the most consistent gross pathological lesion were experienced by farmed tilapia fingerlings at two different locations in Northern Europe. In this study such outbreaks of granulomatous disease were diagnosed as francisellosis with a genus-specific PCR, and 10 new isolates of the bacterium including the one named STIR-GUS-F2f7, were recovered on a new selective "cysteine blood-tilapia" agar and cysteine heart agar with bovine haemoglobin. Ultrastructural observations of the pathogen in Nile tilapia (*O. niloticus*) tissues suggested the secretion of outer membrane vesicles (OMVs) by the bacterial cells during infection in these fish. This represented the first documented report of isolation of pathogenic *Francisella* strains from tilapia in Europe. The phenotypic characterisation indicated that isolates recovered were able to metabolise dextrin, N-acetyl-D glucosamine, D-fructose, α-D-glucose, D-mannose, methyl pyruvate, acetic acid, α-keto butyric acid, L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-glutamic acid, L-proline, L-serine, L-threonine, inosine, uridine, glycerol, D L-α-glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate. The predominant structural fatty acids of the isolates were 24:1 (20.3%), 18:1n-9 (16.9%), 24:0 (13.1%) 14:0 (10.9%), 22:0 (7.8%), 16:0 (7.6%) and 18:0 (5.5%). Anti-microbial resistance analyses indicated that STIR-GUS-F2f7 was susceptible to neomycin, novobiocin, amikacin, ciprofloxacin, imipenem, gatifloxacin, meropenem,

tobramycin, nitrofurantoin, and levofloxacin using the quantitative broth micro-dilution method, while the qualitative disc diffusion method indicated susceptibility to enrofloxacin, kanamycin, gentamicin, tetracycline, oxytetracycline, florfenicol, oxolinic acid and streptomycin. The use of the following housekeeping genes: mdh, dnaA, mutS, 16SrRNA-ITS-23SrRNA, prfB putA rpoA, rpoB and tpiA indicated 100% similarity with other isolates belonging to the subspecies *F. noatunensis orientalis* (*Fno*). Koch's postulates were successfully fulfilled by establishing an intraperitoneal injection (IP) challenge model with STIR-GUS-F2f7 in Nile tilapia. Moreover, the challenge model was used to investigate the susceptibility of 3 genetic groups of tilapia to STIR-GUS-F2f7. The lowest amount of bacteria required to cause mortality was 12 CFU/ml and this was seen as early as only 24 hours post infection in the red Nile tilapia and in the wild type after 26 days, no mortalities were seen in the species *O. mossambicus* with this dose. The mortality in red *O. niloticus* was significantly higher than that of the other two tilapia groups when 12 and 120 CFU/fish were injected. It was also observed that when a dose of 1200 CFU/ml was used, the mortality in *O. niloticus* wild type was significantly lower than that of the other two tilapia groups and no differences were seen among the 3 groups when the highest dose (1.2×10^5 CFU/fish) was used. The median lethal dose (LD_{50}) of *O. niloticus* wild type was the most stable during the experiment (values around 10^4 CFU/ml) and the highest of the three groups after day 25 post infection. At the end of the experiment (day 45) the LD_{50} was 30 CFU/ml in the red Nile tilapia, 2.3×10^4 CFU/ml for the wild type and 3.3×10^2 CFU/ml for *O. mossambicus*. This pattern, where the LD_{50} of the red tilapia was lower than that of the other two groups, was observed during the whole experiment. The outcomes of these experiments suggested that the red Nile tilapia family appeared to be the most susceptible while the wild type Nile tilapia family the most

resistant. The complete genome of STIR-GUS-F2f7 was sequenced using next generation sequencing (NGS) Illumina® Hi-Seq platform™, and the annotation of the assembled genome predicted 1970 protein coding sequences and 63 non-coding rRNA sequences distributed in 328 sub-systems. The taxonomy of the species *Francisella noatunensis* was revised using genomic-derived parameters form STIR-GUS-F2f7 and other strains in combination with a polyphasic approach that included ecologic, chemotaxonomic and phenotypic analyses. The results indicated that STIR-GUS-F2f7 and all the other strains from warm water fish represent a new bacterial species for which the name *Francisella orientalis* was assigned. Moreover the description of *F. noatunensis* was emended and the creation of a new subspecies within this *taxon* i.e. *Francisella noatunensis* subsp. *chilense* was proposed. The results of this study led to the development of a highly efficacious vaccine to protect tilapia against francisellosis.

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List of Abbreviations

%	Percentage
~	Approximately
µl	Microliters
µm	Micrometres (microns)
µS	Micro Siemens
µM	Micromolar (pmol/ml)
ARF	Aquatic Research Facility
ATCC	American Type Culture Collection
BLASTN	Nucleotide-nucleotide basic local alignment search tool
BSA	Bovine serum albumin
C	Carbon
comb. nov.	*combinatio nova* “new combination”
CFU	Colony forming units
CHA	Cysteine heart agar
CHAH	Cysteine heart agar with 2% bovine haemoglobin
CHTB	Cysteine heart agar with 5% tilapia blood
CLSI	Clinical and Laboratory Standards Institute
Cm	Centimetre
DNA	Deoxyribonucleic acid
DDH	DNA-DNA hybridisation technique
DDBJ	DNA DataBank of Japan
dNTP	Deoxynucleotide triphosphate
dpi	Days pots infection
dpv	Days post vaccination

DSMZ	German Culture Collection of Microorganisms and Cell Cultures
DVM	Doctor in Veterinary Medicine
e.g.	*exempli gratia* “for example”
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
et al.	*et alii* “and others”
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FLB	*Francisella*-like bacteria
Fe	*Francisella endociliophora*
Fg	*Francisella guangzhouensis*
Fh	*Francisella hispaniensis*
Fh_{al}	*Francisella halioticida*
Fn	*Francisella noatunensis*
Fnn	*Francisella noatunensis noatunensis*
Fno	*Francisella noatunensis orientalis*
Fo	*Francisella orientalis*
Fp	*Francisella philomiragia*
Ft	*Francisella tularensis*
Fth	*Francisella tularensis holarctica*
Ftm	*Francisella tularensis mediasitica*
Ftn	*Francisella tularensis novicida*
Ftt	*Francisella tularensis tularensis*
GC	Gas chromatography
GGD	Genome to genome distance

g	Gravities
gDNA	Genomic DNA
GE	Genome equivalent
h	Hours
hpi	Hours post infection
H&E	Haematoxylin and eosin
HLB	Hydrophilic-lipophilic balance
HRP	Horseradish peroxidase
i.e.	*id est* “that is”
IoA	Institute of Aquaculture
IMM	Immersion
IP	Intraperitoneal
ITS	16SrRNA—23SrRNA intergenic spacer gene
KCl	Potassium chloride
Kbp	Kilo base pairs
LSWB	Low salt wash buffer
l	Litres
LD_{50}	Median lethal dose or lethal dose 50%
m	Metres
MAS	Motile aeromonas septicemia
MEGA	Molecular Evolutionary Genetic Analyses software
MAb	Monoclonal antibody
$MgCl_2$	Magnesium chloride
MIC	Minimal inhibitory concentration
min	Minutes
ml	Millilitres

mm	Millimetres
mM	Millimolar
Mm	Molar mass
MMHB	Modified Mueller-Hinton II cation adjusted broth
MMLA	Modified Martin Lewis agar
MTMA	Modified Thayer-Martin agar
MOPS	3-[N-morpholino] propanesulfonic acid
NCBI	National Centre for Biotechnology Information
ng	Nanograms
nm	Nanometres
NVI	Norwegian Veterinary Institute
O/F test	Oxidation/fermentation of glucose test
^{o}C	Degrees Celsius
OD	Optical density
OD_{600}	Optical density at 600 nanometres
OMVs	Outer membrane vesicles
pmol	Picomole
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Power or potential of hydrogen
rpm	Revolutions per minute
RPS	Relative percent survival
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Svedberg subunit
s	Seconds

SEM	Scanning electron microscopy
sp.	Species
spp.	Species plural
subsp.	Subspecies
ssp.	Subspecies plural
sp. nov.	*species nova* “new species”
TA	Tropical aquarium
TE Buffer	Tris-EDTA buffer
TEM	Transmission electron microscopy
TPQ	Tricaine Pharmaq (tricaine methanesulfonate or MS-222)
Tris	Trisaminol or trisamine
Tris-HCl	Tris-hydrochloride
TS	Type strain
TSA	Trypticase soy agar
TSB	Trypticase soy broth
UK	United Kingdom
UoS	University of Stirling
USA	United States of America
v/v	Volume/volume
VBNC	Viable but not culturable

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List of Presentations in Conferences

Ramírez-Paredes JG, E Soto, C Öhrman, K Jolley, M Maiden, HJ Busse, RH Richards, DJ Colquhoun, P Larsson and A Adams. **The use of "taxo-genomics" to clarify the nomenclature and taxonomical status of fish pathogenic *Francisella* spp**. 17th International Conference on Diseases of Fish and Shellfish/ European Association of Fish Pathologists. Canary Islands Convention Centre. Las Palmas de Gran Canaria, Spain. 7-11th of September 2015. (Accepted). **2015 EAFP Student Award granted for attendance**.

Ramírez-Paredes JG, Mendoza-Roldan M, Metselaar M, Soto E, Larsson P, Colquhoun DJ, Adams A and Richards RH. **Studies on Francisellosis in red Nile tilapia *(Oreochromis niloticus)*** The 4th PhD Research Conference 2015, Institute of Aquaculture, University of Stirling. 18th February 2015.

Ramírez-Paredes JG, Thompson KD, Mendoza-Roldan M, Soto E, Lopez-Jimena B, Metselaar M, Penman DJ, Richards RH, Colquhoun DJ and Adams A. **Francisellosis in tilapia**. The 9th Symposium on Diseases in Asian Aquaculture (DAA9). Rex Hotel 24th-28th of November 2014, Ho Chi Minh City, Vietnam.

Mendoza-Roldan M, **Ramírez-Paredes JG**, Metselaar M, , Thompson KD and Adams A. **Efficacy of an autologous vaccine against Piscine *Francisellosis* in red Nile Tilapia (*Oreochromis niloticus*).** European Association of Fish Pathologists UK and Ireland Branches Inaugural meeting. Keele University, 15-16th September 2014. Keele United Kingdom.

Ramírez-Paredes JG Metselaar M, Soto E, Penman DJ, Doudu S, Southgate P, Thompson KD, Öhrman C, Larsson P, Colquhoun DJ, Adams A and Richards RH. **Isolation and characterisation of *Francisella noatunensis orientalis* in Europe and its elevation to the rank of species as *F. orientalis* comb. nov., sp. nov.** Aquaculture UK Conference 2014. Macdonald Highland Resort, 28th and 29th of May 2014. Aviemore, United Kingdom.

Ramírez-Paredes JG, Metselaar M, Soto E, Penman DJ, Doudu S, Southgate P, Thompson KD, Orhman C, Larsson P, Colquhoun DJ, Adams A and Richards RH. **A polyphasic approach for the characterisation of *Francisella noatunensis*: a baseline for a proposal of minimal standards within the genus *Francisella?*** Bergey's International Society for Microbial Systematics (BISMiS) Conference 2014, "Defining Microbial Diversity in the Genomic Era". Apex International Hotel, April 7th-10th, 2014. Edinburgh, United Kingdom.

Ramírez-Paredes JG, Soto E, Metselaar M, Thompson KD, Penman DJ, Adams A, Richards RH. **First Isolation and characterisation of *Francisella noatunensis orientalis* from farmed Nile tilapia (*Oreochromis niloticus*) in Europe**. Lunch Time Seminar, Institute of Aquaculture, University of Stirling, 27th of March 2014. Stirling, United Kingdom.

Ramírez-Paredes JG, Soto E, Metselaar M, Thompson KD, Penman DJ, Adams A and Richards RH. **Piscine Francisellosis around the British Isles**, UK Fish Veterinary Society Spring Meeting 2014. Norton House Hotel, 25th and 26th of March 2014. Edinburgh, United Kingdom. Novartis small grant awarded for attendance.

Ramírez-Paredes JG, Soto E, Metselaar M, Southgate P, Penman DJ, Thompson KD, Adams A, Richards RH, Duodu S, Colquhoun DJ Öhrman C, Myrtennäs K, Larsson P. **Whole genome sequencing and comparison of several strains of the aquatic pathogenic bacteria *Francisella noatunensis***. Basics Bioinformatics for Marine Microbial Genomics (Workshop). May 27-31, 2013. Max Planck Institute for Marine Microbiology, Bremen, Germany. FP7 Coordination and Support Action "Marine Genomics for Users" (MG4U) grant awarded for attendance.

Ramírez-Paredes JG, Soto E, Thompson KD, Penman DJ, Adams A and Richards RH. **Isolation and characterisation of *Francisella noatunensis* affecting red tilapia hybrids in Thailand**. Microbial community management in aquaculture (PhD course & Training school). Ghent University, Belgium August 20-22 2012. Ghent University scholarship (European Community's 7th Framework Programme) awarded for attendance.

Ramírez-Paredes JG, Turner WA, Kledmanee K, Thompson KD, Penman DJ, Adams A, Soto E, Richards RH. **Molecular diagnosis, isolation and characterisation of *Francisella noatunensis* affecting tilapia in Thailand**. (Poster) The 3th PhD Research Conference 2012, Institute of Aquaculture, University of Stirling. 24th October 2012.

Ramírez-Paredes JG, Thompson KD, Penman DJ, Adams A and Richards RH. **Francisellosis an emerging disease in tilapia aquaculture**. 8th Postgraduate Research Conference 2012, The Stirling Graduate School, University of Stirling. 9th May 2012.

GRANTS AWARDED FOR THIS PROJECT

The Santander Doctoral Travel Awarded £2000.00 to visit the Interdisciplinary Centre for Aquaculture Research and the Laboratory of Biotechnology and Aquatic Genomics in “Universidad de Concepcion” Chile. Project title: **Spleen and head kidney transcriptomics of red Nile tilapia (*Oreochromis niloticus*) in response to vaccination and infection with *Francisella orientalis*.**

The Marine Alliance for Science and Technology for Scotland (MASTS) small grant scheme Awarded £500.00 to visit the Swedish Defence Research Agency. Project title: **Comparative genome analysis of the marine pathogenic bacteria *Francisella noatunensis orientalis*.**

The EAFP Small grant Scheme (Co-Author/Co-supervisor). Awarded €1000.00 for the internship project: **Implementation of a real time quantitative PCR assay to quantify bacterial loads in spleen of vaccinated and not vaccinated tilapia challenged with *Francisella orientalis***.

Chapter One

General introduction and aims of this study.

1.1 Introduction

1.1.1 World aquaculture and farmed fish production

Fish for food production (fisheries and aquaculture) has kept growing steadily worldwide during the last 50 years at an average rate of 3.2% overtaking world population growth by 1.6%. Thanks to this, the global fish consumption *per capita* has increased worldwide from 9.9kg in the 1960s to 19.2kg in 2012 (FAO, 2014).

The aquaculture sector itself continues to grow at an incredible pace and now provides almost half of all fish for human consumption, in fact the worldwide "food fish" production from aquaculture, where the term refers to finfishes, crustaceans, molluscs, amphibians, freshwater turtles and other aquatic animals (such as sea cucumbers, sea urchins, sea squirts and edible jellyfish) produced for the intended use as food for humans, has expanded from 32.4 million tonnes in 2000 to 66.6 million tonnes in 2012 (average annual rate of 6.2%) making of aquaculture one of the fastest-growing food-producing sectors in the world and one of the most important sources of high quality protein for human consumption (FAO, 2014; Tidwell and Allan, 2001).

According to FAO (2014) it is estimated that a portion of 150g of fish can provide about 50–60 percent of an adult's daily protein requirements and that in 2010, fish accounted for 16.7% of the global population's intake of animal protein and 6.5% of all protein consumed. Within the group of finfishes the farming of tilapias is the most widespread type of aquaculture in the world with official statistics from 135 countries and territories on all continents, however it is estimated that the true number of producer countries is

higher because commercially farmed tilapias are yet to be reflected separately in national statistics in Canada and some European countries (FAO, 2014)

1.1.2 Tilapia aquaculture

The term tilapia refers to a group of cichlids from Africa that comprises more than 100 species from 3 genera: *Oreochromis*, *Tilapia* and *Sarotherodon* (Fitzsimmons and Watanabe, 2010). In aquaculture, tilapia is the second largest production in volume after carps and is also the most extensively farmed group of fish in the world (Norman-López and Bjørndal, 2009).

Thanks to their great adaptability to different farming systems tilapia is becoming one of the most successful sectors in aquaculture, with figures from the last decade indicating that tilapia production has increased from 2.6 million tons in 2005 to 4.5 million tonnes in 2012 (FAO, 2014) and according to Fitzsimmons *et al.* (2014) it is expected that it will reach the 4.8 or 5 million tonnes between 2014 and 2015.

Nile tilapia (*Oreochromis niloticus*) is recognised as the most commercially important tilapia species, as its production accounts for ~80% of the total, tilapia production worldwide. Other important tilapia species in aquaculture are: Mozambique tilapia (*O. mossambicus*), blue tilapia (*O. aureus*) and a variety of hybrids produced by the crossing of these species (FAO, 2010).

Particular significance have the red tilapia hybrids, produced and cultured in several countries in Asia and Latin America to extend the production into brackish and sea water

and to satisfy the demand of consumers that relate the red tilapia with marine and reef fishes of similar hue (Castillo-Campo, 2013; Ramírez-Paredes *et al.*, 2012a).

In Europe, Nile tilapia production can be found in, France, Germany, the Netherlands Great Britain, which has the biggest market for this industry, Sweden, Latvia, Norway and Poland. To cope with the climate of these regions and to satisfy the high demand of consumers, farmers are using recirculating systems with hyper intensive culture conditions (high stocking densities, poor water quality and excessive handling).

Unfortunately the use of these intensive culture systems without adequate biosecurity has brought several drawbacks to this industry and one of the main problems is the occurrence of infectious diseases, particularly those caused by bacterial pathogens, which usually led to massive financial losses (Bebak, 1998).

Disease-related financial losses in aquaculture are divided in market and direct losses. The market losses are associated with a reduction in the quality of survivors (i.e. resulting from reduced growth rates or reduced product quality) and poor market assessments due to a decrease in the facility's reputation. This last one is takes and added importance in cases or regions where the farming of a specific species is incipient like the case of tilapia in Europe. The direct losses relate to mortalities (sometimes the loos of the whole productive cycle), facility closure, restriction of fish movement and inability to replace stock especially in cases where intracellular pathogens with vertical transmissions are presented (Bebak, 1998).

In tilapia aquaculture, bacterial diseases are considered to be a major cause of losses to the industry and they occur when rearing or farming conditions are poor as it frequently happens in highly or hyper intensive farming (Plumb and Larry, 2010).

1.1.3 Bacterial diseases of tilapia

Tilapia are in general considered to be resistant to infectious diseases, especially when they are farmed under extensive conditions in their natural ecosystem; however as the production intensifies their susceptibility to pathogenic microorganisms increases (Fitzsimmons and Watanabe, 2010; Plumb and Larry, 2010).

Several bacterial diseases can affect tilapia including streptococcosis, caused by S*treptococcus agalactiae* and *S. iniae*; francisellosis caused by the facultative intracellular bacterium *Francisella noatunensis orientalis*; edwardsiellosis by *Edwardsiella tarda* and *E. ictaluri;* vibriosis resulting from different *Vibrio* spp.; columnaris disease caused by *Flavobacterium columnare* and motile aeromonas septicemia (MAS) associated with several opportunistic *Aeromonas* spp. including *A. hydrophila* (Austin, 2011; Austin, 2012; Plumb and Larry, 2010).

Of these diseases, streptococcosis and francisellosis have been reported as the most economically important, with both having a worldwide distribution and affecting fish at a range of sizes, in fresh, brackish and sea water. However, while streptococcosis has been studied in tilapia since the 1970s and there are commercial treatments for its prevention, francisellosis is still considered an emergent disease in several countries and no treatments are available for its prevention or cure.

Francisellosis does not only affect the tilapia industry worldwide, but also affects other important aquaculture sectors such as ornamental fish (Camus *et al.,* 2013; Hsieh *et al*., 2007; Lewis *et al.*, 2014; Soto *et al.*, 2014a), the salmon industry in Chile (Birkbeck *et al.*, 2007; Birkbeck, 2011; Bohle *et al.*, 2009; Ibieta e*t al*., 2011), cod farming in Norway (Birkbeck *et al.*, 2011; Colquhoun and Doudu, 2011; Nylund *et al*., 2006; Ottem *et al*., 2008) and abalone production in Japan (Brevik *et al.*, 2011; Kamaishi *et al.*, 2010).

1.1.4 Francisellosis in tilapia and other aquatic organisms

1.1.4.1 Aetiological agents (host specificity and reservoirs)

The *Francisella* species that have been identified as causative agents of natural or experimental disease in aquatic organisms include the following species:

***Francisella noatunensis noatunensis* (*Fnn*),** in Europe it has been found in wild and farmed Atlantic cod (*Gadus morhua*) in Sweden, Denmark, Norway and Ireland (Birkbeck *et al.*, 2011; Colquhoun and Doudu, 2011) and farmed Atlantic salmon (*Salmo salar*) in Norway (Ottem *et al.*, 2008). Experimental infections have been successfully reproduced in cod (Ellingsen *et al*., 2011; Gjessing *et al*., 2011; Inami, 2011) and zebrafish (*Danio rerio*) (Brudal *et al.*, 2014a). Additionally it has been retrospectively detected by quantitative real time PCR (qPCR) in wild cod sampled from the coasts of the UK and in Norway in wild fish: saithe (*Pollachius virens*), pollock (*Pollachius pollachius*), poor cod (*Trisopterus minutes*), mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), megrim (*Lepidorhombus whiffiagonis*), angler-fish (*Lophius piscatorius*) and flounder (*Platichthys flesus*), as well as in invertebrates such as blue mussel (*Mytilus edulis*) and edible crab (*Cancer pagurus*) (Ottem *et al.*, 2008;

Wangen, 2009; Wangen *et al*., 2012). In Chile natural outbreaks in farmed Atlantic salmon and and common galaxias or jollytail (*Galaxias maculatus*) (Birkbeck *et al*., 2007; Birkbeck *et al*., 2011; Bohle *et al*., 2009; Cvitanich *et al*., 1995; Enríquez *et al*., 1998; Pharmaq Norway, 2006).

Successful experimental infections in Chile with *Fnn* have been reported in Atlantic salmon and to a less extent in coho salmon (*Oncorhynchus kisutch)* (Brossard-Andrade, 1999; Cárdenas-Villaroel, 1998; Gacitúa-Sandoval, 1999; Hausdorf-Volke, 2009; Schulze-Barrientos, 2010; Thibaut-Páez, 2009; Urra-Sáez, 2010) and according to Cárdenas-Villaroel (1998) experimental infections in rainbow trout (*Onchorhyncus mykiss*) were not successful.

***Francisella noatunensis orientalis* (*Fno*)** is associated with natural outbreaks in farmed tilapia (*Oreochromis* spp., *Sarotherodon melanotheron, Tilapia* spp. and hybrids of these species) (Colquhoun and Doudu, 2011); Three line grunt or Isaki fish (*Parapristipoma trilineatum*) (Kamaishi *et al*., 2005); Hybrid striped bass (*Morone chrysops* x *M. saxatilis*) (Ostland *et al*., 2006); Indo-Pacific reef fish species (fairy wrasses including orangeback, (*Cirrhilabrus aurantidorsalis*), solor (*C. solorensis*), exquisite (*C. exquisitus*), Lubbock's (*C. lubbocki*) and blue-green damselfish (*Chromis viridis*) (Camus *et al*., 2013); Ornamental marine grunts (French grunt *Haemulon flavolineatum* and Caesar grunt *Haemulon carbonarium*) (Soto *et al*., 2014a); Ornamental Malawi cichlids: *Nimbochromis venustus, N. linni*, *Aulonocara stuartgranti* "maleri", *Placidochromis* sp. "blue hongi", *Protomelas* sp., *Naevochromis chryosogaste*r, *Copadichromis mloto* and *Otopharynx tetrastigama* (Lewish *et al*., 2014).

It has been possible to experimentally infect Isaki fish, tilapia and zebra fish with *Fno* (Brudal *et al.*, 2014; Kamaishi *et al.*, 2005; Soto *et al.*, 2009a; Vojtech *et al.*, 2009; Vojtech *et al.*, 2012).

Additionally, *Fno* has been detected by conventional polymerase chain reaction (PCR) and real time quantitate PCR (RT q-PCR) in wild guapote tigre (*Parachromis managuensis*); ornamental cichlids: firebird (*Aulonocara rubescens*), elegans (*Pseudotropheus elegans*), zebra cichlid (*Pseudotropheus zebra*), Rhodes's chilo (*Chilotilapia rhoadesii*), Malawi eyebiter (*Dimidiochromis compressiceps*), brown discus (*Symphysodon aequifasciatus*), deep-water hap (*Haplochromis electra*), electric blue hap (*Sciaenochromis fryeri*), blue-white labido (*Labidochromis caeruleus*), *Placidochromis milomo*, Frontosa cichlid (*Cyphotilapia frontosa*), barracuda (*Sphyraena barracuda*), farmed Hawaiian Chinese catfish (*Clarias fuscus*) and barramundi (*Lates calcarifer*) farmed in Hawaii (Colquhoun and Duodu, 2011; Camus *et al.*, 2013; Hsieh, *et al.*, 2007; Kamaishi *et al.* 2005; Klinger-Bowen *et al*., 2015; Lewisch *et al.*, 2014; Ostland *et al.*, 2006; Soto *et al.*, 2012a; Soto *et al.*, 2014a).

***Francisella halioticida* (*Fh*)** is associated with natural outbreaks in Japanese farmed giant abalone *Haliotis gigantea* and experimental infections in Japanese black abalone *Haliotis discus discus* and *H. gigantea* (Brevik *et al.*, 2011; Kamaishi *et al.*, 2010).

***Francisella tularensis novicida* (*Ftn*):** experimental infections were successfully established with the strain U112 in zebrafish (Brudal *et al.*, 2014a). Unsuccessful experimental infections via intraperitoneal injection were reported in rainbow trout, northern pike (*Esox Lucius*), black bullhead (*Ameirus melas*), black crappie (*Pomoxis*

nigromaculatus), largemouth bass (*Huro salmoides*) and yellow perch (*Perca flavescens*) with *Francisella tularensis tularensis* (*Ftt*) more than 70 years ago where no mortalities or clinical signs of francisellosis were observed in the fish (Morgan, 1947).

1.1.4.2 History and taxonomy

The first report of disease outbreaks in fish involving *Francisella* sp. was from Japan in 2005 (Kamaishi *et al.*, 2005). Previous to this study, there were several cases of granulomatous infections where no isolation or molecular methods were used to confirm the identity of the causative agents.

Most of these outbreaks occurred between 1981 and 2005 affecting the following fish species: wild Atlantic cod captured around the English costs of the UK during 1981-1983 (Van Banning, 1987); wild dragonets (*Callionymus lyra*) from Welsh coasts of the UK in 1986 (Davies, 1986); farmed tilapia in Taiwan, Hawaii, Jamaica, Indonesia and continental USA (southern California, Florida and South Carolina) during the 1990s (Chern and Chao, 1993; Chen *et al.*, 1994; Mauel *et al.*, 2003; Mauel and Miller, 2002); ornamental blue-eyed plecostomus (*Panaque suttoni*) from Colombia in 1995 (Khoo *et al.*, 1995); imported three-line grunt from China into Japan from 1999-2001(Fukuda *et al.*, 2002); hatchery-reared juvenile white seabass (*Atractoscion nobilis*) in southern California USA (Chen *et al*., 2000); farmed grouper (*Epinephelus melanostigma*) in Taiwan in 2000 (Chen *et al.*, 2000); snakehead fish (*Ophiocephalus argus*) in China between 2001-2002 (Guo *et al.*, 2004), tilapia farmed in the USA (Florida, California and South Carolina) during 2001-2003 (Mauel *et al.*, 2005) and farmed Atlantic salmon and common galaxias or jollytail fish in Chile (Birkbeck *et al*., 2007; Birkbeck *et al*.,

2011; Bohle *et al.*, 2009; Cvitanich *et al*., 1995; Enríquez *et al*., 1998; Pharmaq Norway, 2006).

In most of these reports the presence of intracellular bacteria *Piscirickettsia salmonis* and/or *Mycobacterium* spp. was discarded by molecular methods and the authors assigned the terms rickettsia like organisms (RLOs), *Piscirickettsia*-like organisms (PRLOs) to the possible causative agents and mycobacteriosis-like or piscirickettsiosis-like disease to the pathogeny.

The first report confirming *Francisella* sp. as the causative agent of mortalities in farmed fish was presented in 2005 (Kamaishi *et al.*, 2005). After that, researchers started to use conventional and quantitative PCR, and selective synthetic media to investigate cases of granulomatous disease in aquatic organisms and a number cases in tilapia (Soto *et al.*, 2009a), Atlantic salmon (Birkbeck *et al.*, 2007), Atlantic cod (Olsen *et al.*, 2006), ornamental fish (Camus *et al.*, 2013; Lewisch *et al.*, 2014; Soto *et al.* 2014a) and hybrid striped bass (Ostland *et al.*, 2006) were reported. Additionally with the use of these techniques it was also possible to retrospectively confirm some of the cases previously reported as mycobacteriosis-like or piscirickettsiosis-like disease as piscine francisellosis. (Hsieh *et al.*, 2007; Mauel *et al.*, 2007; Mauel *et al.* 2003; Soto *et al.*, 2011a; Zerihun *et al.*, 2011)

It was not until 2007 that the first attempt to taxonomically classify the *Francisella* spp. recovered from fish was published (Ottem *et al.*, 2007b). In that study the comparative characterisation of one strain recovered from farmed Norwegian Atlantic cod and the

type strain of *Francisella philomiragia* indicated that the first should constitute a new species, for which the authors gave the name *Francisella piscicida*.

Later the same year, Mikalsen *et al.* (2007) compared seven cod isolates against four strains of *F. philomiragia* and classified the cod isolates as a subspecies of the latter and named it as *F. philomiragia* subsp. *noatunensis*.

The name *F. piscicida* was published as valid in January 2008 (Euzeby, 2008). In that report, it was suggested that the bacterium could constitute a heterotypic synonym of *F. philomiragia* subsp. *noatunensis* thus according to the rule of priority, if the later had to later be elevated to the rank of species the epithet "*noatunensis*" should remain over "*piscicida*". Some months later in 2008, the type strains of *F. piscicida* and *F. philomiragia noatunensis* were compared against five isolates of *F. philomiragia* including the type strain. Additionally, one strain from diseased fish farmed in warm water environment "Ehime-1" and DNA from one isolate recovered from Indonesian tilapia were included (Ottem *et al.*, 2009).

As a result of these comparisons *F. piscicida* was proved to be a heterotypic synonym of *F. philomiragia noatunensis* and both were elevated to the rank of species as *F. noatunensis,* while the single isolate Ehime-1 was described as the type strain of the subspecies *F. noatunensis orientalis* on the basis of a few phenotypic traits (Ottem *et al.* 2009). These results appeared to elucidate the new name for the various isolates, but were not considered as valid until finally were published in the "list of new names and new combinations previously effectively, but not validly, published" in 2009 (Euzeby, 2009a; Euzeby, 2009b).

In September 2009, an “ahead of print electronic publication” of the International Journal of Systematic and Evolutionary Microbiology (IJSEB) appeared online. In this study a Chilean strain named “PQ1106”, DNA from the Japanese strain Ehime-1 and a strain from tilapia farmed in Costa Rica were analysed. The result of this study also elevated the rank *F. philomiragia noatunensis* to the species level as *F. noatunensis* and additionally described the Costa Rican and Japanese strains as a new species for which the authors gave the name *F. asiatica* (Mikalsen and Colquhoun, 2009).

Since *F. noatunensis* had already been published as a valid name, this electronic version of this paper was later withdrawn by the IJSEB and the name *F. asiatica* was never considered as valid or even effectively published. After this no other investigations took place to clarify the taxonomic rank *F. noatunensis orientalis*.

1.1.4.3 Morphological and biochemical characteristics

All the fish pathogenic *Francisella* strains are Gram negative, non-motile highly pleomorphic coccobacilli cells, with a size ranging from 0.2 to 0.4µm in width and 0.4 to 1.9µm in length, catalase positive, oxidase negative, with requirement of cysteine to grow and agglutinate with polyclonal antiserum against *Fnn* (strains GM2212 and NVI 5330) but not with monoclonal or polyclonal antiserum against *F. tularensis*.

Micrographs in Figure 1.1 show the ultrastructure of *Fno* in tilapia tissues. The biochemical characteristics of fish pathogenic *Francisella* strains is summarised in Table 1.1. The table is adapted from the results presented by (Ottem, 2011; Birkbeck *et al.*, 2011).

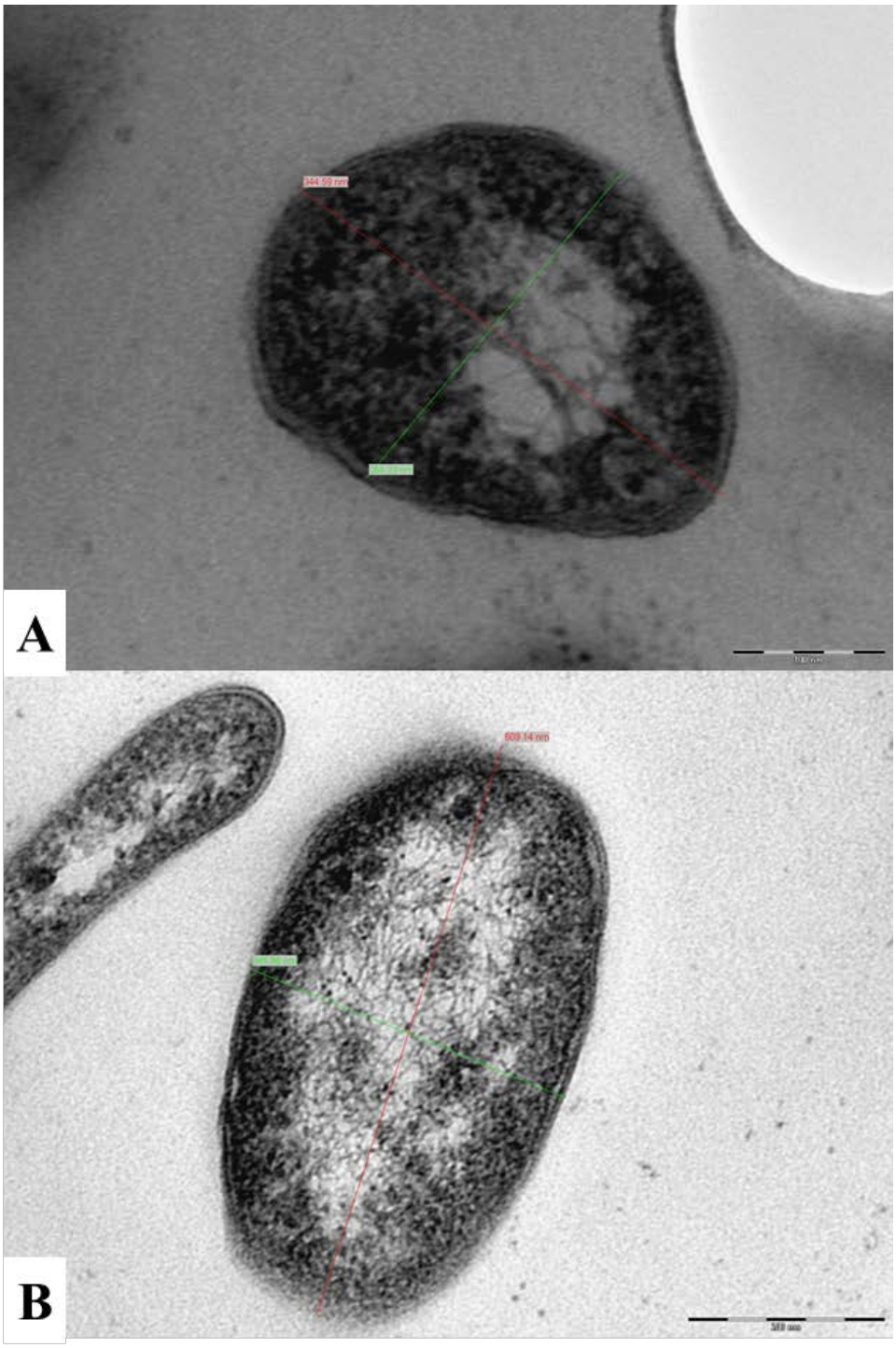

Figure 1.1 Micrograph of *F. noatunensis orientalis* in tilapia tissues. The shape and size of the cell varies according to life cycle stage and localisation in the host. **A** Bacterial cell size is 344.59nm (red line) X 264.23nm (green line). Scale bar=100nm. **B** Bacterial cell size is 609.14nm (red line) X 345.86nm (green line). Scale bar=200nm.

Table 1.1. Biochemical and physiological characteristics of fish pathogenic *Francisella* spp. and the *F. philomiragia* type strain

Characteristic	*Fno*	*Fnn* from Europe	*Fnn* from Chile	*F. philomiragia*
Optimal temperature °C	28.5	22	22	15-37
Cysteine required for growth	+	+	+	-
Motility	-	-	-	-
Oxidase	-	-	-	-
Catalse[ŧ]	+	+	+	+
Indole[ŧ]	+	+	+	+
Alkaline phosphatase	+	+	+	+
Esterase C4	+	+	+	+
Esterase lipase C8	+	+	+	+
acid phosphatase	+	+	+	+
H_2S-production	+	+	+	+
β-Lactamase	+	+	+	+
Naphtol-AS-BI phosphohydrolase	+	-	w+	+
Proline arylamidase	+	+	-	+
Phenylalanine arylamidase	+	-	-	+
Tyrosine arylamidase	-	w+	w+	+
Alanine arylamidase	w+	+	w+	+
Histidine arylamidase	-	w+	w+	+
InDoxyl phosphate (IDP)	+	-	-	+
Arginine arylamidase	+w	+w	+w	+
D-Glucose	[+]	+	+	+
Mannose	-	-	-	+

Data presented are based on combined results published by Birkbeck *et al*. (2011) and Ottem (2011); W=weakly positive;

In green: differences between *Fno* and *Fnn*; In orange the differences between *Fnn* from Chile and Fnn from continetal Europe;

The result in [ ] is shown as negative and ŧ as weekly positive in Birkbeck *et al*. (2011);

There are minor differences between the results shown in Table 1.2 and those stated in the valid description of *F. noatunensis* (Ottem *et al.*, 2009) (previously *F. philomiragia noatunensis* (Mikalsen and Colquhoun, 2009)). In both reports API Kits were used to characterise the strains, however in the description of the species it is stated that *F. noatunensis* is positive for Naphtol-AS-BI phosphohydrolase and degradation of mannose, and negative for tyrosine arylamidase, histidine arylamidase and arginine arylamidase.

According to the currently valid description (Ottem *et al.*, 2009) there are only four differences between *Fnn* and *Fno* and they are presented in Table 1.2.

Table 1.2. Phenotypic differences between *Fno* and *Fnn* according to their currently valid description.

Characteristic	*Fno*	*Fnn*
Phenylalanine arylamidase	+	-
InDoxyl phosphate	+	-
Arginine arylamidase	+	-
Mannose	-	+

The few differences between strains that have such a dissimilar geographical and ecological niche (i.e. farmed tilapia in Central America and wild cod caught in Scandinavia) and the inconsistencies between studies indicate that the API Kits are not a good option to biochemically and phenotypically analyse fish pathogenic *Francisella* strains or any other members of this genus. These results also highlight the need of reliable methodologies to characterise these bacteria and eventually establish minimal standards for the delineation of species and subspecies within this genus.

1.1.4.4 Clinical signs and gross pathology in tilapia

The clinical signs of francisellosis in tilapia can be variable and are not considered specific for the disease. The most frequently found include enlargement of the abdominal cavity due to ascites and swollen organs, pale/darkened skin colour, pale gills, skin haemorrhages (petechial on the sides), ulcers/excess of mucus in the skin, lethargic behaviour, frayed fins, exophthalmia and abnormal swimming (fish may float at the surface and/or show vertical or circular movements) (Chern and Chao, 1993; Chen *et al*., 1994; Colquhoun and Doudu, 2011; Mauel *et al*., 2003; Mauel *et al*., 2007; Mauel and Miller, 2002; Soto *et al*., 2009a; Soto *et al*., 2011a).

At necropsy, the organs in the abdominal cavity are enlarged and haemorrhagic, white nodules can be found mainly in the spleen and kidney, but also in the liver, gills, stomach, intestine and mesenteric fat (Chern and Chao, 1993; Chen *et al*., 1994; Mauel *et al*., 2003; Mauel *et al*., 2007; Mauel and Miller, 2002; Soto *et al*., 2009a; Soto *et al*., 2011a).

A transparent fluid can be extracted from moribund fish with advanced degrees of ascites and centrifugation of the fluid reveals the presence of bacteria in extracellular phase (personal observation).

Figure 1.2 and 1.3 show clinical signs and lesions of experimentally infected Mozambique tilapia via intraperitoneal injection.

Figure 1.2 Clinical signs and gross pathology of experimentally induced francisellosis in Mozambique tilapia. **A** Anaemic gills and pale skin. **B** Bilateral exophthalmia. **C** Varying degrees of ascites. **D** Extraction of transparent fluid from ascites. **E** Haemorrhagic gills and enlargement of liver and intestines.

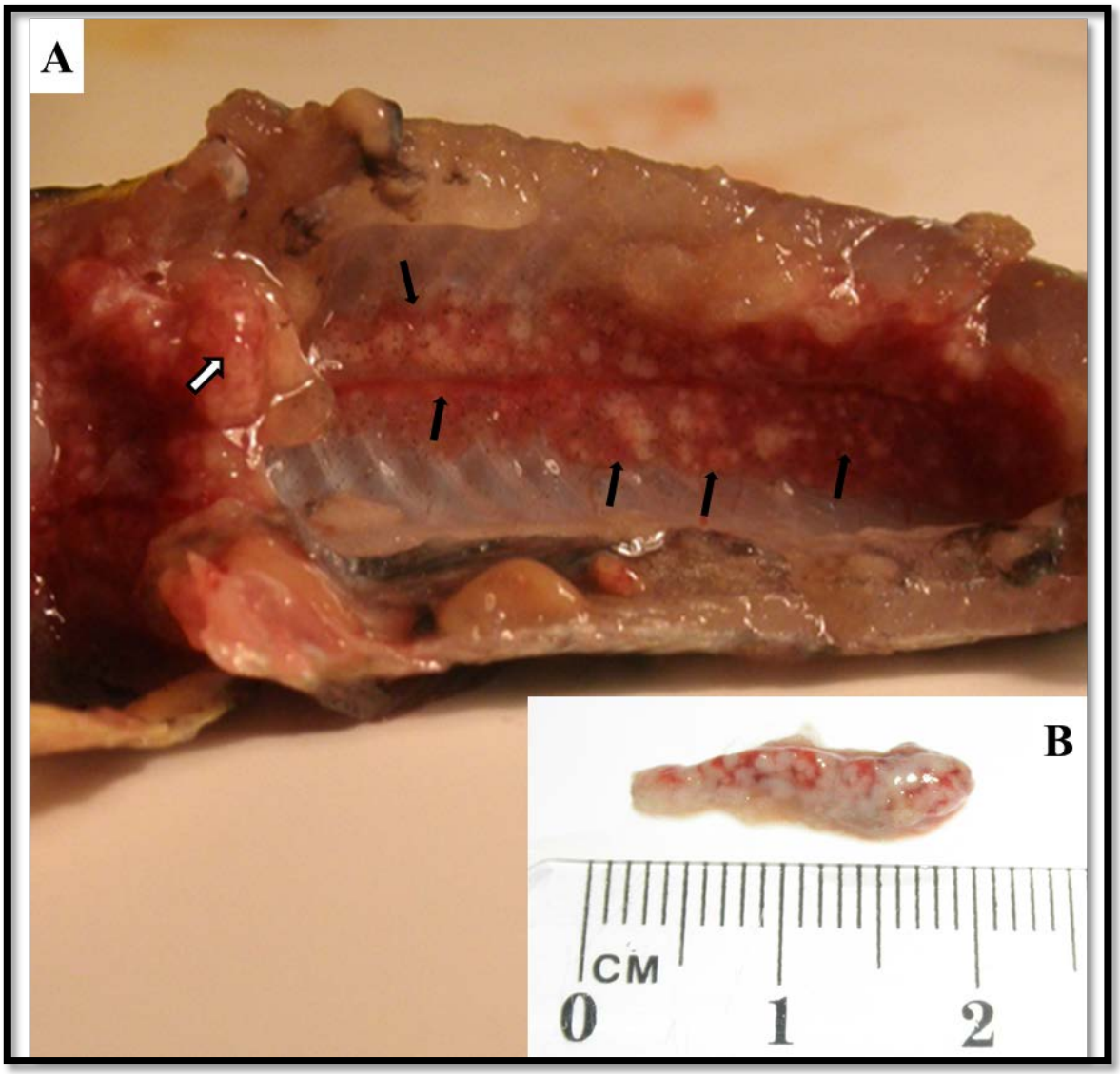

Figure 1.3 Kidney and spleen of Mozambique tilapia with experimentally induced francisellosis. **A** White arrow points to the enlarged head kidney, black arrows point multiple white nodules in posterior and middle kidney. **B** The enlarged spleen with parenchyma covered by multiple coalescent white nodules. The fish is the same individual shown in Figure 1.2.

1.1.3.5 Haematology and histopathology in tilapia

According to Chen *et al.* (1994) the haematocrit value (percentage of the packed cell-volume) in infected fish was around 66% less (10.5 ± 5.2) than that of the healthy fish (31.5 ± 2.8). In that study blood smears stained of infected fish stained with Giemsa, Gram stain and periodic acid-Schiffs showed apparent neutrophilia and 36 to 42% less protein and monocytes than the healthy fished. The authors also found basophilic granules within the cytoplasmic vacuoles of macrophages stained in the blood smears. Large amounts of Gram-negative coccobacilli with polymorphic shapes were found intracellularly in organ smears stained with Liu's stain and Gram stain.

In haematoxylin and eosin (H&E) sections, severe granulomatous formation can be systemically seen with fibrin or thrombi in the small blood vessels of organs (larger blood vessels are involved in the kidney and liver) and perivascular necrosis with disseminated intravascular coagulation. The granulomas consist of large vacuolated macrophages encircled by thin fibrous capsules and small cuffs of lymphocytes. Melanomacrophages are frequently found associated with the infiltrates. Infected macrophages often have obliterated and the nucleus displaced to the periphery, while the vacuoles contain enlarged and numerous, pleomorphic, basophilic intracytoplasmic coccobacilli cells. The centre of the granuloma can be necrotic or with small areas of necrosis associated with individual or small clusters of the pleomorphic coccobacilli cells. (Birkbeck *et al.*, 2011).

According to Hsieh *et al.* (2006), who histologically examined 120 tilapias, the most affected organs with granuloma formation were the spleen (100%), kidneys (100%), liver (85.8%), gills (72.5%), gonads (63.3%), gastrointestinal tract (56.7%), heart (47.5%),

swim bladder (40%) and eyes (30.8%). The brain and the muscle tissue can also present granulomatous lesions with necrosis and chronic inflammatory cell infiltration and this is linked with the abnormal swimming behaviour observed (Birkbeck *et al.*, 2011; Mauel *et al.*, 2005)

Some of the histopathological findings reported in specific tissues include necrosis and ulcers in the intestine and stomach (found in the muscular layer) and focal chronic inflammation in the submucosa, lamina propria or serosa; in advanced cases generalised coagulation and necrosis invading from serosa through the mucosa can also be observed. In gills some fish may show epithelial hyperplasia with consolidation or fusion of secondary lamellae or "loss" of intralamellar spaces. Granulomatous proliferative response can also be presented at the base of the gill arch. In the heart, epicarditis, endocarditis and myocarditis can be observed with fragmentation of myocardial fibres, and granulomas can also be detected. In kidney, necrosis and degeneration of renal tubules can be seen indicating the formation of hyaline droplets. Invasion of inflammatory cells in anterior and posterior kidney is commonly observed replacing the renal tissue. The nonreplaced renal epithelium generally shows degeneration. The spleen generally shows multi-granulomatous formation, and replacement of normal lymphoid tissues by diffuse chronic inflammatory cells. The replacement can be found in almost the entire normal splenic architecture depending on the severity of the infection. In liver degeneration of hepatocytes within macrophages containing bacteria in cytoplasmic vacuoles and hepatic lipidosis are frequently noted (Chen *et al.*, 1994; Chern and Chao 1993; Mauel *et al.*, 2005; Soto *et al.*, 2009a). Typical histopathological findings in kidney, spleen and heart of tilapia are shown in Figures 1.4 to 1.6.

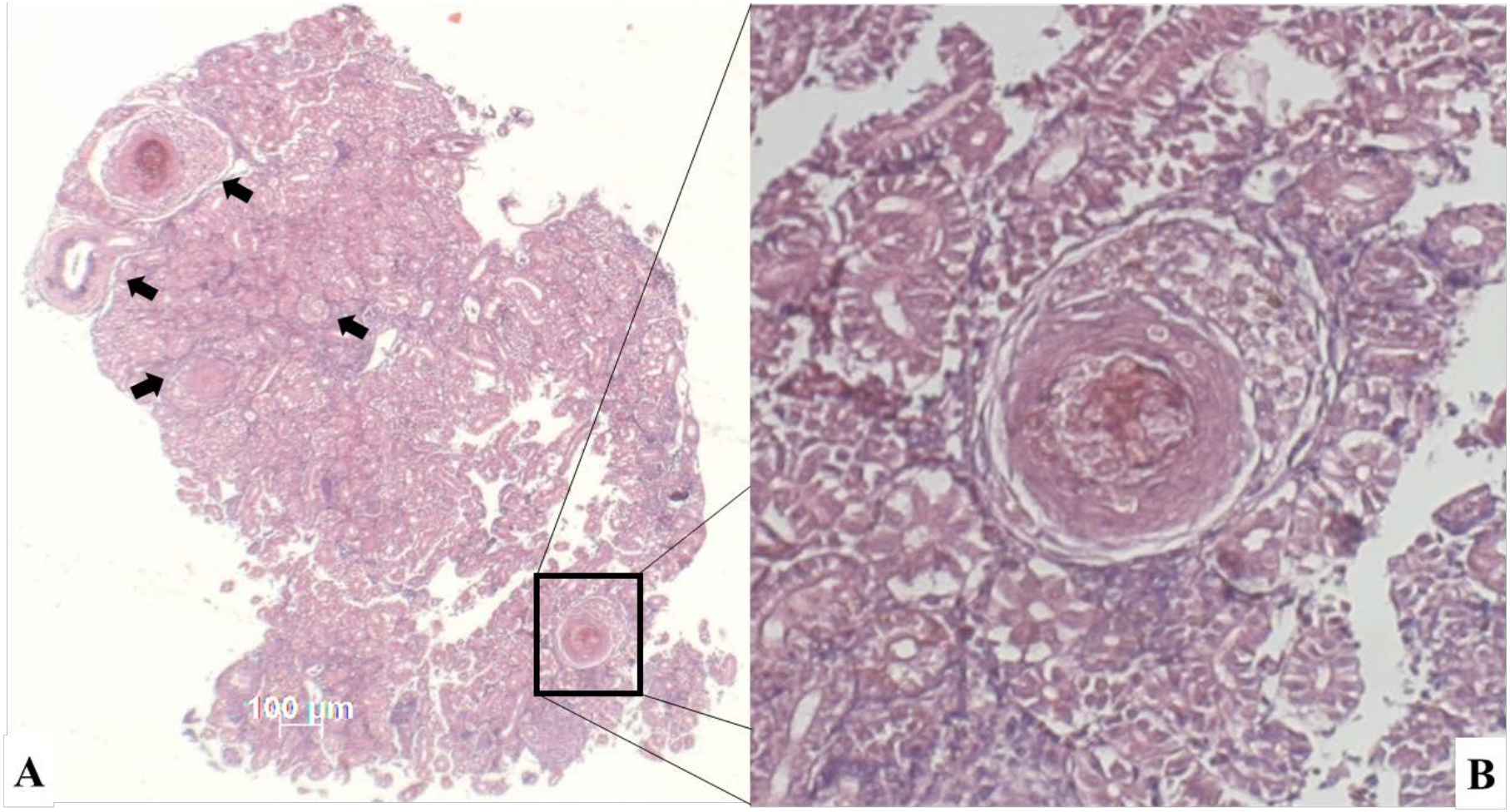

Figure 1.4 Histological sections stained with H&E of the kidney of tilapia experimentally infected with francisellosis. **A** The arrows indicate the granuloma formation. Scale bar=100µm. **B** Zoom to one of the granulomas 100X.

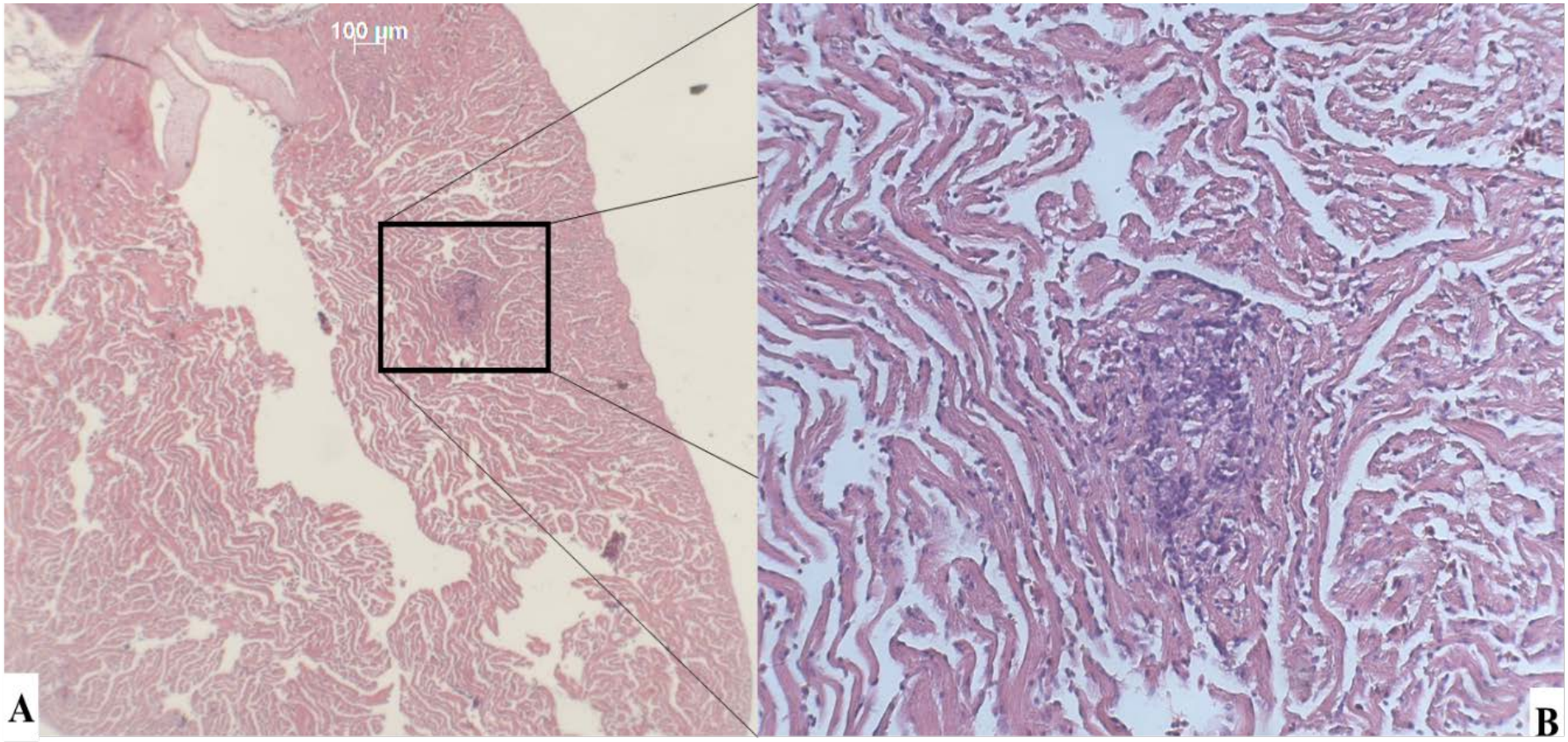

Figure 1.5 Histological sections stained with H&E of the heart of tilapia experimentally infected with francisellosis. **A** The square indicates the granuloma formation and fragmentation of myocardial fibres. Scale bar=100µm. **B** Zoom to the granuloma 200X.

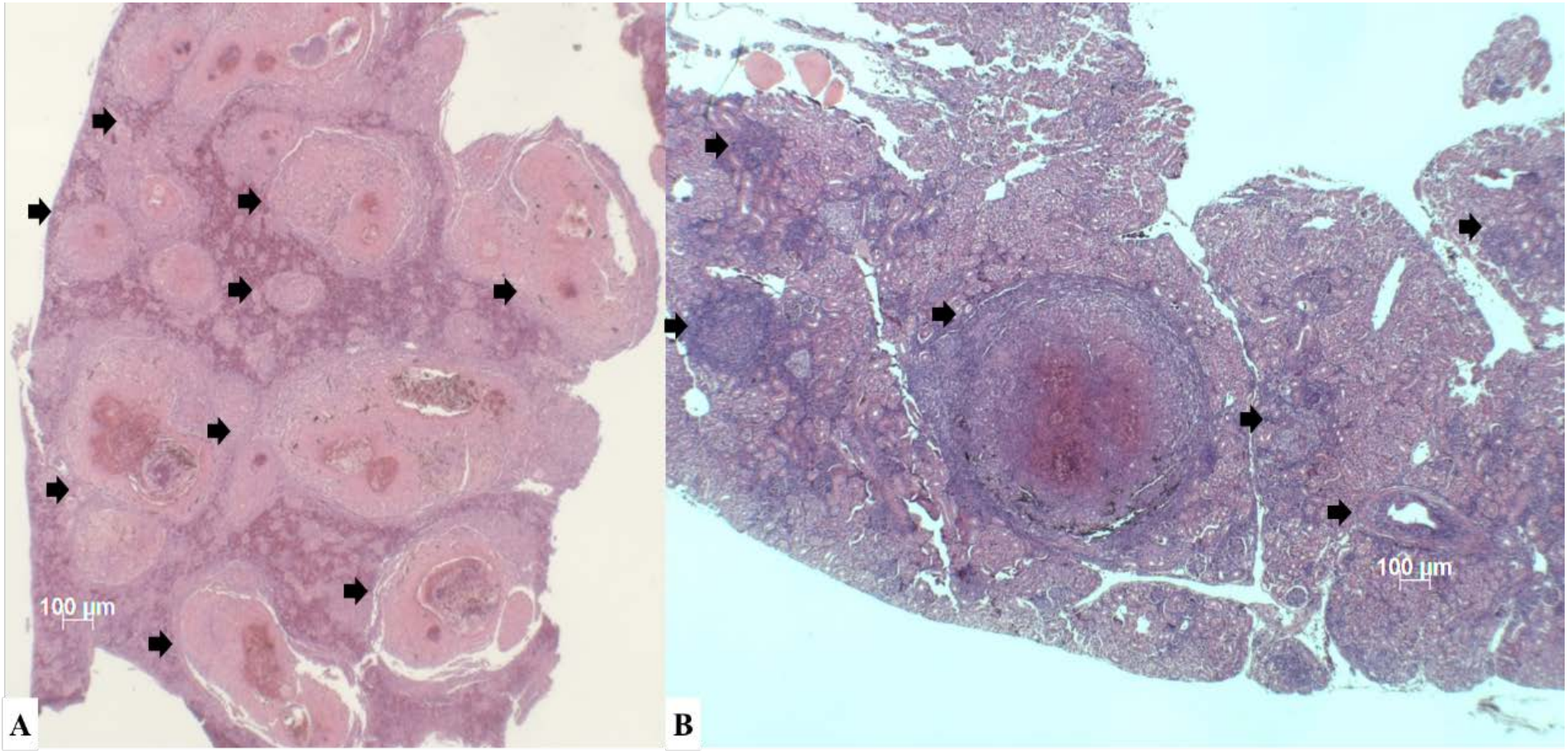

Figure 1.6 Histological sections stained with H&E of the spleen and kidney of tilapia experimentally infected with francisellosis. **A** Spleen. Arrows point coalescent granulomas replacing haematopoietic tissue. Scale bar=100µm. **B** Kidney. The arrows indicate formation of granulomas. Scale bar=100µm.

1.1.4.6 Pathogenesis of Francisella noatunensis orientalis

Soto *et al.* (2011b) showed that *Fno* replicates in the skin mucus and enters the fish via gills and skin lesions. According to Chen *et al.* (1994) the bacterium then spreads to the endothelial cells of capillary beds via the blood where acute vasculitis occurs. This is followed by perivascular leucocytic inflammatory infiltration and these lesions lead to focal necrosis and chronic infiltration of inflammatory cells.

Some of the genes associated with the ability of *Fno* to survive and replicate within the phagocytic cells of the fish have been identified as homologues of those previously reported in *F. tularensis* like the intracellular growth ABCD operon (*iglABCD*) (Soto *et al.*, 2009b). According to the authors the iglC gene is necessary for intra-macrophage survival in tilapia, but plays no role in the resistance to normal and heat inactivated tilapia serum (Soto *et al.*, 2010a).

In adult zebrafish intraperitoneally infected with *F. noatunensis orientalis* a pro-inflammatory response with up regulation of inter-leukin-1β (IL-1β), gamma interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α) was detected in the gills 6h post infection and it lasted for up to 7 days (Vojtech *et al.*, 2009).

In another experiment the same researchers used *Fno* to show that during experimental infections primary leukocytes from adult zebrafish display caspase-1-like activity that results in IL-1β processing demonstrating for the first time the processing and secretion of this interleukin in zebrafish (Vojtech *et al.*, 2012).

A different research group also found a robust proinflammatory immune response, dominated by increased transcription of TNF-α and IL-1 β in zebrafish embryos injected with *Fno* (Brudal *et al.*, 2014a). In this study authors also found the induction of the anti-inflammatory cytokine IL-10. According to the authors, a lack of IL-10 production in systemic infections can result in higher levels of pro-inflammatory cytokines, more tissue damage, and earlier death, while excessive production causes decreased ability to clear pathogens, resulting in chronic or lethal infections.

A recent study has demonstrated that temperature plays a key role on the pathogenesis of the disease in tilapia during experimental infections. When fish are kept at 25°C mortalities and spleen bacterial loads were significantly higher than those observed when the fish was maintained at 28°C. This study also demonstrated that water salinity has no effect on the course of the disease (Soto *et al.*, 2012c). In contrast to tilapia, all the experimental infections in zebrafish have been successfully achieved when the fish is maintained at 28°C.

1.1.4.7 Diagnosis and culture

The clinical signs, lesions and histopathological findings described in Section 1.3.4 are valuable as a first approach to the diagnosis of piscine francisellosis. However, since many other disease like mycobacteriosis and piscirickettsiosis develop similar patterns other techniques preferably molecular approaches should be used to confirm the presence of the pathogen in disease outbreaks.

Among the different options for molecular diagnosis of piscine francisellosis, the conventional genus specific PCR developed by Forsman *et al.* (1994) is a useful,

inexpensive tool that allows the identification of *Francisella* spp. in the fish tissues without the need of DNA sequencing. Since quantification of the bacterium is limited using this technique a number of real time quantitative PCR assays have been published in the last ten years.

In 2008, Ottem *et al.* (2008) published a multiplex assay that combined two real time PCR assays to detect low levels of *Fnn*, although the sensitivity of these assays was good it was not capable of differentiating between *Fnn* and other *Francisella* spp. without multiplexing or combining different genes. A year later Soto *et al.* (2010b) published a quantitative PCR assays for diagnosis of *Fno* in tilapia, this protocol had a lower (better) power of detection but like the previous assays this was not considered specific for bacteria belonging to the *taxon Fno*.

In 2012 Doudu *et al.* (2012) published two highly sensitive real time PCRs. These assays were developed with specific *Fnn* and *Fno* gene sequences, and therefore are considered subspecies specific, but could not distinguish between *Fnn* strains. The limit of detection of the assays was only of 10 genome equivalents. This assay is supposed to have the lowest limit of detection for the diagnosis of piscine francisellosis however it would be recommended to develop a comparative study, such as that recently presented by Monaghan *et al*. (2015) for viral detection in carp (*Cyprinus carpio*), to analyse its real sensitivity and specificity in comparison with all the other PCR assays.

The main drawback of conventional and real time PCRs is that they require the use of specialised lab equipment and cannot be performed at the farm side. A solution to these problem were addressed by Caipang *et al.* (2010), who proposed a loop-mediated

isothermal amplification (LAMP) reaction to identify *Fnn* in cod tissues. According to the authors, the method was 100 times more sensitive than the conventional PCR and highly specific for *Fnn*.

The LAMP approach has not been attempted for detection of *Fno* in tilapia aquaculture and further research is required to develop non-lethal diagnostic methods that can be performed at the farm site.

Prior to the first identification of *Francisella* spp. in fish, the isolation of these pathogens had only been achieved using cell culture. The cell lines that were successfully used to recover *Fnn* were: salmon head kidney (SHK-1), Atlantic salmon kidney cells (ASK) and rainbow trout gonad cell line RTG-2, (Bohle *et al*., 2009; Cvitanich *et al.*, 1995; Nylund *et al*., 2006) while *Fno* was isolated using Chinook salmon embryo (CHSE-214) cells lines (Hsieh *et al.*, 2006).

After the confirmation of *Francisella* spp. in the fish outbreaks, several synthetic selective media haven used to isolate *Fnn* and *Fno*. The formulation of these agars is based on media used to isolate *F. tularensis* and environmental *Francisella* spp. Soto *et al.* (2009a) reported the following agars as suitable for secondary passages of pure *Fno*: chocolate Agar/Improved Thayer-Martin biplate, Chocolate II Agar, and the Mueller Hinton base supplemented with 3% foetal bovine serum, 1% glucose and 0.1% cysteine. Table 1.3, adapted from (Colquhoun and Duodu, 2011), shows a summary of the agars that have been successfully used to recover *Fnn* and *Fno*. The broth or liquid media that have been successfully used to grow purified *Fnn* and *Fno* are: Bacto Eugon Broth with $FeCl3{\cdot}6H_2O$, Growth medium B1817 and modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX™ (Ottem *et al.*, 2007a; Soto *et al.*, 2009a).

Table 1.3 Media used for primary isolation of *Francisella* spp. from diseased fish

Bacterium	Fish species	Media type	Reference
Fnn	Atlantic cod	cysteine heart agar + 5% ovine blood	Olsen *et al.* (2006)
		blood agar + 0.1% cysteine + 1% glucose (BCG-plates)	Nylund *et al.* (2006)
		MacConkey agar	Ottem *et al*. (2006)
		growth medium B1817 in agar	Ottem *et al*. (2007)
		cysteine heart agar + 5% ovine blood+10% vancomycin	Ruane *et al*. (2015)
	Atlantic salmon	cysteine heart agar + 5% ovine blood	Birkbeck *et al*. (2007)
		agar Eugon + 0.1% L-cysteine, 1% glucose 6% (v/v) yeastolate and 10% (v/v) bovine foetal serum	Bohle *et al*. (2009)
Fno	Isaki	cysteine heart agar + 1% bovine haemoglobin	Kamaishi *et al.* (2005)
	Tilapia	Thayer-Martin agar	Hsieh *et al*. (2006)
		modified Thayer-Martin agar	Soto *et al.* (2009a)
		cysteine heart agar + 2% bovine haemoglobin	Soto *et al.* (2009a)
		cysteine heart agar + 2% bovine haemoglobin + polymixin B (100 units/ml) and ampicillin (50 µg/ml)	Soto *et al.* (2009a)
		cysteine heart agar + 5% rabbit blood	Soto *et al.* (2009a)
	French and Caesar grunts	modified Thayer-Martin agar	Soto *et al.* (2014a)
	Malawi cichlids	cysteine heart agar + 2% laked horse blood + ampicillin (50 µg/ml)	Lewisch *et al*. (2014)

1.1.4.8 Treatment of piscine francisellosis

In 1994, Chen *et al.* (1994) reported on the effectiveness of using oxytetracycline (OTC) to treat 75g Mozambique tilapia at a dose of 30mg/kg given for a period of 10-14 days when the fish were held at 30°C.

In 2003, Mauel *et al.* (2003) also reported effectiveness of OTC in 10g Nile tilapia when it was mixed with the food at a dose of 4g/2.2kg and Ostland *et al.* (2006) had good results with OTC in hybrid striped bass, but with a dose of 3.8g per 0.5kg of food when feeding at 3% of the body weight for 10 days.

Nylund *et al.* (2006) and Ottem *et al.* (2007a) were the first to report on the susceptibility of *Francisella* spp. against a variety of antibiotics *in vitro* with the strains showing resistance to erythromycin, sulfamethoxazole/trimethoprim, penicillin, ampicillin, cefuroxime erythromycin and susceptibility to ceftazidime tetracycline gentamicin ciprofloxacin and oxolonic acid.

Bohle *et al.* (2009) reported the following minimum inhibitory concentrations (MIC) for *Francisella* spp. obtained from Atlantic salmon in Chile: florfenicol 1.0 µg/ml; flumequine 0.25 0 µg/ml; oxolonic acid 0.25 0 µg/ml; oxytetracycline 0.5 0 µg/ml; amoxicillin >64.00 µg/ml.

Also using isolates collected from Chile, Muller *et al.* (2011) reported on the effectiveness of a commercial product Duplalim® (quaternary ammonium and glutaric aldehydes) (Veterquimica, Santiago, Chile) used at a dilution of 1:50 for 5 min to disinfect facilities that had problems with francisellosis in salmon.

In 2010, Soto *et al.* (2010c) investigated the *in vitro* and *in vivo* efficacy of florfenicol to control francisellosis in tilapia, determining the MIC values for *Fno* with this antibiotic using the broth dilution method. They also examined the susceptibility of the bacterium to antibiotics *in vitro* in tilapia head kidney macrophages and the efficacy of using medicated-feed against experimental infections using immersion challenges. The results indicated that florfenicol administered during early stages of infection had the potential to treat the disease by penetrating the fish cells and clearing the bacteria.

Soto *et al.* (2012b) examined the susceptibility of *Fno* to a variety of antibiotics *in vitro* and reported it to be susceptible to enrofloxacin, gentamycin, neomycin, oxytetracycline (0.25 µg/ml), tetracycline, florfenicol, streptomycin, novobiocin, amikacin, ciprofloxacin (0.5 1.0 µg/ml), imipenem, atifloxacin, meropenem, tobramycin, nitrofurantoin, oxolinic acid, and levofloxacin; and resistant to penicillin, amoxicillin, ampicillin, piperacillin, oxacillin, erythromycin, novobiocin, tylosin tartrate, clyndamycin, sulphathiazole, sulphadimethoxine, trimethropin/sulfamethoxazole, piperacillin/tazobactam constant 4, ticarcillin/clavulanic acid constant 2, ampicillin/sulbactam 2:1 ratio, aztreonam, ceftiofur, cefazolin, cefepime, cefotetan Na, ceftriaxone, ceftazidime, cefoxitin, cefuroxime and vancomycin.

Shortly after Isachsen *et al.* (2012) analysed *Fnn* from cod and determined the following MIC values to these antibiotics: oxytetracycline 64µg/ml, trimethoprim/sulfadiazine 64-128µg/ml, erythromycin 16µg/ml, ciprofloxacin 8µg/ml and streptomycin 32-128µg/ml, florfenicol0.5µg/ml oxolinic acid 0.25µg/ml, flumequine 0.25µg/ml and rifampin 0.25-1.0µg/ml.

Soto *et al.* (2013a) subsequently determined the most efficacious concentration of florfenicol administered in feed to control experimentally induced infections of *Fno* in Nile tilapia. Their results indicated that doses of 15 and 20 mg/kg body weight per day for 10 days significantly reduced mortalities.

Taking into account all the *in vitro* and *in vivo* studies performed so far, florfenicol and oxytetracycline seem to be the most appropriate antibiotics to fish in farms with piscine francisellosis as they have provided positive results in preliminary trials and are authorised antibiotics for use in food fish in Europe and the USA.

1.1.4.9 Prevention (vaccine development)

There are currently no commercial products to prevent piscine francisellosis. The first attempt to develop an autologous vaccine against piscine francisellosis were performed in Norway and the results were presented in 2007 (Krossøy *et al.*, 2007). On that study different oil-adjuvants were tested to prepare whole cell inactivated vaccines with *Fnn* isolates from Cod and the possible side effects at different time points post vaccination were assessed.

Two years later, also in Norway, Schrøder *et al.* (2009) performed a similar experiment to study the mechanisms of the adaptive immune response of cod, immunising the fish against *Fnn, Vibrio anguillinarum* and *Aeromonas salmonicida* and subsequently experimentally infecting the fish to assess the efficacy of the vaccine preparations. In 2011 results of the immune gene expression analysed by microarray technique in Cod immunised with these formulations were presented by Müller *et al*. (2011).

Soto *et al.* (2009b, 2011b) developed a live attenuated strain, with a deletion mutation at the intracellular growth *locu*s C gene (iglC), which was shown to confer a relative percentage survival (RPS) of 87.5% in Nile tilapia after experimental infection by immersion. Later on, it was demonstrated that temperature and size at vaccination are important factors when implementing immunization prophylaxis in cultured tilapia under laboratory conditions (Soto *et al.*, 2014) with this vaccine.

More recently Brudal *et al*., (2014b) developed a vaccine based on "outer membrane vesicles" (OMVs), which contain similar structures to those found on Gram negative bacteria that contain virulence factors and which have been shown to protect zebrafish embryos against *Fnn*. The challenges with high doses of live pathogenic *Fnn* indicated that the vesicles have potential for further research in vaccine development against piscine francisellosis.

1.2 Aims of this study:

- To diagnose outbreaks of piscine francisellosis and isolate new strains of fish pathogenic *Francisella* spp. from diseased Nile tilapia farmed in Europe.

- To develop a methodology for the phenotypic and genetic analyses of fish pathogenic *Francisella* spp. strains and integrate a polyphasic approach for their characterisation.

- To sequence the complete genome of the novel isolates using next generation sequencing technology.

- To clarify the taxonomic status of the novel strains and other *Francisella* spp. from fish using genomic derived parameters and the polyphasic approach proposed.

- To establish an *in vivo* challenge model via intraperitoneal injection in different species of tilapia with one of the novel isolates.

- To investigate the *in vivo* susceptibility/resistance of difference genetic groups of tilapia to one of the novel isolates using the *in vivo* challenge model proposed.

- To develop an autologous vaccine against piscine francisellosis with one of the novel isolates and assess its efficacy in red Nile tilapia.

Chapter Two

First isolation and characterisation of *Francisella noatunensis orientalis* from red Nile tilapia (*Oreochromis niloticus* L.) farmed in Europe.

2.1 Introduction

Francisella noatunensis (*Fn*), (genus *Francisella* family *Francisellaceae*) is a Gram negative, non-motile, non-sporulating, aerobic, coccobacillar, facultative intracellular bacterium and the causative agent of the emerging disease "piscine francisellosis", reported to affect several farmed and wild (marine and fresh water) fish species worldwide. (Colquhoun *et al.*, 2014)

Currently *Fn* is divided into two subspecies: *noatunensis* (*Fnn*) and *orientalis* (*Fno*) (Euzeby, 2009a; Euzeby, 2009b; Ottem *et al.*, 2009). *Fnn* is responsible for causing the disease in cold water fish species, mainly farmed and wild Atlantic Cod (*Gadus morhua*) (Birkbeck *et al.*, 2011; Colquhoun and Duodu, 2011) and farmed Atlantic salmon (*Salmo salar*) in fresh and sea water (Birkbeck *et al.*, 2007; Ottem *et al.*, 2008).

Fno is the causative agent of the disease in a range of warm, marine and freshwater fish including: guapote tigre (*Parachromis managuensis*), Hawaiian Chinese catfish, barramundi, three line grunt or Isaki fish (*Parapristipoma trilineatum*), hybrid striped bass (*Morone chrysops* x *M. saxatilis*), tilapia (*Oreochromis* spp.) and a number of ornamental species belonging to more than 18 different genera (Camus *et al.*, 2013; Colquhoun and Duodu, 2011; Hsieh *et al.*, 2007; Kamaishi *et al.*, 2005; Lewisch *et al.*, 2014; Ostland *et al.*, 2006; Soto *et al.*, 2012a; Soto, *et al.*, 2014a).

In farmed fish, francisellosis occurs as an acute or chronic disease, with non-specific clinical signs such as erratic swimming, lethargy, frayed fins, pale or dark skin, exophthalmia, ascites, emaciation, pale or haemorrhagic gills, and haemorrhagic or white skin nodules, with levels of mortality ranging from 5% up to 95%. Disease progression,

mortality rates and clinical signs presented, vary depending on the fish species, water temperature and rearing system (Colquhoun and Duodu, 2011).

As facultative intracellular pathogens, *Fno* and *Fnn* are capable of avoiding the immune system of their hosts by entering macrophages and other phagocytic cells, to eventually form granulomas (white nodules), which are the most frequent finding at necropsy. These structures are regularly found in the splenic and renal (haematopoietic) tissues of diseased fish, but they can also be systemically distributed (Soto *et al.*, 2011a). *F. noatunensis* ssp. are highly fastidious pathogens that grow slowly (up to 5 days) and require the use of cell lines or artificial media enhanced with supplements and enrichments for their isolation from diseased fish. Moreover, they are able to enter into a viable but not culturable (VBNC) state (similar to the Gram positive bacterial spores) that allows them to persist in the environment and fish tissues causing clinical signs, without being isolated on culture media (Duodu and Colquhoun, 2010). Additionally the antagonism of secondary bacterial infections and the use of antibiotics to kill them, frequently contributes to complicate the recovery of *Fn* from the fish. At present there are no reports of *Fn* isolation from sources other than diseased fish i.e. from environmental samples or plants.

Due to these difficulties, the isolation and characterisation of *Francisella* pathogens from fish has historically represented a challenge for researchers. Several non-cultured and therefore uncharacterised *Francisella* spp. and *Francisella*-like bacteria (FLB) have been reported based only on molecular diagnosis such as conventional polymerase chain reaction (PCR) (Jeffery and Stone, 2010; Krajangwong *et al*., 2011;Ostland *et al.*, 2006; Ramírez-Paredes *et al*., 2012b).

In Europe the presence of *Fnn* affecting fish has been reported in cod and salmon in: Norway (Mikalsen *et al.*, 2007), Sweden (Ottem *et al.*, 2008), United Kingdom (UK) (Zerihun *et al.*, 2011), Denmark (Ottem *et al.*, 2007a) and Ireland (Ruane *et al.*, 2013), with reports of successful isolation in Denmark, Sweden, Norway, and Ireland, whereas *Fno* has been diagnosed in tilapia in the UK (Jeffery and Stone, 2010) and isolated from ornamental Malawi cichlids in Austria (Lewisch *et al.*, 2014).

Consistent with their fastidious nature, the *Fn* strains are biochemically unreactive, a condition that complicates the use of methodologies that rely on bacterial growth like standardised biochemical test kits, to assess their physiological response to different metabolites and environments (phenotypic characterisation). Recent reports document isolation of novel *Fn* strains, particularly *Fno* using commercial Thayer-Martin agar and 5% sheep blood or 2% bovine haemoglobin enriched cysteine heart agar (CHA) (Leal *et al.*, 2014; Lewisch *et al.*, 2014; Nguyen *et al.*, 2014). However, these studies lack in-depth methodology for the integrated characterisation of novel isolates and their descriptions are based only on a few primary phenotypic and genetic characteristics.

The present study describes the diagnosis of francisellosis in farmed tilapia and the isolation of *Fno* strains using a novel selective (5%) tilapia blood enriched CHA and the development of a comprehensive methodology (based on a polyphasic approach) for the phenotypic and genetic characterisation of this highly fastidious bacterium. Additionally, an experimental infection model was established in Nile tilapia to fulfil Koch's postulates and to investigate pathogenesis in this species. This study is thought to constitute the first documented report of isolation and characterisation of *Fno* from Nile tilapia in Europe.

2.2 Materials and methods

2.2.1 Presumptive diagnosis

2.2.1.1 Diseased fish history

During 2011 and early semester of 2012, chronic disease episodes with non-specific clinical signs and mortalities of up to 60% were experienced in farmed tilapia fingerlings at two different farms in Northern Europe.

In July 2012, five individual fish from different sections of each farm (ten fish in total) were euthanized with a lethal overdose of Tricaine Pharmaq 1000mg/g (TPQ) anaesthetic (Pharmaq, Hampshire, UK) i.e. 3x the normal dose for anaesthesia (300mg/l) and sampled for diagnosis.

The gills, heart, kidney, liver and spleen were fixed for at least 24h at 4 °C in 10% neutral buffered formalin for histologic analyses, 2.5% (v/v) glutaraldehyde in 100mM sodium cacodylate buffer (pH 7.2) for electron microscopy and 96% ethanol for molecular diagnosis.

Views of the production systems in the farms and pictures of the sampled fish are shown in Figure 2.1.

Figure 2.1 European tilapia farms with francisellosis outbreaks. **A** and **B** Facilities and fish sampled at Farm 1; **C** and **D** Recirculating systems at the second farm.

2.2.1.2 Histopathological analyses

Formalin fixed tissues were processed using standard histological methods, stained with haematoxylin and eosin (H&E) and examined under light microscopy.

2.2.1.3 Transmission scanning and electron microscopy analyses

The spleen and head kidney, previously fixed in 2.5% glutaraldehyde in 100mM sodium cacodylate buffer, were cut to obtain 2mm samples that were then post-fixed in 1% osmium tetroxide in cacodylate buffer and rinsed three times in distilled water 10 minutes each time to remove excess fixative.

For TEM the tissues were dehydrated in a gradient cold acetone series (50–100%), embedded in Epon812, and cut into ultra-thin sections. The sections were observed under an FEI Tecnai Spirit G2 Bio Twin transmission electron microscope. For SEM the tissues were dehydrated in an ethanol series and critical point dried using a Bal-Tec 030 critical point drier. Samples were then attached to aluminium stubs, which were gold sputter coated using an Edwards S150B sputter coater, and visualised using a Jeol JSM6460LV scanning electron microscope.

2.2.1.4 Molecular diagnosis using a genus specific PCR

A PCR able to amplify a specific DNA sequence from bacteria belonging to the genus *Francisella* (Forsman *et al.,* 1994) was performed using genomic DNA (gDNA) extracted from the fish tissues as a template. The gDNA was extracted from the ethanol fixed samples using a Nucleo Spin Tissue® kit (Macherey&Nagel, Düren, Germany), according to the manufacturer's instructions.

A negative control was included using gDNA from tilapia reared at the Tropical Aquarium (TA) IoA UoS a source confirmed in this study to be free of francisellosis after sampling 25 fish for biological (culture in plates) and molecular (PCR) diagnosis.

The PCR was performed using Illustra PuReTaq Ready-To-Go Beads™ (200 μM each dNTP in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 1.5 mM $MgCl_2$) (GE Healthcare, Chalfont St. Giles, UK) reconstituted to a final volume of 25 μl with: 5μl of DNA template (~80ng/μl), 2.5 μl of each primer (F11 5'-TAC CAG TTG GAA ACG ACT GT-3' and F5 5'-CCT TTT TGA GTT TCG CTC C-3') at a concentration of 10pmol/ml (10μM) and 15μl of ultrapure water. Cycling conditions consisted of an initial denaturation step of 2min at 93ºC, followed by 35 cycles of: 1min at 94ºC, 1min at 65ºC and 1min at 72ºC, and a final extension step of 5min at 72ºC in a Biometra TGradient Thermocycler (Biometra, Göttingen, Germany). Amplification products were visualized on ethidium bromide stained 1% agarose gel after electrophoresis for 35 minutes at 90 volts.

2.2.2 Bacterial isolation and identification

2.2.2.1 Primary isolation and purification

After preliminary results involving PCR and electron microscopy indicated the presence of *Francisella* spp. in the diseased fish, a follow up visit to the farms took place in November 2012 with the aim of isolating the *Francisella* strains involved and other pathogens that could be associated with the outbreak. For this purpose, ten fish were randomly selected from each farm, euthanized with a lethal overdose of TPQ and sampled. During necropsy, spleens were aseptically collected and homogenised in 1ml

of 1X sterile phosphate buffered saline (PBS) using a Cordless Motor Pellet Pestle (Sigma-Aldrich, Dorset, UK).

For primary isolation of the pathogens, 20µl loopfuls of the homogenates were streaked onto 5 different media: CHA (BD, Oxford, UK) with 2% bovine haemoglobin solution (BD, Oxford, UK) (CHAH); modified Martin Lewis agar (BD, Oxford, UK) (MMLA); modified Thayer-Martin Agar, (BD, Maryland, USA) (MTMA); trypticase soy agar (Oxoid Ltd., Hampshire, UK) (TSA) and cysteine heart agar with 5% tilapia blood (CHTB). The CHAH and CHTB were prepared with and without the following antibiotics: polymyxin B sulphate salt 100units/ml (Sigma-Aldrich, Dorset, UK) and ampicillin readymade solution 50µg/ml (Sigma-Aldrich, Dorset, UK). All plates were incubated at 28^{o}C for 10 days. Dominant colonies consistent with *Francisella* morphology were sub-cultured up to two times on CHAH for purification using the culture conditions described. Basic phenotypic characteristics of bacteria from *Francisella*-like isolates of both farms were determined using primary identification tests such as: Gram stain, catalase, oxidase, oxidation/fermentation of glucose (O/F test) and motility.

2.2.2.2 Molecular identification

To confirm the identity of *Francisella*-like colonies, gDNA was released using the boiling technique as outlined by Seward *et al.* (1997) with modifications. Briefly, five colonies were re-suspended in a 1.5ml Eppendorf tube containing 100µl of Tris-EDTA (TE) buffer and heated for 10min at 99^{o}C. The suspensions were then cooled on ice for 5min and centrifuged at 15,800 gravities (g) in a benchtop IEC Microlite Centrifuge

(Thermo Electron Corporation, Massachusetts, USA) for 1min. The supernatant containing the crude extracted gDNA was then transferred into a fresh 0.5ml tube and stored at -20^{o}C. This was subsequently used as a template in the PCR protocols described in Section 2.1.4. Once the identity of the isolates was established, bacteria were resuspended in liquid medium consisting of modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX™ (BD, Oxford, UK) and 0.1% D-(+)-glucose ACS reagent (Sigma-Aldrich, Dorset, UK) (MMHB). The broth cultures were grown overnight at 28^{o}C in a shaker incubator at 175rpm and stored frozen at -80^{o}C with 20% sterilised glycerol (BDH Prolabo-VWR International Eurolab, Leuven, Belgium).

2.2.3 Bacterial phenotypic characterisation

2.2.3.1 Bacterial isolates and growth conditions

Of the ten *Fno* isolates recovered in the present study, STIR-MATT-F1-f6 (from fish 6 from the first farm) and STIR-GUS-F2f7 (recovered from fish 7 from the second farm) were selected as the representatives of each farm. The *Fno* type strain Ehime-1 was purchased from the German Culture Collection of Microorganisms and Cell Cultures "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DSMZ) collection in Braunschweig Germany, while isolate PQ1104 from tilapia farmed in Costa Rica was kindly provided by Dr. Duncan Colquhoun at the Norwegian Veterinary Institute (NVI) in Oslo Norway. A quality control bacterium *Escherichia coli* ATCC 25922, was obtained from the Bacterial Culture Collection, Bacteriology Laboratory, Institute of Aquaculture (IoA), University of Stirling (UoS). The *Fno* isolates recovered and used in the present study are presented in Table 2.1.

Table 2.1 *Francisella noatunensis orientalis* isolates used and recovered in this study

ISOLATE	Location	Year	Diseased fish
Ehime-1♣	Japan	2001	three line grunt or Isaki fish (*Parapristipoma trilineatum*)
PQ1104	Costa Rica	2006	tilapia (*Oreochromis* sp.)
STIR-AVU-F1f5	Europe farm 1 fish 5	2012*	red Nile tilapia (*O. niloticus*)
STIR-MATT-F1f6	Europe farm 1 fish 6	2012*	red Nile tilapia (*O. niloticus*)
STIR-AVU-F1f7	Europe farm 1 fish 7	2012*	red Nile tilapia (*O. niloticus*)
STIR-AVU-F1f9	Europe farm 1 fish 9	2012*	red Nile tilapia (*O. niloticus*)
STIR-AVU-F1f10	Europe farm 1 fish 10	2012*	red Nile tilapia (*O. niloticus*)
STIR-GUS-F2f7	Europe farm 2 fish 7	2012*	red Nile tilapia (*O. niloticus*)
STIR-AVU-F2f9	Europe farm 2 fish 9	2012*	red Nile tilapia (*O. niloticus*)
STIR-AVU-F2f10	Europe farm 2 fish 10	2012*	red Nile tilapia (*O.s niloticus*)

* From present study; ♣ indicates the *Fn*o type strain.

For both the phenotypic and genetic characterisation the *Fno* isolates were grown on CHAH and MMHB, while the quality control bacterium was grown on TSA and trypticase soy broth (Oxoid Ltd., Hampshire, England) (TSB). *Fno* was cultured on solid media for 96h and the quality control for 24h. The broth cultures were incubated in 15ml aliquots for 18 to 21h (i.e. mid log phase of the growth curve) in a shaker incubator at 150rpm. All isolates were grown at 28ºC (optimal *in vitro* temperature) in both agar and broth.

2.3.3.2 Optimal growth temperature and growth curves in vitro

The optimal *in vitro* growth temperature of each *Fno* isolate was investigated on agar by plating triplicates of six 20µl drops containing the same bacterial concentration (dilution 10^{-6} with an optical density of 0.4 at 600 nanometres (nm) (OD_{600})) and incubating them at 5ºC, 15ºC, 18ºC, 21ºC, 22ºC, 24ºC, 26ºC, 28ºC, 29ºC, 30ºC, 32ºC, 33ºC and 37ºC for 10 days.

Growth curves for STIR-GUS-F2f7 and Ehime-1 were established by inoculating and incubating triplicate culture flasks containing 99ml of MMHB with 1ml of starting culture (OD_{600} 1.0) for 72h at 28^{o}C, on an orbital shaker incubator at 150rpm. To monitor the bacterial growth, a sample of 1ml was taken every 3h and the optical density recorded. The bacterial growth curve was produced by plotting the density OD_{600} against time (h).

2.3.3.3 Carbohydrate fermentation and enzymatic activity

The enzymatic activity and fermentation of carbohydrates of all *Fno* isolates and the quality control *Escherichia coli* ATCC 25922 were assessed in triplicate using API 20E and API ZYM kits (BioMerieux, Marcy l'Etoile, France). The kits were used as described by the manufacturer with the following modifications: the bacteria were grown in CHAH as described in Section 2.4.1.2, the API ZYM kit-strips were visually read at 4, 8 and 24h post inoculation and the API20E strips after 24h.

2.3.3.4 Carbon metabolism (metabolic fingerprint)

To assess the metabolic activity (carbon utilisation) of the novel *Fno* strains, the Biolog GN2 micro plates (Biolog Inc., California, USA) were used. These plates contain an array of 95 different carbon (C) sources i.e. carbohydrates, lipids, sugars, amino acids, nucleic acids and other metabolites. The C sources are coupled to a redox (tetrazolium) dye that is immediately reduced when metabolic activity starts, producing a change in colour that can be colourimetrically measured to develop a metabolic fingerprint.

The GN2 Biolog micro plates were set up and analysed according to the following protocol: bacteria were grown, as described in Section 2.4.1. For each isolate three GN2 Biolog micro plates were used and three 50ml centrifuge tubes containing 15ml of

MMHB were used. After incubation at 28 ºC for 18 to 21h the cultures were centrifuged in a Sigma 4K15C refrigerated benchtop centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 4ºC for 15min at 2602g.

The bacterial pellets were then washed by resuspending them in 20ml of 1X sterile PBS (a sterile disposable 5µl loop was required to break the pellets) and vortexing for 5min. The tubes were centrifuged as before and the PBS discarded. Each pellet was then suspended in 3ml of GN inoculating fluid (Biolog Inc., Hayward, California, USA) in one 50ml centrifuge tube. The OD_{600} of the suspension was adjusted to the desired bacterial concentration by adding GN inoculating fluid.

The plates were inoculated with 150µl of the adjusted bacterial suspension per well, using a multichannel pipette and a sterile reservoir (Biolog Inc., California, USA). After inoculation, the Biolog GN2 micro plates were kept at 28ºC for 24h and the colour change recorded every 6h by visual inspection, computer scanning with an Epson Perfection V370 Photo scanner (Epson, London, UK) and absorbency measurements at 450nm using a 96-well plate spectrophotometer (Biotek Instruments, Friedrichshall, Germany).

In order to optimise the protocol and find the optimal inoculation density, a gradient of densities, was prepared for type strain Ehime-1 and STIR-GUS-F2f7. The optical densities tested were 0.36, 0.46, 0.56, 0.66, 0.76, 0.86, 0.96, 1.06 and 1.5 and subsequently the remaining *Fno* isolates were tested at an OD_{600} 0.85.

2.3.3.5 Chemotaxonomic analyses (cellular fatty acids methyl esters)

The cellular fatty acid methyl esters (FAME) composition of *Fno* was analysed by gas chromatography (GC) in the Lipids Laboratory of the IoA, UoS.

The two novel *Fno* isolates and the type strain Ehime-1 were grown in MMHB as described in Section 2.4.1, using three 50ml centrifuge tubes containing 20ml of MMHB per isolate, and incubated for 43h; after incubation, the OD_{600} of the cultures was determined and the bacterial suspension centrifuged at 4ºC for 15min at 2602g. The resulting bacterial pellets were then washed by suspending them in 5ml of sterile 1X PBS and vortexing them for 5min.

The lipid content was extracted from the pellets by suspending them in 5ml of ice-cold chloroform/methanol (2:1 v/v) using a disposable glass Pasteur pipette according to Folch *et al*. (1957) and quantified gravimetrically. FAME from lipid were prepared by acid-catalysed trans-esterification at 50ºC for 17h (Christie, 1993).

FAME were extracted (not purified) as described previously (Tocher and Harvie, 1988) and separated and quantified by GC using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30m x 0.32mm x 0.25mm ZB-wax column (Phenomenex, Cheshire, UK) 'on column' injection and flame ionization detection. Hydrogen was used as carrier gas with an initial oven thermal gradient from 50ºC to 150ºC at 40ºC min to a final temperature of 230ºC at 2ºC min. Individual FAME were identified by comparison to known standards (Supelco™ 37-FAME mix; Sigma-Aldrich, Dorset, UK) and published data (Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows version 1.19 (Thermoquest Italia SpA., Milan, Italy).

2.3.3.6 Antibacterial susceptibility tests

a) Quantitative assay "broth microdilution method"

The minimal inhibitory concentration (MIC) of 39 different antimicrobial compounds to *Fno* and the quality control *E. coli* ATCC25922 were investigated using GN2F and AVIAN1F Sensititre Plates (Trek Diagnostic System, West Sussex, UK). This procedure was performed in duplicate following the manufacturer's instructions and previously published protocols for *Fno* and *Francisella tularensis* (Baker *et al.*, 985; Brown *et al.*, 2004; García del Blanco, 2004; Soto *et al.* 2012b; Urich and Petersen, 2008).

The media preparation, inoculation densities, incubation temperature, quality-control organism and interpretation of results were performed in compliance with the Clinical and Laboratory Standards Institute (CLSI) values (CLSI, 2006a).

Briefly, the *Fno* isolates and *E. coli* ATCC 25922 were plated on CHAH agar and incubated as described in Section 2.4.1. Colonies were suspended in 1X sterile PBS to a McFarland standard 0.5. This suspension was diluted 100-fold (*Fno*) or 1000-fold (*E.coli* ATCC25922) in MMH and 50µl added with a multichannel pipette to each well of the sensititre plates. The plates were then incubated at 28^{o}C and the bacterial growth visually checked at 48h (*Fno* isolates) or 24h (*E. coli* ATCC 25922) post inoculation. The MIC value was defined as the lowest concentration exhibiting no visible growth. The MIC assay for the quality control *E. coli* ATCC 25922 was determined utilizing MMHB at 28^{o}C following the CLSI protocol (CLSI, 2006a).

b) Qualitative assay disc diffusion (Kirby–Bauer) method

The susceptibility or resistance of *Fno* isolates to 16 different antibiotics in agar plates was investigated following the protocol established by Soto *et al.* (Soto *et al.*, 2012b). Briefly, *Fno* bacteria were harvested after incubation in CHAH for 72h at 28^{o}C as described in Section 2.4.1, and suspended in 1X PBS to achieve a turbidity equivalent to McFarland standard 0.5. A fresh set of CHAH plates were inoculated with 100µl of the suspension using sterile disposable Drigalski spatulas. After 60min when the plates were dried, the antibiotic discs were dispensed using a self-tamping antimicrobial susceptibility disc dispenser (Oxoid Ltd., Hampshire, England). Plates were incubated at 28^{o}C for 96h and results recorded after 72h by measuring the diameter of the inhibition zones. The antibiotic discs were commercially obtained (Oxoid Ltd., Hampshire, England).

2.2.4 Bacterial genetic characterisation

2.2.4.1 Bacterial genomic DNA extraction

The total bacterial gDNA from the representative isolate of the second European farm STIR-GUS-F2f7 was released by the boiling method as described in the Section 2.2.2. The purity and concentration of the crude DNA was assessed by 260/280 and 260/230 ratios with a NanoDrop^{TM} ND1000 (ThermoScientific, Delaware, USA).

2.2.4.2 Housekeeping genes (selection and sequencing)

Twelve housekeeping genes were selected for amplification and sequencing: 16SrRNA, 16SrRNA-23SrRNA intergenic spacer (ITS), 23SrRNA, malate dehydrogenase (mdh), chromosomal replication initiator protein alpha subunit (dnaA), DNA mismatch repair

protein (mutS), phospho-glucomutase (pgm), peptide chain release factor 2 beta subunit (prfB), bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase alpha subunit (putA), DNA-directed RNA polymerase alpha subunit (rpoA), DNA-directed RNA polymerase beta subunit (rpoB) and triose-phosphate isomerase alpha subunit (tpiA). The suitability of these genes for phylogenetic analysis has been previously reported for the genetic characterisation of novel *Francisella* spp. recovered from farmed aquatic organisms (Bohle *et al.*, 2009; Brevik *et al.*, 2011; Nylund *et al.*, 2006; Ottem *et al.*, 2009).

To design the primers for amplification of the housekeeping genes, the complete genome sequence of *Fno* Toba04, GenBank® accession number NC_017909 (Sridhar *et al.*, 2012) and the software Primer3 (Untergasser *et al.* 2012) were used. The primers were *in silico* tested using the software developed by Bikandi *et al.* (2004) available at http://insilico.ehu.es/ and their attributes are presented in Table 2.2

The PCR was performed using the ready to use 2x MyTaq™ HS Mix, (Bioline, London, UK), each reaction contained 25μl of the mix, 1.0 μl of both forward and reverse primers (20μM), 200ng of the DNA template (~4μl) and ultrapure water to a total volume of 50μl. Cycling conditions consisted of an initial denaturation step of 1min at 95^{o}C, followed by 35 cycles of: 15s at 95^{o}C, 15s at 66^{o}C and 10s at 72^{o}C performed in a Biometra TGradient Thermocycler (Biometra, Göttingen, Germany).

Table 2.2 Overview of the primers designed for amplification and sequencing of selected housekeeping genes from *Fno* STIR-GUS-F2f7

Gene short name	Primer	Sequence	Position in Toba04	Tm °C	GC-Content %	Expected amplicon (bp)
dnaA	dnaA-F1	AAAACCTTTCTACGTTTGAAT	56-76	50.1	28.6	
dnaA	dnaA-R1	GCAACTCATAATCATCAGAT	1471-1452	51.2	35	1415
mutS	mutS-F1	TAGTTCAAAGAGTATATTTAG	703305-703325	54.0	38.1	
mutS	mutS-R1	TGACATTGAAAGGATAATTTCTC	704814-704792	57.3	50	
mutS	mutS-F2	GGCGTCTGATATACTCAGCT	704375-704394	53.5	30.4	
mutS	mutS-R2	AGAAGTTTCAAATCACACACC	705831-705811	48.1	23.8	2526
pgm	pgm-F1	TGGCTATTCAGACTGTATCTAC	1084092-1084113	56.5	40.9	
pgm	pgm-R1	CAGTCATTCCTGTCAGAGAT	1085705-1085686	55.3	45	1613
prfB	prfB-F1	TTGAATCTTTACGGGACTAT	141503-141522	56.5	40.9	
prfB	prfB-R1	AACTTATCTAAATCACCATCTA	142512-142491	57.1	39.1	1009
putA	putA-F1	CTTATTGAACCATTCAGGTGAG	1737813-1737834	56.5	40.9	
putA	putA-R1	GCTTGGTAAGAAACATCTGTATG	1738970-1738948	57.1	39.1	
putA	putA-F2	AGAGCAAGGCTTAGCAGGTT	1738905-1738924	57.3	50	
putA	putA-R2	GAGTTTGTTCAGCAGGTTTA	1739995-1739976	53.2	40	
putA	putA-F3	GACTTCGCAGTGTTAGCAGA	1739407-1739426	57.3	50	
putA	putA-R3	GACCAAATTGTTCTCTACCAA	1740613-1740593	54.0	38.1	
putA	putA-F4	TGATTGTTGGTGCGATGAAAG	1740371-1740391	55.9	42.9	
putA	putA-R4	CGATAGTATCAACACTGACAG	1741828-1741808	55.9	42.9	4015
rpoA	rpoA-F1	CAGGAATTTGTACCTAATATAC	220361-220382	52.8	31.8	
rpoA	rpoA-R1	TGAACACCTAGAGATAAGTTG	221274-221254	54.0	38.1	913
rpoB	rpoB-F1	ATTCGCAAAGAGTTTGGGGT	616228-616247	55.3	45	
rpoB	rpoB-R1	GCTGAAACCATCTGCTTAGC	618194-618175	57.3	50	
rpoB	rpoB-F2	ATTCTTTACATCTGGTGCTT	617718 -617737	51.2	35	
rpoB	rpoB-R2	CTGGAGTACCATCTTCCATA	619519-619500	55.3	45	
rpoB	rpoB-F3	GGCGCGTAAAGATTTCGATG	619068-619087	57.3	50	
rpoB	rpoB-R3	TTCCTCAGATGAGTAGTCAA	620268-620249	53.2	40	4040
tpiA	tpiA-F1	TGGGAAATTGGAAAATGAAT	553453-553472	49.1	30	
tpiA	tpiA-R1	CAATCAAGCCACCATCAAC	554127-554109	54.5	47.4	674
mdh	mdh-F1	ACTTTCTGCCATTTGAATAC	1769342-1769361	53.2	40	
mdh	mdh-R1	GCTTATTGGTGCTGGTAATA	1770056-1770037	52.1	35	714
16srRNA+ITS+23srRNA	16-23s-F1	GTTTCCCTTTCCACTGCG	461307-461324	53.7	50	
16srRNA+ITS+23srRNA	16-23s-R1	GTGTTAATCTGCGATAAG	462231-462214	53.7	50	
16srRNA+ITS+23srRNA	16-23s-F2	CACTTCGCTCGCCACTACTA	462036- 462055	57.3	50	
16srRNA+ITS+23srRNA	16-23s-R2	TGGTAGTCCACGCTGTAAAC	463338-463319	59.4	55	
16srRNA+ITS+23srRNA	16-23s-F3	ACCAGGTAAGGTTCTTCG	463144-463161	49.1	38.9	
16srRNA+ITS+23srRNA	16-23s-R3	AACGGTAACAGGTCTTCG	464059-464042	56.00	55.60	2752

The location of the primers is given in relation to to the open reading frame (ORF) of the protein encoding and rRNA genes from the *Fno* reference genomeToba04, NCBI accesion number NC_017909.

Amplification products were visualized on a 1% agarose gel stained with ethidium bromide after electrophoresis. The PCR products were purified for sequencing with the QIAquick PCR purification kit (QiaGen, Valencia, California USA) as directed by the manufacturer's instructions and sent for Sanger sequencing to GATC Biotech (GATC Biotech, Cologne, Germany).

The quality of the resulting sequences from both primers was visually checked against the ABI chromatogram using the The BioEdit® software version 7.1.11 (Hall 1999), assembled using the Multiple sequence comparison by Log-Expectation (MUSCLE) application of the MEGA (Molecular Evolutionary Genetic Analyses) package version 5 (Tamura *et al.*, 2011) and the overlapped region (consensus sequence) deposited in the GenBank database (Benson et al., 2013) of the National Centre for Biotechnology Information (NCBI) **(**http://www.ncbi.nlm.nih.gov/**)** with the accession numbers and lengths shown in Table 2.3.

Table 2.3 The GenBank accession number and final length of the sequenced genes from *Francisella noatunensis orientalis* STIR-GUS-F2f7

Gene	accesion number	Length (bp)
dnaA	KP657905	1331
mutS	KP657899	2429
prfB	KP657900	991
putA	KP657901	3929
rpoA	KP657902	852
rpoB	KP657903	3900
tpiA	KP657904	651
mdh	KP657898	696
16SrRNA+ITS+23SrRNA	KP657897	2679

2.2.4.3 Sequence homology and phylogenetic analyses

The nine sequences (16SrRNA, ITS and 23SrRNA genes were considered as a concatenated sequence) were individually compared for homology (as percentage of

similarity) with those stored in GenBank database using the nucleotide-nucleotide basic local alignment search tool BLASTN® (Zhang *et al.*, 2000) program from NCBI.

For every house keeping gene, the most similar sequences available from members of the genus *Francisella* were retrieved from GenBank and the BioEdit software version 7.1.11 (Hall, 1999) was used to convert the sequences from .txt into .fas formats.

The rRNA concatenated sequence was aligned as DNA and the coding sequences as codons with the Unweight Pair Group Method with Arithmetic Mean (UPGMA) using the MUSCLE application (Edgar, 2004) of the MEGA package version 6 (Tamura *et al.*, 2013). All the sequences retrieved were indicated with their NCBI accession number.

The alignments were manually adjusted by trimming excess sequences and their suitability for phylogenetic analyses double checked by computing the pairwise and the overall mean distances in MEGA 6 software (Tamura *et al.*, 2013). The nine alignments were used to build phylogenetic trees and analyse the evolutionary relationship of *Fno* STIR-GUS-F2f7 with its closest members in the genus.

Additionally, following the same procedure, the partial 16SrRNA gene (1425bp) of STIR-GUS-F2F7 was selected from the 16SrRNA-ITS-23SrRNA concatenated sequence to build a phylogenetic tree with homologous sequences from 47 other members of the genus *Francisella*, including all the currently validly described species and subspecies, and the fish pathogens *Edwardsiella piscicida* C07-087 and *Piscirickettsia salmonis* AL10015 as outgroups.

The ten evolutionary analyses were constructed in MEGA6 software (Tamura *et al.*, 2013) using the Maximum Likelihood (ML) (Nei and Kumar, 2000) approach with

complete deletion of gaps and missing data. The method chosen for every tree was the best combination of model and rates among sites, and this was investigated for each alignment using the default settings of the "find best DNA/protein model" option. The reliability of the trees (reproducibility) was tested using the bootstrap method with 2000 replications. In all the analyses the nearest-neighbour-interchange was chosen as the ML heuristic method.

2.2.5 Experimental infections

To fulfil Koch's postulates and further investigate the pathogenesis of *Fno* in Nile tilapia, an intraperitoneal (IP) injection challenge model was established with STIR-GUS-F2f7 in Nile tilapia. During the trials, mortalities were monitored and recorded at least 4 times per day, and dead and moribund fish were sampled for bacterial recovery and histopathology.

2.2.5.1 Standard curve for bacterial quantification of Fno STIR-GUS-F2f7

A standard curve was produced to determine the number of bacteria present at different optical densities of a solution prepared in 1X PBS. The biomass or number of cells was expressed as colony forming units (CFU) and the turbidity of the suspension measured by OD_{600}. The bacterium was grown in triplicate in broth as previously described in Section 2.2.3.1, and the pellets washed and adjusted to an initial OD_{600} of 1.0 with PBS. This solution was subsequently diluted to yield optical density values of 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1. Each sample was then cultured on CHAH after preparing 10 fold series of dilutions from 10^{-1} until 10^{-7} to perform drop counts using the method reported by Miles *et al.* (1938) to enumerate the number of viable bacterial cells at every

OD. The average number of cells per ml (CFU/ml) was plotted against the corresponding OD.

2.2.5.2 Experimental fish origin

Healthy red and wild type Nile tilapia fingerlings, 6-7 months/~11g (7-13g), were obtained from the Tropical Aquarium (TA) at the IoA UoS a source previously confirmed to be free of francisellosis as indicated in section 2.2.1.1.

To perform the infections, the fish were moved into a flow-through system in the Aquatic Research Facility (ARF), IoA, UoS, for an acclimation period of ten days at 23 ± 2 ^{o}C to replicate the natural epidemiological conditions at which the disease occurs. A high stocking density and air bubbles were used to prevent the hierarchical aggression commonly presented in this species. During periods of acclimation and infection, fish were fed twice a day at a rate of 2% of their biomass.

2.2.5.3 IP challenge model

Sixteen fish were placed in a 2 litre plastic tank with a water volume adjusted to 1.8 litres. On the day of the challenge, the fish were anaesthetised with MS-222 (100mg/l) and IP injected with 0.1ml of a bacterial suspension at an OD_{600} of 0.4 (~$1.0x10^{9}$ CFU/ml).

2.2.5.4 Ethics

These experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and complied with local institutional regulations. Due to the nature of the experiment and to comply with the British legislation no replicate nor control tanks were used.

2.3 Results

2.3.1 Presumptive diagnosis

2.3.1.1 Diseased fish examination

During the follow up visit to the farms experiencing the outbreaks, the examined fish showed frayed fins, scale loss, pale skin, white gills and emaciation. At necropsy most of the organs were enlarged and either haemorrhagic or pale. In some fish, the anterior kidneys were hyperaemic, protruding and up to 3X enlarged, resembling a raspberry in appearance. Most of the fish presented white nodules in the spleen, posterior kidney and liver, and in some severe cases, these covered over 60% of the tissue surface. Figure 2.2.

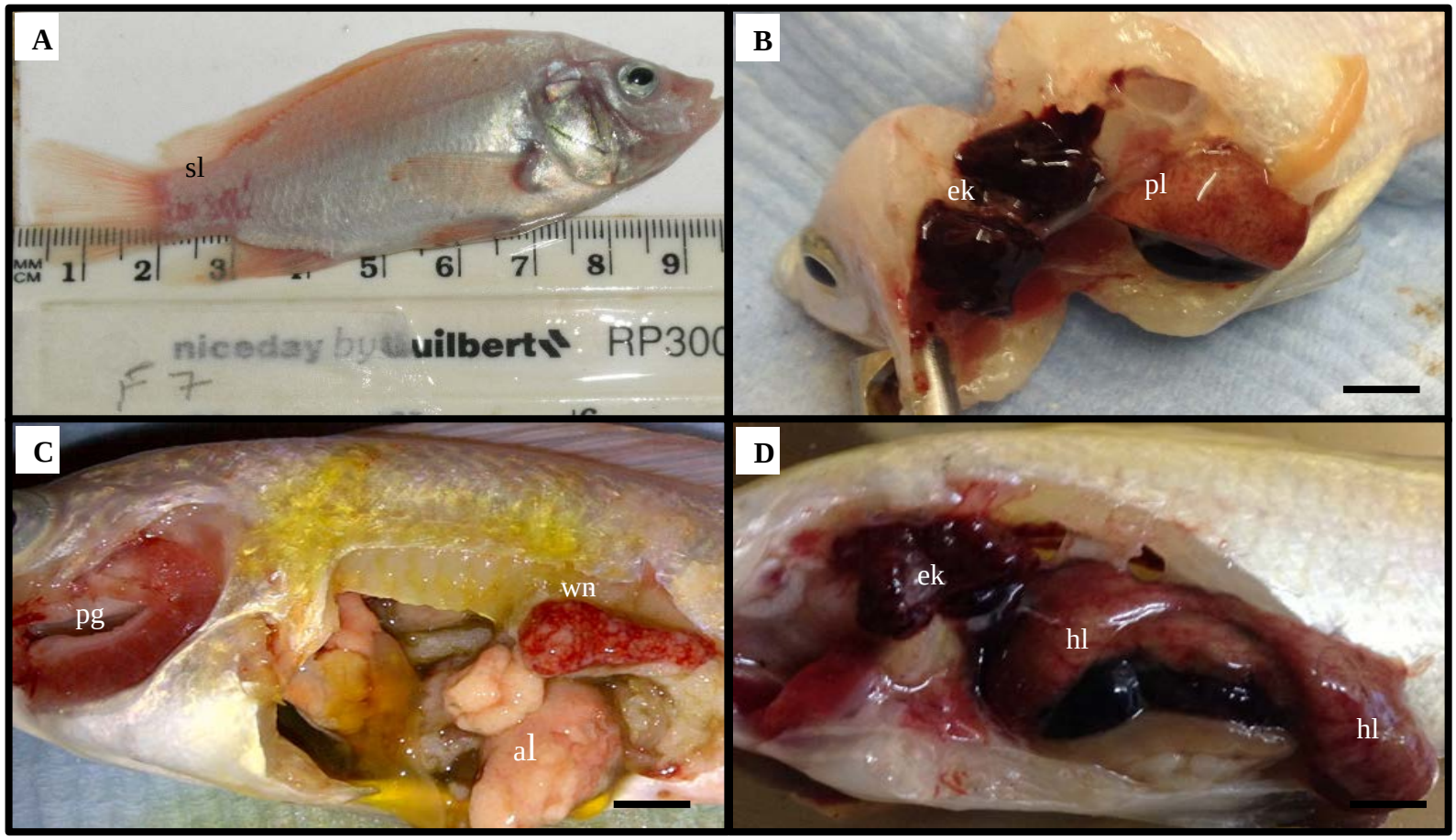

Figure 2.2 Diseased red Nile tilapia (*O. niloticus*) examined at the farms. **A** Fish seven from the second farm "F2f7" (size of average sampled fish) sl indicates scale loss. **B** Enlarged and haemorrhagic head kidney (ek) and pale liver (pl). **C** Enlarged spleen with white nodules (wn), pale gills (pg), atrophic liver (al). **D** Enlarged head kidney with raspberry appearance (ek) and enlarged-haemorrhagic liver (hl). Scale bar=1.0 cm.

2.3.1.2 Histopathological analyses

Histological observations revealed the presence of extensively distributed granulomatous inflammation. The affected tissues showed necrotising vasculitis and infiltration of mononuclear cells and neutrophils. The granuloma content was dominated by hypertrophied macrophages, fibroblasts and leukocytes. Most granulomas exhibited necrotic cores. The most severely affected tissues were the spleen and the anterior kidney, with granuloma structures covering up to 60% of the parenchyma. Granuloma formation was also seen in heart, liver, gills (Figure 2.3).

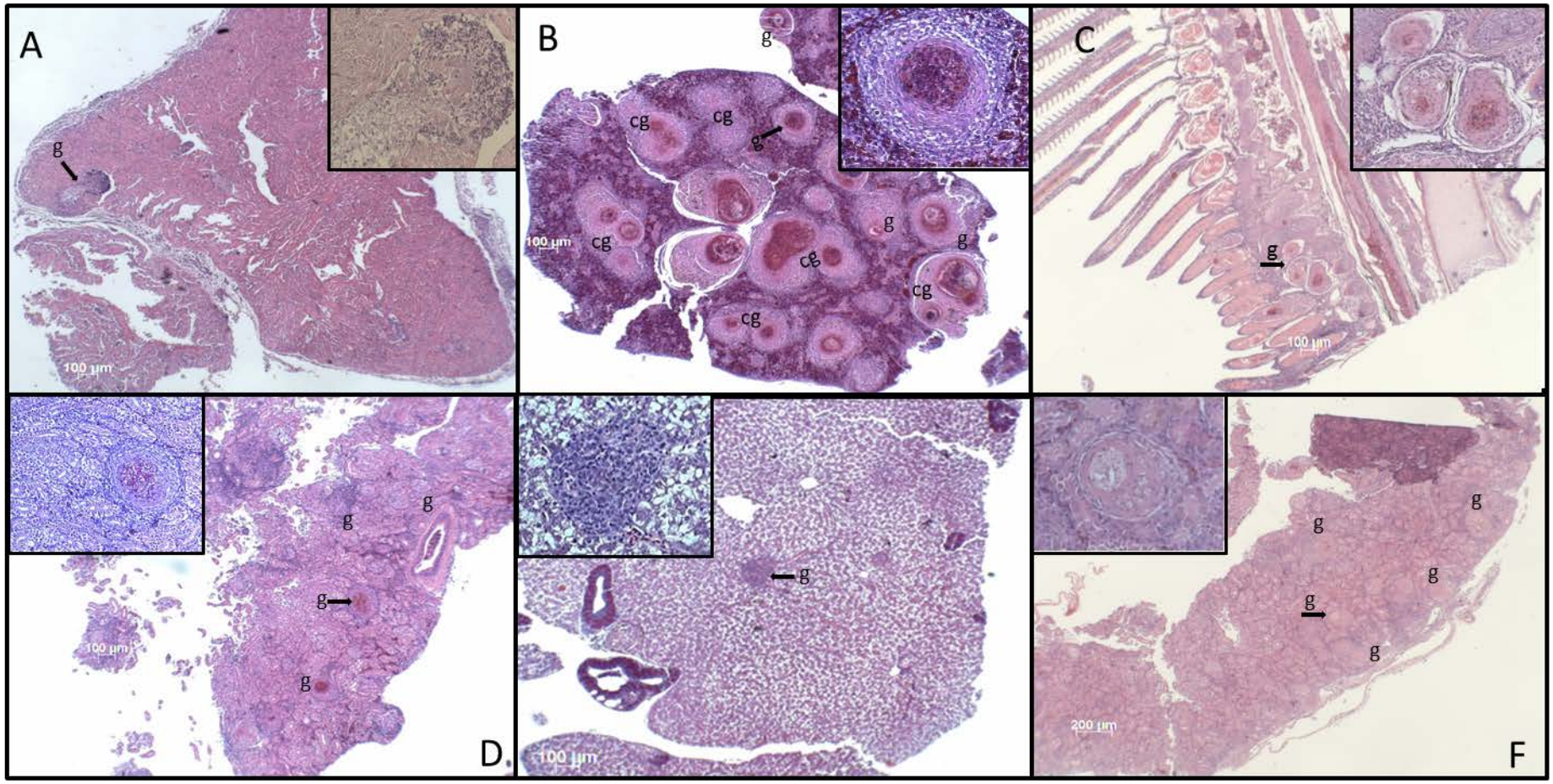

Figure 2.3 Histopathological findings of diseased red Nile tilapia (*O. niloticus*) using H&E technique. **A** Heart; **B** Spleen; **C** Gill; **D** Head kidney; **E** Liver; **F** Posterior kidney. Letter g indicates the presence of granuloma and cg coalescence of granulomas. The micrographs were taken at 40X magnification. The arrows indicate the section amplified in the black upper squares which show pictures taken at 200X magnification.

2.3.1.3 Transmission scanning and electron microscopy analyses

By TEM, it was possible to find pleomorphic coccobacillary bodies ranging in size from 0.2-0.4µm (width) to 0.4-1.7µm (length) Figure 2.4. These structures could be observed inside the phagocytic cells, sometimes free in the cytoplasm and most frequently within vacuoles surrounded by an electron lucent membrane.

For the SEM analyses the tissues were dry-fractured after being gold sputter coated to release the content of the granulomas. On the SEM micrographs the 3 dimensional structure of the bacteria resembled the shape of a corn grain and was visible in both samples from the spleen and head kidney Fig. 2.5.

Additionally, the presence of vesicles with electron dense membranes detaching from the bacterial cells was detected in TEM. The size of these structures was 60-80nm width for 90-100nm length Figure 2.6. Similar ultra-structures have been recently described by Brudal *et al.* (2014b) as “outer membrane vesicles” (OMVs) in *Fnn* grown *in vitro* and *in vivo* in zebrafish and in that study the structures contained virulence factors that played a key role in pathogenesis. More research is required to confirm if the structures here described in STIR-GUS-F2f7 are OMVs with similar functions to those presented in *Fnn*.

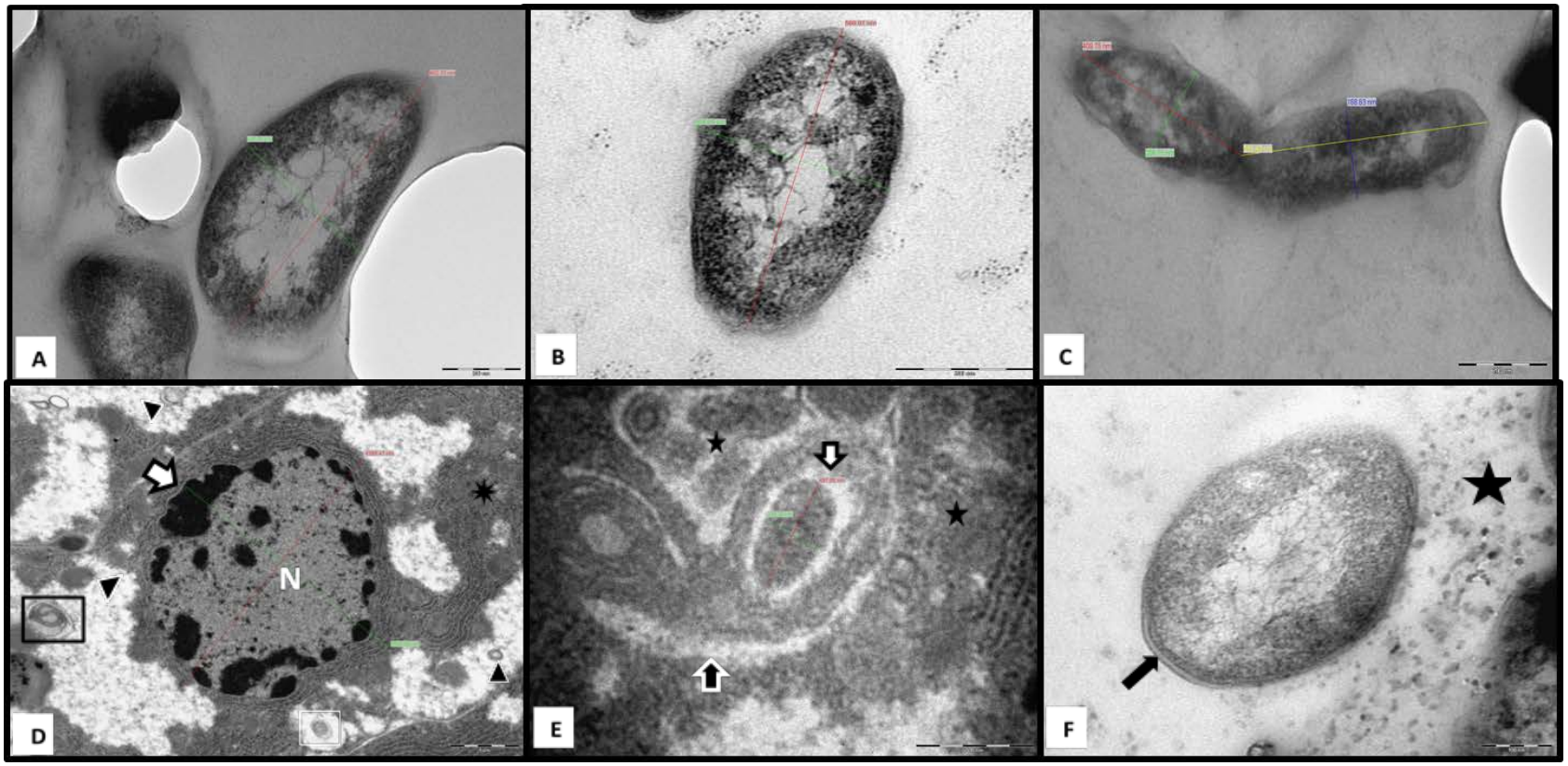

Figure 2.4 Transmission electron micrographs of diseased Nile tilapia head kidney. **A** to **C** Show extracellular localisation of pleomorphic *Francisella*-like structures. C Shows the replication of bacteria. In A to C Scale bars=200nm. **D** to **F** Intracellular localisation of *Francisella*-like cells. **D** Infected macrophage showing early signs of apoptosis, cytoplasmic condensation (asterisk), chromatin aggregation and marginalisation (white arrow), nucleus (N), black square (bacterial replicating within a phagosome), white square (bacteria surrounded by electron lucent space), black arrowhead (bacteria free in the cytoplasm). Scale bar=1µm. **E** intra-phagosomal replication of bacteria, black arrow phagosome membrane, white arrow (electron lucent space surrounding the bacteria), stars (vesicular and membranous bodies). Scale bar=500nm. **F** Bacterial cell free in the cytoplasm, black arrow (cell wall), star vesicular bodies. Scale bar=100nm.

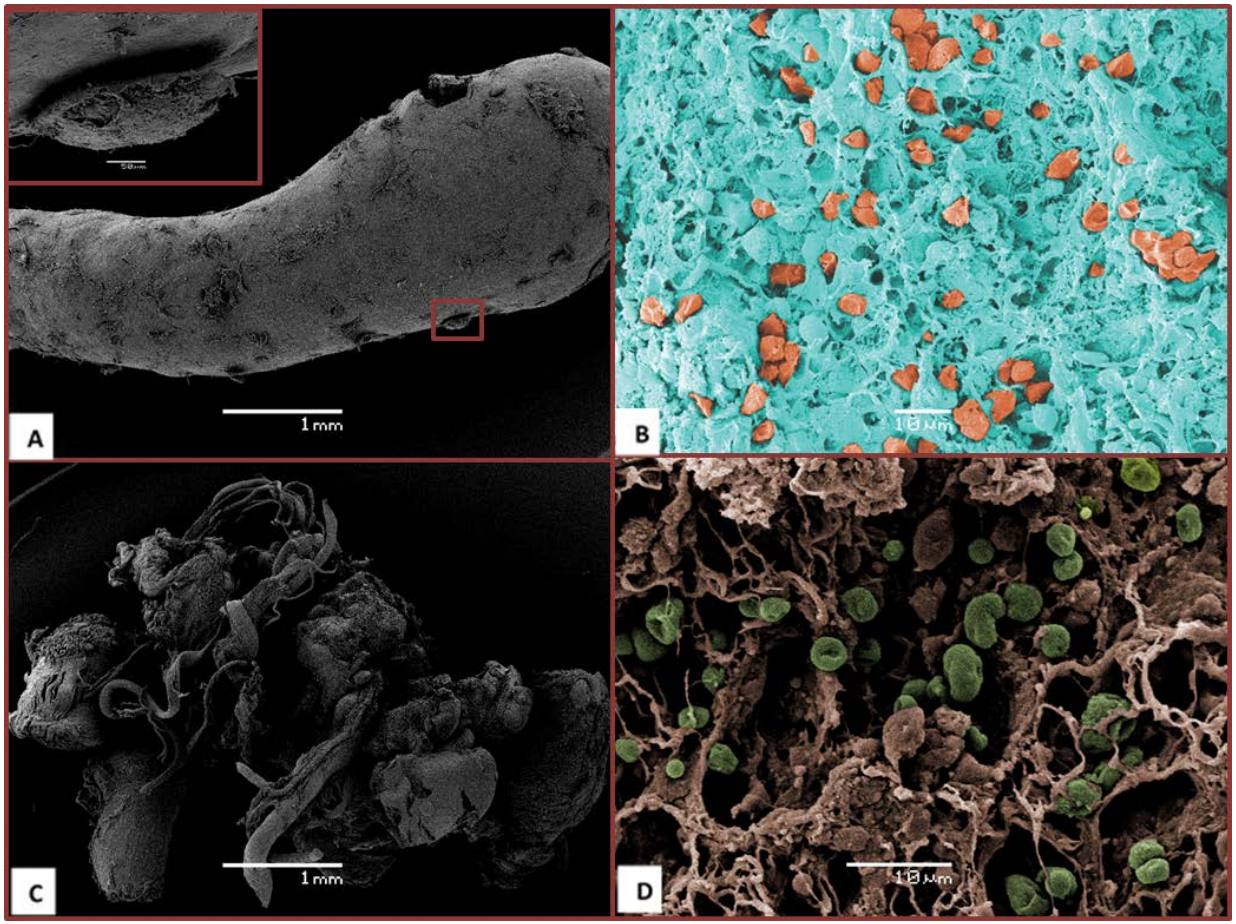

Figure 2.5 Scanning electron micrographs of moribund Nile tilapia spleen and head kidney. **A** Enlarged spleen with several granuloma formations. Red square shows single granuloma presented at a higher magnification where the scale bar=50µm. **B** Content of the spleen after fracturing the tissue, several *Francisella*-like structures coloured in orange. **C** Enlarged head kidney with multiple granulomas. **D** Interior of the kidney tissue, pleomorphic *Francisella*-like cells coloured in green.

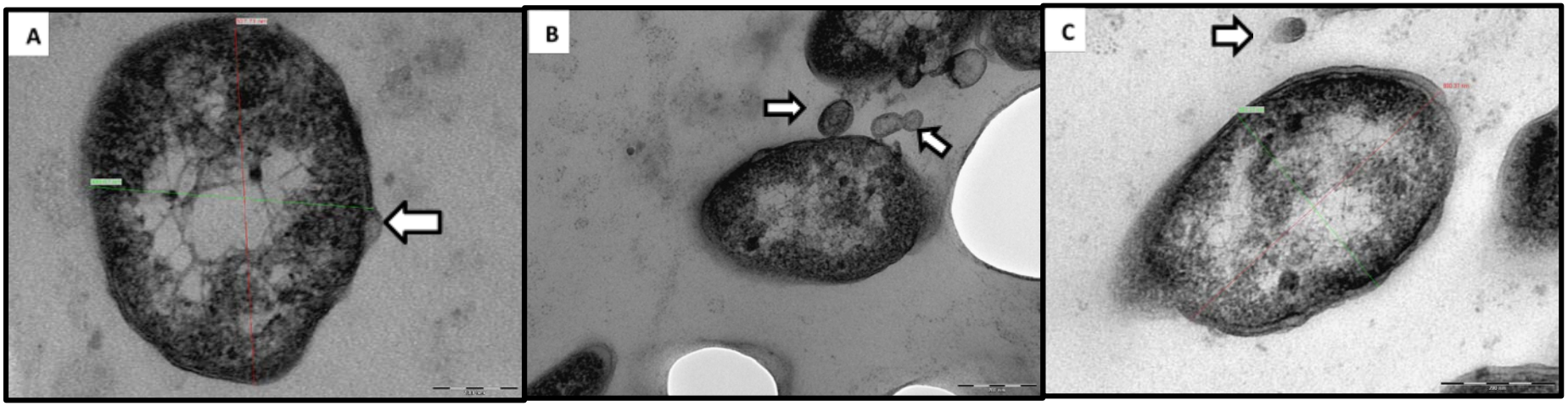

Figure 2.6 Transmission electron micrographs of OMV-like structures. **A** to **C** Extracellular localisation of *Francisella*-like cells, white arrows indicate the OMV-like structures. **A** Bacterial membrane blebbing (arrowed). Scale bar=100nm. **B** Budding and secretion of vesicular membranous bodies (arrowed). Scale bar=200nm. **C** Bacterial cell next to a free floating vesicle (arrowed). Scale bar=200nm**.**

2.3.1.4 Molecular diagnosis using a genus specific PCR

For molecular diagnosis, the PCR used to amplify a genus *Francisella* specific sequence, yielded a product of approximately 1.2Kbp in five of the ten samples tested, confirming the presence of a *Francisella* sp. in the tissues of the diseased fish. The band intensity was high in lanes 4, 9 and 11, moderate in lane 5 and almost imperceptible in lane 7 Figure 2.7.

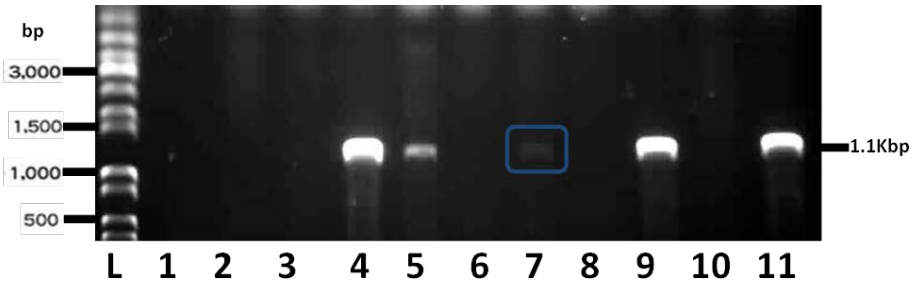

Figure 2.7 Molecular diagnosis of piscine francisellosis. PCR amplification of a genus *Francisella* specific sequence. L 1Kb ladder; 1 negative control (water); 2-6 samples from Farm-1. 7-11 samples from Farm-2, blue square, a weak band from sample six.

2.3.2 Bacterial isolation and identification

After molecular identification and purification, a total of 10 new *Francisella* sp. isolates (seven from the first farm and three from the second farm) were recovered and preserved (Table1). Of the different culture media tested to isolate the pathogen, the CHAH, CHATB and the commercial MTMA were successful for primary recovery of novel isolates. No noteworthy differences were observed between the CHATB plates with and without antibiotics, and no dominant or notable colonies were observed on TSA. The commercial MMLA failed to isolate any bacterium directly from fish tissues and although some growth was observed after plating pure *Fn*, this was not sufficient to be

considered an option for culturing these isolates. The results obtained with the spleen homogenates of the 9th fish 2nd farm (STIR-AVU-F2f9) are presented in Figure 2.8.

The recipe to prepare 100ml of the new 5% tilapia blood enriched CHA without antibiotics is: 95ml of water, 5ml of tilapia blood and 5.1g of CHA. The blood can be heparinised and should be added after autoclaving the agar when it has cooled down to ~52°C.

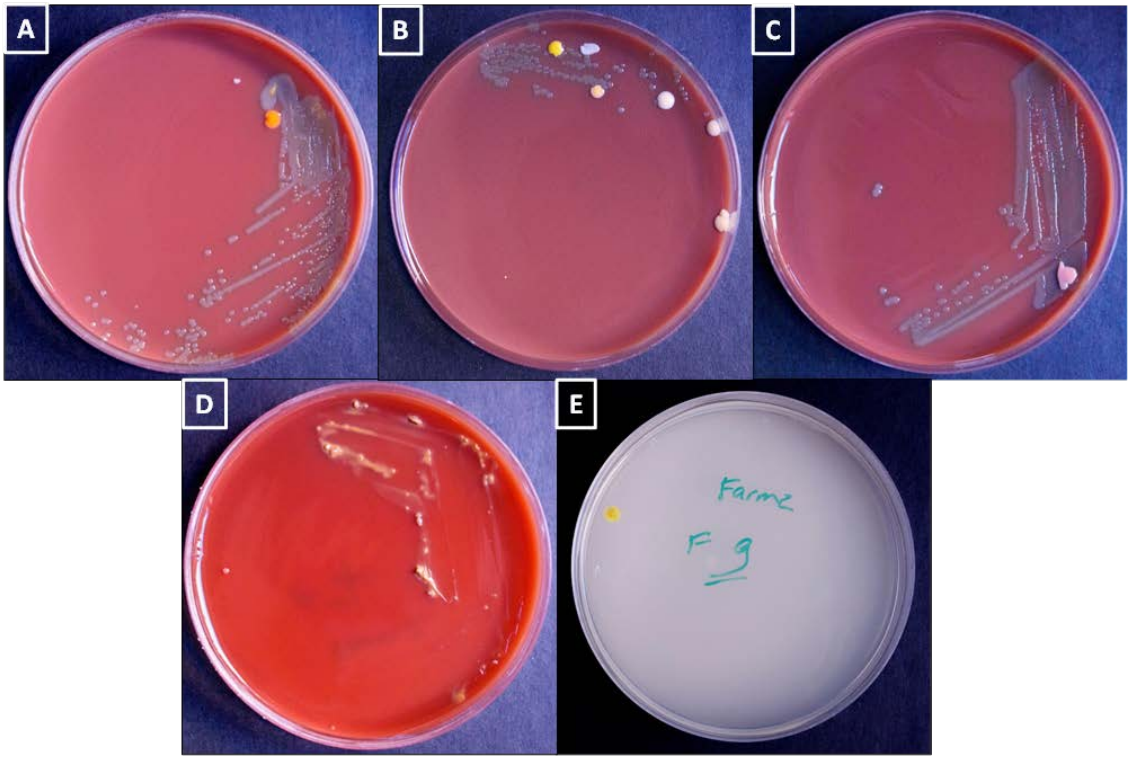

Figure 2.8 Media used for the primary isolation of *Francisella* sp. from Nile tilapia. **A** Cysteine heart agar + bovine haemoglobin. **B** Modified Thayer Martin agar. **C** Cysteine heart agar tilapia blood. **D** Modified Martin Lewis agar. **E** Trypticase soy agar. In all the plates the colonies were convex, smooth and with a grey-greenish colour.

2.3.3 Bacterial phenotypic characterisation

2.3.3.1 Optimal growth temperature and growth curves in vitro

The optimal culture temperature *in vitro* was 28.0-28.5 °C on agar plates for all *Fno* isolates tested, at this temperature the colonies appeared after 64h. No growth was observed at temperatures of 18°C or lower, nor at 33°C or higher. Visible colonies appeared after 120h at 22°C, 87h at 24°C and 69h at 26°C.

Although growth started to appear after only 48h on plates incubated above 28°C, colonies on these plates were only visible after 72h at 29°C, 75h at 30 °C and 144h at 32°C. The growth of the novel isolate STIR-GUS-F2f7 occurred a few hours faster than the type strain Ehime-1.

Under the conditions described, the exponential phase of the growth curve started after 15 hours, the mid log phase was between 18 to 23 hours and the stationary phase was reached after 30 hours.

2.3.3.2 Carbohydrate fermentation and enzymatic activity

Using the API20E kit, only the CIT (citrate), VP (Voges–Proskauer) and GEL (gelatinase) panels showed a positive reaction. This demonstrated the ability of the strains to utilise the citrate as a carbon source, produce acetoin from sodium pyruvate and hydrolyse gelatine. No differences were observed between the novel *Fno* isolates and the type strain Ehime-1.

The use of the APIZYM kit revealed an identical profile among the different *Fno* isolates where eight of the twenty enzymes were reactive. These enzymes are (in decreasing order of intensity): acid phosphatase, naphthol-AS-BI-phosphohydrolase, esterase lipase (C8), alkaline phosphatase, esterase (C4), Lipase (C14), α-chymotrypsin, and β-galactosidase.

2.3.3.3 Carbon metabolism (metabolic fingerprint)

According to the inoculum gradient, an OD_{600} of 0.86 was optimal to test the *Fno* isolates in the Biolog GN2 micro plates. With this density several false negatives appeared now as positives and the plates developed a full profile after only six hours post inoculation with the control well remaining clearly as negative. The use of higher densities led to the appearance of colour in the control well making the interpretation of these results unreliable or biased. For this reason although D-cellobiose (A12), mono-methyl-succinate (C12), α-keto glutaric acid (E4), D,L-lactic acid (E6), succinic acid (E12), bromo succinic acid (F1), glycyl-Lglutamic acid (F12), L-ornithine (G4) and Thymidine (H4) appeared to be positive using OD_{600} of 0.96 and 1.06 they were recorded as negative. The digitalised images of the scanned STIR-GUS-F2f7 Biolog GN2 plates at an OD_{600} 0.86 and 0.96 are presented in Figure 2.9.

The results of the spectrophotometer did correlate in most of the plates with the colour change in the wells. The numeric value of the control well was subtracted from the rest of the wells to normalise the results and a control reading was taken immediately after inoculating. These figures were useful to support the findings when they were correlated with the visual inspection of the plates but they are not shown in this study since a high OD was presented in some wells in with no colour change i.e. well A10.

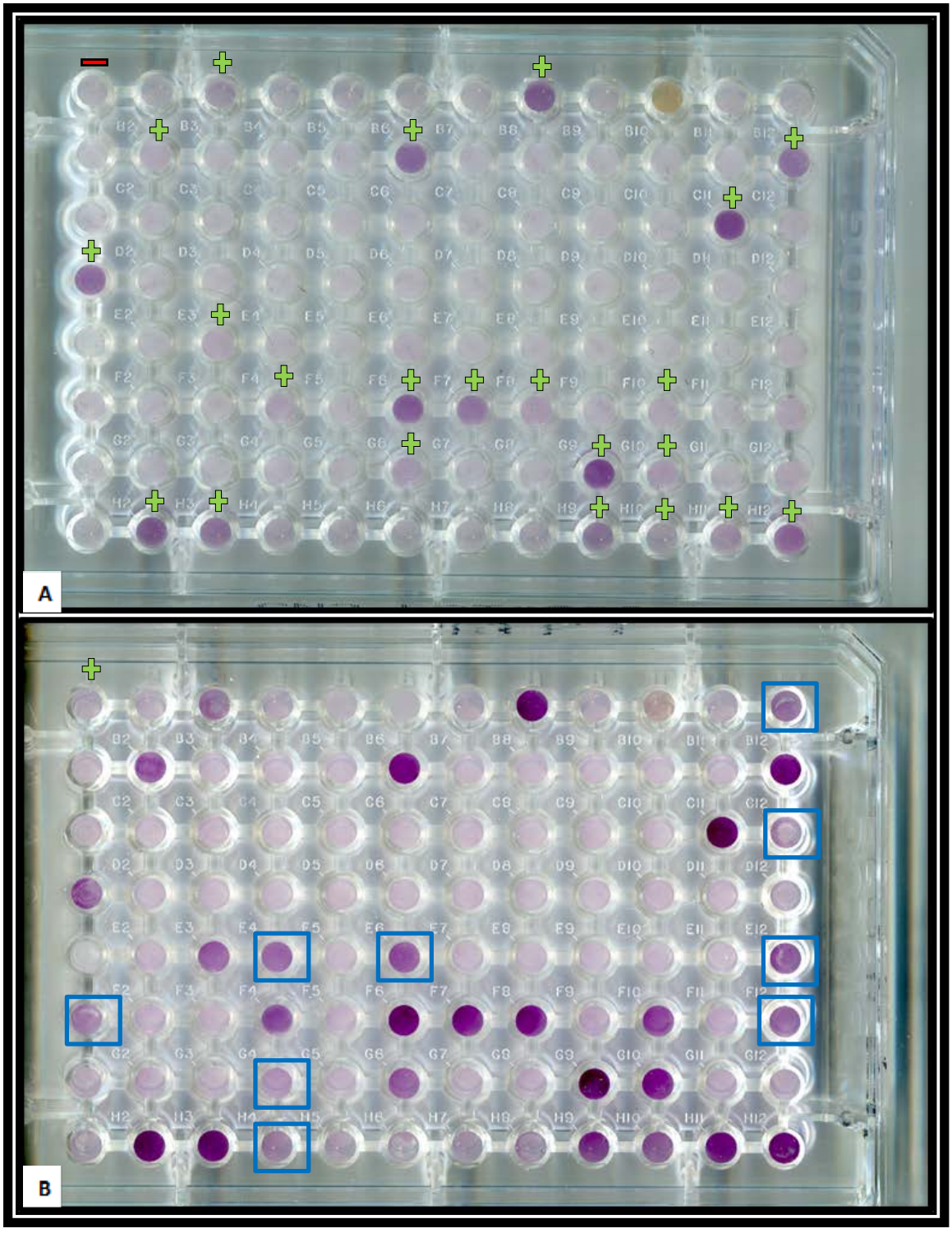

Figure 2.9 Phenotypic fingerprint of *Fno* STIR-GUS-F2f7. **A** Photograph of the plates with an OD_{600} 0.86. Well A1 is negative, green plus symbol (+) indicate positive wells where colour has changed. **B** Photograph of the plate with an OD_{600} 0.96. Blue squares indicate ambiguous results when control well A1 appears to be positive.

The phenotypic fingerprints of all the isolates tested in this study at an OD_{600} of 0.86 are presented in Table 2.4. No differences were observed between the metabolic fingerprints of the isolates recovered from tilapia in the present study and the type strain Ehime-1. The isolate PQ1104 from Costa Rica had an almost identical profile to the other *Fno* isolates with only in 1 difference of the 95 carbon sources tested (i.e. acetic acid).

Table 2.4 Metabolic fingerprint of the different *Fno* isolates at an OD_{600} of 0.86

CARBON SOURCE TEST		*Fno* 0.86			
Well		1	2	3	4
A1	Water	-	-	-	-
A3	Dextrin	+	+	+	+
A8	N-Acetyl-Dglucosamine	+	+	+	+
B2	D-Fructose	+	+	+	+
B6	α-D-Glucose	+	+	+	+
B12	D-Mannose	+	+	+	+
C11	Methyl Pyruvate	+	+	+	+
D1	Acetic Acid	+	+	-	+
E3	α-Keto Butyric Acid	+	+	+	+
F4	L-Alaninamide	+	+	+	+
F6	L-Alanine	+	+	+	+
F7	L-Alanylglycine	+	+	+	+
F8	L-Asparagine	+	+	+	+
F10	L-Glutamic Acid	+	+	+	+
G6	L-Proline	+	+	+	+
G9	L-Serine	+	+	+	+
G10	L-Threonine	+	+	+	+
H2	Inosine	+	+	+	+
H3	Uridine	+	+	+	+
H9	Glycerol	+	+	+	+
H10	D,L-α-Glycerol Phosphate	+	+	+	+
H11	Glucose-1-Phosphate	+	+	+	+
H12	Glucose-6-Phosphate	+	+	+	+

Isolates: 1, Ehime-1; 2, STIR-GUS-F2f7; 3 PQ1104; 4, STIR-MATT-F1f6.

2.3.3.4 Chemotaxonomic analyses (cellular fatty acids methyl esters)

The FAME profiles were similar for all the *Fno* isolates tested and no differences in the order of the major components among the isolates were observed. The predominant fatty acids for the novel strain STIR-GUS-F2f7 were: 24:1 (20.3%), 18:1n-9 (16.9%), 24:0 (13.1%) 14:0 (10.9%), 22:0 (7.8%), 16:0 (7.6%) and 18:0 (5.5). An overview of the relative composition of the *Fno* strains here analysed is presented in Table 2.5

Table 2.5 Relative fatty acid composition (%) of *Fno* STIR-GUS-F2f7 and the other *Fno* isolates after 43h incubation in Modified Muller Hinton broth

Fatty acid	STIR-MATT-F1f6	STIR-GUS-F2f7	EHIME-1	*Fno* avergae
14:0	10.96 ± 0.03	10.92 ± 0.30	9.72 ± 0.52	11
Iso 15:0	0.03 ± 0.03	0.06 ± 0.04	0.13 ± 0.03	0
15:0	0.27 ± 0.05	0.35 ± 0.05	0.37 ± 0.02	0
16:0	7 ± 0.18	8 ± 0.51	8 ± 0.12	8
17:0	0.45 ± 0.01	0.52 ± 0.04	0.54 ± 0.03	1
18:0	6.03 ± 0.09	5.50 ± 0.20	5.26 ± 0.12	6
19:0	0.17 ± 0.01	0.19 ± 0.01	0.25 ± 0.01	0
20:0	4.01 ± 0.10	3.61 ± 0.27	3.50 ± 0.07	4
16:0 3-OH	0.37 ± 0.05	0.52 ± 0.01	0.49 ± 0.02	0
21:0	0.09 ± 0.00	0.10 ± 0.01	0.15 ± 0.01	0
17:0 3-OH	0.18 ± 0.03	0.22 ± 0.09	0.39 ± 0.00	0
22:0	8 ± 0.09	8 ± 0.35	8 ± 0.06	8
18:0 3-OH	1.95 ± 0.22	2.67 ± 0.16	2.58 ± 0.16	2
23:0	0.42 ± 0.01	0.50 ± 0.04	0.63 ± 0.05	1
24:0	13 ± 1.38	13 ± 1.13	12 ± 0.09	13
25:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0
26:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0
Total saturated	53.61 ± 1.61	53.67 ± 1.22	51.39 ± 0.51	53
16:1n-9	0.29 ± 0.09	0.21 ± 0	0.24 ± 0.03	0
16:1n-7	0.54 ± 0.08	0.72 ± 0.18	0.66 ± 0.12	1
17:1	0.54 ± 0.02	0.78 ± 0.14	0.79 ± 0.05	1
18:1n-9	18 ± 0.28	17 ± 0.09	17 ± 0.31	17
18:1n-7	0.10 ± 0.06	0.12 ± 0.07	0.18 ± 0.01	0
19:1	0.17 ± 0.01	0.23 ± 0.02	0.24 ± 0.02	0
20:1	1.68 ± 0.03	1.63 ± 0.11	1.42 ± 0.03	2
21:1	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.00	0
22:1n-11	3.55 ± 0.19	4.40 ± 0.83	3.93 ± 0.39	4
23:1	0.75 ± 0.02	0.98 ± 0.10	1.23 ± 0.07	1
24:1	21.0 ± 1.15	20 ± 0.82	23 ± 0.91	21
Total monounsaturated	46.32 ± 1.61	46.25 ± 1.21	48.46 ± 0.52	47
18:2n-6	0.07 ± 0.0	0.08 ± 0.01	0.29 ± 0.01	0
Total n-6 PUFA	0.07 ± 0	0.08 ± 0.01	0.29 ± 0.01	0
TOTAL	100 ± 0	100 ± 0	100 ± 0	100

2.3.3.5 Antibacterial susceptibility tests

a) Broth microdilution method

There were no differences between the MIC of the antimicrobial compounds to STIR-GUS-F2f7 and the other *Fno* isolates, including type strain Ehime-1, and these results were consistent among replicates performed for all the strains. From the values obtained for the quality control, 37 of 39 fell within the range given by the CLSI (CLSI, 2006a) validating the use of the broth for this test and appropriateness of the assay. The antimicrobial MIC values obtained with the GN2F and AVIAN1F Sensititre Plates are presented in Table 2.6 including the range of variability observed.

b) Disc diffusion method

The inhibition zones observed on CHAH were clear, consistent and repeatable for all isolates. No substantial differences were found when comparing STIR-GUS-F2-f7 with the other *Fno* isolates. The largest zones of inhibition were found with enrofloxacin (5µg), kanamycin (30µg), and gentamicin (2µg); the results obtained using the Kirby–Bauer method are summarised in Table 2.7.

Table 2.6 Minimal inhibitory concentration (MIC) of the *Francisella noatunensis orientalis* isolates and the quality control *Escherichia coli* ATTC 25922 by the broth micro-dilution method to 39 antimicrobial compounds

Antimicrobial tested (concentration)	PQ1104		STIR-GUS-F2f7		Ehime-1		STIR-MATT-F1f6		*Fno*	*E. coli* ATCC 25922	
	1	2	1	2	1	2	1	2	range	present results	CLSI range
Enrofloxacin (2–0.12 mμ/ml)	<0.12	<0.12	0.25	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	0.008-0.03
Gentamycin (8–0.5 mμ/ml)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.5	0.25-1
Ceftiofur (4–0.25 mμ/ml)	>4	4	>4	2	>4	4	>4	4	2 to >4	0.5	0.25-1
Neomycin (32–2 mμ/ml)	<2	<2	<2	<2	<2	<2	<2	<2	<2	8	0.5-2
Erythromycin (4–0.25 mμ/ml)	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	N/A
Oxytetracycline (8–0.5 mμ/ml)	4	0.5	4	<0.25	1	0.5	2	0.5	<0.25 to 4	2	0.5-2
Tetracycline (8–0.25 mμ/ml)	4	0.5	4	0.25	2	1	2	0.5	0.25 to 4	8	0.5-2
Amoxicillin (16–0.25 mμ/ml)	>16	>16	>16	>16	2	2	16	2	2 to >16	4	4.0-16.0
Spectinomycin (64–8 mμ/ml)	32	<8	16	<8	32	16	32	<8	<8 to 32	>64	8.0-64.0
Sulphadimethoxine (256–32 mμ/ml)	>256	128	256	<128	256	256	>256	128	128 to >256	>256	8.0-64.0
Trimethoprim/sulfamethoxazole (2/38–0.5/9.5 mμ/ml)	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	<0.5/9.5	<0.5/9.5
Florfenicol (8–0.25 mμ/ml)	4	<1	4	<1	2	<1	2	<1	<1 to 4	8	4.0-16.0 2-8
Sulphathiazole (256–32 mμ/ml)	>256	64	256	64	>256	64	256	64	64 to >256	>256	N/A
Penicillin (8–0.06 mμ/ml)	>8	>8	>8	4	>8	4	>8	4	4 to >8	>8	N/A
Streptomycin (1024–8 mμ/ml)	<8	<8	<8	<8	<8	<8	<8	<8	<8	16	4.0-16.0
Novobiocin (4–0.5 mμ/ml)	2	1	2	<0.5	2	1	1	<0.5	<0.5–2	>4	N/A
Tylosin tartrate (20–2.5 mμ/ml)	>20	20	>20	20	>20	20	>20	20	20 to >20	>20	>32
Clyndamycin (4–0.5 mμ/ml)	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	N/A
Amikacin (64–8 mμ/ml)	<8	<8	<8	<8	<8	<8	<8	<8	<8	16	0.5-4.0
Ampicillin (32–4 mμ/ml)	>32	<4	>32	<4	>32	<4	16	<4	<4 to >32	<4	2.0-16.0 2-8
Ampicillin/sulbactam 2:1 ratio (4/2–32/16 mμ/ml)	32/16	< 4/2	>32/16	< 4/2	>32/16	< 4/2	>32/16	< 4/2	<4/2 to >32/16	<4/2	2/1-8/4
Aztreonam (32–8 mμ/ml)	>32	32	>32	16	>32	32	>32	16	16 to >32	<8	0.06-0.25
Cefazolin (32–4 mμ/ml)	>32	32	>32	32	>32	32	>32	32	32 to >32	<4	1.0-4.0
Cefepime (32–4 mμ/ml)	>32	<4	>32	<4	>32	<4	>32	<4	<4 to >32	<4	0.015-0.12
Cefotetan Na (32–8 mμ/ml)	>32	>32	>32	>32	>32	>32	>32	>32	>32	<8	0.12-1
Ceftriaxone (64–1 mμ/ml)	>64	<1	>64	<1	>64	4	>64	<1	<1 to >64	<1	0.03-0.12
Ceftazidime (32–1 mμ/ml)	>32	<1	>32	<1	>32	<1	>32	<1	<1 to >32	<1	0.06-0.5
Cefuroxime (32–4 mμ/ml)	>32	32	>32	32	>32	32	>32	32	32 to >32	8	2.0-8.0
Ciprofloxacin (4–0.5 mμ/ml)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.004-0.015
Gatifloxacin (8–1 mμ/ml)	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	0.008-0.03
Meropenem (8–1 mμ/ml)	>8	4	>8	2	8	4	>8	2	2 to >8	<1	0.008-0.06
Imipenem (16–2 mμ/ml)	>16	8	>16	16	>16	16	>16	<2	<2 to >16	<2	0.06-0.25
Nitrofurantoin (128–16 mμ/ml)	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	4.0-16.0
Cefoxitin (32–4 mμ/ml)	>32	>32	>32	>32	>32	>32	>32	>32	>32	8	2.0-8.0
Piperacillin (128–16 mμ/ml)	>128	<16	>128	<16	>128	>128	>128	<16	<16 to >128	<16	1.0-4.0
Piperacillin/tazobactam constant 4 (128/4–16/4 mμ/ml)	>128 /4	<16/4	>128 /4	<16/4	>128 /4	<16/4	>128 /4	<16/4	<16/4 to >128/4	<16/4	1/4-4/4
Ticarcillin/clavulanic acid constant 2 (64/2–16/2 mμ/ml)	>64/2	<16/2	>64/2	<16/2	>64/2	<16/2	>64/2	<16/2	<16/2 to >64/2	<16/2	4/2-16/2
Tobramycin (8–4 mμ/ml)	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	0.25-1
Cefpodoxime (16–2 mμ/ml)	>16	<2	>16	<2	>16	<2	>16	<2	<2 to >16	<2	0.25-1

Table 2.7 Antimicrobial susceptibility test by the disc-diffusion (Kirby–Bauer) method for STIR-GUS-F2f7 and other *Fno* isolates in CHAH after 72h incubation, the mean zone diameters (mm) and standard deviation are presented.

Antimicrobial (concentration)	STIR-GUS-F2f7	Ehime-1	STIR-MATT-F1f6	PQ1104
Enrofloxacin (5µg/disk)	42 ± 3.46	40.5 ± 1	42.5 ± 1.91	43.5 ± 3.42
Kanamycin (30µg/disk)	35.3 ± 4.16	34.5 ± 4.12	38 ± 1.63	42.5 ± 1.91
Gentamicin (2µg/disk)	32 ± 2	29 ± 3.83	32.5 ± 1.91	33 ± 5.03
Tetracycline (30µg/disk)	22 ± 2	20 ± 2	23 ± 1.41	20 ± 2
Oxytetracycline (30µg/disk)	29.5 ± 4.43	26 ± 4.9	28.5 ± 1.91	27.5 ± 1.91
Florfenicol (30µg/disk)	22.8 ± 1.1	25.3 ± 1.15	24.8 ± 5.4	24.8 ± 2.28
Oxolinic acid (2µg/disk)	20.5 ± 4.73	18.5 ± 3.79	18.5 ± 4.12	21.5 ± 1.91
Streptomycin (10µg/disk)	27.5 ± 1.91	23 ± 1.41	22 ± 2	23 ± 1.41
Penicillin G (10units)	0	0	0	0
Lincomycin (15µg/disk)	0	0	0	0
Oleandomycin (15µg/disk)	0	0	0	0
Amoxycillin (10µg/disk)	0	0	0	0
Carbenicillin (100µg/disk)	0	0	0	0
Polymyxin B (300 units)	0	0	0	0
Ampicillin (10 µg/disk)	0	0	0	0
Sulphamethoxazole/ trimethoprim 19:1 (25µg/disk)	0	0	0	0

2.3.4 Bacterial genetic characterisation

2.3.4.1 Bacterial genomic DNA extraction

According to the NanoDrop™ data, the crude gDNA from STIR-GUS-F2f7 had an initial concentration of 622ng/µl and values of 2.1 and 1.8 for the 260/280 and 260/230 ratios respectively. The initial concentration of the DNA was adjusted to 50ng/µl prior to its use in the PCRs.

2.3.4.2 Housekeeping genes (selection and sequencing)

Of the 18 pairs of primers tested for amplification of the 12 housekeeping genes, 17 yielded the product size expected Figure 2.10. The primers for the gene pgm did not amplify the expected product in the PCR and this gene was therefore not sequenced nor further analysed.

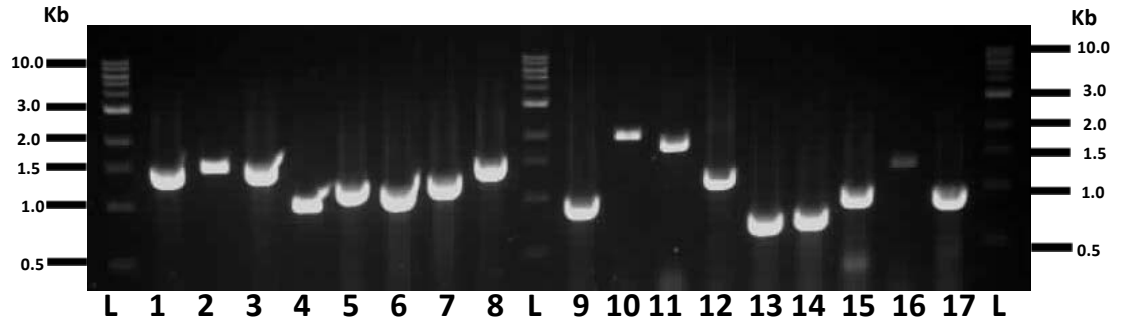

Fig. 2.10 PCR amplification of *Fno* STIR-GUS-F2f7 housekeeping genes. L's 1Kb ladders. 1 dnaA. 2 mutS-1. 3 mutS-2. 4 prfB. 5 putA-1. 6 putA-2. 7 putA-3. 8 putA-4. 9 rpoA. 10 rpoB-1. 11 rpoB-2. 12 rpoB-3. 13 tpiA. 14 mdh. 15 16SrRNA-ITS-23SrRNA-1. 16 16SrRNA-ITS-23SrRNA-2. 17 16SrRNA-ITS-23SrRNA-3.

2.3.4.3 Sequences homology

The nine STIR-GUS-F2F7 gene sequences shared an identity of 99-100% with those from other *Fno* strains. After *Fno*, the closet genetically related sequences were those belonging to members of *Fnn* and *Fp*, followed by sequences from the 4 *Ft* subspecies, *F. halioticida* and *F. guangzhouensis*. The similarity in percentage (%) between the sequences from STIR-GUS-F2f7 and the sequences from these *taxa* is presented in Table 2.8.

Table 2.8 Range of gene sequence similarity in percentage (%) between *Fno* STIR-GUS-F2f7 and the validly described members of the genus *Francisella*

Gene sequences	*Fno*	*Fnn*	*Fp*	*Ft* ssp.*	*Fhal*	*Fg*
dnaA	99-100	99	94-95	85-86	84	n/a
mutS	100	93-94	92-93	76-77	75	n/a
prfB	99-100	94	93-95	84-86	81	n/a
putA	99	96	96-97	80-81	75	n/a
rpoA	99	99	92-93	83-84	83	77
rpoB	99	91	92	86	84	87
tpiA	99-100	98	94-96	78-80	79	n/a
mdh	100	98-99	96	84-86	85	84
16SrRNA+ITS+23SrRNA	99-100	98-99	99	97	98	94-95

* Includes data from isolates of the four *F. tularensis* subspecies
n/a: not sequences were available in the GeneBank database

2.3.4.5 Phylogenetic analyses

The 16SrRNA tree (Fig. 2.11) was constructed with several sequences from all the validly described *Francisella* species and subspecies; this tree illustrates the evolutionary history and current taxonomy of the genus while. The trees generated with the nine housekeeping genes (Fig. 2.12) depict the evolutionary relation of STIR-GUS-F2f7 with its closest genetically related *taxa*: *Fnn* and *Fp*.

In both figures (2.11 and 2.12) the NCBI accession numbers are indicted next to the sequences retrieved. The final number of nucleotides in the STIR-GUS-F2f7 sequences were 16SrRNA 1339, dnaA 1299, mdh 576, mutS 2327, prfB 882, putA 2340, rpoA 496, rpoB 3042, tpiA 507 and 16SrRNA-ITS-23SrRNA 2679 after trimming and manually adjusting them.

The optimal models determined for the phylogenetic analyses were Kimura 2-parameter (K2) (Kimura, 1980) for 16SrRNA tree; Tamura 3-parameter (T92) (Tamura, 1992) for dnA, mdh rpoA and tpiA; Tamura-Nei (TN93) (Tamura and Nei, 1993) model for mutS and rpoB; and Hasegawa-Kishino-Yano (HKY) (Hasegawa *et al.*,1985) for prfB, putA and the concatenated 16SrRNA-ITS-23SrRNA. A summary of the specific model used, rates among sites, number of positions on the final datasets and number of sequences used on each phylogenetic tree are presented in Table 2.9.

Table 2.9 Evolutionary models used on the phylogenetic analyses

Gene-sequence	Model	Rates among sites	positions	sequences
16SrRNA	K2	Discrete gamma distribution	1350	50
dnaA	T92	Discrete gamma distribution	1298	12
mdh	T92	Uniform rates	576	12
mutS	TN93	Discrete gamma distribution	2326	13
prfB	HKY	Discrete gamma distribution	880	12
putA	HKY	Discrete gamma distribution	2333	12
rpoA	T92	Discrete gamma distribution	464	17
rpoB	TN93	Discrete gamma distribution	3042	13
tpiA	T92	Discrete gamma distribution	507	15
16SrRNA+ITS+23SrRNA	HKY	Uniform rates	2676	9

In all the phylogenetic trees, the novel isolate STIR-GUS-F2f7 was seen to group within the *Fno* clade and the evolutionary divergence between the two *Fn* subspecies seemed as distant as, or even bigger than, that between them and the *F. philomiragia* species (*Fp*).

In 8 of the 11 trees a subclading was observed within the *Fn* taxon, this subdivision clustered the sequences of isolates recovered in the northern hemisphere mainly from diseased cod in Norway and separated them from those isolated from diseased salmon in Chile. This branching was well supported with bootstrap values ranging from 70% in the 16SrRNA tree to 97% in the mdh, putA and rpoB trees.

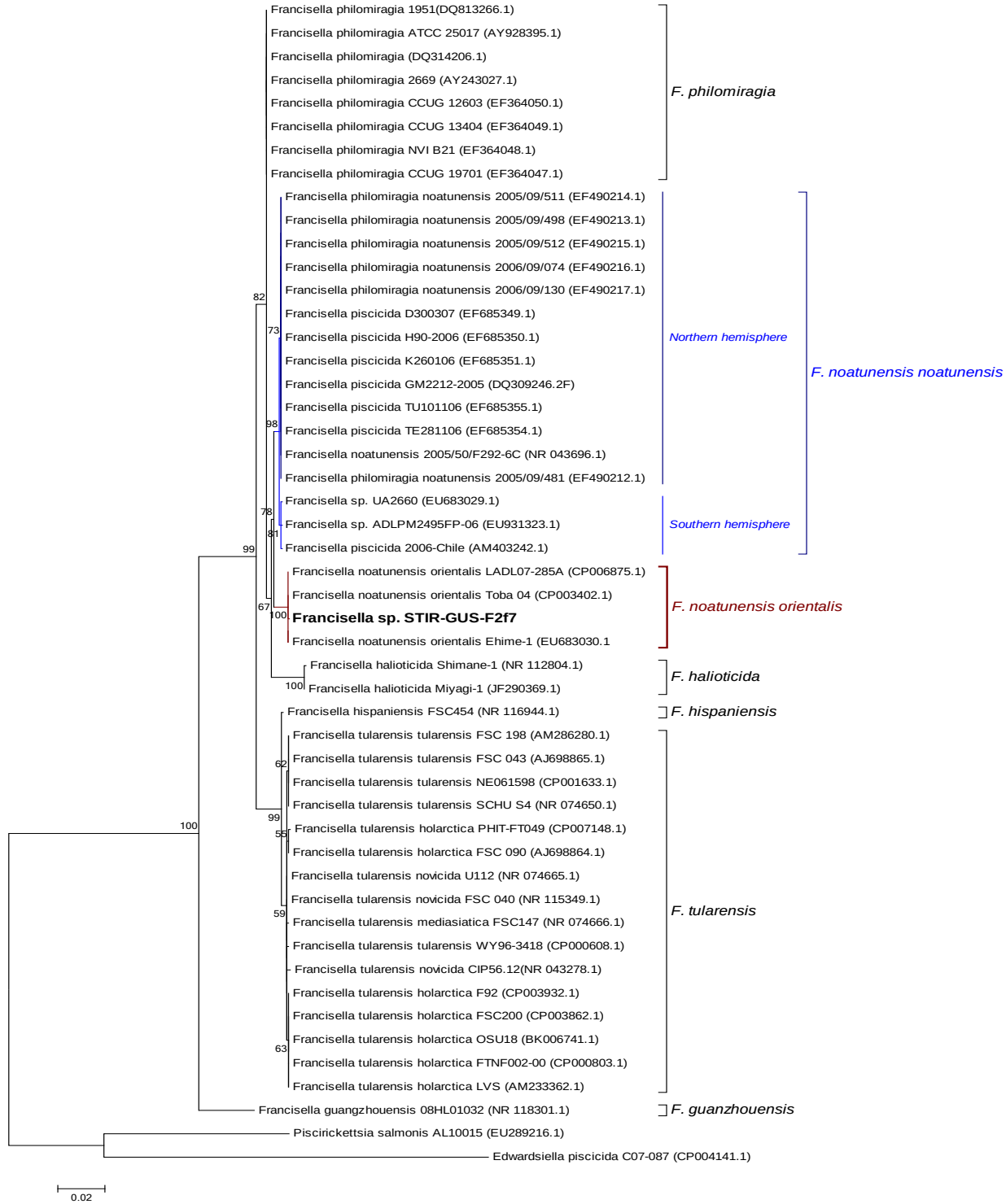

Figure 2.11 Molecular phylogenetic analysis of the genus *Francisella* based on 50 partial 16SrRNA sequences. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-4004.5670) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach.

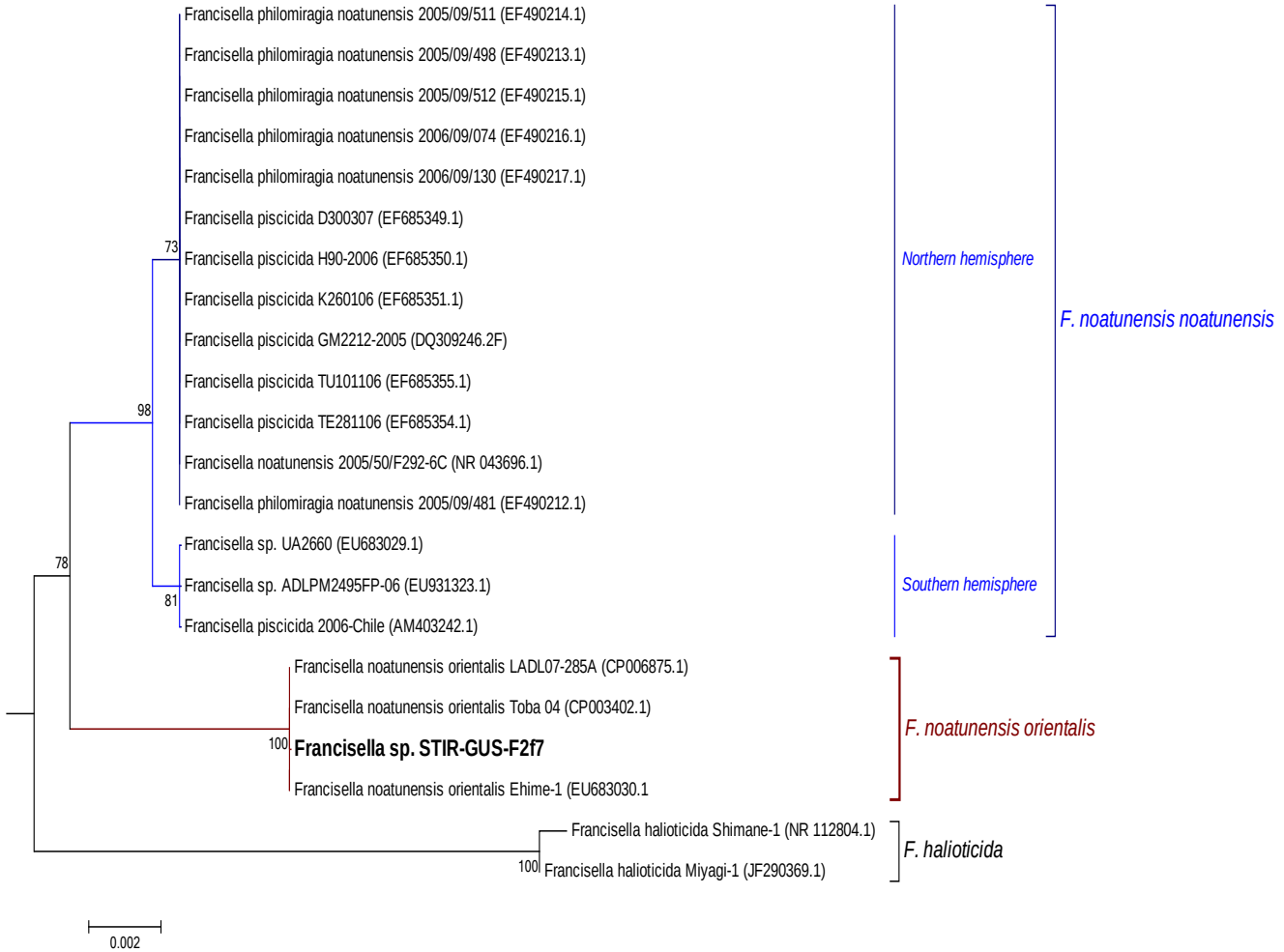

Figure 2.11 Molecular phylogenetic analysis of the genus *Francisella* based on 50 partial 16SrRNA sequences (continued). View of the subtree clustering pathogenic isolates from fish and molluscs.

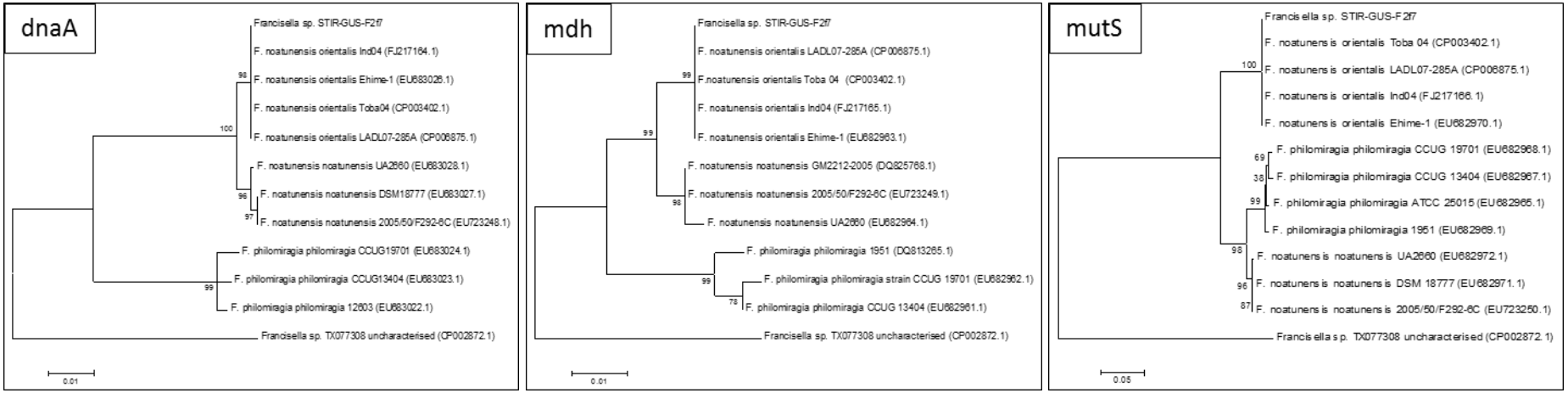

Figure 2.12 Maximum Likelihood trees for all the housekeeping gene sequences. The evolutionary history was inferred using the Maximum Likelihood method based on the model and rate differences among sites specified for each gene on Table 2.9. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

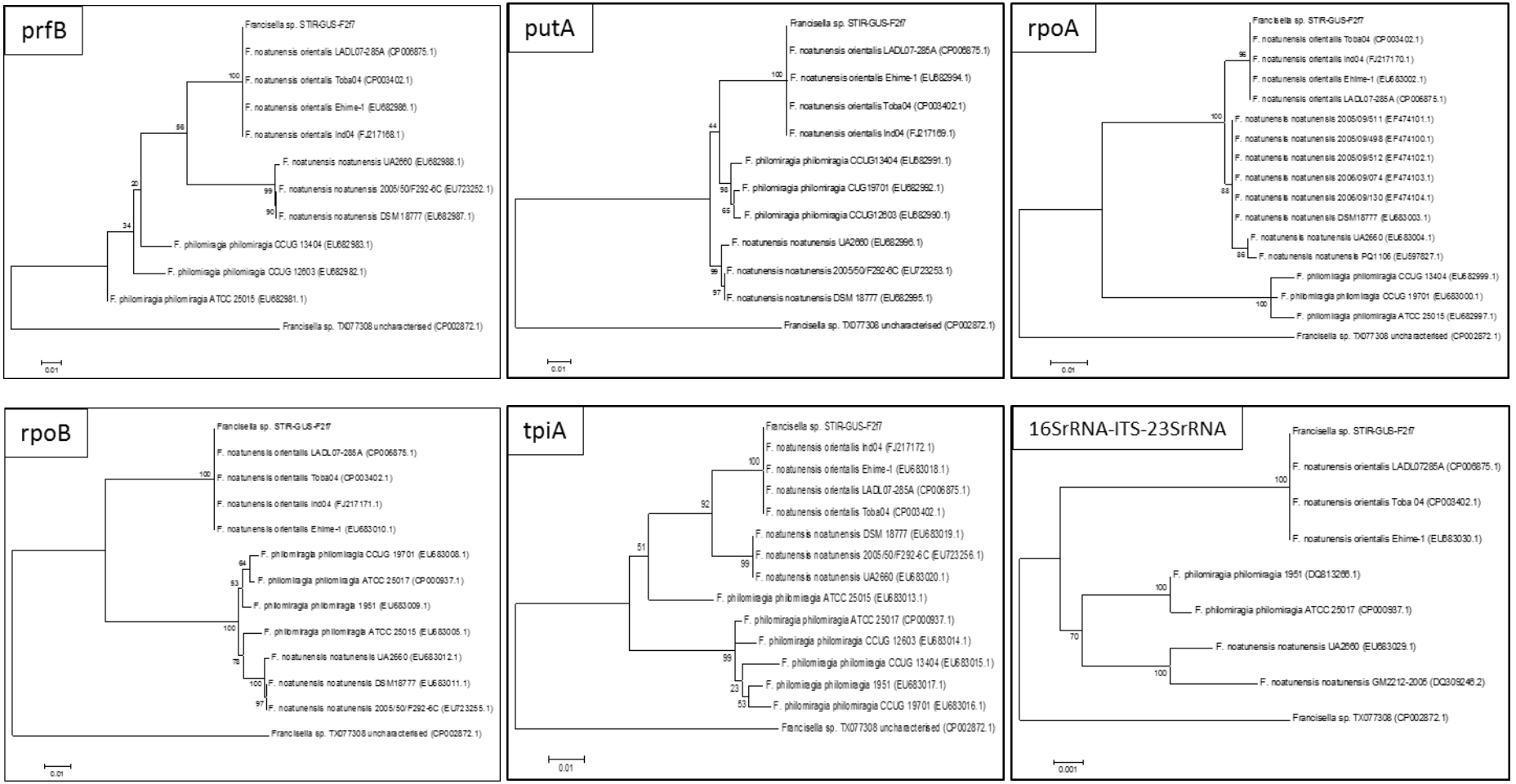

Figure 2.12 Maximum Likelihood trees for all the housekeeping gene sequences (continued).

2.3.5 Experimental infections

Koch's postulates were successfully fulfilled in both the red and the wild type Nile tilapia as the infections resulted in the death of fingerlings with widespread multifocal white nodules/granuloma formations and pure cultures of *Francisella* sp. (confirmed by colony morphology and conventional PCR) were recovered from the moribund fish Figure 2.13. The standard curve indicated that ~$1.1x10^9$ CFU were presented in 1ml of 1X PBS with an OD_{600} of 0.4 (r^2=0.98). In both Nile tilapia groups the clinical signs observed were consistent with those presented at the farms.

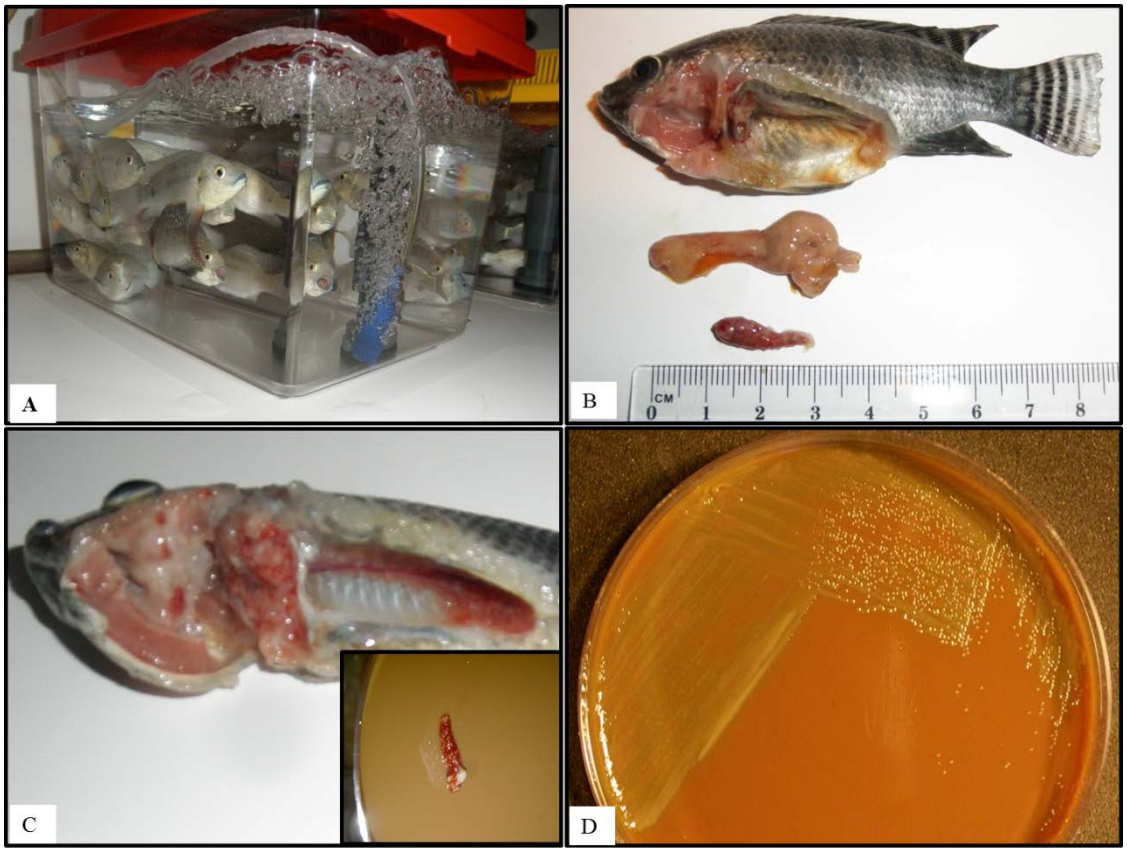

Figure 2.13 Fulfilment of Koch's postulates in naïve Nile tilapia (wild type). **A** Naïve tilapia fingerlings. **B** to **C** Reproduction of clinical signs and gross lesions (mortalities). **C** to **D** Isolation of pure colonies from the spleen of experimentally infected fish.

2.4 Discussion

During the last ten years the application of molecular techniques has facilitated the identification of *Francisella* spp. causing severe mortalities in farmed fish. Unfortunately, despite the use of enriched selective media and established protocols, the causative agent has not always been isolated and little or almost no existing information is available regarding the phenotypic properties of this pathogens.

In the present study, the anamnesis, gross pathology and histopathological findings at the farms were similar to previous reports of chronic francisellosis in tropical aquaculture (Colquhoun and Duodu, 2011; Leal *et al.*, 2014; Soto *et al.*, 2013b). This clearly warranted inclusion of this disease as a differential diagnosis, which subsequently led to isolation and comprehensive characterisation of the pathogen.

The electron microscopy observations of heavily infected fish tissues allowed the visualisation of extracellular pleomorphic bacteria as well as cytoplasmic bounded bacterial like cells within macrophages. Interestingly, ultra-structures consistent with those recently described by Brudal *et al.* (2014b) as “outer membrane vesicles” (OMVs) in *Fnn* grown *in vitro* and *in vivo* in zebrafish embryos were also observed. While that study and previous work in other members of the genus *Francisella* (Burke and Nano, 1991; Golovliov *et al.*, 2003; Pierson *et al.*, 2011) strongly suggest that OMVs may also be presented in *Fno*, further research is required to confirm this. If the vacuoles observed in STIR-GUS-F2f7 are OMVs that contain virulence factors that play a key role during the pathogenesis of the bacterium, these could have a potential application in the development of vaccines against francisellosis in tilapia (Brudal *et al.*, 2014b).

Although the conventional PCR developed by Forsman *et al.* (1994) has the advantages of being cost effective, and was easy and rapid to perform, its specificity and sensitivity are limited. In the present study 4 of 10 fish yielded a positive result and the intensity in one of these bands was almost undetectable. This was thought to be due to the fact that some of the fish tested were older and the PCR could have failed to detect the small amounts of bacterial DNA that are normally presented in adults or carrier fish.

If economic and technical resources at the farm level are limited, the conventional PCR still remains a good option for diagnosis but preferably younger or smaller fish should be tested. However if detection of carrier or asymptomatic fish is necessary (especially prior to the international movement of fish or the introduction of new stocks) a more sensitive assay e.g. a quantitative real time PCR such as that recently proposed by Duodu *et al.* (2012) should be considered.

The primary isolation of the bacterium was successful in 10 of the 20 fish sampled and 7 isolates were obtained from the first farm. It is believed that this may be due to the higher number of brood stock sampled in the second farm, where 50% (n=5) of the fish were not fingerlings. These results correlate with the findings at necropsy and histopathology, where the most severely affected fish were from the on growing facilities.

Of the culture medium tested, MMLA was included as an alternative to the MTMA which is not readily available in Europe. The difference in their composition is minimal, only in the components added to inhibit Gram positive bacteria and yeasts: in MMLA the concentration of vancomycin was increased slightly from 3.0 to 4.0µg/ml and nystatin (1250 units) was substituted for anisomycin (2.0mg/plate). Despite being almost

identical, the MMLA failed to support the growth from fish tissues and pure cultures, and is therefore not considered to be an option to isolate or culture *Fno*.

On the other hand, the medium containing cysteine heart agar and tilapia blood with and without antibiotics proved successful for isolating and growing the bacterium, and even preliminary results of antimicrobial sensitivity tests with the disc diffusion method (data not presented) were successful using this agar.

The use of tilapia blood in agars to culture fish pathogens has been previously reported and discussed by Pasnik *et al.* (2005). In addition the use of CHTB for francisellosis could be useful for the production of vaccines in countries where the use of bovine by-products is banned from veterinary vaccine development due to the bovine spongiform encephalopathy disease or where the accessibility to these products is limited.

The growth characteristics of the bacterium on CHAH and MMHB were the same for all *Fno* isolates including the type strain Ehime-1, with growth from 21-33°C and an optimal *in vitro* growth temperature of 28°C-28.5°C. These results differ to reports by Ottem *et al.* (2009) and Mikalsen and Colquhoun (2009) where growth at 18°C was reported and 22°C was indicated as the optimal *in vitro* growth temperature for the TS Ehime-1.

The API ZYM profiles were identical between all *Fno* isolates, and these results were similar to those previously reported for *Fno* by Ottem *et al.* (2009) and Mikalsen and Colquhoun (2009). These kits have the disadvantage that they are not designed for the characterisation of intracellular bacteria, but instead for the identification of less fastidious pathogens. Consequently, although some information can be obtained from

them, the interpretation of the results should be used with caution and is clearly limited for differentiation between species or subspecies of *Fn*.

In contrast to the API tests, the Biolog GN2 micro plates do not depend upon bacterial growth for reaction because their chemistry is based on the reduction of tetrazolium, as a response to the metabolism of the carbon source rather than to metabolic by-products.

There are no reports of the use of the Biolog GN2 micro plates to characterise *Fno* isolates, but these plates have been used for the automated identification of several *Francisella tularensis* strains (Elkins *et al.*, 2010; Gyuranecz *et al.*, 2010a; Gyuranecz *et al.*, 2010b; Kreizinger *et al.*, 2013; Urich and Petersen, 2008; Versage *et al.*, 2003; Whipp *et al.*, 2003) using either the OmniLog™ System or Biolog MicroStation® plate readers and the GN database provided by the manufacturer. This database can separate *Fp* from *Ft* strains, but cannot differentiate the *Ft* subspecies.

Gyuranecz *et al.*, (2010a) used the Biolog GN2 micro plates to characterise several *Francisella tularensis* strains but instead of using the automated system, the plates were manually read at 4h and 24h post inoculation. When they compared the results with the information provided in the GN2 database an important number of discrepancies were found and the authors were then able to consistently differentiate between subspecies of *Francisella tularensis*.

These plates have been also manually read, for characterisation and description of *F. hispaniensis,* and one strain of *F. philomiragia* and one of *Fnn* were also included (Huber *et al.*, 2010).

The biggest advantage of the Biolog systems is that there are alternate uses for them to satisfy a broad range of needs e.g. characterising slow growing fastidious pathogens, and experimenting with different cell densities, incubation times and reading system, which may be appropriate in particular cases (Shea *et al.*, 2012).

Considering that premise and the special features of *Fno* (small size and fastidious nature) a gradient of bacterial densities was used in this study to identify the optimal inoculum density and prevent false negatives caused by the use of low bacterial concentrations. Additionally the plates were incubated at 28°C, the optimal *in vitro* growth temperature of the pathogen. Similar to the procedure performed by Gyuranecz *et al.* (2010a), the plates were also manually read at different time points but in contrats to their results, in the present study 6h was shown to be the earliest time to achieve a fully developed profile.

The gradient of densities revealed a number of positive reactions which would otherwise have been reported as negative using the inoculant concentrations recommended by the manufacturer. Conversely when higher densities than the optimal were used, an apparent positive reaction was presented in 9 carbon sources.: D-cellobiose (A12), mono-methyl-succinate (C12), α-keto glutaric acid (E4), D,L-lactic acid (E6), succinic acid (E12), bromo succinic acid (F1), glycyl-Lglutamic acid (F12), L-ornithine (G4) and Thymidine (H4).

Due to the saturation of the control well, these results were considered ambiguous and not included as a part of the final metabolic fingerprint. However further research must be performed to rule out whether *Fno* cells have the capability or not to metabolise those

sources. In spite of the high number of false negative results unveiled and the small number of ambiguous results, the Biolog GN2 plates still remain the most useful (practical and powerful) tool for phenotypical characterisation of *Fno*.

Given the results obtained in this study, it is proposed to standardise the use of the Biolog GN2 micro plates as a useful tool for the characterisation of fastidious *Francisella* spp. isolates by determining the optimal inoculum density for every type strain and incubating the plates at the optimal *in vitro* growth temperature, especially for comparative studies between different species and subspecies.

As with the Biolog GN2 micro plates, there are no previous reports of FAME profiling reported for any *Fno* isolate. The results obtained in this study for STIR-GUS-F2f7 and the other *Fno* isolates are consistent with the FAME signature of the genus *Francisella* (Jantzen *et al.*, 1979; Nichols and Mayberry, 1985).

On comparison of the FAME profiles obtained for *Fno* in this study and those available for the closest members of the genus *F. philomiragia* and *Fnn* (Huber *et al.*, 2010; Ottem *et al*., 2006; Ottem *et al*., 2007b) the most dissimilar groups are *Fno* and *Fp* with 8 fatty acids having at least 3% of difference between them. Unexpectedly more differences were found between *Fno* and *Fnn* than between *Fno* and *Fp*.

The most divergent fatty acids between the *Fno* here reported and those available for *Fnn* in (Ottem *et al.*, 2007a; Ottem *et al.*, 20007b) are: 24:1, 20-23% for *Fno* vs 1% for *Fnn*; 18:1n-9, 17-18% for *Fno* vs 10-12% for *Fnn*; 24:0, 12-13% for *Fno* vs 5-6% for *Fnn*; 16:0, 7-8% for *Fno* vs 4% for *Fnn*; while only two differences over 3% were seen between *Fnn* and *F philomiragia*: 22:0 5-6% vs 2% and 18:0 3-5% vs 1%.

The differences between the *Fno* strains studied here and those presented by Ottem *et al.* (2007a and 2007b), for *F. philomiragia* are: 24:1, 20-23% vs 0%; 24:0, 12-13% vs 2%; 22:0 8% vs 2%; 22:1n-11 4% vs 9%; 18:1n-9 17-18% vs 11%; 18:0 5-6% vs 1%; 16:0 7-8% vs 4% and 20:0, 4% vs 1%;

Based on these comparisons, it is possible that FAME can be used as phenotypic marker for *Fno*, *Fnn* and *Fp* if special attention is paid to the fatty acids 24:1, 24:0, 18:1n-9, 16:0, 22:0 and 18:0 as they could be useful to group and separate isolates even at the subspecies level.

As the FAME profile of *Fnn* is more similar to *Fp* than to *Fno* and *Fno* is completely different to *Fp*, further phenotypic studies using other techniques like metabolic profiles, polar lipids, antimicrobial susceptibility etc. should be performed with more *Fn* and *Fp* strains to investigate whether these dissimilarities are stable among these groups.

Regarding the antimicrobial susceptibility studies, due to the recent emergence of the disease, there are currently no standards or methodologies established to test *Fn* strains. In order to standardise and compare the present results with other reports, the methodology of the analyses performed in the current study, was based on those recently presented by Soto *et al.* (2012b), and for these reason the commercial AVIAN and GN2F micro plates were chosen for the broth micro-dilution assay together with the disc diffusion method.

The broth micro-dilution results indicated that the addition of the supplements in the broth did not interfere with the results of the quality control as 37 out of the 39 results were found in the rank established by the CLSI (CLSI, 2006a). These results are in

accordance with those presented by Soto *et al.* (2012b), but inconsistent with Baker *et al.* (1985) for whom the use of IsoVitaleX™ affected their results.

All the isolates tested in the present study had a similar profile in the broth microdilution method, and after comparing the range of results of these strains with the range of those obtained by Soto *et al.* (2012c), minor discrepancies were found in 17 of the 39 antimicrobials. Such inconsistencies may have been caused by the differences in the number of bacterial cells inoculated into the plates. In order to prevent this, it is suggested here that the inoculum densities should be standardised at a specific OD_{600} rather than a McFarland standard.

Based merely on the MIC results, it appears that the isolates may be susceptible to neomycin, novobiocin, amikacin, ciprofloxacin, imipenem, gatifloxacin, meropenem, tobramycin, nitrofurantoin, and levofloxacin.

According to Soto *et al.* (2012b) although the composition of the CHAH influences the disc diffusion assay enough to prevent it from being in compliance with the CLSI (CLSI, 2006b), this technique still provides useful information for the classification of bacteria tested on it if clear and consistent diameters of inhibition are presented.

In this study 8 of the 16 antimicrobials tested developed clear and repeatable zones of inhibition and these were similar among the *Fno* strains. From these, enrofloxacin, gentamicin, tetracycline and florfenicol were also used by Soto *et al.* (2012b) with almost identical results. This is the first time that a *Francisella* ssp. isolated from fish has been tested with kanamycin.

Although there are currently no established diameters to classify *Fno* as resistant, intermediate or sensitive, it can be suggested from the large inhibition zones that the isolates tested in this study are susceptible to enrofloxacin, kanamycin, gentamicin, tetracycline, oxytetracycline, florfenicol, oxolinic acid and streptomycin. This is also supported by the fact that the 6 compounds of this list that were tested by the broth microdilution method had very low MIC values. Though oxolinic acid was not tested in the micro plates, previous results for *Fnn* (Bohle *et al.*, 2009; Ottem *et al.*, 2007; Isachsen *et al.*, 2012) and *Fn*o (Soto *et al.*, 2012b) support the present findings.

From the combination of both methods, the broth micro-dilution and the disc diffusion, it can be established that the isolates tested here are resistant to penicillin G, lincomycin, oleandomycin, amoxicillin, carbenicillin, polymyxin B, ampicillin and sulphamethoxazole/ trimethoprim.

From the compounds that inhibited the *Fno* isolates, florfenicol and oxytetracycline seemed to be the most adequate to treat the outbreaks as these two are authorised antibiotics for use in fish in most state members of the European Union and previous reports have documented their kinetics in live fish treating FLB and *Fn* infections (Chern and Chao 1993; Mauel *et al.*, 2003; Ostland *et al.*, 2006; Soto *et al.*, 2010c; Soto *et al.*, 2013a).

The genetic analyses indicated that the identity of the novel isolate STIR-GUS-F2f7 was 100% similar to that of other isolates belonging to the subspecies *Fno*. This was also supported by the phylogenetic trees generated with the house keeping genes that grouped all of them together. Surprisingly, when the closest members of the genus *Fp* and *Fnn*

were included in the trees, the clade of *Fno* appeared to be distant enough from the clade that groups *Fnn* to be considered a separate species, indicating a possible taxonomic misplacement of the *Fno* group.

This could explain the results observed when comparing the FAME and Biolog GN2 profiles with those available in the literature for *Fnn* and *Fp* (Huber *et al.*, 2010; Ottem *et al*., 2006; Ottem *et al*., 2007b). The possible taxonomic misplacement of the taxon *Fno* has also been suggested by other authors (Soto *et al.*, 2009a; Ottem, 2011). However to clarify this, further research based on a suitable polyphasic approach with several members of *Fno*, *Fnn* and *Fp* simultaneously analysed would be required.

The establishment of the experimental infection models mimicking conditions presented at the farms allowed fulfilment of the third and fourth Koch's postulates, showing that isolate STIR-GUS-F2f7 was able to cause the disease in naïve Nile tilapia and its re-isolation in pure culture from the experimentally infected fish confirmed that this bacterium was the causative agent of the disease outbreaks. What is more, the challenge model established here could be further used to investigate the pathogenesis of this bacterium in different genetic groups of tilapia and to develop strategies for its control and prevention.

2.5 Conclusion

In the present study, the diagnosis of francisellosis in tilapia was confirmed by different methods and several isolates of *Fno* including STIR-GUS-F2f7 were recovered from the diseased fish. To achieve a successful primary isolation of the *Fno* pathogen, the kidney of diseased or moribund fish should be aseptically collected and homogenised in 0.5ml of 1X PBS. The homogenate should be streaked with sterile loops in CHAH or CHATB and the plates should be incubated at 28-29 °C for 3 days. Electron microscopy observations indicated that *Fno*, like other members of the genus *Francisella*, may secrete OMVs. A suitable methodology for the characterisation of the isolates collected here and other *Fno* is presented. This approach is proposed for further taxonomic studies of the genus *Francisella* as the results of its use provided insight into several phenotypical characteristics of *Fno* including antimicrobial susceptibility, and suggested a possible taxonomic misplacement of the *taxon Fno*. Finally, experimental infections with *Fno* STIR-GUS-F2f7 were successfully established in naïve tilapia, confirming it as the causative agent of the disease outbreaks and opening a research avenue to investigate the pathogenesis of the bacterium in this group of fish.

2.6 Acknowledgments

Follow up visit to the farms, antimicrobial analyses and consumables to grow the pathogens were supported by the Fish Vet Group.

The isolate PQ1104 from Costa Rica was kindly provided by Dr. Duncan Colquhoun, Norwegian Veterinary Institute (NVI) in Oslo Norway.

FAME analyses were supported by James Dick at the Lipids Laboratory-Nutrion Group, Institute of Aquaculture, University of Stirling.

Chapter Three

Complete genome sequence of *Francisella noatunensis orientalis* STIR-GUS-F2f7 a highly virulent strain isolated from diseased red Nile tilapia farmed in Europe.

3.1 Introduction

A highly virulent strain of *Francisella noatunensis orientalis* "STIR-GUS-F2f7" was isolated from the spleen of a moribund red Nile tilapia farmed in Europe (Chapter 2). In this communication the complete genome sequencing of this bacterium is reported.

Francisella noatunensis orientalis (*Fno*) is a Gram negative, non-motile, non-sporulating, aerobic, fastidious, pleomorphic coccobacillus (size 0.2-1.7 μm) bacterium associated with disease of fish farmed in tropical aquatic environments.

This pathogen is the causative agent of the disease piscine francisellosis recently described in several fresh and sea water fish species causing a significant negative economic impact in the aquaculture industry for which there are currently no commercially available products for the treatment or prophylaxis (Colquhoun and Duodu, 2011).

In November 2012, STIR-GUS-F2f7, a highly virulent strain of *Fno*, was recovered from farmed red Nile tilapia using a novel 5% tilapia blood enriched cysteine heart agar after homogenising the spleen of the fish in sterile 1X phosphate-buffered saline (PBS) (Chapter 2). The diseased fish (~10 grams/5.5cm) had been reared in a recirculating system with fresh water at 25°C and the mortalities were around 60% during the outbreak. During experimental infections *in vivo*, only as few as 12CFU per fish were required to cause mortality in red and wild type Nile tilapia fingerlings maintained at 23°C ± 2°C. The optimal *in vitro* temperature for culturing this isolate was 28-29°C in both, agar plates and supplemented modified Mueller-Hinton II cation adjusted broth.

3.2 Materials and methods

3.2.1 Genome sequencing and assembling

The genomic DNA (gDNA) of STIR-GUS-F2f7 was extracted as delineated in section 2.2.3.2 The purity, integrity and concentration of the crude DNA was assessed by electrophoresis in 1% agarose gel stained with ethidium bromide at 0.001%, 260/280 and 260/230 indices determined with a NanoDrop™ ND1000 (ThermoScientific, Delaware, USA) and Qubit® 2.0 fluorometer (Invitrogen, Life Technologies, California, USA).

Thereafter, three aliquots with 2µg of DNA were sent for high-throughput sequencing to the NGI Uppsala (SNP&SEQ Technology Platform) Science for Life Laboratory, at Uppsala University in Uppsala Sweden, where the *Fno* STIR-GUS-Ff7 genome was sequenced using Illumina® HiSeq2500™ platform (Illumina, California, USA).

The parallel, paired-end sequence assembler ABySS (Assembly By Short Sequences) version 1.3.4 (Simpson *et al.*, 2009) was used to *de novo* assemble a total of 10 sequence reads, all of them which had a length longer than 100bp.

3.2.3 Genome annotation

The genome of *Fno* STIR-GUS-F2f7 consists of 1,887,347 base pairs distributed in 10 contigs assembled in one single circular chromosome with no plasmids and a G+C content of 32.4% as visualised with the DNA browser Artemis version 16.0.0 (Carver *et al.*, 2012) and the software DNAPlotter (Carver *et al.*, 2009) (Figure 3.1).

3.3 Results

3.3.1 Genome annotation

The RNAmmer programme (Lagesen *et al.*, 2007) predicted 19 copies of rRNA genes: seven of 5S and six of 16S and 23S. The annotation of the genome was performed using the RAST server (Overbeek *et al.*, 2014) which predicted a total of 1970 protein coding sequences and 63 non coding rRNA sequences in 328 subsystems. (Figure 3.2.)

Of the protein coding genes, 510 (25.8%) were annotated as hypothetical proteins. From the 1460 non-hypothetical genes, 239 were predicted as being involved in protein metabolism, 199 in synthesis of amino acids and derivatives, 122 in carbohydrate catabolism, 116 in cofactors, vitamins, prosthetic groups, or pigment production, 100 in RNA metabolism, 95 in fatty acid, lipid, and isoprenoid synthesis, 82 in DNA metabolism, 79 in respiration, 74 in capsule and cell wall synthesis, 71 in stress response, 59 in nucleoside and nucleotide synthesis, 54 in membrane transport, 32 in virulence, disease and defence mechanisms, 24 in cell division and cell cycle, 17 in potassium metabolism, 16 in regulation and cell signalling, 11 in phosphorus metabolism, 10 in iron acquisition and metabolism, 10 in sulphur metabolism, 6 in the metabolism of aromatic compounds, 5 in secondary metabolism, 5 in nitrogen metabolism, 1 in dormancy and 34 as miscellaneous proteins.

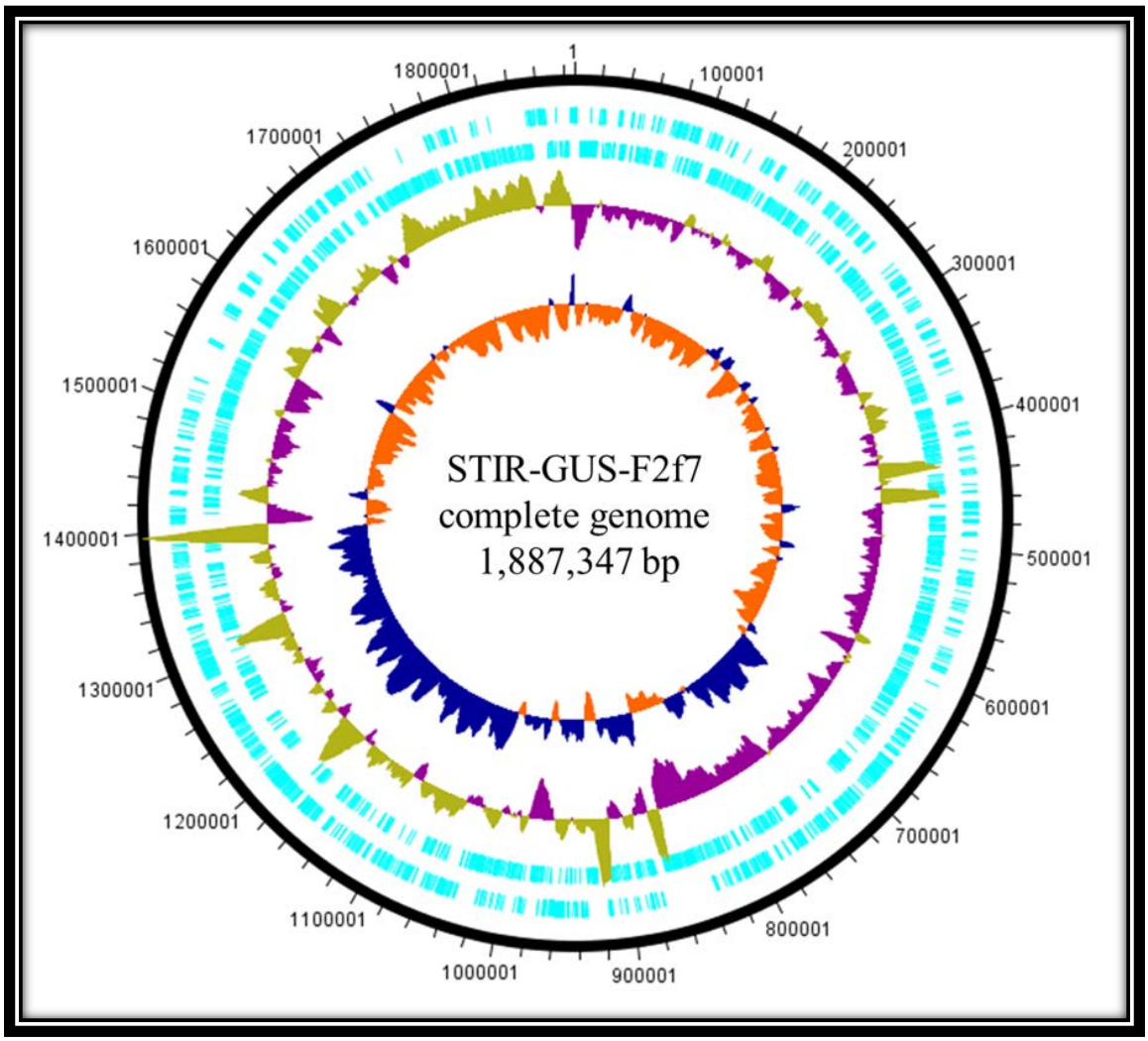

Figure 3.1 Circular map of the genome of *Francisella noatunensis orientalis* strain STIR-GUS-F2f7. The outer scale is marked in base pairs. From outer to inner: First circle (black) all the nucleotides. Second circle (light blue) coding sequences (CDS) in forward strand. Third circle (light blue) CDS in reverse strand. The innermost circles show: GC skew (orange/blue) and G+C content (purple and green).

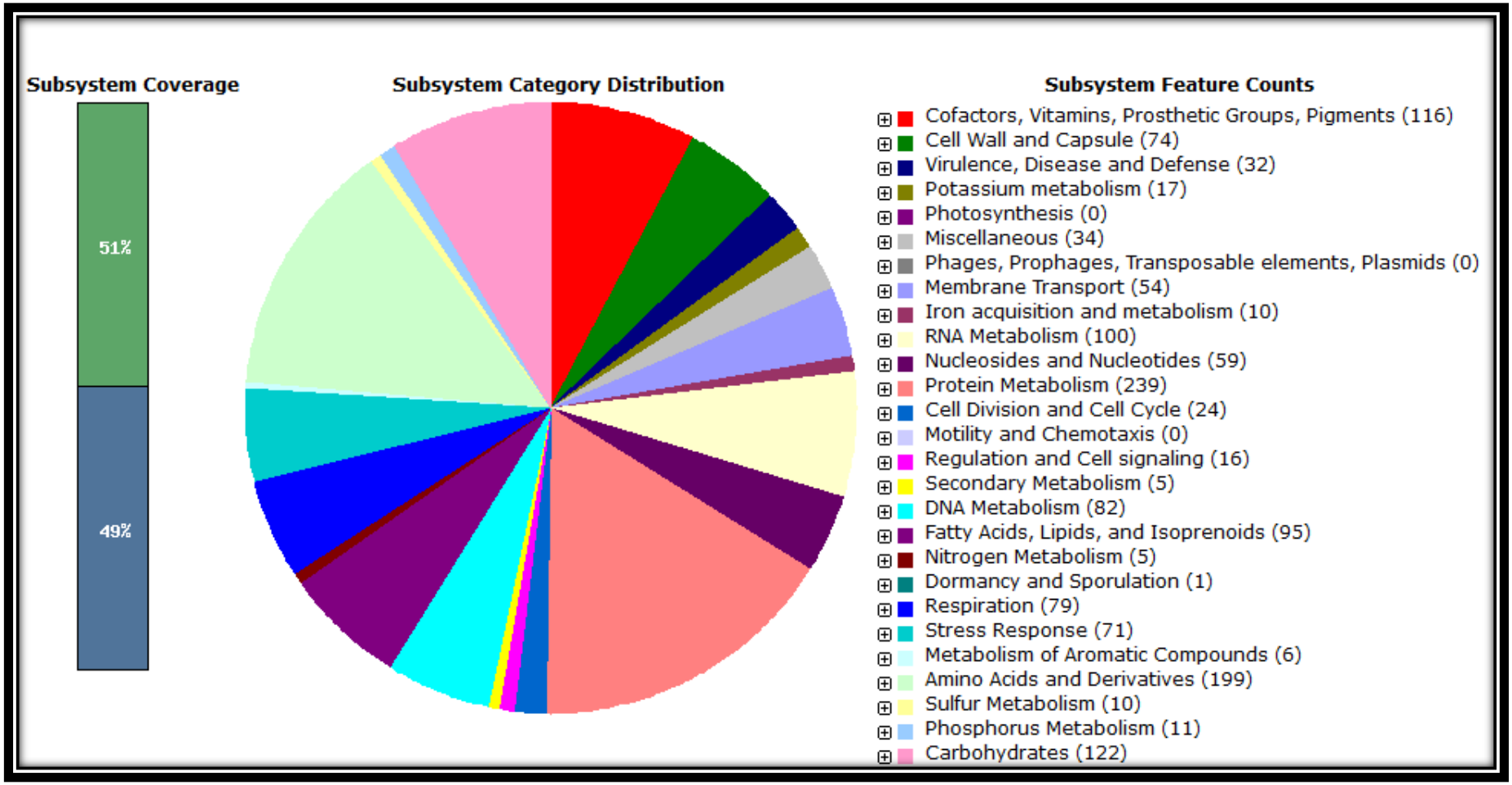

Figure 3.2 Annotation of *Fno* STIR-GUS-F2f7 genome using RAST server. The graph shows the subsystem features distribution (number of genes in brackets) by categories.

3.3.2 Nucleotide accession number

This genome sequence has been deposited at DDBJ/EMBL/GenBank databases under the accession number: XXX-XXX-XXX

3.4 Discussion and conclusions

Currently there are only two fully sequenced genomes of this *taxon* readily available in public databases, TOBA04 (Sridhar *et al.*, 2012) and LADL-07-285A (Soto *et al.*, 2009a), however neither of those strains have been characterised and no reports are available of the isolation or characterisation of the first strain.

This genome sequence is important because STIR-GUS-F2f7 has been comprehensively characterised and several of its metabolic and chemotaxonomic properties have been described in Chapter 2. Thus, accessibility to this sequence will allow further investigation on the genes involved on metabolic and evolutionary pathways and comparative analyses with other *Francisella* spp. including the closely related human pathogens *F. tularensis* and *F. philomiragia*.

Additionally the availability of this genome will favour the identification of mechanisms of pathogenicity towards the development of vaccines against piscine francisellosis in both tropical and cold water fish species and we anticipate it will contribute to the clarification of the intricate taxonomy of the genus *Francisella* particularly those isolates recovered from diseased fish.

3.5 Acknowledgements

Visit to Sweden for genome sequencing and analysis was supported by Division of CBRN Security and Defence, FOI–Swedish Defence Research Agency, Umeå, Sweden.

Sequencing was performed with support of the SNP&SEQ Technology Platform, Science for Life Laboratory at Uppsala University, a national infrastructure supported by the Swedish Research Council (VRRFI) and the Knut and Alice Wallenberg Foundation.

Chapter Four

Elevation of *Francisella noatunensis* subsp. *orientalis* (Ottem *et al.*, 2009) to the rank of species as *Francisella orientalis* comb. nov., sp. nov., and description of *Francisella noatunensis* subsp. *chilense* subsp. nov.

4.1 Introduction

Members of the genus *Francisella* family *Francisellaceae*, order *Thiotrichales*, class *Gammaprotobacteria*, phylum *Proteobacteria* are Gram negative, non-motile, non-sporulating, aerobic, pleomorphic coccobacillar prokaryotic cells (size 0.2-1.7 μm), that are widely distributed in nature with a well-established association with aquatic environments (Colquhoun *et al.*, 2014).

Following the rules established in "The International Code of Nomenclature of Prokaryotes" (Lapage *et al.*, 1992) there are currently six validly described species and six subspecies described within the genus *Francisella*: *F. tularensis* (*Ft*) with its four subspecies, *tularensis* (*Ftt), holarctica* (*Fth*), *mediasiatica* (*Ftm*) and *novicida* (*Ftn*)*; F. philomiragia* (*Fp*)*; F. hispaniensis* (*Fh*)*; F. noatunensis* (*Fn*) with two: subspecies *noatunensis* (*Fnn*) and *orientalis* (*Fno*); *F. halioticida* (Fh_{al}) and *F. guangzhouensis* (*Fg*). With the exception of *F. guangzhouensis* the rest of the species have been described as facultative endosymbionts of eukaryotic cells with the ability to cause severe diseases in their hosts (Colquhoun *et al.*, 2014).

Francisella noatunensis is one of the most recently recognised species of the genus and is the causative agent of piscine francisellosis, an emerging disease reported worldwide affecting several farmed or captured fish species (Birkbeck *et al.*, 2011; Colquhoun and Duodu, 2011).

Since first described, as with other *Francisellaceae* members, the nomenclature of *Fn* has been under constant rearrangement. Initially in July 2007, a comparative characterisation of a single *Francisella* sp. strain recovered from farmed Norwegian Atlantic cod (*Gadus morhua*) was made with the type strain of *F philomiragia*, in that

study the cod isolate was classified as a novel species named *F. piscicida* (Ottem *et al.*, 2007).

A few months later in September 2007, after the name *F. piscicida* had been published but not yet validated, Mikalsen *et al.* (2007) compared seven cod isolates against four strains of *Fp* (including the type strain) and validly published a new nomenclature of the bacterium i.e. *F. philomiragia* subsp. *noatunensis.*

In January 2008, *F. piscicida* was published as a valid species (Euzeby, 2008) and because of the similarity of its 16S rRNA gene the bacterium was considered to be a heterotypic synonym of *F. philomiragia* subsp. *noatunensis* thus, according to the rule of priority, the epithet "*noatunensis*" remained over "*piscicida*".

Later the same year, the current taxonomical status of fish *Francisella* was revised. In that study, the type strains of *F. piscicida* and its heterotypic synonym *F. philomiragia noatunensis* were compared against each other and five isolates of *Fp* including the type strain. Additionally, one strain from a diseased fish farmed in warm water environment in Japan called Ehime-1 and DNA from one strain recovered in Indonesia (Ind04/Toba04) were included (Ottem *et al.*, 2009).

As a result of those comparisons *F. piscicida* was shown to be the heterotypic synonym of *F. philomiragia* subsp. *noatunensis* and both were elevated to the rank of species as *F. noatunensis*, while the strain Ehime-1 was described as the type strain of the new subspecies *F. noatunensis orientalis* on the basis of a few phenotypic traits (Euzeby, 2009a; Euzeby, 2009b; Ottem *et al.*, 2009).

Since the validation of *F. noatunensis*, no further studies have been perfomed to investigate the phenotypic characteristics of this bacteria. Recently, a novel strain of *Fno* STIR-GUS-F2f7 was recovered from moribund red Nile tilapia farmed in Europe, and a new methodology for the characterisation of *Fno* strains was proposed (Chapter 2),

This approach proved to be successful as it provided insight into the phenotypic variability of the isolates analysed, and the results obtained demonstrated a high degree of phenotypic and genetic association within the strains belonging to the subspecies *Fno*, however it also suggested the possible misplacement of this *taxon* within the species *F. noatunensis* and highlighted the need for standardised descriptions of species and subspecies within the genus *Francisella*.

The present study characterise and revise the taxonomical status of more than 30 strains of the species *Francisella noatunensis*. The isolates analysed were recovered at different laboratories from several diseased fish species over a period of 15 years. In addition the type strain of *Fp*, the closest related member of the genus, was included in all the comparisons. The characterisation of the strains was performed using the methodology previously proposed in Chapter 2, which comprises basic phenotyping, metabolic fingerprinting, and chemotaxonomic analyses in combination with DNA-DNA hybridization comparisons and analyses of geographical, ecological and high quality genomic data.

4.2 Materials and methods

4.2.1 Bacterial strains

A total of 33 strains, recovered from diseased fish (16 *Fno* and 16 *Fnn*) of different geographical origin and the type strain of *Fp*, were phenotypically characterised. The genetic characterisation was based on DNA-DNA hybridisation of the type strains and several genome sequence derived parameters.

In this project the whole genome (WG) of nine *F. noatunensis* subsp. *orientalis* (*Fno*) and one *F. noatunensis* subsp. *noatunensis* (*Fnn*) strains was sequenced as per the method described in Section 3.2. In total 13 *Fno*, 7 *Fnn* and 6 *F. philomiragia* (*Fp*) WG sequences were included; additionally the WG sequences of each of the type strains of other validly described members of the genus *Francisella* were included. At the time of this study no genome sequences of *F. halioticida* were available in public databases. The metadata of the 47 strains that were phenotypically and/or genetically characterised are presented in Table 4.1. The accession number of the new genomes and those publicly available are presented in the section “Bioproject” of such table.

Table 4.1 Strains of *Francisella* spp. analysed in this study.

Species and subspecies	Designation	Genome Bioproject	PRESENT ID	NVI	FOI	DSM	CCUG	BCCM/LMG	OTHER	Country	Location	Year	Host	
F. noatunensis orientalis	Ehime-1^{T}	PRJNA73447	Fo1	5887	FSC 771	21254^{T}	-	LMG 24544^{T}	-	Japan	Ehime prefecture	2001	Three-line grunt (Isaki)	*Parapristipoma trilineatum*
F. noatunensis orientalis	F. Victoria	n/a	Fo2	-	FDC 191	-	-	-	-	Unknown	Unknown	Unknown	Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	RUSVM-LA1	n/a	Fo3	-	FDC 192	-	-	-	-	Unknown	Latin America	2012	Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	F. CAL2	n/a	Fo4	-	FDC 193	-	-	-	-	USA	California	Unknown	Hybrid striped bass	*Morone chrysops x M. saxatilis*
F. noatunensis orientalis	F. CAL1	n/a	Fo5	-	FDC 194	-	-	-	-	USA	California	Unknown	Hybrid striped bass	*Morone chrysops x M. saxatilis*
F. noatunensis orientalis	LADL-07-285A	n/a	Fo6	-	FDC 195	-	-	-	-	Costa Rica	Alajuela	2007	Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	LADL-11-060	n/a	Fo7	-	FDC 196	-	-	-	-	USA	Texas	Unknown	Tilapia	*Oreochromis* spp.
F. noatunensis orientalis	LADL-01-100	n/a	Fo8	-	FDC 197	-	-	-	-	USA	Midwest	2010	Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	LADL 10-075 #5	n/a	Fo9	-	FDC 198	-	-	-	-	USA	Midwest	2010	Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	Franc-COS1	-	Fo10	9535	-	-	-	-	-	Mexico	Queretaro	2013	Blue tilapia	*Oreochromis aureus*
F. noatunensis orientalis	Austria	-	Fo11	9449	-	-	-	-	-	Austria	Vienna	2013	Malawi cichlid	*Aulonocara maleri*
F. noatunensis orientalis	STIR-GUS-F2f7	n/a	Fo12	-	FDC 410	-	-	-	-	Unknown	Europe	2012	Red Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	STIR-MATT-F1f6	-	Fo13	-	-	-	-	-	-	Unknown	Europe	2012	Red Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	PQ1104	PRJNA73389	Fo14	5409	FSC 770	-	-	-	-	Costa Rica	Unknown	2006	Tilapia	*Oreochromis* spp.
F. noatunensis orientalis	Cefas	n/a	Fo15	8373	FDC 190	-	-	-	-	UK	England	Unknown	Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	STIR-AVU-F2f9	-	Fo16	-	-	-	-	-	-	Unknown	Europe	2012	Red Nile tilapia	*Oreochromis niloticus*
F. noatunensis orientalis	Toba04	PRJNA82619	Fo17	-	-	-	-	-	-	Indonesia	Lake Toba	2004	Mozambique tilapia	*Oreochromis mossambicus*
F. noatunensis noatunensis	NVI 5330^{T}	PRJNA73397	Fn1	5330	FSC 769	12596	-	LMG 23800^{T}	NCIMB 14265^{T}	Norway	Hordaland County	2005	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 5340	-	Fn2	5340	-	-	-	-	-	Norway	Sogn og Fjordane	2005	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 5396	-	Fn3	5396	-	-	-	-	-	Norway	Rogaland County	2006	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 5394	-	Fn4	5594	-	-	-	-	-	Norway	Møre og Romsdal	2006	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	PQ1106	PRJNA73449	Fn5	5888	FSC 772	-	-	-	-	Chile	Lake Llanquihue	2006	Atlantic salmon	*Salmo salar*
F. noatunensis noatunensis	NVI 6086	n/a	Fn6	6086	FDC 189	-	-	-	-	Norway	Sogn og Fjordane	2008	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 6214	-	Fn7	6214	-	-	-	-	-	Norway	Møre og Romsdal	2008	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 6422	-	Fn8	6422	-	-	-	-	-	Norway	Møre og Romsdal	2008	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 6471	-	Fn9	6471	-	-	-	-	-	Norway	Møre og Romsdal	2008	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 6572	-	Fn10	6572	-	-	-	-	-	Norway	Sogn og Fjordane	2008	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 6684	-	Fn11	6684	-	-	-	-	-	Norway	Nordland County	2009	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 7127	-	Fn12	7127	-	-	-	-	-	Norway	Rogaland County	2008	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	F/134/09A	PRJNA73465	Fn13	7061	FDC 178	-	-	-	-	Ireland	Waterford	2009	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 8087	-	Fn14	8087	-	-	-	-	-	Norway	Møre og Romsdal	2011	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	SVA 74/04	PRJNA73463	Fn15	5518	FSC 846	-	-	-	-	Sweden	Southern Skagerrak	2004	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	NVI 6577	-	Fn16	6577	-	-	-	-	-	Norway	Sogn og Fjordane	2008	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	GM2212 (NVI)	PRJNA73457	Fn17	5865	FSC 774	18777	-	-	-	Norway	Rogaland County	2004	Atlantic cod	*Gadus morhua*
F. noatunensis noatunensis	GM2212 (FOI)	PRJNA73459	Fn18	-	FSC 775	18777	-	-	-	Norway	Rogaland County	2004	Atlantic cod	*Gadus morhua*
F. philomiragia	O#319L^{T}	PRJNA22416	Fp1	5411	-	7535^{T}	19700^{T}	LMG7903^{T}	ATCC 25015^{T}	USA	Utah	1959	Muskrat	*Ondrata zibethica*
F. philomiragia	O#319-029	PRJNA73371	Fp2	-	FSC 037	-	-	-	ATCC 25016	USA	Utah	1960	Water	-
F. philomiragia	O#319-036	PRJNA27853	Fp3	5598	-	-	19701	-	ATCC 25017	USA	Utah	1960	Water	-
F. philomiragia	O#319-067	PRJNA73373	Fp4	-	FSC 039	-	-	-	ATCC 25018	USA	Utah	1960	Water	-
F. philomiragia	CCUG12603	PRJNA73377	Fp5	5596	FSC 145	-	12603	-	-	Sweden	Gothenburg	1982	Human	*Homo sapiens*
F. philomiragia	Swiss	PRJNA73381	Fp6	5597	FSC 154	-	13404	-	CDC E6588	Switzerland	Zurich	1979	Human	*Homo sapiens*
F. tularensis holarctica	GIEM 503^{T}	PRJNA16087	Ft1	-	FSC 200^{T}	-	-	-	GIEM 503^{T}	Sweden	Ljusdal	1998	Human	*Homo sapiens*
F. tularensis mediasiatica	GIEM 543^{T}	PRJNA19571	Ft2	-	FSC 147^{T}	-	-	-	GIEM 543^{T}	Kazakhstan	Alma-Alta region	1965	Mid-day gerbil	*Meriones meridianus*
F. tularensis tularensis	SCHU S$_4$$^{\sharp}$	PRJNA239340	Ft3	-	FSC 237	-		-	-	USA	Ohio	1938	Human	*Homo sapiens*
F. tularensis novicida	U112^{T}	PRJNA16088	Ft4	-	FSC 040^{T}	-	33449^{T}	-	ATCC 15482^{T};	USA	Utah	1951	Water	-
F. hispaniensis	FhSp1^{T}	PRJNA73391	Fh1	-	FSC 454^{T}	22475^{T}	5802^{T}	-	FnSp1^{T}; F62^{T}	Spain	Unknown	2003	Human	*Homo sapiens*
F. guangzhouensis	08HL01032^{T}	PRJNA271279	Fg1	-	-	-	60119^{T}	-	NCTC 13503^{T}	China	Guangzhou city	2008	Water	-

(n/a) not available yet; NVI: Norwegian Veterinary Institute; FOI: Swedish Defence Research Agency; DSM: German Culture Collection of Microorganisms and Cell Cultures; CCUG Culture Collection, University of Göteborg, Sweden; Belgian Coordinated Collections of Microorganisms; NCIMB: National Collection of Industrial, Food and Marine Bacteria; ATCC: American Type Culture Collection; CDC: Centers for Disease Control and Prevention; GIEM: Gamalei Institute of Epidemiology and Microbiology; NCTC:National Collection of Type Cultures;

(-) not applicable; $^{\sharp}$ Is the prototypic virulent strain not the type strain.

4.2.2 Phenotypic characterisation

All the strains phenotypically characterised were grown on CHAH and MMHB prepared as indicated in Section 2.2.2.The optimal *in vitro* temperature of *Fno*, *Fnn* and *Fp* was confirmed by incubating the type strains on agar plates at a wide range of temperatures as indicated in Section 2.2.3. The mid logarithmic phase of the type strains was investigated by constructing a growth curve on MMHB at the optimal *in vitro* temperature as indicated in Section 2.2.3.1 The bacterial biomass for the different phenotypic techniques was produced at the optimal *in vitro* temperature of each *taxon* and the cells were harvested at the mid log phase.

4.2.2.1 Colony morphology and basic phenotype

The colony morphology of the strains used was compared on CHAH and the basic phenotypic characteristics were confirmed using primary identification tests such as: Gram stain, catalase, oxidase and motility.

4.2.2.2 Metabolic fingerprint

To assess the metabolic activity of the isolates Fp1, Fo2-Fo16 and Fn1-Fn14, the Biolog GN2 micro plates (Biolog Inc., California, USA) were used. These plates contain an array of 95 different carbon (C) sources i.e. carbohydrates, lipids, sugars, amino acids, nucleic acids and other metabolites to test the capability of the bacterial cells to metabolise them. To avoid appearance of false negatives the GN2 Biolog micro plates were set up according to the manufacturer's instructions with the modifications indicated in Section 2.2.3.1.

After inoculation, the microplates were incubated at the optimal *in vitro* temperature of each *taxon*. The results were visually read at six and twelve hours and recorded for posterior comparisons with a computer photo scanner model Epson Perfection V370 Photo scanner (Epson, London, UK).

4.2.2.3 Chemotaxonomic analysis

The cellular fatty acid methyl ester (FAME) composition of the strains Fp1, Fn1, F12-F14, Fn1-Fn12 and Fn14-Fn16 was investigated at the Lipids Laboratory IoA, UoS as per the protocol described in Section 2.2.3.1 with slight modifications. Briefly, the FAME extracted were purified on HPTLC (high performance thin layer chromatography) plates (10cm X 10cm X 0.15mm) pre-coated with silica gel 60 (without fluorescent indicator) *(*Merck KGaA, Darmstadt, Germany*)*.

In order to investigate if the percent composition of individual fatty acids could be used as a suitable tool to differentiate between *Fno, Fnn* and *Fp*, statistical analyses were performed using Minitab® v.16.1.0 software (Minitab Inc., Coventry, UK) considering each isolate as a biological replicate of the respective *taxon*. Briefly, the data obtained were assessed for normality with the Kolmogorove-Smirnov test and for homogeneity of variances by Bartlett's test and then transformed using the natural logarithm or arcsine transformation. Only the data of those FAME with normal distribution were compared by one-way analysis of variance (ANOVA), with post hoc comparisons using Tukey's test (Zar, 2007). A significance of $P < 0.05$ was applied to all statistical tests performed.

4.2.3 Genetic characterisation

4.2.3.1 DNA-DNA hybridisation (DDH)

The gold standard for species delineation was performed in duplicate experiments at 62°C at the German Culture Collection of Microorganisms and Cell Cultures "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DSMZ) lab in Braunschweig Germany. The *F. noatunensis orientalis* strain Fo14 was compared against the type strain of *Fnn*.

Three grams of wet biomass of the strains were grown at the NVI (Norwegian Veterinary Institute) and sent suspended in 1:1 isopropanol and water solution (v/v) at room temperature to the DSMZ. At arrival, bacterial cells were disrupted by using a Constant Systems TS 0.75 KW cell disruptor (IUL Instruments, Braunschweig, Germany) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA-DNA hybridization was carried out as outlined by de Ley *et al.* (1970) with modifications described by Huss *et al.* (1983) using an ultraviolet visible spectrophotometer Cary100 Bio UV/VIS (CaryBio, California, USA) equipped with a Peltier-thermostate multicell changer and a temperature controller with *in situ* temperature probe.

4.2.3.2 16S rRNA gene sequence similarity and phylogeny

The complete 16S rRNA gene sequence was retrieved from all the genomes doing an *in silico* PCR with the universal primers EUBB 5'AGAGTTTGATCMTGGCTCAG'3 and EUBA 5'AAGGAGGTGATCCANCCRCA'3 described by Suzuki and Giovannoni (1996) using the Sequence Manipulation Suite platform (Stothard, 2000) with

modifications in the script for an input limit of 3,000,000 characters. After extraction the sequences were aligned as DNA with the Unweight Pair Group Method with Arithmetic Mean (UPGMA) using the MUSCLE application (Edgar, 2004) of the MEGA package version 6 (Tamura *et al.*, 2013).

After alignment the nucleotide composition of the sequences and their evolutionary divergence (pairwise distances) were estimated using the software MEGA version 6 (Tamura *et al.*, 2013). These values and the length of the aligment were used to calculate the pairwise percent similarity of the sequences.

A phylogenetic tree was constructed for each alignment using MEGA6 software (Tamura *et al.*, 2013) with the Maximum Likelihood (ML) (Nei and Kumar, 2000) approach and complete deletion of gaps and missing data. The model used was a combination of the Hasegawa-Kishino-Yano method (HKY) (Hasegawa *et al.*, 1985) with a discrete gamma distribution of rates among sites. The reliability of the tree (reproducibility) was tested using the bootstrap method with 1000 replications while the nearest-neighbour-interchange was chosen as the ML heuristic method.

4.2.3.3 Whole genome average nucleotide identity (wg-ANI$_m$)

Pairwise comparisons were performed within and between the *taxa* analysed using the programme JSpecies (Richter and Rosselló-Móra, 2009) with the MUMmer algorithm (Kurtz *et al.*, 2004) to calculate the ANI$_m$ across the genome sequences.

4.2.3.6 Genome to genome distance (GGD) and in silico DDH

The GGD between all the types trains of the genus as well as the correspondent DDH value for those comparisons were estimated using the genome to genome distance calculator version 2.0 (Meier-Kolthoff *et al.*, 2013) available at the DSMZ website http://ggdc.dsmz.de/

4.2.3.6 Multilocus sequence analysis (MLSA)

This analysis was performed with the following eight housekeeping genes: malate dehydrogenase (mdh), chromosomal replication initiator protein alpha subunit (dnaA), DNA mismatch repair protein (mutS), peptide chain release factor 2 beta subunit (prfB), bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase alpha subunit (putA), DNA-directed RNA polymerase alpha subunit (rpoA), DNA-directed RNA polymerase beta subunit (rpoB) and triose-phosphate isomerase alpha subunit (tpiA).

The suitability of these genes for phylogenetic analyses was previously reported in section 2.3.4.5 when they were used as single sequences to infer the evolutionary relationship of *Francisella noatunensis orientalis* STIR-GUS-F2f7 with other bacterial pathogens of fish. In this study the 8 housekeeping genes were analysed as a concatenated sequence.

The housekeeping genes were retrieved, aligned, and concatenated from the genome sequences according to the following procedure. For each genome, the gene models and fasta sequences of all the predicted were obtained using the programme Prodigal version 2.6.2 (Hyatt *et al.*, 2010).

The house-keeping genes of each genome were subsequently identified using NCBI BLASTN version 2.2.28+ (by default settings) and the corresponding sequences from the STIR-GUS-F2f7 genome as query by retaining the best hit for each genome. The predicted genes were blasted against prototypic *Francisella* genomes using NCBI BLASTN v. 2.2.28+ with the blastn task selection and an e value of 0.01.

Resulting hits were filtered to exclude alignments shorter than 80% of the query genes and a lower identity than 75% for genomes representing *F. noatunensis* and *F. philomiragia*; 70% for genomes representing *F. tularensis* and *F. hispaniensis* and 65% for the sole representative of *F. guangzhouensis*. Hits corresponding to the previous criteria were saved in GFF format and subsequently used for visualization in the DNAplotter (Carver *et al.*, 2009) software packaged within Artemis v.16.0 (Carver *et al.*, 2012).

All sequences for each gene were aligned subsequently separately using MUSCLE (Edgar, 2004) and visually inspected to ensure a correct selection of gene sequences and good performance of the alignment.

The aligned gene sequences corresponding to each genome were subsequently concatenated using an in-house script developed with the programing language Perl available at www.perl.org. Finally, pairwise percent similarity values were estimated using the final length of the aligment and the pairwise distances as inferred using MEGA6 (Tamura *et al.*, 2013).

The phylogenetic tree for the concatenated sequence was constructed using the Neighbour Joining (NJ) algorithm and previously the suitability of the data for this

analysis was confirmed using the average pairwise Jukes-Cantor (JC) distance (Nei and Kumar, 2000).

4.2.3.9 Whole genome G+C content and phylogeny

The genomes were aligned using a novel objective score called "a sum-of-pairs breakpoint score" available with the progressive Mauve software (Darling *et al.*, 2004) version 2.4.0 development snapshot (2015-02-13). Due to poor performance using all genomes in a single multiple alignment, multiple alignments were performed in sets of 12/13 genomes using tree genomes commonly represented in all three alignments (*F. philomiragia* ATCC25017, *F. tularensis holarctica* FSC200 and *F. noatunensis orientalis* LADL-07-285A).

Using an in-house script, the resulting alignments were processed to match nucleotide positions in the LADL-07-285A genome sequence, enabling merging of the sub alignments into a single multiple alignment of all genomes. Processing included extending gap positions by 15bp in both the 5' and 3' directions to limit the potential impact of misalignments that may arise in such regions. Prior to the alignment the genome sequences of the strains Fh1, Ft2 and Fp1 were reassembled using the parallel assembler for short read sequence data ABySS (Assembly By Short Sequences) version 1.3.4 (Simpson *et al.*, 2009).

The nucleotide composition of the genomes and the WG phylogenetic tree were computed using the software MEGA version 6 (Tamura *et al.*, 2013). A phylogenetic tree was built using the Neighbour Joining (NJ) algorithm and the suitability of the data for

this analysis was confirmed using the average pairwise Jukes-Cantor (JC) distance (Nei and Kumar, 2000).

An un-rooted phylogenetic network was constructed using the software SplitsTree4 version 4.12.3 (Huson and Bryant, 2006) with the default settings and the alignment previously generated in Mega 6. A circular map was generated for each of the type strains genomes and STIR-GUS-F2f7 using the genome viewer and annotation tool Artemis 16.0.0 (Carver *et al.*, 2012) and the software DNAPlotter (Carver *et al.*, 2009).

4.3 Results

4.3.1 Colony morphology and basic phenotype

As expected the basic phenotypic analyses confirmed all the isolates to be Gram negative and non-motile. *Fnn* and *Fno* were oxidase negative and catalase positive while the *Fp* type strain was positive in both tests.

Colonies of *Fno* on CHAH were greenish-greyish, circular, convex and smooth, with complete margins and slightly mucoid appearance. The optimal growth temperature was 28-29°C and at this temperature pin-point colonies appeared after 59h and were clearly visible (~1mm) after 65-75h; colonies integrate with each other after 90h and couldn't be recovered after 4 weeks. No growth was observed at temperatures of 18°C or lower or at 33°C or higher. Visible colonies appeared after 120h at 22°C, 87h at 24°C and 69h at 26°C. The pellets harvested from broth by centrifugation were particularly sticky and mechanical disruption was required with sterile disposable loops as they could not be dissolved by vortexing.

The *Fnn* isolates recovered from Scandinavia were white, slightly translucent, with a clearly mucoid appearance, and their optimal temperature was at 22°C when the colonies appeared after 4 day of incubation. No growth was observed at 15°C or lower temperatures or at 28°C or higher. The pellets of these strains were easy to disrupt by vortexing. Replication of this bacterium was slower as indicated not only by the incubation time, but also by the concentrations required in the starting cultures to produce similar amounts of biomass as *Fno*.

The *Fnn* isolate recovered from Ireland was slightly more greyish. In contrast the colour of the Chilean isolate was grey-greenish somehow resembling the *Fno* isolates colour and it also had a particular odour not presented in the *Fnn* strains and different to the *Fno* particular smell.

Interestingly, when *Fnn* and *Fno* type strains were cultured overnight in the same broth and drops of the mixed culture were plated, the cells of *Fnn* grouped with each other to form well defined white colonies that emerged among the *Fno* colonies Fig. 4.1 letter E.

The colonies of the *Fp* type strain were white but not translucent, with a bigger size than *Fnn* and *Fno* and a faster growth rate; they were able to grow at a similar pace in a range of temperature from 15°C to 37°C. Overnight cultures at 34°C were able to produce as much as twice the biomass of *Fnn* and *Fno* (as measured by OD_{600}). A plate of the colony morphology of the 3 type strains is presented in Fig 4.1.

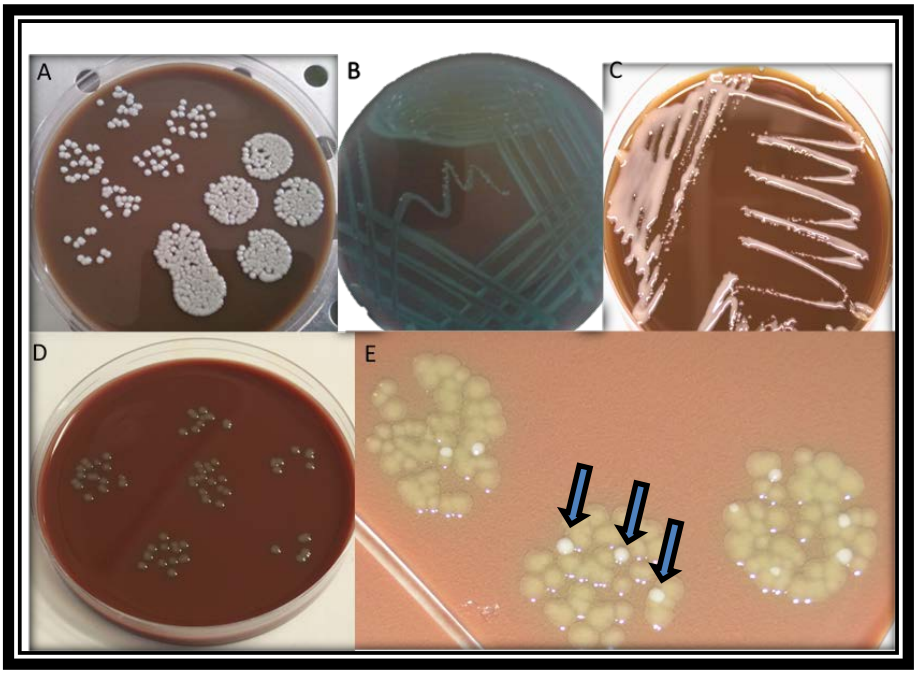

Figure 4.1 Colony morphology on CHAH of the *taxa* analysed. **A** *Fp*, **B** *Fno*, **C** *Fnn*. **D** Drop count of *Fno*. **E** Drop count of mixed *Fnn* and *Fno* culture, arrows indicate growing *Fnn* and were distinct from *Fno*.

4.3.2 Phenotypic (metabolic) fingerprint

The cultures used to inoculate the Biolog GN2 micro plates with *Fno* and *Fnn* were OD_{600} 0.85, while 0.65 was adequate for *Fp*. A picture of the phenotypic fingerprint of *Fnn* (Fn1) at OD_{600} 0.85 and 1.06 is shown in Figure 4.2.

There were 24 differences between *Fp* and *Fnn*, 17 between *Fno* and *Fp*¸ 10 between *Fnn* and *Fno* and additionally some differences were seen within the *Fnn taxon* where the isolates recovered from Chile, Ireland and Sweden showed differences with those from Norway. The largest number of differences within this *taxon* was seen between the *Fnn* from Chile and the strains recovered from Norway, where a total of 9 differences were seen. Finally, as with the Norwegian *Fnn* strains, the comparison of the Chilean *Fnn* with the *Fp* type strains also indicated 24 differences.

The number of positive reactions recorded was 39 for *Fp*, 22 for *Fno*, 17 for the Irish *Fnn*, 16 for Norwegian *Fnn* and 15 for the Chilean and Swedish strains. A summary of the results obtained with the modified protocol of the Biolog GN2 plates is presented in Table 4.2.

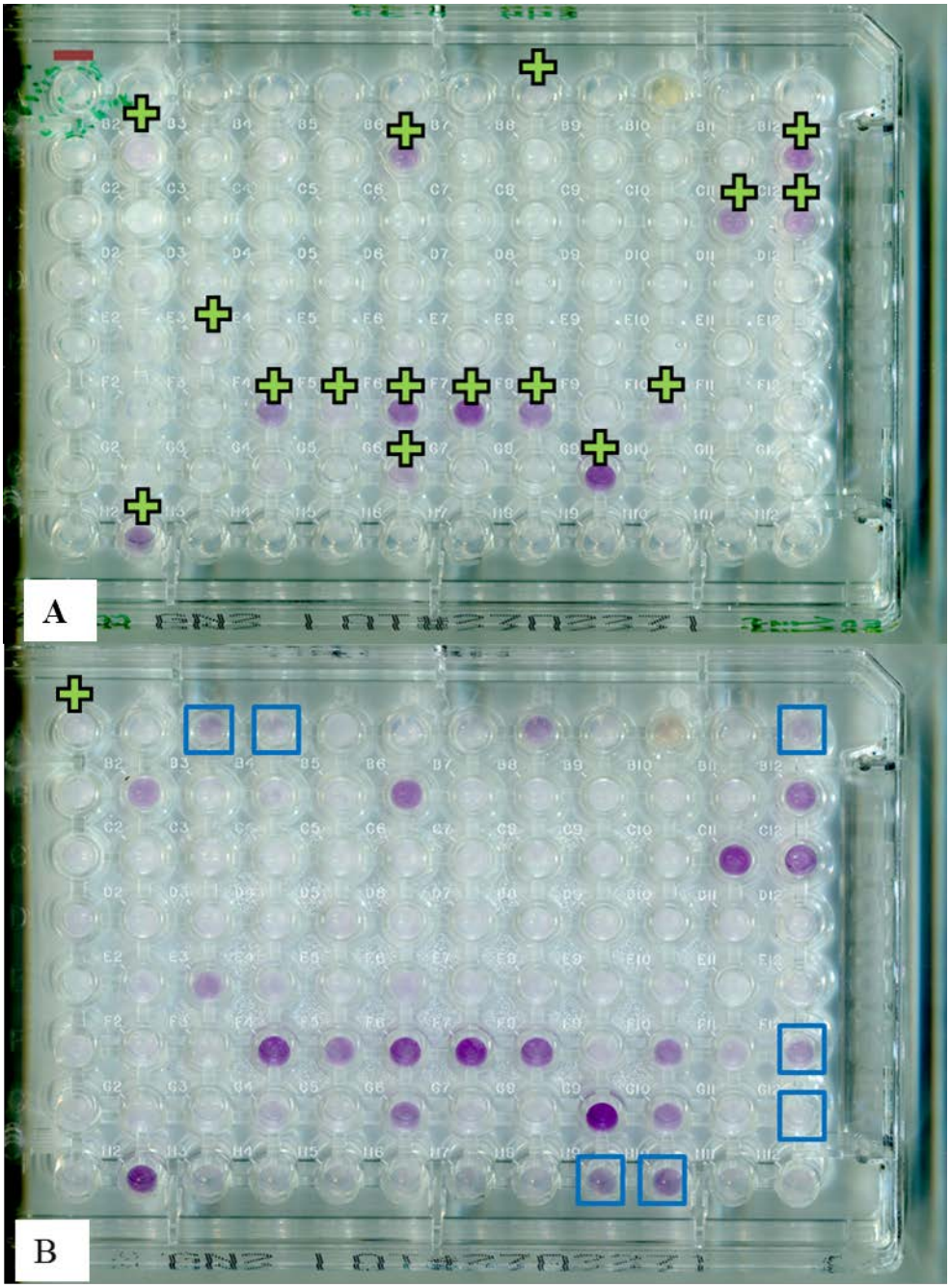

Figure 4.2 Phenotypic fingerprint of *F. noatunensis noatunensis* (Fn1). **A** Photograph of the plate with an OD_{600} 0.85. Well A1 is negative and green + is used to indicate positive wells (where colour has changed). **B** Photograph of the plate with an OD_{600} 1.06 the blue squares indicate ambiguous results when well A1 appears to be positive.

Table 4.2 Metabolic fingerprint of the different *Francisella* spp. analysed in this study

WELL	CARBON SOURCE	*Francisella noatunensis orientalis*															*Francisella noatunensis noatunensis*														
		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
A3	Dextrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A4	Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A8	N-Acetyl-Dglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
A12	D-Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B2	D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B4	D-Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B6	α-D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B10	Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B12	D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C3	D-Psicose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C7	Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C8	D-Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C9	Turanose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C11	Methyl Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C12	Mono-Methyl- Succinate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
D1	Acetic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-
D10	α-Hydroxy Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D11	β-Hydroxy Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E3	α-Keto Butyric Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E6	D,L-Lactic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
F4	L-Alaninamide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
F5	D-Alanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-
F6	L-Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F7	L-Alanylglycine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
F8	L-Asparagine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F9	L-Aspartic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F10	L-Glutamic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F12	Glycyl-LGlutamic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G6	L-Proline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G7	L-Pyroglutamic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G9	L-Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G10	L-Threonine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
H2	Inosine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H3	Uridine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	Thymidine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-
H10	D,L-α-Glycerol Phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H11	Glucose-1-Phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	Glucose-6-Phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1= Fp; 2-16= Fo1-Fo15; 17-31=Fn1-Fn15; In green phenotypic differences between *Fno* and *Fnn*; In red differences within *Fnn* strains.

4.3.3 Chemotaxonomic analyses

The FAME results per strain showed homogeneity within the *Fno taxon*, but variations were seen amongst the *Fnn* strains where differences of up to 11% were seen between Fn11 and Fn12 on the total content of saturated fatty acids. The FAME content per isolate is shown in Table 4.3.

Table 4.3 Relative fatty acid composition (%) per isolate of the *Francisella* spp. analysed in this study

Fatty acid	*F. philomiragia*			*F. noatunensis orientalis*				*F. noatunensis noatunensis*												
	Fp1	Fp3[§]	Fp6[§]	Fo1	Fo12	Fo13	Fo14	Fn1	Fn2	Fn3	Fn4	Fn6	Fn7	Fn8	Fn9	Fn10	Fn11	Fn12	Fn14	Fn16
14:0	7	10	7	8	8	6	10	10	11	9	11	10	9	11	7	8	9	12	9	9
16:0	10	10	12	8	8	6	9	9	10	7	10	9	9	9	8	9	8	9	8	9
17:0	0	0	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	0	0	1
18:0	5	3	4	7	6	7	6	4	5	4	5	5	5	5	5	5	5	5	5	5
20:0	5	3	4	4	4	4	4	4	4	5	5	4	4	4	5	5	5	4	5	5
22:0	9	4	6	9	9	11	9	9	7	14	9	9	7	9	15	11	12	7	11	10
23:0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0
24:0	2.3	1	2	11	13	11	11	5	5	8	4	4	4	5	9	5	9	1	7	5
26:0	1	0	0	1	2	1	1	1	0	1	0	0	0	0	1	0	1	0	1	0
Total saturated	39	32	37	52	53	48	53	42	42	50	45	43	39	45	50	44	50	39	45	44
16:1n-7	1	1	1	0	0	0	0	1	2	1	1	1	1	1	1	1	1	1	1	1
17:1	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
18:1n-9	17	19	19	15	16	17	16	17	17	15	20	18	18	17	15	18	14	18	17	19
20:1n-9	2	4	3	2	2	2	2	3	3	3	4	3	3	3	3	4	3	3	3	3
20:1n-7	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0
22:1	5	10	7	3	3	4	3	7	7	6	8	6	8	6	6	7	6	7	6	7
23:1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
24:1n-9	30	29	28	23	21	25	21	26	23	20	18	24	26	25	22	23	23	28	24	23
26:1	5	4	3	2	2	2	2	3	2	4	2	2	3	2	2	2	2	3	2	2
Total monounsaturated	61	68	62	48	47	52	47	58	57	50	55	57	60	55	50	56	50	61	55	56
18:2n-6	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

[§]Isoltes were donated for this comparison by Dr. Duncan Colquhoun at the The Norwegian Veterinary Institute.
Strains are labelled according to Table 4.1.

The results per *taxon* showed that levels of 24:0, and the total amount of saturated and total monounsaturated FAMEs were significantly different from each other. The content of 24:1n-9 in *Fp* was significantly higher than in *Fnn* and in *Fno* and the content of 22:1 was lower in *Fno* than in *Fp* and *Fnn.* The relative FAME composition per *taxon* is presented in Table 4.4.

Table 4.4 Relative fatty acid composition (%) of the *taxa* analysed in this study

Fatty acid	*Fp*	*Fno*	*Fnn*	Average
14:0	8 ± 2	8 ± 2	10 ± 1	9
16:0	11^{a} ± 1	8^{b} ± 1	9^{b} ± 1	9
18:0	4 ± 1	7 ± 1	5 ± 0	5
20:0	4 ± 1	4 ± 0	5 ± 1	4
22:0	6^{b} ± 2	9^{ab} ± 1	10^{a} ± 3	9
24:0	2^{c} ± 1	11^{a} ± 1	6^{b} ± 2	6
Total saturated	36^{c} ± 3	51^{a} ± 2	44^{b} ± 4	44
18:1n-9	18 ± 1	16 ± 1	17 ± 2	17
20:1n-9	3^{a} ± 1	2^{b} ± 0	3^{a} ± 0	3
22:1	7^{a} ± 2	3^{b} ± 0	7^{a} ± 1	6
24:1n-9	29^{a} ± 1	23^{b} ± 2	23^{b} ± 3	25
26:1	4 ± 1	2 ± 0	2 ± 1	3
Total monounsaturated	63^{a} ± 4	49^{c} ± 2	55^{b} ± 4	56
Total	100	100	100	100

Data are presented as mean + SD (range per *taxon*).
Green colour suitable markers for species diferentiation.

4.3.4 DNA-DNA hybridisation (DDH)

The DDH value between *Fno* and *Fnn* strain was 60% (62.1 in round one and 58.40 in round two), clearly indicating that the two fish pathogens constitute separate bacterial species. Table 4.5 shows these results with other wet lab DDH values available in the literature for fish pathogenic *Francisella* spp.

Table 4.5 DNA-DNA hybridisation values (%) between members of the genus *Francisella*

Species	Strains*	Round 1	Round 2	Average	70% criteria	Temperature
Fno vs *Fnn*	Fo14 vs Fn1	62.1	58.40	60	different species	62°C
†*Fno* vs *Fnn*	Fo1 vs Fn17	64.0	73.4	69	different species	63°C
♫ *Fno* vs *Fnn*	Fo1 vs Fn17	69.7	70.1	70	same species	62°C
¥*Fnn* vs *Fp*	Fn1 vs Fp1	65.2	70.8	68	different species	62°C
♯*Fnn* vs *Fp*	Fn17 vs Fp1	69.5	70.8	70	same species	61°C
♫*Fnn* vs *Fp*	Fn17 vs Fp1	68.0	74.0	71	same species	62°C
♫*Fnn* vs *Fp*	Fnn§ vs Fp1	74.5	67.0	70	same species	62°C
†*Fno* vs *Fp*	Fo1 vs Fp1	57.3	64.3	61	different species	63°C
Fno vs *Fp*	Fo1 vs Fp1	76.5	68.7	73	same species	62°C
♫ *Fnn* vs *Fnn*	Fnn§ vs Fn17	87.2	81.0	84	same species	62°C
♣*Fp* vs *Fp*	Fp1 vs Fp1	-	-	100	same species	65°C
♣*Fp* vs *Fp*	Fp1 vs Fp2	-	-	77	same species	65°C
♣*Fp* vs *Fp*	Fp1 vs Fp3	-	-	83	same species	65°C
♣*Fp* vs *Fp*	Fp1 vs Fp4	-	-	78	same species	65°C
♣*Fp* vs *Fp*	Fp1 vs Fp6	-	-	79	same species	65°C
♣*Fp* vs *Ftn*	Fp1 vs Ft4	-	-	51	different species	50°C
♣*Fp* vs *Ftt*	Fp1 vs Ft3	-	-	35	different species	50°C
♦*Fh* vs *Ft*	Fh1 vs Ft1	57	38	48	different species	-
♦*Fh* vs *Ft*	Fh1 vs Ft2	61	35	48	different species	-
♦*Fh* vs *Ftn*	Fh1 vs Ft4	52	54	53	different species	-
♦*Fh* vs *Fp*	Fh1 vs Fp1	39	43	41	different species	-
♦*Fh* vs *Fnn*	Fh1 vs Fn17	47	28	38	different species	-
¶*Fhal* vs *Fp*	Fhal vs Fp1	58.4	63.5	61	different species	62°C
¶ *Fhal* vs *Fnn*	Fhal vs Fn1	49.8	48.5	49	different species	62°C
¶ *Fhal* vs *Fno*	Fhal vs Fo1	59.6	53.3	56	different species	62°C
♆*Fg vs Fp*	Fg vs Fp1	-	-	38	different species	-

*As identified in this study (Table 4.1); Same colour are parallel studies with members of the same *taxa* ;

§Refers to the *Fnn* Chilean strain UA2660 not analysed in this study;

¥ Mikalsen *et al*. (2007); † Ottem *et al* . (2008); ♯ Ottem *et al* . (2007); ♫Ottem *et al* . (2011);

♣ Hollis *et al* . (1989); ♦ Huber *et al* . (2010); ¶ Brevik *et al* . (2011); ♆ Qu *et al* . (2013);

4.3.5 16S rRNA gene similarity and phylogeny

The length of the 16S rRNA of the *Fno* and *Fnn* strains was 1520 nucleotides, which was 2bp smaller than in all the *Fp* strains. The nucleotide composition of the 16S rRNA gene of each type strain analysed is presented in Table 4.6.

Table 4.6 Nucleotide composition (%) of the 16S rRNA gene of the *Francisella* type strains

Strain	T(U)	C	A	G	GC%	Length (bp)
Fo1	26	31	22	21	52	1520
Fn1	26	31	22	21	52	1520
Ft1	26	31	22	21	52	1520
Ft2	26	31	22	21	52	1520
Ft4	26	31	22	21	52	1520
Fh1	26	31	22	21	52	1520
Ft3	26	31	22	21	52	1519
Fg1	27	30	23	20	51	1519
Fp1	26	31	22	21	52	1518

The final length of the alignment was 1516 nucleotides. The average of differences between *Fno* and *Fnn* was 15bp, between *Fno* and *Fp* 13bp and between *Fnn* and *Fp* 10bp. The estimates of evolutionary divergence and the percent similarity of these genes are shown in Table 4.7. The phylogenetic tree generated for this gene is presented in Figure 4.3.

Table 4.7 Estimates of evolutionary divergence and percent similarity of the 16S rRNA gene

-	Fo1	Fo2	Fo3	Fo4	Fo5	Fo6	Fo7	Fo8	Fo9	Fo12	Fo14	Fo15	Fo17	Fn1	Fn5	Fn6	Fn13	Fn15	Fn17	Fn18	Fp1	Fp2	Fp3	Fp4	Fp5	Fp6	Ft1	Ft2	Ft3	Ft4	Fh1	Fg1
Fo1	-	100	100	100	100	100	100	100	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo2	0	-	100	100	100	100	100	100	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo3	0	0	-	100	100	100	100	100	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo4	0	0	0	-	100	100	100	100	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo5	0	0	0	0	-	100	100	100	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo6	0	0	0	0	0	-	100	100	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo7	0	0	0	0	0	0	-	100	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo8	0	0	0	0	0	0	0	-	100	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo9	0	0	0	0	0	0	0	0	-	100	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo12	0	0	0	0	0	0	0	0	0	-	100	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo14	0	0	0	0	0	0	0	0	0	0	-	100	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo15	0	0	0	0	0	0	0	0	0	0	0	-	100	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fo17	0	0	0	0	0	0	0	0	0	0	0	0	-	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.2	99.2	97.8	97.8	97.9	98.0	98.0	95.1
Fn1	12	12	12	12	12	12	12	12	12	12	12	12	12	-	99.8	100	100	100	100	100	99.5	99.3	99.3	99.3	99.4	99.4	98.0	98.0	98.2	98.2	98.2	95.3
Fn5	13	13	13	13	13	13	13	13	13	13	13	13	13	3	-	99.8	99.8	99.8	99.8	99.8	99.4	99.2	99.2	99.2	99.3	99.3	98.0	98.0	98.1	98.1	98.2	95.1
Fn6	12	12	12	12	12	12	12	12	12	12	12	12	12	0	3	-	100	100	100	100	99.5	99.3	99.3	99.3	99.4	99.4	98.0	98.0	98.2	98.2	98.2	95.3
Fn13	12	12	12	12	12	12	12	12	12	12	12	12	12	0	3	0	-	100	100	100	99.5	99.3	99.3	99.3	99.4	99.4	98.0	98.0	98.2	98.2	98.2	95.3
Fn15	12	12	12	12	12	12	12	12	12	12	12	12	12	0	3	0	0	-	100	100	99.5	99.3	99.3	99.3	99.4	99.4	98.0	98.0	98.2	98.2	98.2	95.3
Fn17	12	12	12	12	12	12	12	12	12	12	12	12	12	0	3	0	0	0	-	100	99.5	99.3	99.3	99.3	99.4	99.4	98.0	98.0	98.2	98.2	98.2	95.3
Fn18	12	12	12	12	12	12	12	12	12	12	12	12	12	0	3	0	0	0	0	-	99.5	99.3	99.3	99.3	99.4	99.4	98.0	98.0	98.2	98.2	98.2	95.3
Fp1	13	13	13	13	13	13	13	13	13	13	13	13	13	8	9	8	8	8	8	8	-	100	100	100	100	100	98.5	98.5	98.7	98.7	98.7	95.6
Fp2	14	14	14	14	14	14	14	14	14	14	14	14	14	11	12	11	11	11	11	11	3	-	100	100	100	100	98.6	98.6	98.7	98.7	98.8	95.5
Fp3	14	14	14	14	14	14	14	14	14	14	14	14	14	11	12	11	11	11	11	11	3	0	-	100	100	100	98.6	98.6	98.7	98.7	98.8	95.5
Fp4	14	14	14	14	14	14	14	14	14	14	14	14	14	11	12	11	11	11	11	11	3	0	0	-	100	100	98.6	98.6	98.7	98.7	98.8	95.5
Fp5	12	12	12	12	12	12	12	12	12	12	12	12	12	9	10	9	9	9	9	9	1	2	2	2	-	100	98.5	98.5	98.6	98.6	98.7	95.6
Fp6	12	12	12	12	12	12	12	12	12	12	12	12	12	9	10	9	9	9	9	9	1	2	2	2	0	-	98.5	98.5	98.6	98.6	98.7	95.6
Ft1	33	33	33	33	33	33	33	33	33	33	33	33	33	30	31	30	30	30	30	30	22	21	21	21	23	23	-	99.9	99.9	99.9	99.6	95.1
Ft2	33	33	33	33	33	33	33	33	33	33	33	33	33	30	31	30	30	30	30	30	22	21	21	21	23	23	2	-	99.9	99.9	99.6	95.1
Ft3	32	32	32	32	32	32	32	32	32	32	32	32	32	28	29	28	28	28	28	28	20	19	19	19	21	21	2	2	-	99.9	99.6	95.2
Ft4	31	31	31	31	31	31	31	31	31	31	31	31	31	28	29	28	28	28	28	28	20	19	19	19	21	21	2	2	2	-	99.7	95.2
Fh1	30	30	30	30	30	30	30	30	30	30	30	30	30	27	28	27	27	27	27	27	19	18	18	18	20	20	6	6	6	4	-	95.3
Fg1	74	74	74	74	74	74	74	74	74	74	74	74	74	71	74	71	71	71	71	71	66	68	68	68	66	66	75	75	73	73	72	-

The evolutionary divergence is shown below the diagonal where the number of base differences per sequence from between sequences are shown. The % similarity is shown above the diagonal.
In pink *Fno* vs *Fn* ; In purple *Fp* vs *Fno* and in blue *Fnn* vs *Fp* ; In yellow, differences and % similarity within the 4 *Ft* ssp. (below) and between the Chilean and the other *Fnn* strains (above).

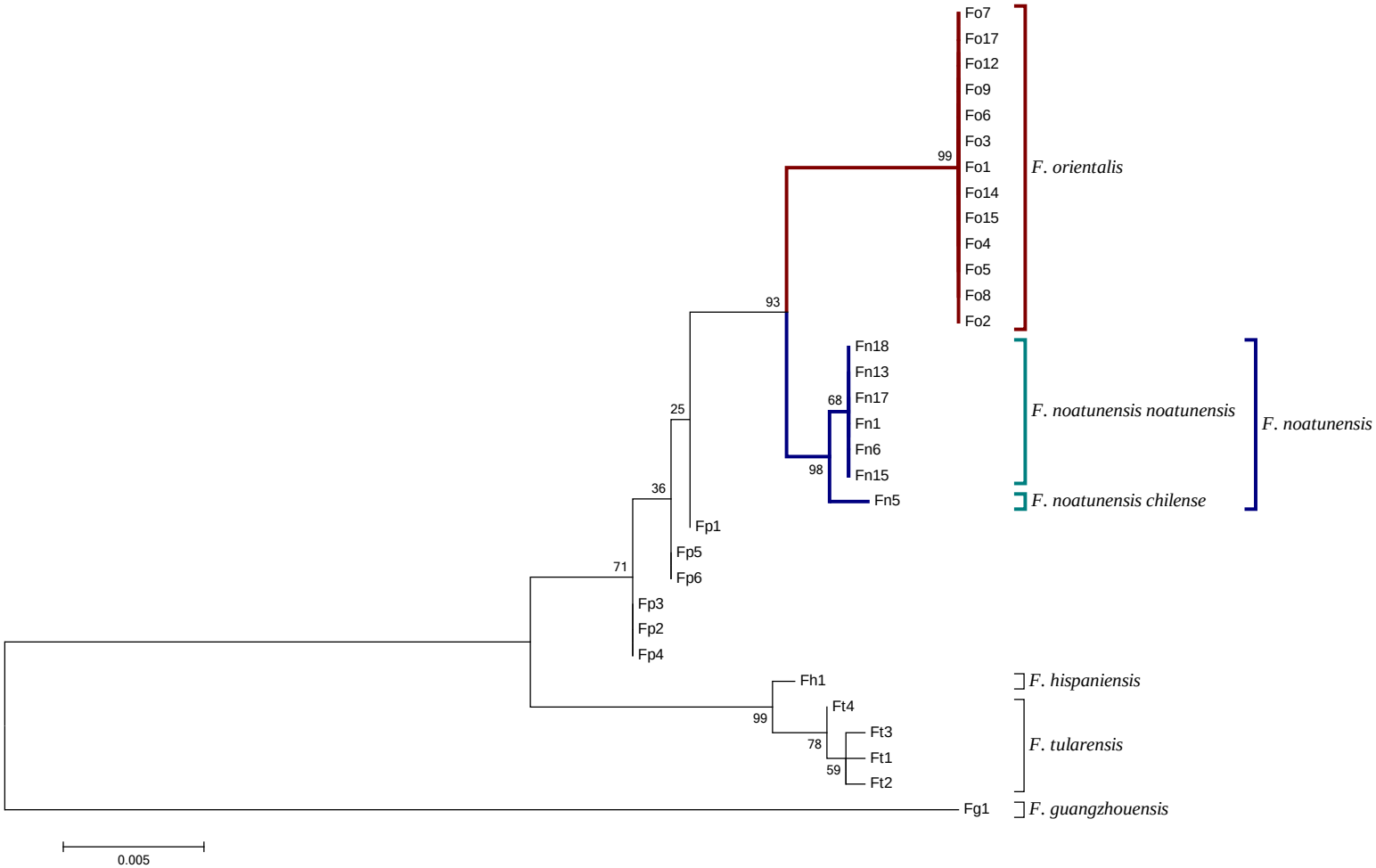

Figure 4.3 Phylogenetic analysis based on the complete 16S rRNA sequence of the 32 genomes analysed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated *taxa* clustered together is shown next to the branches. The strains analysed are identified according to Table 4.1.

4.3.6 Whole genome average nucleotide identity (wg-ANI_m)

Whole genome ANI estimated with the software JSpecies (Richter and Rosselló-Móra, 2009) and the MUMmer algorithm (Kurtz *et al.*, 2004) indicated 100% similarity within the *Fno* strains; with the exception of the Chilean strain, the similarity within the *Fnn taxon* was also 100%. The Chilean strain and the rest of the *Fnn* strains have an ANI of 99.5%. The similarity among the four *F. tularensis* subspecies varied from 98.1 to 99.4%.

The ANI between *Fno* and *F. philomiragia* was 93% and between *Fp* and *Fnn* was 95% indicating that *Fp* is more closely related to *Fnn* than to *Fno*. Finally the ANI between *Fno* and *Fnn* was 94-95% confirming that these *taxa* constitute separate species. The results of the whole genome average nucleotide identity including the other validly described members of the genus analysed are presented in Table 4.8.

Table 4.8 Whole genome average nucleotide identity (%) of the genus *Francisella*

	Fo1	Fo2	Fo3	Fo4	Fo5	Fo6	Fo7	Fo8	Fo9	Fo12	Fo14	Fo15	Fo17	Fn1	Fn5	Fn6	Fn13	Fn15	Fn17	Fn18	Fp2	Fp3	Fp4	Fp5	Fp6	Ft1	Ft2	Ft3	Ft4	Fh1	Fg1
Fo1	---																														
Fo2	100	---																													
Fo3	100	100	---																												
Fo4	100	100	100	---																											
Fo5	100	100	100	100	---																										
Fo6	100	100	100	100	100	---																									
Fo7	100	100	100	100	100	100	---																								
Fo8	100	100	100	100	100	100	100	---																							
Fo9	100	100	100	100	100	100	100	100	---																						
Fo12	100	100	100	100	100	100	100	100	100	---																					
Fo14	100	100	100	100	100	100	100	100	100	100	---																				
Fo15	100	100	100	100	100	100	100	100	100	100	100	---																			
Fo17	100	100	100	100	100	100	100	100	100	100	100	100	---																		
Fn1	94	95	95	95	95	95	95	95	95	95	94	95	94	---																	
Fn5	94	94	94	94	94	94	94	94	94	94	94	94	94	99.5	---																
Fn6	94	94	94	94	94	94	94	94	94	94	94	94	94	100	99.5	---															
Fn13	94	94	94	94	94	94	94	95	94	95	94	95	94	100	99.5	100	---														
Fn15	94	95	95	95	95	95	95	95	95	95	94	95	94	100	99.5	100	100	---													
Fn17	94	95	94	94	95	95	95	95	95	95	94	95	94	100	99.5	100	100	100	---												
Fn18	94	95	94	94	95	95	95	95	95	95	94	95	94	100	99.5	100	100	100	100	---											
Fp2	93	93	93	93	93	93	93	93	93	93	93	93	93	95	95	95	95	95	95	95	---										
Fp3	93	93	93	93	93	93	93	93	93	93	93	93	93	95	95	95	95	95	95	95	100	---									
Fp4	93	93	93	93	93	93	93	93	93	93	93	93	93	95	95	95	95	95	95	95	100	100	---								
Fp5	93	93	93	93	93	93	93	93	93	93	93	93	93	95	95	95	95	95	95	95	98	98	98	---							
Fp6	93	93	93	93	93	93	93	93	93	93	93	93	93	95	95	95	95	95	95	95	98	98	98	98	---						
Ft1	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	---					
Ft2	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	99.3	---				
Ft3	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	85	99.3	99.4	---			
Ft4	85	86	85	86	86	86	86	86	86	86	86	86	85	85	85	85	85	85	85	85	86	86	86	86	86	98.1	98.1	98.2	---		
Fh1	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	86	92	92	92	92	---	
Fg1	84	85	84	85	85	85	85	85	85	85	84	85	84	84	84	83	84	84	84	84	84	84	84	84	84	83	84	83	84	84	---

In green: similarity between strains belonging to the *taxon Fno* ; In orange similarity between strains belonging to the *taxon Fnn* (in yelow *Fnn* Chilean strain);
In blue: similarity between *Fno* and *Fnn* ; In purple: similarity within the 4 *F. tularensis* subspeceis;
In pink: similarity of *F. philomiragia* to *Fno* and *Fnn* ;

4.3.7 Genome to genome distance (GGD) and *in silico* DDH

The genome to genome distances of the type strains and their respective DDH values are shown in Table 4.9.

The values of the *in silico* DDH between *Fno* and the strains of *Fnn* (62-63%) showed that these *taxa* represent separate species. The results confirmed that all the *F. tularensis* strains tested belong to the same species with values ranging from 92-99%. The association value (98%) of the Chilean strain with the rest of the *Fnn* strains was lower than that observed within the *F. tularensis* strains confirming that this isolate represents a new subspecies of *F. noatunensis*.

F. philomiragia had higher values of association with *Fnn* (65%) than with *Fno* (56%). All the other strains had values ranging from 21-49%, which confirms their status as independent species.

Table 4.9 *In silico* DNA-DNA hybridisation and GGD of the type strains

	Fo1	Fn1	Fn5	Fp1	Ft1	Ft2	Ft3	Ft4	Fh	Fg
Fo1	-	63	62	56	24	24	24	24	25	21
Fn1	0.407	-	98	65	24	24	24	24	25	21
Fn5	0.409	0.076	-	65	24	24	24	24	25	21
Fp1	0.467	0.388	0.391	-	25	25	25	25	25	21
Ft1	0.890	0.893	0.894	0.880	-	99	99	92	46	21
Ft2	0.896	0.893	0.893	0.880	0.060	-	99	92	47	21
Ft3	0.895	0.890	0.889	0.879	0.055	0.045	-	92	47	21
Ft4	0.892	0.889	0.890	0.868	0.164	0.163	0.164	-	49	21
Fh	0.870	0.871	0.871	0.863	0.561	0.553	0.551	0.531	-	21
Fg	0.966	0.966	0.965	0.963	0.961	0.961	0.961	0.960	0.963	-

GGD values are shown below the diagonal and DDH values (%) above.
In blue strains that belong to the same species according the 70% threshold.

4.3.8 Multilocus sequence analysis (MLSA)

The total length of the alignment of the concatenated housekeeping genes was 15750 positions. The pairwise percent similarity of the sequences is presented in Table 4.10. The estimates of average evolutionary divergence overall sequence pairs using the Jukes-Cantor model indicated that the average was 0.09. The phylogenetic tree constructed with the neighbour joining method is presented in Figure 4.4. The Perl scrip created to concatenate the sequences is:

```
#!/usr/bin/perl -w
#
#use Sort::Key::Natural qw(natkeysort);
die "concatenateFromFastas.pl <suffix> <file_Of_strains>\n" if (!(@ARGV));
die "concatenateFromFastas.pl <suffix> <file_Of_strains>\n" if ( $#ARGV != 1 );
chomp($suff = $ARGV[0]);
chomp($file = $ARGV[1]);
open(FH1, "<$file");
chomp(@list = <FH1>);
close(FH1);
@fastas = <*.$suff>;
foreach $genome (@list) {
   $genome =~ s/\s+//g;
   $hash{$genome} = "";
}
foreach $fasta (@fastas) {
   open(FH2, "<$fasta");
   chomp(@tmp = <FH2>);
   close(FH2);
   foreach $row (@tmp){
            $row =~ s/\s+//g;
            if($row =~ />(\S+)/){
               $genome = $1;
               $genome =~ s/_\d+$//g;
               print "$genome\n";
            }
            else{
               if(exists($hash{$genome})){
                        $concats{$genome} .= $row;
               }
            }   }
   foreach $genome (sort { $a cmp $b } keys(%concats)) {
            $concats{$genome} .= "--nn--nn--";
   }
}
$outfile = "concat.genes2.fas";
open (FH2, ">$outfile");
foreach $genome (sort { $a cmp $b } keys(%concats)) {
   print FH2 ">" . $genome . "\n";
   until(length($concats{$genome}) == 0){
            print FH2 substr($concats{$genome}, 0, 80, "") . "\n";
   }
}
close(FH2);
```

Table 4.10 Pairwise percent similarity of the concatenated housekeeping genes.

	Fo1	Fo2	Fo3	Fo4	Fo5	Fo6	Fo7	Fo8	Fo9	Fo12	Fo14	Fo15	Fo17	Fn1	F5	Fn6	Fn13	Fn15	Fn17	Fn18	Fp1	Fp2	Fp3	Fp4	Fp5	Fp6	Ft1	Ft2	Ft4	Ft3	Fh	Fg
Fo1	-																															
Fo2	100	-																														
Fo3	100	100	-																													
Fo4	100	100	100	-																												
Fo5	100	100	100	100	-																											
Fo6	100	100	100	100	100	-																										
Fo7	100	100	100	100	100	100	-																									
Fo8	100	100	100	100	100	100	100	-																								
Fo9	100	100	100	100	100	100	100	100	-																							
Fo12	100	100	100	100	100	100	100	100	100	-																						
Fo14	100	100	100	100	100	100	100	100	100	100	-																					
Fo15	100	100	100	100	100	100	100	100	100	100	100	-																				
Fo17	100	100	100	100	100	100	100	100	100	100	100	100	-																			
Fn1	95	95	95	95	95	95	95	95	95	95	95	95	95	-																		
F5	95	95	95	95	95	95	95	95	95	95	95	95	95	99.7	-																	
Fn6	95	95	95	95	95	95	95	95	95	95	95	95	95	100	100	-																
Fn13	95	95	95	95	95	95	95	95	95	95	95	95	95	100	99.7	100	-															
Fn15	95	95	95	95	95	95	95	95	95	95	95	95	95	100	99.7	100	100	-														
Fn17	95	95	95	95	95	95	95	95	95	95	95	95	95	100	99.7	100	100	100	-													
Fn18	95	95	95	95	95	95	95	95	95	95	95	95	95	100	99.7	100	100	100	100	-												
Fp1	94	94	94	94	94	94	94	94	94	94	94	94	94	97	97	97	97	97	97	97	-											
Fp2	94	94	94	94	94	94	94	94	94	94	94	94	94	97	97	97	97	97	97	97	98.6	-										
Fp3	94	94	94	94	94	94	94	94	94	94	94	94	94	97	97	97	97	97	97	97	98.6	100	-									
Fp4	94	94	94	94	94	94	94	94	94	94	94	94	94	97	97	97	97	97	97	97	98.6	100	100	-								
Fp5	94	94	94	94	94	94	94	94	94	94	94	94	94	97	97	97	97	97	97	97	98.5	98.9	98.8	98.8	-							
Fp6	94	94	94	94	94	94	94	94	94	94	94	94	94	97	97	97	97	97	97	97	98.6	98.8	98.8	98.8	98.9	-						
Ft1	83	83	83	83	83	83	83	83	83	83	83	83	83	84	84	84	84	84	84	84	84	84	84	84	84	84	-					
Ft2	83	83	83	83	83	83	83	83	83	83	83	83	83	84	84	84	84	84	84	84	84	84	84	84	84	84	99.6	-				
Ft4	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	99	99	-			
Ft3	83	83	83	83	83	83	83	83	83	83	83	83	83	84	84	84	84	84	84	84	84	84	84	84	84	84	99.7	99.8	99	-		
Fh	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	94	94	94	94	-	
Fg	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	-

In green *Fno* vs *Fnn* (95%); In orange *Fp* vs *Fno* (94%); In light blue *Fp* vs *Fnn* (97%); Dark blue the 4 *Ft* ssp.; Red the Chilean isolate with respect to the other *Fnn*.

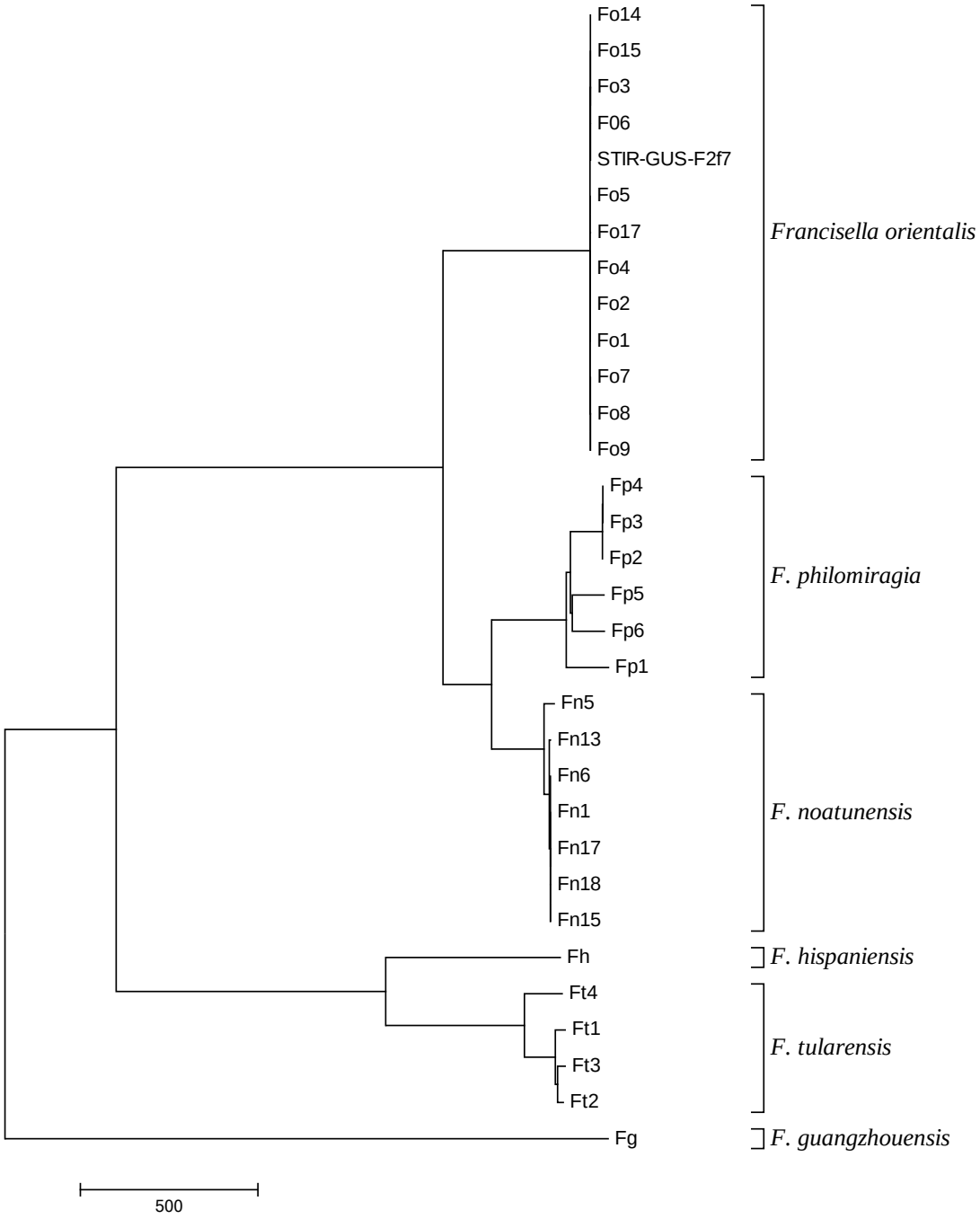

Figure 4.4 Phylogenetic analysis based on the concatenated sequences of the 8 housekeeping genes. The strains analysed are identified according to Table 4.1. The optimal tree with the sum of branch length = 0.44353318 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 15750 positions in the final dataset.

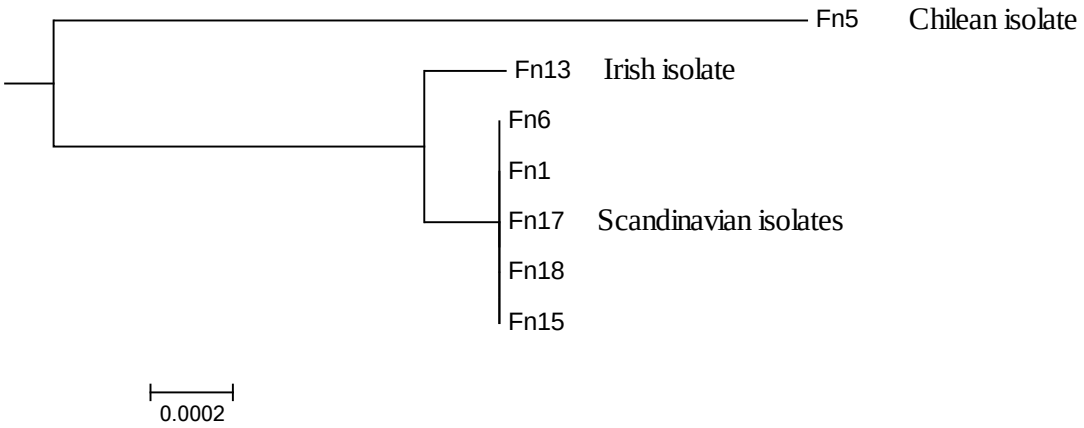

Figure 4.4 Phylogenetic analysis based the concatenated sequences of the 8 housekeeping genes (continued). View of the subtree clustering isolates of *Fnn*.

4.3.9 Whole genome G+C content and phylogeny

The amino acid composition (%), total size (bp) and G+C content (%) of each genome analysed are presented in Table 4.11. The average percent of G+C content for the *taxon Fno* was 32.3, for *Fnn* 32.5, for *Fp* 32.6 and for Ft 32.3. The WG phylogenetic tree is presented in Figure 4.5 and the WG phylogenetic network is shown in Figure 4.6. A diagram comparing the circular chromosome maps of the whole genome sequences of the type strains of the genus *Francisella* and STIR-GUS-F2f7 is presented in Figure 4.7.

The Perl script to run the progressive Mauve alignment was:

```
#!/usr/bin/perl -w

die "usage: run_progressiveMauve.pl <files postfix> \n" if (!defined(@ARGV));
die "usage: run_progressiveMauve.pl <files postfix> \n" if ( $#ARGV != 0 );

chomp($pf = $ARGV[0]);

@files = <*.$pf>;

$genomes = join(' ', @files);

system("/home/parlar/bin/progressiveMauve --output=alignment.xmfa $genomes");
```

Table 4.11 Overall composition (%) and G+C content (%) of the genomes analysed

Strain♣	A	C	G	T(U)	Total (bp)	G+C	average per taxon
Fo1	33.8	15.5	16.7	34.0	1846774	32.2	
Fo2	33.6	14.6	17.7	34.0	1914623	32.3	
Fo3	33.6	14.5	17.7	34.1	1882841	32.2	
Fo4	33.6	14.6	17.7	34.0	1904264	32.3	
Fo5	33.6	14.6	17.7	34.0	1930364	32.3	
Fo6	33.9	16.2	16.1	33.8	1858986	32.3	
Fo7	33.6	14.6	17.7	34.1	1878063	32.3	
Fo8	33.6	14.6	17.8	34.0	1890246	32.4	
Fo9	33.6	14.7	17.7	34.1	1907963	32.3	
Fo12	33.7	17.1	15.4	33.9	1887347	32.4	
Fo14	33.9	15.6	16.6	33.9	1875075	32.2	
Fo15	33.6	14.6	17.8	34.0	1886598	32.4	
Fo17	33.7	14.6	17.7	34.1	1847202	32.2	32.3
Fn1	33.9	16.1	16.4	33.6	1719713	32.5	
Fn5	33.7	16.2	16.2	33.9	1701861	32.4	
Fn6	33.9	16.1	16.2	33.8	1902019	32.3	
Fn13	33.6	16.2	16.2	34.0	1712403	32.4	
Fn15	33.8	16.5	16.1	33.7	1699540	32.5	
Fn17	33.7	16.5	16.2	33.6	1640505	32.7	
Fn18	33.5	16.0	16.6	33.9	1652624	32.6	32.5
Fp1	33.7	16.4	16.2	33.7	2017400	32.6	
Fp2	33.7	16.7	15.8	33.7	1985122	32.5	
Fp3	33.8	16.1	16.4	33.6	2045775	32.6	
Fp4	33.9	16.4	16.1	33.7	1910031	32.5	
Fp5	33.8	16.8	15.8	33.6	1916998	32.6	
Fp6	33.5	15.4	17.3	33.8	1880223	32.6	32.6
Ft1	33.8	15.9	16.3	34.1	1894157	32.2	
Ft2	34.0	16.0	16.3	33.7	1893886	32.3	
Ft3	34.0	15.9	16.3	33.7	1892775	32.3	
Ft4	33.9	16.4	16.1	33.7	1910031	32.5	32.3
Fh	34.0	16.2	15.9	33.9	1919170	32.1	32.1
Fg	33.9	16.0	16.0	34.1	1658482	32.0	32

♣ Strains are identified according to Table 4.1

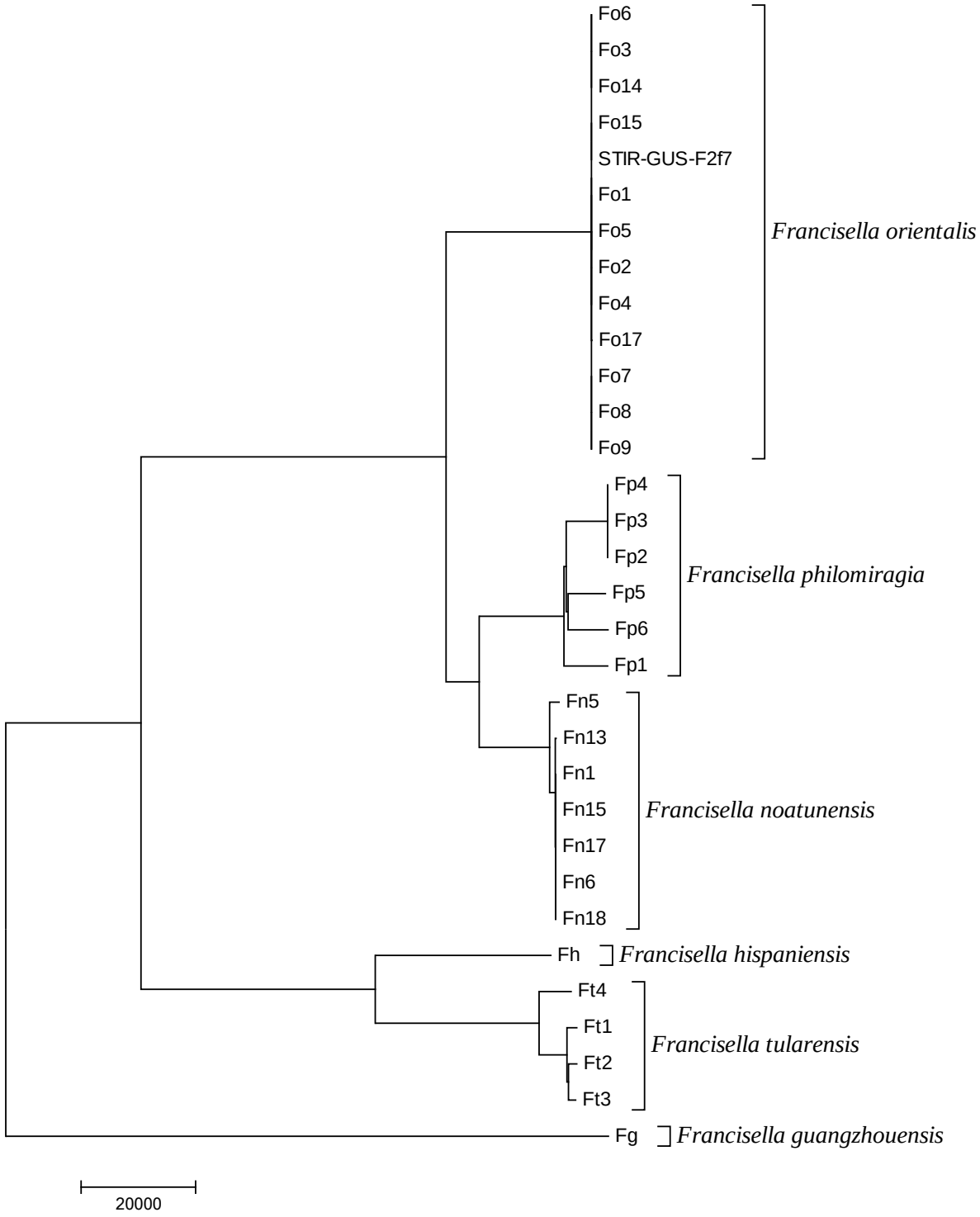

Figure 4.5 Evolutionary relationship of the genus *Francisella*.The evolutionary history was inferred using the Neighbor-Joining method. There were a total of 841918 positions in the final dataset.

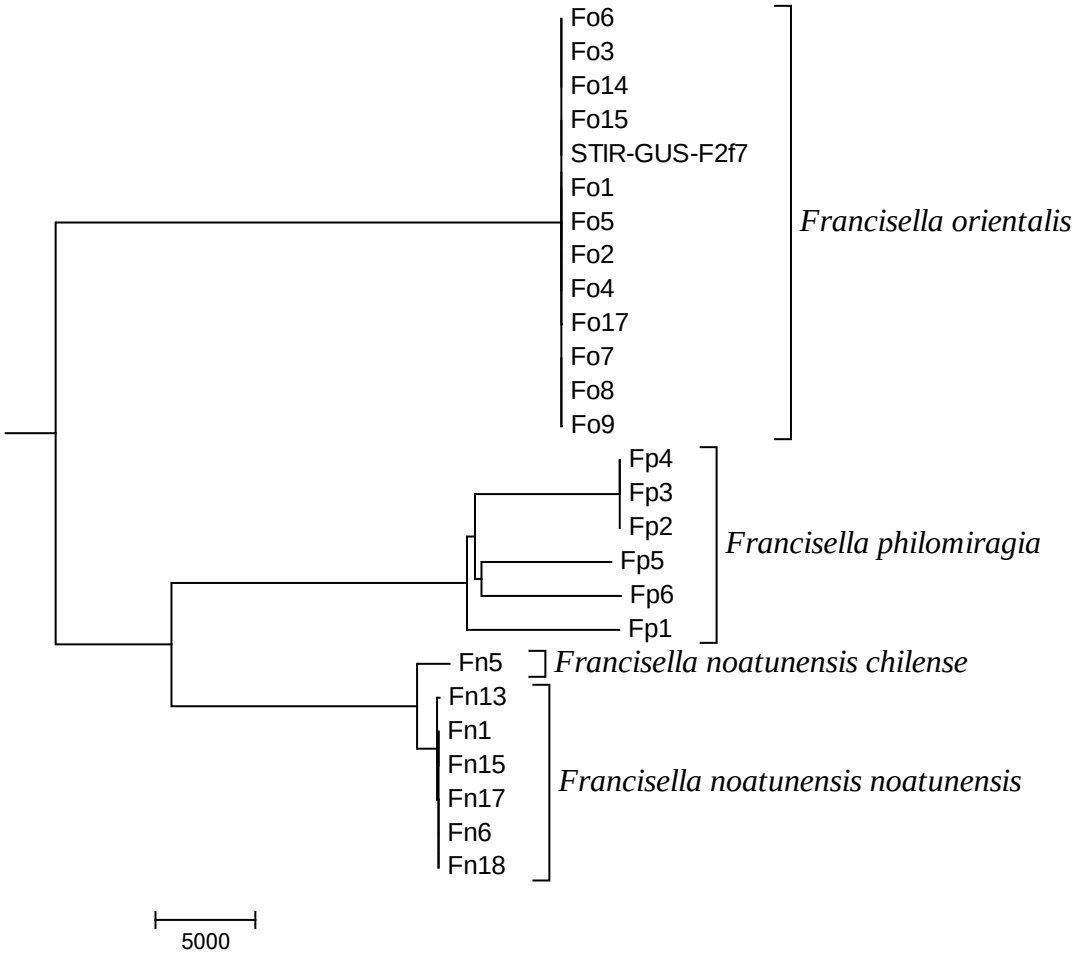

Figure 4.5 Evolutionary relationship of the genus *Francisella* (continued). Close up of the subtree clustering fish pathogenic strains.

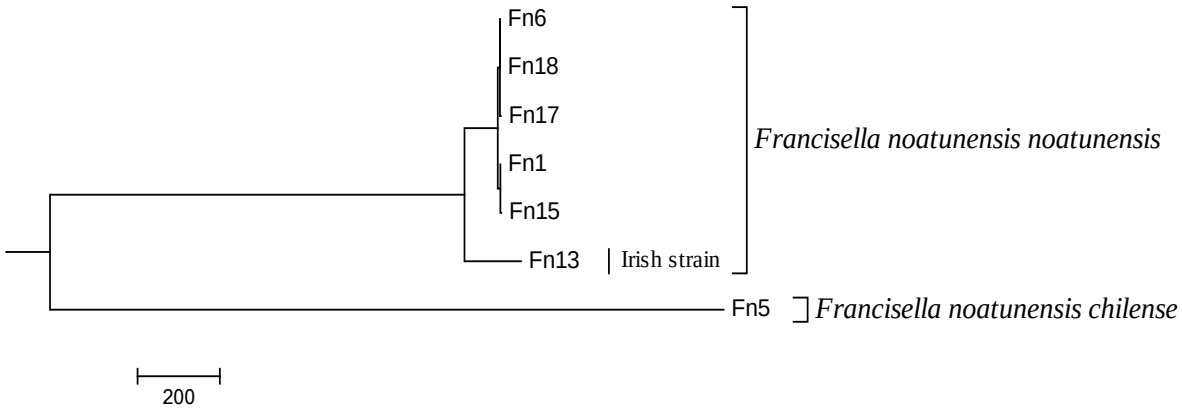

Figure 4.5 Evolutionary relationship of the genus *Francisella* (continued). Comparison of the subtree clustering pathogenic strains with cold water fish species.

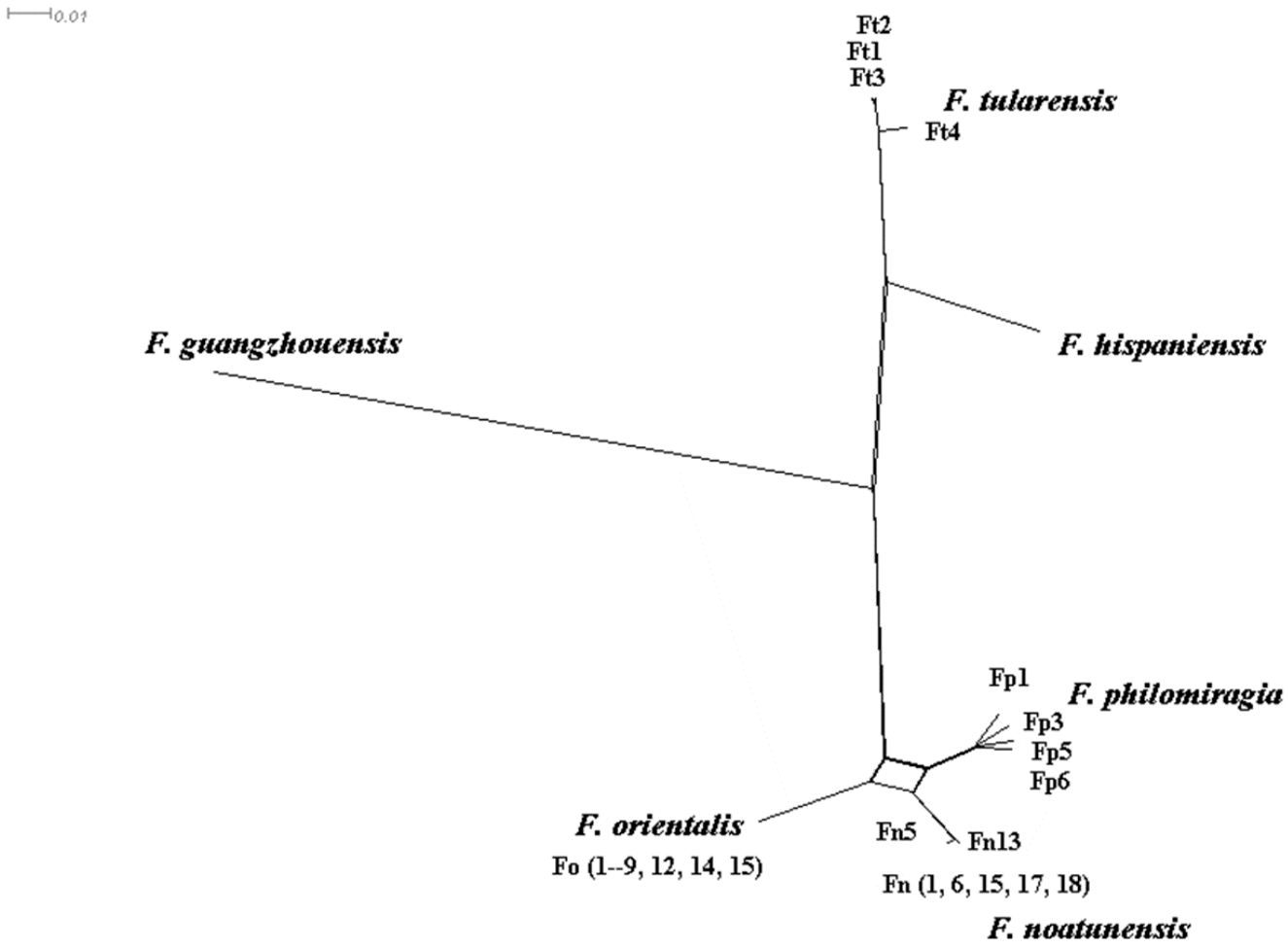

Figure 4.6 Whole genome phylogenetic network of the genus *Francisella*. Split decomposition analysis constructed under neighbour net graph and uncorrected p distance options of the SplitTree programme.

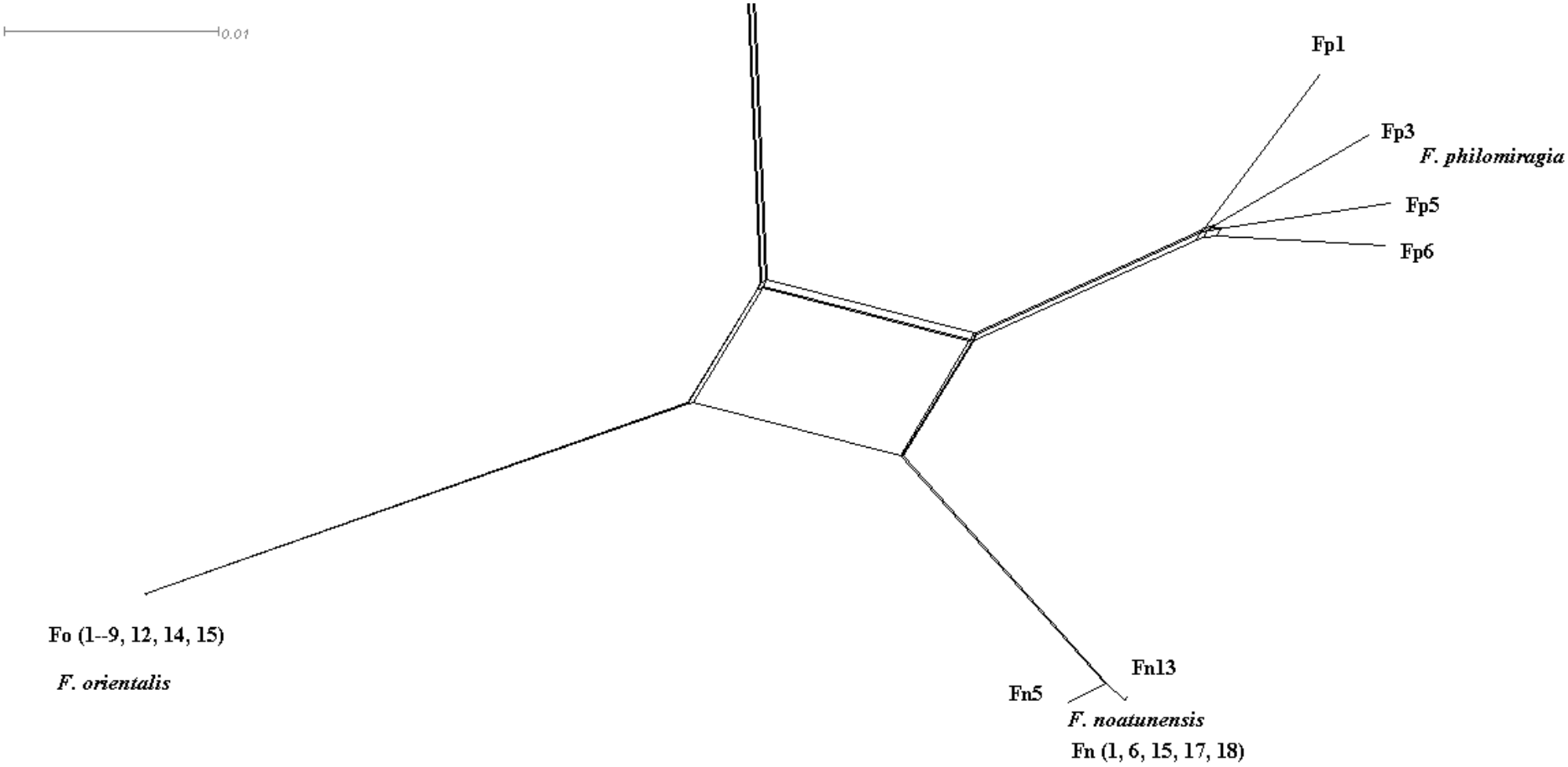

Figure 4.6 Whole genome phylogenetic network of the genus *Francisella* (continued). Close up of the lower branch of the network.

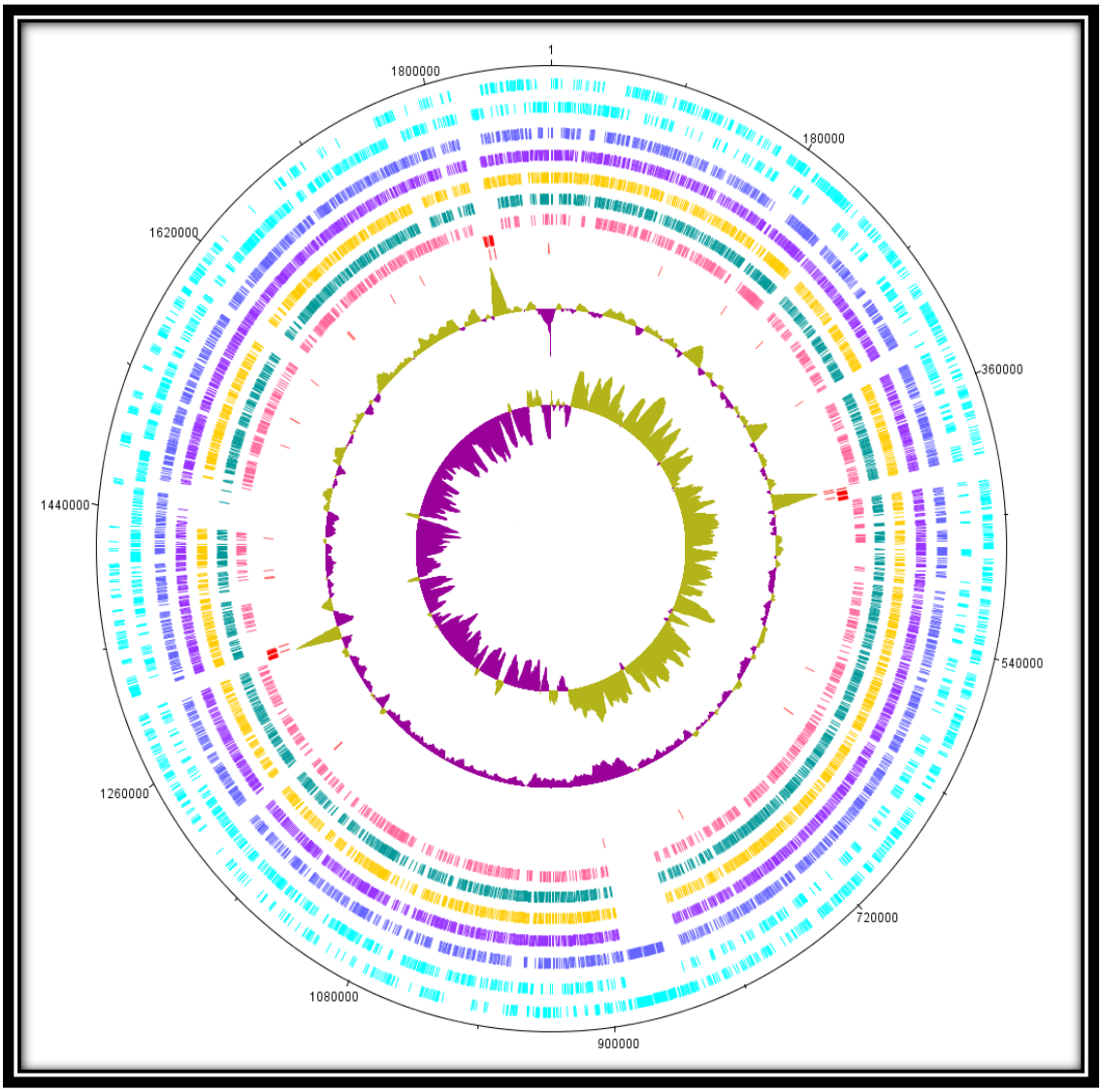

Figure 4.7 Circular map of the genome of the *Francisella* type strains and STIR-GUS-F2f7. The outer scale is marked in base pairs. From outer to inner circles: first (black) all the nucleotides. Second STIR-GUS-F2f7 coding sequences (CDS) in forward strand. Third STIR-GUS-F2f7 CDS in reverse strand. Dark blue *F. noatunensis*^{TS}. Purple *F. philomiragia*^{TS}. Yellow *F. hispaniensis*^{TS}. Green *F. tularensis*^{TS}. Pink *F. guangzohuensis*^{TS}. Red rRNA genes. 10th circle tRNA's. The innermost circles show: GC skew and G+C content.

4.4 Discussion

Recently the taxonomy of the order *Thiotrichales* (class *Gammaprotobacteria* phylum *Proteobacteria,* superkingdom *Bacteria*) was revised by Boscaro *et al.* (2012) and as a result the creation of a new monophyletic *taxon* within the family *Francisellacea* (at the rank of the genus *Francisella*) was proposed to comprise the bacteria *Wolbachia persica, Caedibacter taeniospiralis*, "*Candidatus* Nebulobacter yamunensis" and *Fangia hongkongensis*.

Such a recommendation attempted to "extend" the boundaries of the family allocating the mentioned *taxa* and clarify the taxonomic status of many *incertae sedis* and unculturable microorganisms i.e. *Francisell*a spp. from soil and water (Barns *et al.*, 2005) or *Francisella*-like endosymbionts from ticks (de Carvalho *et al.*, 2011; Dergousoff and Chilton, 2011; Ivanov *et al.*, 2011; Michelet *et al.*, 2013; Montagna *et al.*, 2012; Scoles, 2004; Sréter-Lancz *et al.*, 2009; Szigeti *et al.*, 2014;).

However, since such proposal was based on the rules for nomenclature of uncultivated bacteria (Murray and Schleifer, 1994; Amann *et al.*, 1995; Murray and Stackebrandt, 1995) and the "modern" concept of species "metapopulation lineages" (de Queiroz, 2005), it was not considered as validly published or approved by the International Committee on Systematics of Prokaryotes and its Judicial Commission, but appeared in the List of Changes in Taxonomic Opinion (Euzeby, 2013).

Thus, as per the requirements of the International Code of Nomenclature of Prokaryotes (Lapage *et al.*, 1992) the genus *Francisella* with its six species and six subspecies is the only validly accepted within the family *Francisellacea*.

The requirements and guidelines for the taxonomic nomenclature of prokaryotes indicate that strains thought to constitute a novel *taxa* must be phenotypically and genetically characterised as comprehensively as possible (Tindall *et al.*, 2010). Therefore non-cultured and consequently non-phenotypically characterised (or poorly characterised) strains cannot be described as a new valid *taxon* or assigned into one (Gevers *et al.*, 2005; Kämpfer, 2010; Wayne, 1987)

Since the *Bacterium tularense* was first described in 1912 (McCoy and Chapin, 1912), several taxonomic rearrangements have taken place from the subspecies or lower ranks to the genus level and the ecology, pathogenicity and fastidious nature of the bacteria *Francisella* spp. appears to be the reason and logic explanation as why this has historically happened.

The most recent example of these changes is "*Francisella endociliophora*", a bacterium that was originally identified as a non-isolated endosymbiont or "*Francisella*-like bacterium" of the marine ciliate *Euplotes raikov* (Schrallhamme *et al.*, 2011). This bacterium was partially genetically characterised, but was at that time, uncultured so according to the rules for description of well known (genetically characterised) but yet not isolated (Murray and Stackebrandt, 1995) it was classified as a "candidatus" novel subspecies of *F. noatunensis* i.e. "*Candidatus* F. noatunensis ssp. endociliophora". A few years later it was isolated using acid treatment (Humrighouse, 2011) to release it from the ciliate and its genome was fully sequenced (Sjödin *et al.*, 2014). Although the name *F. endocilipohora* was published in the genome announcement, it is not yet considered as validly published and it is unlikely that it will be validated if the phenotype is not analysed.

Like *F. endociliophora,* there are many other *Francisella* spp. strains that have been isolated and genetically, but not phenotypically characterised (Kugeler *et al.*, 2008; Petersen *et al.*, 2009; Respicio-Kingry *et al.*, 2013; Rydzewski *et al.*, 2014). As mentioned before, this may be explained due to the fastidious nature of such strains i.e. slow growth and low reactivity with standard biochemical identification tests, and perhaps little interest from clinical and environmental researchers towards the taxonomy of their findings.

The phenotypic analyses performed in the current study allowed the differentiation of two of the closest related *taxa* within the genus and suggests that this methodology could be a suitable inexpensive tool to describe novel *Francisella* spp. and those isolated, but not yet described strains in compliance with the International Code of Nomenclature of Prokaryotes and the International Committee on Systematics of Prokaryotes and its Judicial Commission (Lapage *et al.*, 1992).

The morphology of the *Fnn* and *Fno* colonies and the optimal temperature described in this study are in disagreement with that reported by Ottem *et al.* (2009) on the description of *Fno*. In the current experiments, the optimal growth temperature found for this bacterium was 28-29 °C, but not 25°C and the colonies were greenish-greyish but not pale yellowish.

The *Fno* FAME profiles obtained in this study were similar to the results obtained in Section 2.3.3.4 for STIR-GUS-F2f7 and the other two *Fno* strains analysed at that time. In the present study, several strains of each *taxon* were simultaneously used and statistical analyses were performed to compare the content per *taxon*, these analyses indicated that the fatty acid 24:0 was useful to differentiate between the *taxa*. Additionally, the

summations of the saturated and mono saturated fatty acids were significantly different among the groups, confirming that summations and the content of fatty acid 24:0 can be considered as *taxon*-specific chemotaxonomical markers and a reference for future research on *Francisella* spp. taxonomy. The values obtained in the current study for the amount of 24:0 in *Fnn* and *Fp* were almost identical to those presented by Ottem *et al.* (2007).

The use of the modified Biolog GN2 protocol allowed better characterisation of *Fnn* as it was possible to identify 10 positive reactions that appeared as false negatives with the standard manufacturer's guidelines. This is clearly in agreement with the results presented by Huber *et al.* (2010) for strain Fn17, who only found six positive reactions, as in the present study.

However, as with *Fno* in Chapter 2 there were few reactions i.e. dextrin (A3) glycogen (A4), D-cellobiose (A12), glycyl-Lglutamic acid (F12), L-threonine (G12), glycerol (H9) and D-L-α-glycerol phosphate (H10), that appeared to be positive with higher densities in the strains from Norway, and other tests should be considered to confirm whether the cells are capable or not of metabolising those carbon sources. This was not seen in *F. philomiragia*, which grows faster, is more biochemically reactive and is considerably less fastidious.

From the reactions that were clearly positive or clearly negative, no differences were seen within the *Fno* strains, despite their widely different geographical origin. However, several differences were seen between some of the *Fnn* strains. In total there were 22 positive reactions in *Fno* and 17 in *Fnn*.

On comparison of *Fnn* with *Fno*, 10 differences were seen, most notably the inability of *Fnn* to metabolise glucose-1- phosphate (H11), glucose-6-phosphate (H12), and acetic acid (D1) and the inability of *Fno* to metabolise D-alanine (F5).

All of the *Fnn* isolates from Norway had the same profiles and they were slightly different to the Irish isolate, however almost as many differences as those seen between *Fno* anf *Fnn* were seen between the Norwegian and Chilean isolates, this was also reflected in the colony morphology, where the Chilean isolate was greenish-greyish and the others whitish-greyish.

Some of the phenotypic differences (more reproducible assays) that allowed differentiation between species and subspecies in this study are summarised in Table 4.12.

Table 4.122 Differential characteristic of fish pathogenic *Francisella* spp.

Characteristic	*F. orientalis*	*F. noatunensis*		
	F. orientalis	*F. noatunensis noatunensis*		*F. noatunensis chilense*
Origin/ distribution	world wide	Europe (Scandinavia)	Europe (Ireland)	Chile
Optimal temperture °C	28-29	22	22	22
Colony colour on CHAH	greenish-greyish	white	white-greyish	greyish-greenish
Mono-Methyl- Succinate	-	+	+	-
Acetic Acid	+	-	+	+
D,L-Lactic Acid	-	-	-	+
D-Alanine	-	+	-	-
L-Alanylglycine	+	+	+	-
L-Threonine	+	-	-	+
Glycerol	+	-	+	+
Glucose-6-Phosphate	+	-	-	-

In prokaryote taxonomy the gold standards for bacterial species delineation are percent similarity in the 16SrRNA gene and DNA-DNA hybridisation (DDH). The current guidelines indicate that if two strains share a similarity of 98.65% or lower in the 16S rRNA gene they constitute two separate species (Kim *et al.*, 2014). However this rule

cannot be applied to all genera, therefore if there are important phenotypic differences and the 16S rRNA value is higher, wet lab DDH experiments should be performed to clarify the status.

In such experiments the strains should show DNA-DNA re-association values of 70% or higher if they belong to the same species or lower to constitute two separate species. Since the wet lab DDH technique has many drawbacks including the fact that it is time consuming, labour intensive, expensive and not easily reproducible between laboratories (Goris *et al.*, 2007), bacterial taxonomists are looking for new methodologies to replace it.

The new options are based on correlations between DDH values and genome derived information (Ramasamy *et al.*, 2014). Among the different proposals, perhaps the most widely used are average nucleotide identity value (ANI) (Richter and Rosselló-Móra, 2009) and genome to genome distance GGD or "*in silico* DDH" (Meier-Kolthoff *et al.*, 2013). Although these have been demonstrated to be highly reliable they can only be used if the researchers have sequenced the complete genome of their strains.

Given the high level of relatedness between some members of the genus *Francisella*, the current criteria of 98.65% in the 16S rRNA gene is not suitable to investigate the taxonomical status of their members and DDH experiments are usually performed.

In this study the DDH presented for the comparison of *Fno* against *Fnn* was 10% below the 70% threshold confirming that they represent separate species nevertheless the values between *Fnn* and *Fp* and *Fp* and *Fno* available in the literature are ambiguous and inconsistent.

The DDH result shown here (Fo14 in comparison to Fn1) was originally presented in Mikalsen and Colquhoun (2009) however since this report is no longer available in the scientific literature it is included within this study in collaboration with Dr. Duncan Colquhoun.

In order to validate DDH result presented in this study, clarify the ambiguity of previous wet lab DDH values (Table 4.5) and explore the boundaries of species and subspecies delineation within *Francisella noatunensis*, whole genome derived information was included in this study. The inclusion of genomic data for taxonomy "taxo-genomics" was useful not only to comply with the future "gold standards" (GGD and ANI), but also to investigate and compare the phylogeny at three different levels of resolution i.e. single gene, concatenated housekeeping genes and whole genome analyses.

The phylogenetic trees were consistent across the 3 levels of resolution. In all of them, *Fno* and *Fnn* clustered in different branches, and the Chilean isolate separated from the other *Fnn*, but was still contained within the same subtree. The Irish isolate showed some branching separation from the others, but this was only clearly visible at the whole genome scale.

The WG analyses here included were also useful in establishing a threshold for subspecies delineation within *Francisella noatunensis,* where it is proposed that with an ANI of 99.5%, a GGD of 0.076 and an *in silico* DDH value of 98% with respect to the other *Fnn,* the isolates recovered from Chile constitute a new subspecies, but not the isolate from Ireland that even though it showed some differences in the phenotype, had 100% ANI with respect to all the other *Fnn*.

4.5 Conclusion and description of new *taxa*

4.5.1 Conclusion

In this study a polyphasic approach that incorporated several phenotypic features and genome-sequence derived parameters was used to characterise several strains of fish pathogenic *Francisella* ssp. and clarify their taxonomical status.

Based on the results obtained it is proposed to elevate *Francisella noatunensis orientalis* to the rank of species as *Francisella orientalis* comb. nov. sp. nov and amend the description of *Francisella noatunensis*. Furthermore it is proposed to create a new subspecies within *F. noatunensis* for which the name *F. noatunensis* subsp. *chilense* subsp. nov. is proposed. According to the rule 40b of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1992) the creation of this subspecies automatically creates the subspecies *F. noatunensis* subsp. *noatunensis* subsp. nov.

4.5.2 Description of *Francisella orientalis* comb. nov. sp. nov.

Francisella orientalis (o.ri.en.ta‘lis. L. fem. adj. orientalis from the east, referring to where the type strain was isolated).

The description of this species is as that presented for *F. noatunensis* subsp. *orientalis* (Ottem *et al*., 2009) with the following corrections and additions:

The optimal growth temperature is 28-29 °C and the colonies are greenish-greyish on CHAH. The lipase (C14), α-chymotripsin and α-galactosidase tests are weakly positive.

It is capable of metabolising dextrin, N-acetyl-Dglucosamine, D-fructose, α-D-glucose, D-mannose, methyl pyruvate, acetic acid, α-keto butyric acid, L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-glutamic acid, L-proline, L-serine, L-threonine, inosine, uridine, glycerol, DL-α-glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate.

The polyamine profile of the type strain is (μmol/g dry weight): diaminopropane 0.2-1.3, putrescine 2.0, cadaverine 22.6, spermidine 11.5 and spermine 0.2. The quinoe system is conformed by 78.8% ubiquinone Q8 and 21.2% of Q7.

The type strain is Ehime-1 (=DSM21254T = LMG24544T) isolated from the farmed marine fish three-line grunt or isaki (*Parapristipoma trilineatum*) in 2001 in Ehime-prefecture, Japan.

4.5.3 Emended description of the *F. noatunensis* (Ottem *et al.*, 2009)

Francisella noatunensis (no.at.un.en.sis. N.L. n. noatun (enclosure of ships) was the coastal abode of the Norse god of fisheries and seamanship; L. fem. suffix -ensis suffix meaning ‘belonging to’; N.L. fem. adj. noatunensis belonging to the coast/sea.

In addition to the descriptions provided by Mikalsen *et al.* (2007) and Ottem *et al.* (2009) the strains of this species (both subspecies and atypical strains) are capable of metabolising D-fructose, α-D-glucose, D-mannose, methyl pyruvate, α-keto butyric acid, L-alanine, L-asparagine, L-glutamic acid, L-proline, L-serine, inosine. In contrast to *F. orientalis* the strains of both subspecies of this *taxon* are negative for dextrin, uridine, DL-α -glycerol phosphate, glucose-1-phosphate, and glucose-6-phosphate.

The type strain of the species is 2005/50/F292-6C^T (=NCIMB14265^T = LMG23800^T) isolated from Atlantic cod in Norway in Hordaland county Norway in 2005.

4.5.4 Description of *Francisella noatunensis* subsp. *chilense* subsp. nov.

Francisella noatunensis subsp. *chilense* (chi.len′se. N.L. neut adj. *chilense* referring to the country Chile where the type strain was isolated).

This subspecies has only been isolated in the southern hemisphere from diseased farmed or captured fish in fresh or brackish water in Chile. In contrast to the strains of *F. noatunensis noatunensis* the colonies of this subspecies are greenish-greyish and don't grow well with concentrations of NaCl over 2% in the media and is unable to metabolise N-acetyl-Dglucosamine, mono-methyl- succinate, L-alaninamide, D-alanine, L-alanylglycine, but it is positive for glycerol, L-threonine, DL-lactic acid and acetic acid.

The type strain is PQ1106^T NVI5888 ^{T} FSC772 ^{T} isolated from Atlantic salmon in the lake Llanquihue Chile in 2006.

4.5.5 Description of *Francisella noatunensis* subsp. *noatunensis* subsp. nov.

Francisella noatunensis noatunensis (no.at.un.en.sis. N.L. n. noatun (enclosure of ships) was the coastal abode of the Norse god of fisheries and seamanship; L. fem. suffix -ensis suffix meaning 'belonging to'; N.L. fem. adj. noatunensis belonging to the coast/sea.

The strains of this subspecies have only been isolated from diseased fish farmed or captured in northern Europe. The colonies are whitish and can grow in concentrations up to 6% of NaCl. In addition to the emended description of the species *F. noatunensis*

presented here, the strains of this subspecies are also able to metabolise N-acetyl-Dglucosamine, mono-methyl-succinate, L-alaninamide, D-alanine, L-alanylglycine,

Atypical strains: in contrast with the other *Fnn* the atypical “Irish” strain has the ability to metabolise acetic acid and glycerol but incapable to use D-alanine. A strain isolated from Sweden is also incapable to metabolise D-alanine.

The type strain of this subspecies is 2005/50/F292C^T (=NCIMB14265^T=LMG23800^T)

4.6 Acknowledgments

The genome sequencing of the strains was performed by the SNP&SEQ Technology Platform, Science for Life Laboratory at Uppsala University, a national infrastructure supported by the Swedish Research Council (VRRFI) and the Knut and Alice Wallenberg Foundation in collaboration with the Swedish defence research agency (FOI) in Umea Sweden.

The polyamine profile and quinone system of the *Francisella orientalis* type strain Ehime-1 was kindly analysed by Dr. Hans-Jürgen Busse at the Institut für Bakteriologie, Mykologie & Hygiene Veterinärmedizinische Universität Wien.

The strain Fo7 “Franc-COS1” was kindly donated by Dr. Cesar Ortega Santana at the Aquatic Animal Health Laboratory-Centro de Investigación y Estudios Avanzados en Salud Animal Facultad de Medicina Veterinaria y Zootecnia Universidad Autónoma del Estado de México.

The strain Fo11 “Austria” was kindly donated by Dr. Mansour El-Matbouli at the Department for Farm Animals and Vet. Public Health, University of Veterinary Medicine in Vienna Austria.

The visit to Sweden for the genomic analyses was completed with the support of the Grant SG131 awarded by the Marine Alliance for Science and Technology (MASTS) small grant scheme.

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Chapter Five

A whole-cell inactivated *Francisella orientalis* vaccine protects red Nile tilapia against experimental infection via intraperitoneal injection.

5.1 Introduction

Francisellosis is an acute to chronic bacterial disease that can affect a diverse spectrum of animals including mammals, aquatic animals and invertebrates. This disease is caused by members of the genus *Francisella*, which is currently the only fully recognised genus within the family *Francisellacea*e γ-p*roteobacteria* class (Colquhoun and Duodu 2011).

The notable pathogenic species of the genus are *F. tularensis*, causative agent of tularaemia, a fatal disease in humans (Nano and Schmerk 2007) and *Francisella orientalis*, causative agent of francisellosis in warm water fish recently described as a new species (Chapter 4).

Outbreaks of *Fo* have been reported in farmed tilapia in several countries in America, Africa, Asia and Europe. The first record of this disease in tilapia was in Taiwan (Chen *et al.*, 1994; Chern and Chao 1993) but it has subsequently been reported in several cichlids, including a number of ornamental species (Hsieh *et al.*, 2006; Hsieh *et al.*, 2007; Kamaishi *et al.*, 2005; Mauel *et al.*, 2007; Ostland *et al.*, 2006; Soto *et al.*, 2009).

According to FAO (2014) tilapia is the second most important group of freshwater fish by production after carps and the most widespread species in aquaculture. The worldwide production of tilapia increased from 2.6 million tons in 2005 to 4.2 million tons in 2012, and its production is expected to exceed 5 million tons by late 2015 (Fitzsimmons *et al.*, 2014).

Francisellosis in tilapia is characterised by widespread (multifocal) granulomas (white nodules) in the peritoneal cavity, including organs such as the kidney (anterior and posterior), spleen, gastrointestinal tract, liver and mesenteric fat; the clinical signs include

exophthalmia, flashing, petechial haemorrhages in gills or fins; it has a high percentage of morbidity (80-90%) and mortality (50-90%). Microscopically, the characteristic pathological change is granulomatous inflammation in multiple organs, most severe in spleen and kidney, but also present in heart, liver, eyes, gills, brain and gut. The macrophages in the granulomas contain bacteria, but bacteria can also be found extracellularly following release from damaged macrophages.

Although farming of tilapia species is considered one of the most important sectors in aquaculture and *Fo* is one of the most pathogenic bacteria for them (Chapter 2; Soto *et al.*, 2009) there are currently no commercially available products for the treatment or prevention of this disease.

Experimental vaccination trials against piscine francisellosis started in Norway where different oil-adjuvanted, whole cell preparations of *Francisella noatunensis* (*Fn*), the cause of francisellosis in cold water fish species, were tested in Atlantic cod (Krossøy *et al.*, 2007; Müller *et al*., 2011; Schrøder *et al*., 2009).

For *Fo* the only approach for vaccine development was performed by Soto *et al.*, (2011b) using a live attenuated strain, with a mutation at the intra-cellular growth *locu*s C gene (iglC), which was shown to confer a relative percentage survival (RPS) of 87.5% in Nile tilapia after immersion challenge (Soto *et al.*, 2011b). Although such attenuated vaccine demonstrated to be effective under laboratory conditions (Soto *et al.*, 2011b; Soto *et al.*, 2014b), it is unlikely to be licensed for use in Europe due to the fact that regulatory authorities in the European Union do not favour licencing of live vaccines in aquaculture.

These restrictions reflect the inherent risks presented by live vaccines (e.g. reversion to virulence, dissemination into the environment, potential transmission to other aquatic species etc.). Thus there is a need for the development of an inactivated vaccine against this fastidious pathogen

Recently, a novel strain of *Fo* was isolated from moribund red Nile tilapia farmed in Europe. The isolate, STIR-GUS-F2f7, was subjected to a comprehensive phenotypic characterisation based on morphological, physiological and chemotaxonomic analysis (Chapter 2).

The aims of the current study were to investigate the susceptibility of different genetic groups of tilapia to *Fo*, develop an autologous inactivated vaccine against francisellosis and test its efficacy by measuring survival rates, specific antibody production, and differential gene expression during the onset of the infection.

5.2 Materials and methods

5.2.1 Fish, bacteria and experimental models

5.2.1.1 Fish species

Three genetic groups of tilapia i.e. red and wild type *O. niloticus* and *O. mossambicus* were used to determine the LD_{50} of *Fo* isolate STIR-GUS-F2f7, while red Nile tilapia was used for the pre-challenge and the vaccination studies. Healthy naïve tilapia, 6-7 months/~11g (7-13g), were used as described in Section 2.2.5.

5.2.1.2 Bacterial strains

The pathogenic isolate of *Francisella orientalis* STIR-GUS-F2f7, recently isolated from moribund Nile tilapia in Europe, was used. The bacterium was grown on CHA and MMHB, as described previously in Section 2.2.3.1.

5.2.1.3 Virulence and vaccine efficacy testing

In both the virulence assays and the vaccine efficacy testing, an intraperitoneal (IP) injection model was used, as described in Section 2.2.4. For the vaccination procedure, including the safety test, the fish were maintained in the TA at a water temperature of 28°C. For all the infections trials the fish were moved from the TA into a flow-through system at the Aquatic Research Facility (ARF), where the water was maintained at 23±2^{o}C to replicate the natural epidemiological conditions at which the disease occurs. Once moved to the ARF, the fish were given an acclimation period of ten days prior to beginning the experiments.

During periods of acclimation, infection, and vaccination, the fish were fed twice a day at a rate of 2% biomass. In all the experiments the fish were anaesthetised with a dose of 100mg/l of Tricaine Pharmaq 1000mg/g (TPQ) (Pharmaq, Hampshire, UK) prior to injection. All moribund fish observed during the experiment were immediately sacrificed with an overdose of TPQ. All the survivors were sacrificed also with an overdose of TPQ at the end of the trial. All the procedures were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and complied with local institutional regulations.

5.2.2 Lethal dose 50% (LD_{50}) virulence testing

Three genetic groups of tilapia (a total of 188 fish) and 4 doses of bacteria were used. For *Oreochromis niloticus* and *O. mossambicus* 16 fish per tank were used, while for *O. niloticus* wild type the stocking density was increased to 20 fish per tank to prevent the hierarchical aggression initially observed.

The fish were placed in 2 litre plastic tanks with the water volume adjusted to 1.8 litres and containing individual air stones. Four tanks per genetic group were used and each tank was injected with one of 4 different doses of bacteria. The fish were maintained for 45 days post-infection (dpi) and mortalities recorded 4 times per day. All dead and moribund fish were sampled for bacterial recovery and histopathology.

Based on the standard curve for bacterial quantification, shown in Section 2.3.5, a bacterial "stock" suspension with an initial concentration of ~$1.0x10^9$ CFU/ml (OD_{600} of 0.4) was prepared in 1X sterile PBS. This stock was then serially diluted 10-fold and the

dilutions containing 10^2 CFU/ml (dilution 10^{-7}), 10^3 CFU/ml (dilution 10^{-6}), 10^4 CFU/ml (dilution 10^{-5}) and 10^6 CFU/ml (dilution 10^{-3}) selected for the infection doses.

Each fish received an IP injection with 0.1 ml of the respective dilution of bacteria and the actual number of viable bacteria present in the stock solution was estimated on CHAH by drop counts using the method reported by Miles *et al.* (1938). The cumulative percentage mortality recorded was used to establish sigmoidal dose-response curves for each tilapia species and for each dose.

5.2.3 Pre-challenge (LD_{60} confirmation)

A pre-challenge was established by injecting 20 red Nile tilapia fingerlings IP with a dose of 2.4 x10^4 CFU/fish. The dose and fish group were selected based on the results of the LD_{50} experiment. The objective of this experiment was to confirm that this dose would cause around 60% mortality by 21 dpi in naïve red tilapia.

5.2.4 Vaccine preparation and safety test

F. orientalis STIR-GUS-F2f7 was cultured in CHAB and MMHB under culture conditions indicated in Section 2.2.3.1. Inactivation of the bacteria was achieved using formaldehyde at a final concentration of 0.05%. Bacteria were grown overnight (~18h) in 15ml of broth. This culture was then centrifuged at 3500g for 20 min, the bacterial pellet washed 3 times with 1X sterile PBS and adjusted to an OD_{600} of 1.0 (2.63 x10^9 CFU/ml). The bacteria and 40% formalin were mixed and left overnight at 4°C in a sterile glass vessel, with slow stirring. The formaldehyde was neutralized using 10ml per

1000ml (1/100 dilution) of 15% sodium metabisulphite stock solution added 96h after inactivation.

Finally, the solution was washed and the bacterial pellets with dead bacteria were diluted in 1X PBS to obtain an OD_{600} of 1.0. Inactivation of the bacteria was confirmed by inoculating on to CHAHB and incubating at 28°C for 72h.

The commercial oil adjuvant Montanide™ ISA763AVG (SEPPIC, Puteaux Cedex, France) was emulsified with the bacteria. The protocols followed for this were for W/O emulsion in a 30/70 distribution of the continuous and dispersed phases (30% antigen to 70% adjuvant) according to the manufacturer's guidelines. The emulsion was homogenised for 5min with a hand blender at 4000rpm. For the first 3min a constant speed of 4000rpm was used, then the speed was increased to 4500rpm for 30s, after which it was decreased to 4000rpm for 1min and then at the second speed for a final 30s.

The initial concentration of inactivated bacteria was $2.6x10^9$ CFU/ml, and was reduced to $1.3x10^9$ CFU/ml after emulsification. As indicated by the adjuvant manufacturer and (Aucouturier, 2001) the emulsion was stored at 4°C and its stability checked after 24h and 15 and 60 days by performing a visual inspection, droplet test, conductivity readings, particle size assessment and syringeability in and out of the fish.

The final volumes used for the production of the vaccine were 100ml of bacteria in PBS, 500µl of 40% formalin, 1ml of 15% sodium metabisulphite. The total amount of formalin-killed bacteria obtained was 60ml of which 33ml were added to 77ml of adjuvant to obtain a total volume of 110ml of vaccine.

To perform a safety test, thirty fish were IP injected with 0.2ml of vaccine (i.e. double of the vaccination dose) and checked twice a day for 7 days looking for any possible side effects, such as changes in behaviour, toxicity, mortalities or other signs that could be related to the formulation of the vaccine.

5.2.5 Vaccination and infection

5.2.5.1 Experimental design

The experiment was designed to monitor and quantify the production of specific antibodies and assess the efficacy of the vaccine. A total of 678 red Nile tilapia were divided between 16 tanks were used. The first set of tanks consisted of treatments 1-3, with three tanks per treatment and 52 fish per tank; Fish in the first treatment were injected with the vaccine preparation (antigen/adjuvant emulsion), fish in the second treatment were injected with PBS as a control group and fish in the third treatment were injected with only adjuvant (emulsified with1X PBS) with no antigen.

The second set of tanks consisted of treatments 4-6 with three tanks in treatments 4 and 5, and one tank in treatment 6; in this set of treatments 30 fish per tank were used in order to assess granuloma formation in the tissues and investigate the spleen and head kidney transcriptomic profiles of vaccinated and unvaccinated fish shortly after infection. The fish in treatment 4 were vaccinated and then challenged, while the fish in treatment 5 were challenged without being vaccinated and the thirty fish in tank 16 (treatment 6) were not vaccinated/not challenged and kept as a control group.

For the vaccination process, all the fish were IP injected using stainless hypodermic needles 22GX7mm and 21GX8mm (Aqualife Fish Care Professionals, Stirling, UK) with

0.1ml of their respective treatment i.e. vaccine, adjuvant or PBS and kept for 30 days at the TA. For the challenge all the fish were moved to ARF 31dpv and IP injected with 0.1ml of a PBS solution containing $4x10^4$ CFU/ml ($4x10^3$ CFU/fish), a dose expected to produce cumulative mortality between 60% and 80% in the PBS (control) group within the first 35dpi. The experimental design is illustrated in Figure 5.1

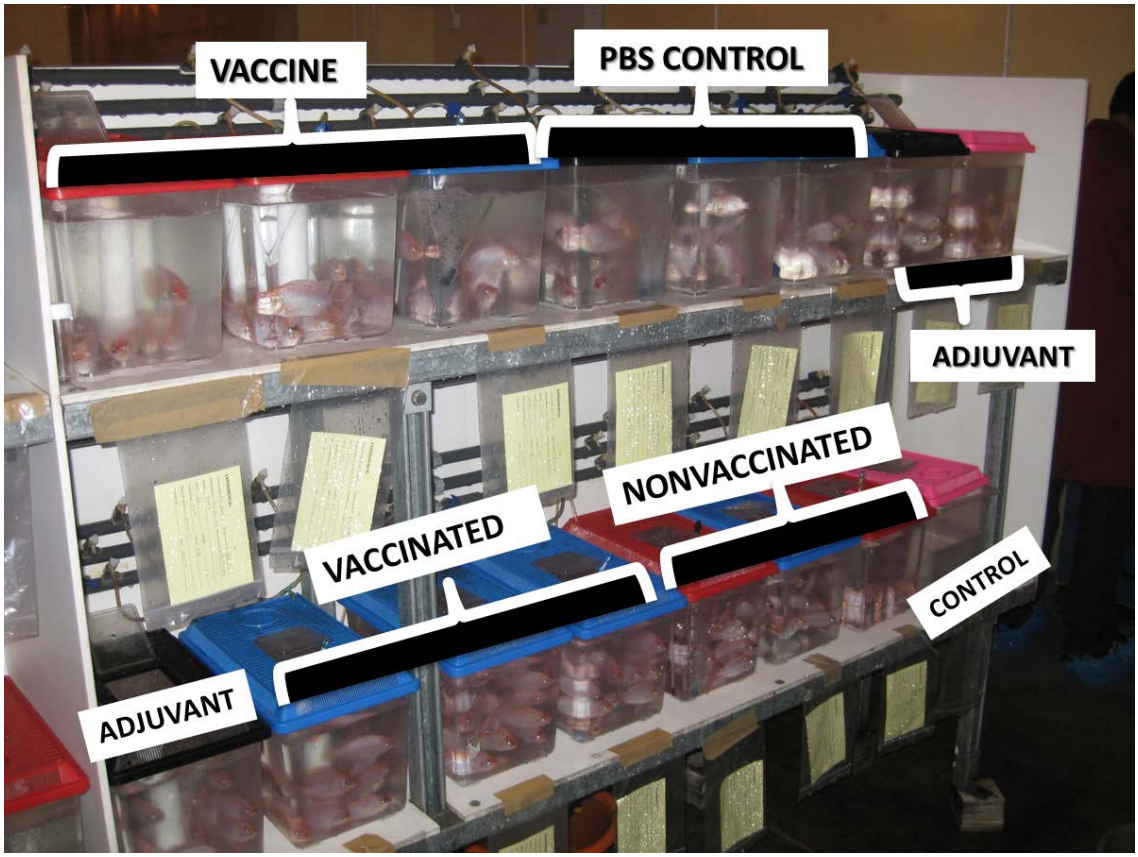


Fig. 5.1 Experimental design of the vaccination-infection experiment. The picture shows the fish after being transferred to the ARF at day 30 post vaccination just before the beginning of the experimental infection.

5.2.5.2 Sampling points

Fish in set one (treatments 1-3) were sampled at 4, 8, 15 and 30 days post vaccination (dpv). At each sampling point, 8 fish per tank were bled from the caudal vein to investigate the kinetics of the serum humoral response (antibody production). The remaining 20 fish of each tank were not handled after the infection and their mortalities were recorded to determine the efficacy of the vaccine. In the second set of treatments the sampling was performed at 24, 48 and 96 hours post infection (hpi). In these tanks the spleen and head kidney of the fish were aseptically removed, placed in RNAlater® stabilization solution (Life technologies, Paisley, UK), snap frozen in liquid nitrogen and stored at -80°C. The spleens of these fish were visually inspected for granuloma formation before placing them in the RNAlater. All the sampling handling was performed after having sacrificed the fish with a lethal overdose (300mg/l) of TPQ.

5.2.5.3 Vaccine efficacy assessment (RPS)

To establish the efficacy of the vaccine as a percentage value, the relative percent survival (RPS) was calculated. This value indicates the proportional relationship between mortality in the vaccinated group (treatment 1) and the unvaccinated group (treatment 2) and was established using the following equation (Amend, 1981):

$$RPS = \left[1 - \left(\frac{\%\ mortality\ in\ vaccinated\ fish}{\%\ mortality\ in\ non\text{–}vaccinated\ fish}\right)\right] \times 100\%$$

The RPS value was also obtained for fish injected only with the adjuvant for comparisons with the value obtained in the vaccinated fish.

5.2.5.4 Specific antibody titration by ELISA

An indirect enzyme linked immunosorbent assay (ELISA) was used to measure the specific antibody response of the tilapia sera against whole cells of *Fo*. The antibody titres were determined according to the protocol outlined by (Adams *et al.*, 1995) with modifications. Briefly, 96-well ELISA plates were coated with 100 µl of 1% w/v poly-L-lysine in carbonate–bicarbonate buffer and incubated for 60min at room temperature (~21°C). Plates were then washed three times with a low salt wash buffer (LSWB) (0.02 mol l-1 Trizma base, 0.38 mol l-1 NaCl, 0.05% (v/v) Tween 20, pH 7.2). Bacteria were added to each well (100µl /well) and plates were incubated overnight at 4°C. For this, bacteria had been cultured overnight and then washed twice with 1X PBS (20 min at 3500×g) and the concentration of the resulting suspension adjusted to an OD_{600} of 1.0.

Fifty microliters of a 0.05 % v/v solution of glutaraldehyde in LSWB was added to fix the bacteria and plates were incubated at 21°C for 20 min before washing three times with LSWB. Non-specific reactions caused by the bacteria were removed by incubating with hydrogen peroxide (1:10 of a 30% stock solution) for 1h, and then non-specific binding sites were blocked by incubating the plates with 5% w/v skimmed milk powder in water at 21°C for 120 min. After washing plates three times with LSWB, 100µl serially diluted fish serum in LSWB (from 1:32 to 1:1024) were added to the plates and incubated overnight at 4°C (Palaksha *et al.*, 2008). Plates were then washed five times with high salt wash buffer (HSWB) (0.02 mol l^{-1} Trizma base, 0.5 mol l^{-1} NaCl, 0.01% (v/v) Tween 20, pH 7.4) and left for 5 min covered in HSBW after the last wash to remove unbound antibodies.

The anti-tilapia IgM monoclonal antibody (MAb) (Aquatic Diagnostics Ltd, Stirling, UK) was then added (100µl per well) and plates were incubated at 21°C for 60 min. Following the subsequent washing of the plates with HSW, as previously described, goat anti-mouse immunoglobulin-G labelled with horseradish peroxidase (HRP) (Sigma-Aldrich, Dorset, UK), diluted 1:4000 in conjugate buffer [1% w/v bovine serum albumin (BSA) in LSW] was added to the wells and incubated for 60min at 21°C. Plates were once again washed with HSW as previously described.

The assays were developed with 100µl well^{-1} of substrate/chromogen (i.e. 15ml substrate buffer containing 5µl hydrogen peroxide and 150µl trimethyl-benzidine (TMB) dihydrochloride) [150 µl (42 mM 3, 3′5,5′-tetramethylbenzidine hydrate dihydrochloride (Sigma-Aldrich, Dorset, UK) added to 6ml of 50% acetic acid) to 15 ml of substrate buffer and 5µl of hydrogen peroxide (H_2O_2).

Following a 10 min incubation at 21 °C, the reaction was terminated with the addition of 50µl well^{-1} of 2 M H_2SO_4 and the absorbance was measured at OD_{450} using a 96-well plate spectrophotometer (Biotek Instruments, Friedrichshall, Germany).

The ELISA antibody titre was defined as the reciprocal of the highest dilution (1/x dilution) showing an OD_{450} at least two times greater than the negative control. Both positive (vaccinated and challenged fish) and negative controls (serum blanks/ naïve fish) were added to each plate.

5.2.5.5 Spleen granuloma score

The spleens of fish in treatments 4 and 5 were macroscopically analysed to count and record the presence of white nodules and/or the extension of the coalescent nodules in

the parenchyma, as well as colour changes and signs of splenomegaly in all the treatments.

5.2.5.6 Transcriptomic profiles

The spleen and kidney samples that were frozen in RNAlater® were sent to the Interdisciplinary Centre for Aquaculture Research (INCAR) in Concepcion University Chile for RNA sequencing using next generation sequencing. At arrival to the lab, the samples were prepared for total RNA extraction.

Two methods were compared for the extraction of total RNA from 10mg of spleen and head kidney of fish from treatments 4 and 5, the RiboPure™ RNA purification kit (Ambion Life Technologies, California, USA) and TRIzol® reagent (Ambion Life Technologies, California, USA).

The total RNA concentration and purity of the samples were initially estimated with a NanoDrop™ ND1000 (ThermoScientific, Delaware, USA) and their integrity verified by electrophoresis in MOPS (3-[N-morpholino] propanesulfonic acid)/formaldehyde, 1.2% agarose gel stained with ethidium bromide at 0.001%.

After comparison of preliminary results of NanoDrop™ and gel electrophoresis the TRIzol® reagent was chosen as the method to complete the extraction of spleen only from fish sampled at 24hpi.

The RNA extracts with 260/280 and 260/230 ratios equal or greater than 2.0 and apparent integral RNA were screened in a Tape Station 2200 system (Agilent Technologies, California, USA) using the R6K reagent kit according to the manufacturer's instructions.

The concentrations given by the NanoDrop were used as a reference to adjust each sample to ~200µg/µl before screening them on the TapeStation. The samples with the best RNA integrity number (RIN) i.e. RIN≥7 were selected (six per treatment) and pooled by mixing 15µl of each sample, the RIN values of the head kidney tissues didn't yield the required values and were not further considered; the RNA concentration of the spleen pools of both treatments was quantified with a Qubit® 2.0 fluorometer (Invitrogen, Life Technologies, California, USA) and their integrity verified on the TapeSation. Finally the concentration of the pools was readjusted to 100ng/µl in a volume of 50µl for cDNA library construction.

The cDNA libraries were prepared according to the TruSeq® Stranded mRNA Sample Preparation Guide (Illumina, California, USA). The final products were quantified by real time PCR with the StepOne™ Real-Time PCR software (Life Technologies Ltd, Paislye, UK) and their integrity was assessed and validated with the Tape Station 2200 system (Agilent Technologies, California, USA) using the D1K screen tape kit according to the manufacturer's instructions

5.2.5.7 Statistical analyses

Comparative susceptibility: due to the different number of fish used in the wild type Nile tilapia population, a Z test for two population proportions with a significance level of 0.05 and one tailed hypothesis was chosen as the statistical method to compare the number of dead fish obtained with each dose. The population's proportions were transformed using the natural logarithm or arcsine transformation prior to the analyses. The LD_{50} at 8 different time points was calculated for each tilapia group with the statistical method described by Reed and Muench (1938).

5.3 Results

5.3.1 Lethal dose 50% (LD_{50}) virulence assay

After quantification by the drop count method, the concentration estimated for the solution with an OD_{600} of 0.9 was $1.2x10^9$ CFU/ml of *F. orientalis*. The cumulative mortalities of the different genetic groups of tilapia challenged by IP injection with different doses of *F. orientalis* are presented in Figure 5.1

Comparisons of cumulative mortalities indicated that mortality in red *O. niloticus* was significantly higher than that of the other two tilapia groups, when 12 and 120 CFU/fish were injected into the fish. It was also observed that when a dose of 1200 CFU/ml was injected into the fish, the mortality in *O. niloticus* was significantly lower than that of the other two species and no differences were seen when the highest dose ($1.2\ x10^5$ CFU/fish) was used. The cumulative mortalities per dose are presented in Figure 5.2.

At 45 dpi the median lethal dose observed for the red Nile tilapia was just 30 CFU/ml, while it was $2.3x10^4$ for the wild type and $3.3x10^2$ for *O. mossambicus*. This result, where the LD_{50} of the red tilapia was lower than that of the other two groups, was maintained during the course of the experiment. The LD_{50} for the wild type was the most stable with values always around 10^4 CFU/ml and the highest of the three groups from 25dpi onwards.

A comparison of the LD_{50} values obtained for the different tilapia groups is presented in Table 5.1. The lowest amount of bacteria required to cause mortality was 12 CFU/ml and this was seen as early as only 24hpi in the red Nile tilapia and in the wild type after 26 dpi. No mortalities were seen with this dose in the species *O. mossambicus*.

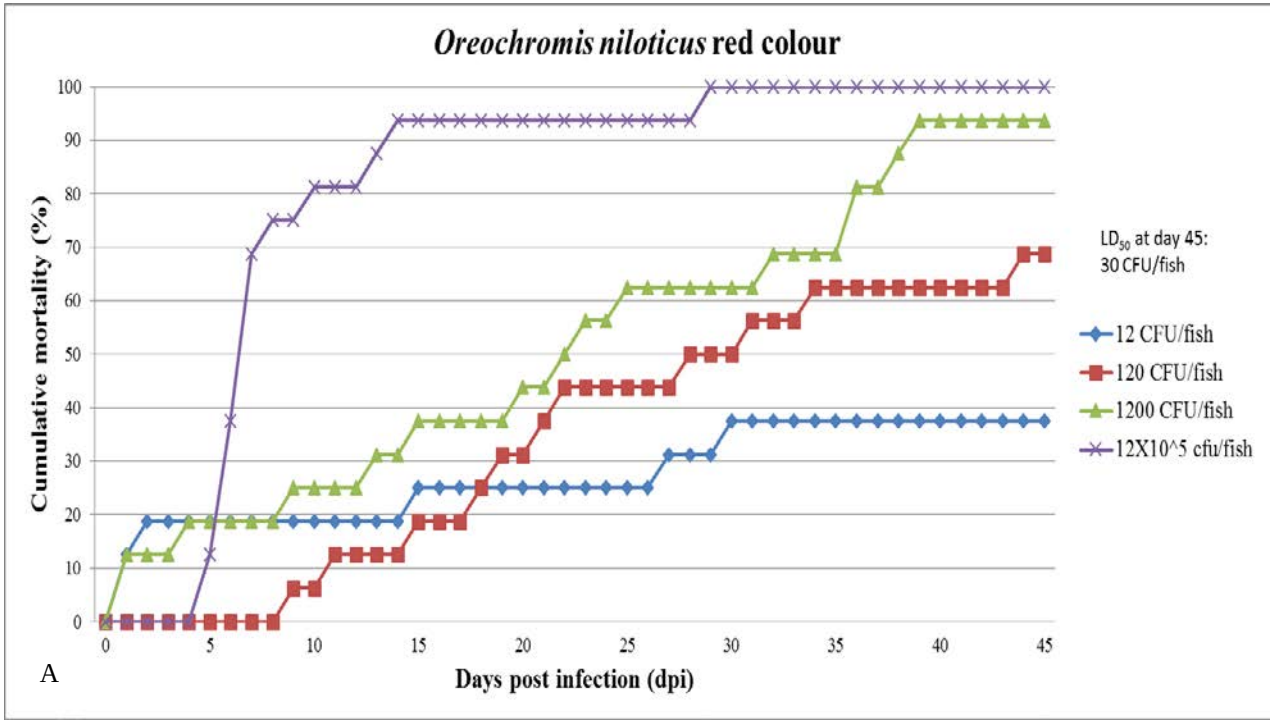


Fig. 5.2 Cumulative percent mortality of different genetic groups of tilapia challenged with *F. orientalis* STIR-GUS-F2f7. **A** Nile tilapia red colour. **B** Nile tilapia wild type. **C** Mozambique tilapia.

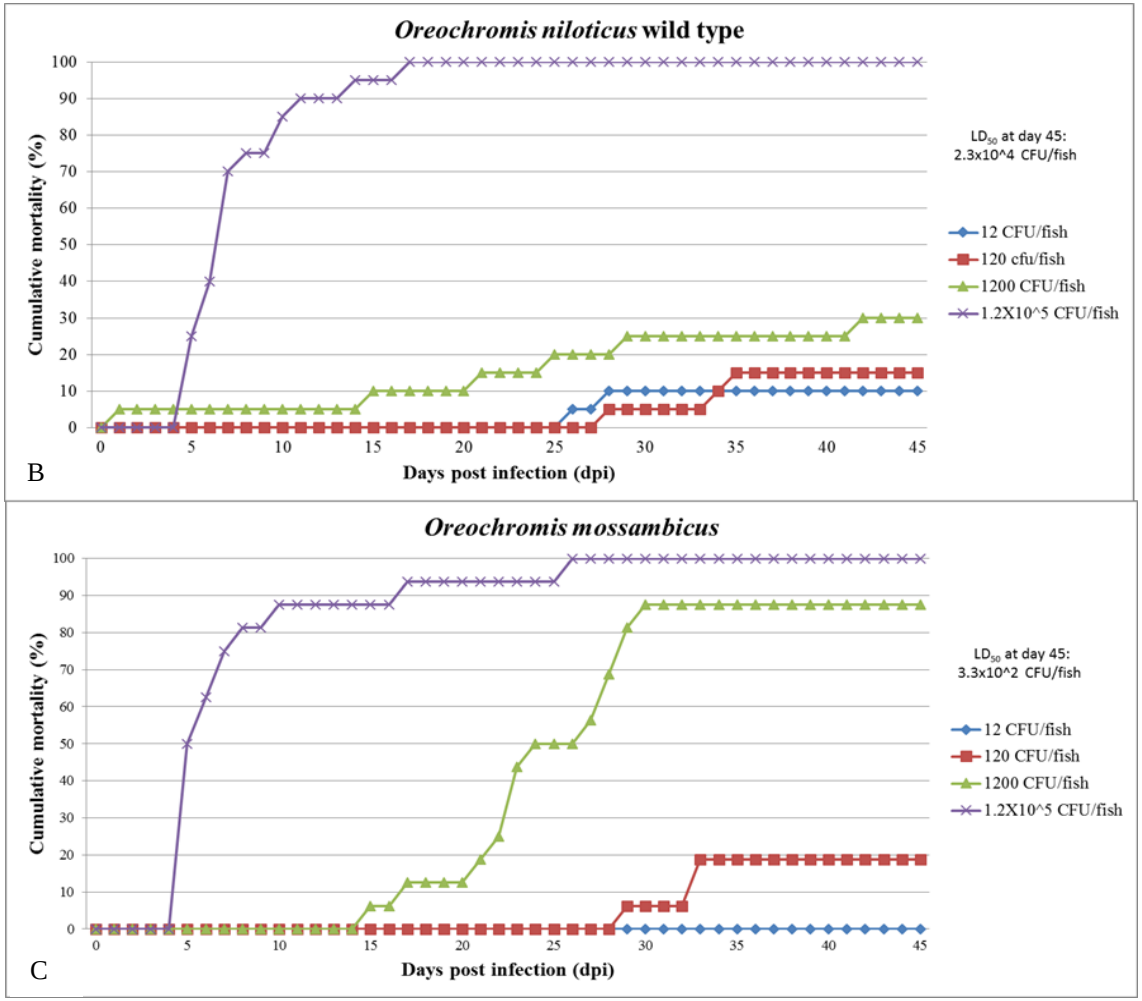


Fig. 5.2 Cumulative percent mortality of different genetic groups of tilapia challenged with *F. orientalis* STIR-GUS-F2f7 (continued). **B** Nile tilapia wild type. **C** Mozambique tilapia.

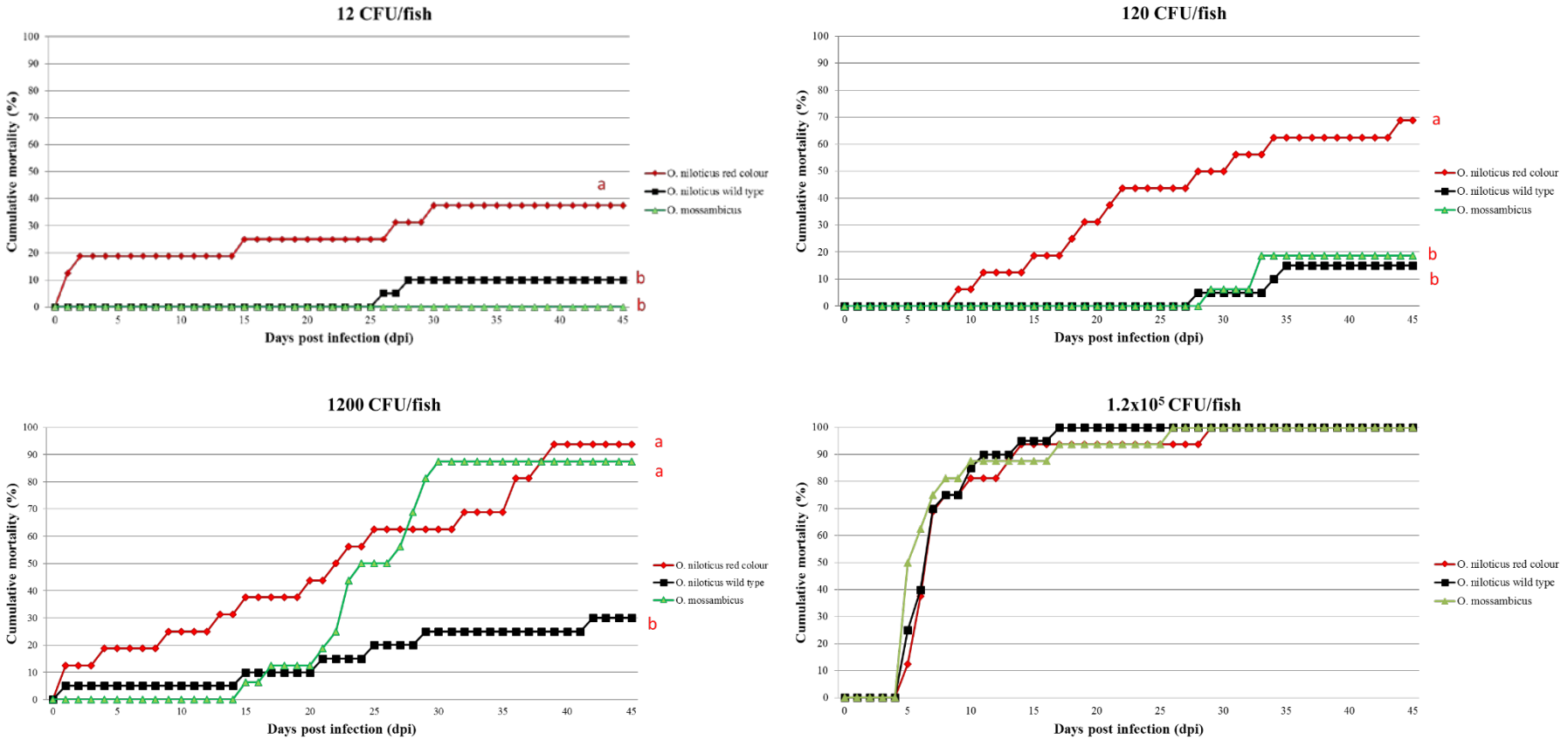


Figure 5.3 Cumulative mortalities observed in the different tilapia species with each of the doses tested. The mortalities bearing different letters are significantly different ($P < 0.05$).

Table 5.1 LD_{50} values (CFU/fish) of different tilapia species during the experiment

Day	*O. niloticus* red	*O. niloticus* wild type	*O. mossambicus*
10	$3.3x10^4$	$4.3x10^4$	$4.4x10^4$
15	$1.9x10^4$	$3.5x10^4$	$4.1x10^4$
20	$1.5x10^4$	$3.3x10^4$	$3.4x10^4$
25	$2.5x10^2$	$2.8x10^4$	$1.2x10^3$
30	$1.2x10^2$	$2.5x10^4$	$4.6x10^2$
35	3.8x10	$2.5x10^4$	$3.3x10^2$
40	30	$2.5x10^4$	$3.3x10^2$
45	30	$2.3x10^4$	$3.3x10^2$

5.3.2 Pre-challenge (LD_{60} confirmation)

The estimated dose of bacteria used for this experiment was $2.4x10^5$ CFU/ml (10^4 CFU/fish). After 21 dpi, 18 of the 20 fish had died, showing that this dose produced a cumulative mortality of 90% (Figure 5.3). As a result of this, the decision was taken to use a dose one log lower (i.e. 10^3 CFU/fish) to challenge the vaccinated fish.

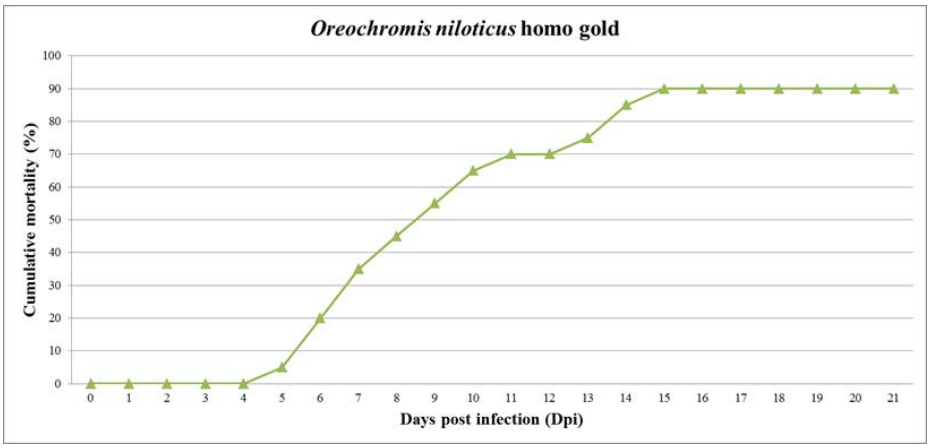


Figure 4 Cumulative mortality of red Nile tilapia challenged with a dose of $2.4x10^4$ CFU/fish of *Francisella orientalis* STIR-GUS-F2f7 after 21 days following infection.

5.3.3 Vaccine stability and safety test

Separation of the components was observed in the water layer in the drop test, between the densities, creating a linear drop where the oily component stayed in the superficial layer, while the dispersed stage of the emulsion went to the bottom of the beaker, a major portion of the drop stayed at the surface. The conductivity measurement was 30µs/cm.

The microscopic particles observed were 1µm in size and were homogenously distributed of the continuous and dispersed phase of the mixture, showing a dense liquid compatible with the 70/30 emulsion. The stability of the emulsion was tested 24h after the emulsification, with no separation of the liquid phases of the solution observed. Thus, after 24h after preparation, the emulsion was still intact indicating that the vaccine was suitably stable for injection in to the fish. The same test, performed after 15, 30 and 60 days post-preparation showed that the stability of the emulsion remained stable with no phase separation.

In the safety test, all 30 fish injected with a double dose (0.2ml) of the vaccine appeared healthy, with no changes in behaviour (such as lethargy, lack of feeding, aggression, gaping, flashing) observed. These fish were dissected at the end of the trial to observe any possible alteration in the peritoneal cavity. No adhesions of the mesenteries or abdominal organs were observed, nor any melanisation. Although 12 fish (40%) had dark pigments within the peritoneal walls and mesenteric fat, this could not be related directly to the vaccine because peritoneal pigmentation is a common feature of tilapia. All the fish had a droplet of the vaccine in the cavity always located dorsal, caudal and lateral to the right (below the swim-bladder, posterior section of the cavity). Smaller droplets could

also be observed distributed randomly within the cavity. The droplets were contained in a smooth soft transparent sac with vascularisation.

5.3.4 Vaccination and infection

As in the pre-challenge, all the fish vaccinated with the normal dose of vaccine remained healthy and no mortalities or signs of toxicity or disease observed within this group during the 30 days of the vaccination period.

During the challenge, differences in colouration and behaviour were seen between treatments. The vaccinated group fish were more active, reactive to stimuli (visual, physical and feeding) and had a red/pink coloration. This was also the case for the adjuvant treated fish. The non-vaccinated fish were more lethargic, had less active feeding behaviour as well as social interaction by hierarchy (aggression), and had pale (white) coloration. These signs started from 4dpi and the first mortality occurred at 8dpi. Moribund fish were gasping, had erratic swimming (no balance) and had swollen abdomens (ascites).

When dead fish were dissected, granulomas were observed in their spleen and kidneys, with varying grades of severity. Some spleens were larger than normal, with dark red coloration and white nodules in 80-90 % of the parenchyma, others were bright red with 90-100 % granulomas and some others shown splenomegaly with large nodules and a white membranous lining of the capsule of the spleen which extended over the majority of the peritoneal cavity. Head kidneys appeared enlarged, haemorrhagic and full of granulomas, in some cases the organ was protruding ventrally towards the anterior

section of the cavity, in contact with the spleen, liver, and gut sections. Other organs were affected such as the gonads, gut and posterior kidney.

Histopathological sections of the affected organs showed the typical granulomatous lesion with the bacterium contained within enlarged macrophages or an accumulation of phagocytic cells enclosing the pathogen covered by fibrin and outlined by inflammatory cells and a concomitant mononuclear infiltration and an increased vascularisation of the affected areas. Non-specific mortalities were ruled out by clinical signs and lack of bacterial recovery, with infected fish showing 100% granulomatous lesions in target organs.

5.3.5 Vaccine efficacy assessment (RPS)

After 40dpi the average mortality in the control group reached 63.3%. In the adjuvant group the mortality was 36.7%. The vaccinated group had no dead fish (0%) in any of the tanks during the whole period of experiment. The RPS value at the end of the experiment was 100% for the vaccinated group and 42% for the adjuvant group (Figure 5.5).

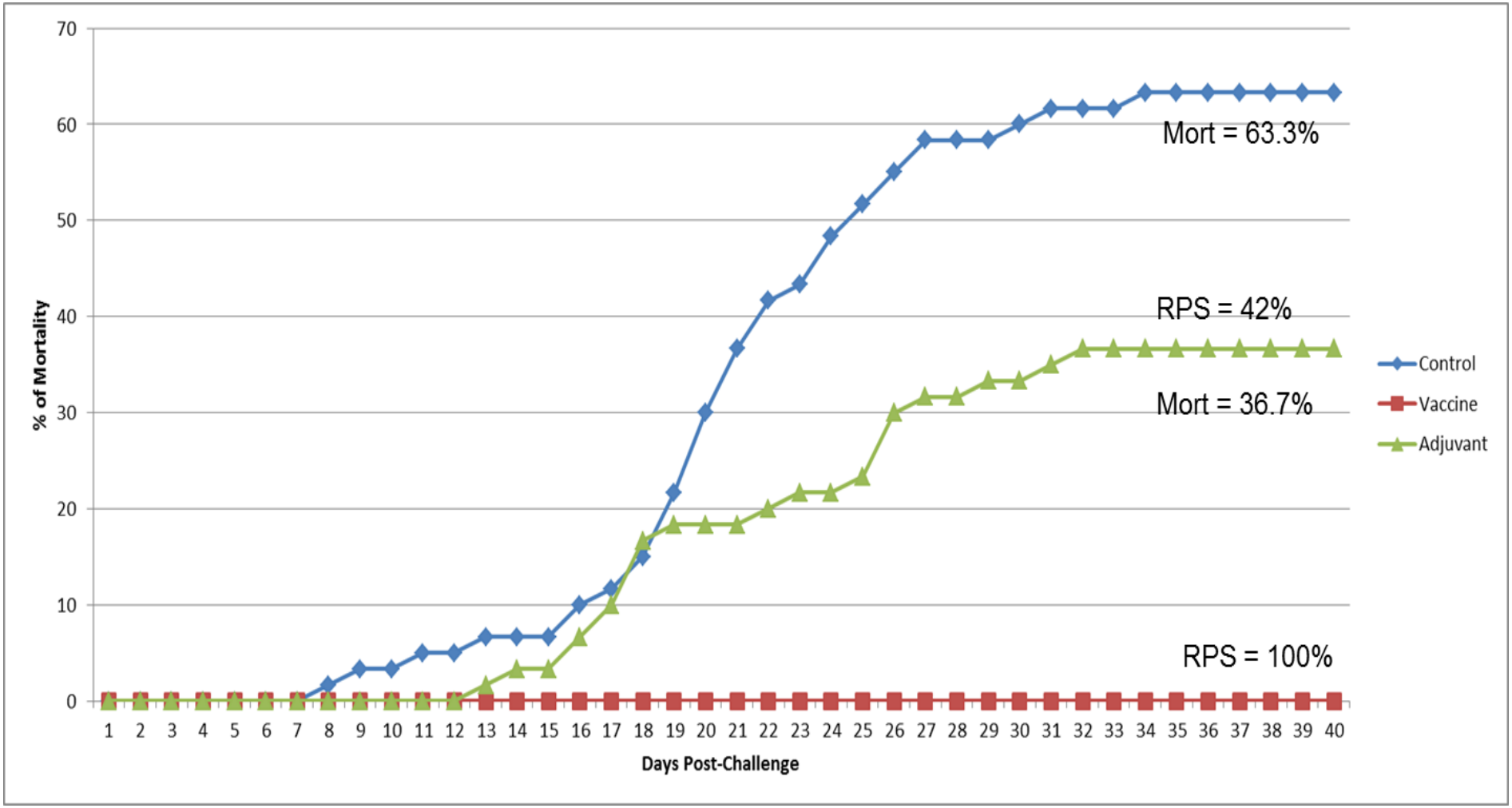


Figure 5.5 Mortality curves during the vaccination-challenge experiment. The graph shows differences in protection of vaccine and adjuvant treatment.

5.3.6 Specific antibody titration by ELISA

To reduce non-specific reactions, hydrogen peroxide was used together with 5% casein or 1% BSA. The dilution of the naïve and immune tilapia serum was evaluated between 1/32 and 1/65536. In addition, the anti-tilapia MAb was tested at 1/33 and 1/66 as well as for the HRP anti-mouse (polyclonal) at 1/1000 and 1/4000.

The first attempt indicated that a dilution of 1/32 gave high non-specific reactions (ODs between 1.05 and 0.6) and no significant difference between the blocking reagents. For the second attempt positive (vaccinated and challenged fish), negative (naïve fish) and PBS controls were used at serum dilutions of 1/64 to 1/1024.

For the blocking of reactions, 5% casein and 2% BSA were used. The anti-tilapia MAb was tested again at 1/33 and 1/66. This resulted in a difference between MAb dilutions but not blocking reagents.

As shown in Figure 5.6 the antibody response was between 1/64 and 1/128 using 1/33 MAb, taking into account the threshold (x3 the average of the PBS control multiplied by 3), providing a threshold of 0.225 (OD_{450}).

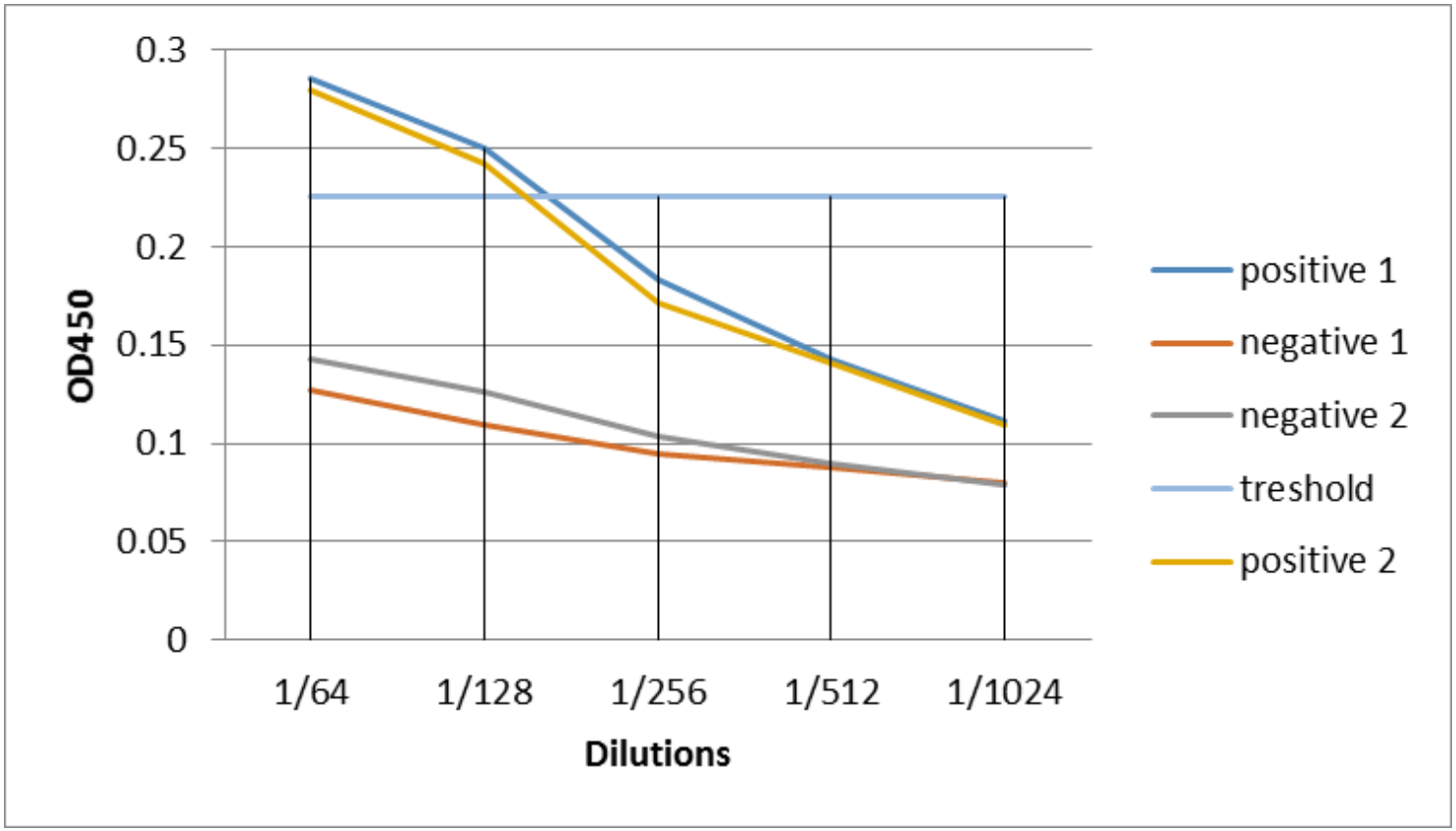


Figure 5.6 Measurement of specific antibody response in red Nile tilapia (*O. niloticus*) to *Francisella orientalis* by ELISA. The graph shows the difference between positive and negative responders over a range of serum dilutions.

The antibody response in the vaccinated fish at three time points post-vaccination is shown in Table 5.2. No specific antibody response was seen in serum sampled from the adjuvant or control groups. In the vaccine group the response remained similar between time points with no significant variability, therefore the pattern of kinetics in the development of the antibody titres was a plateau, with the titre peaking by 30dpv and remaining similar at 45dpv.

Table 5.2 Serum antibody response of experimental fish measured as the mean ±SD of the OD values with its respective mean/range of the antibody titre

Treatments	Day post-immunisation	OD values		Antibody titre		Threshold
		Mean	±SD	Mean	Range	
Vaccinated	15	0.495	0.238	1/245	1/128 - 1/256	0.401
	30	0.773	0.248	1/281	1/256 - 1/512	0.752
	45	0.470	0.245	1/281	1/256 - 1/512	0.337
Control PBS	15	0.319	0.190	-	-	0.392
	30	0.127	0.099	-	-	0.629
Adjuvant	15	0.086	0.093	-	-	0.327
	30	0.167	0.176	-	-	0.525

OD values in vaccinated fish are above the thresholds, indicating specific response to the antigen with a titre between 1/256 and 1/512. The threshold is considered after reduction of the background data (normalized) being two times the average of the PBS wells. The vaccinated groups showed variability between individuals, i.e. non responding fish and high responders (with an endpoint titre over 1/512) (Figure 5.7), but the average OD_{450} remained above the sensitivity threshold showing a clear positive response.

5.3.7 Spleen granuloma score

The macroscopic analyses of the white nodules in the spleens revealed that 90% of the non-vaccinated fish had granulomas, while only 13.4% of the vaccinated fish developed such lesions.

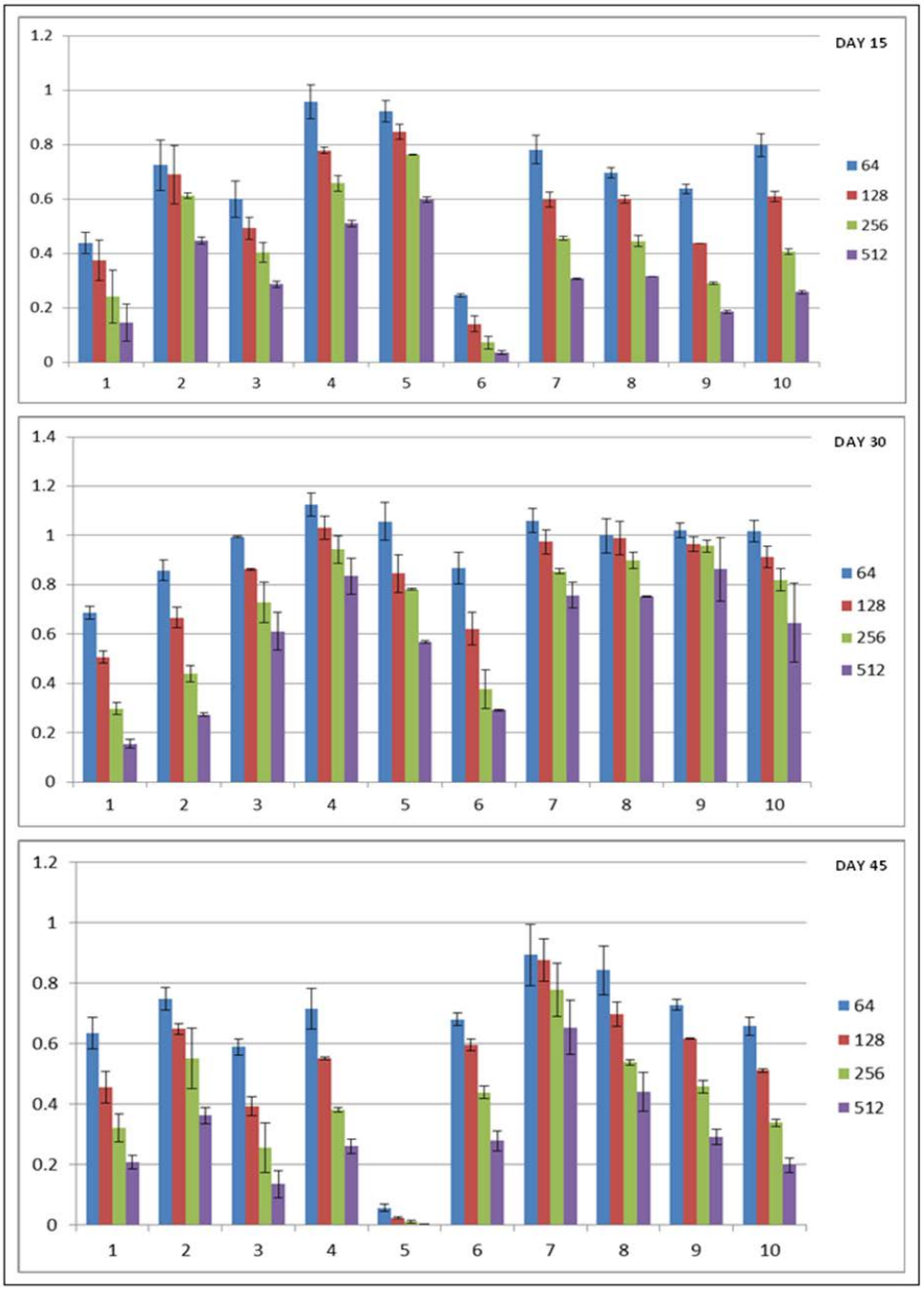


Figure 5.7 Antibody response of 10 vaccinated fish at days 15, 30 and 45 post injection and ±SD. Dotted line indicates sensitivity threshold. Variability per individual is observed, such as non-responders: fish 6(15), 1(30), 5(45) and specific response measured between 1/64 and 1/512. X = The ten fish analysed; Y = OD values.

5.3.8 Transcriptomic profiles

5.3.8.1 RNA integrity and concentration

All the samples tested had RNA integrity values (RIN) below the ideal required (RIN=9) for library preparation. The gel images obtained in the Tape Station with total RNA extracted from the spleens of vaccinated and not vaccinated fish (sampled at 24 hours post infection) are presented in Figure 5.8

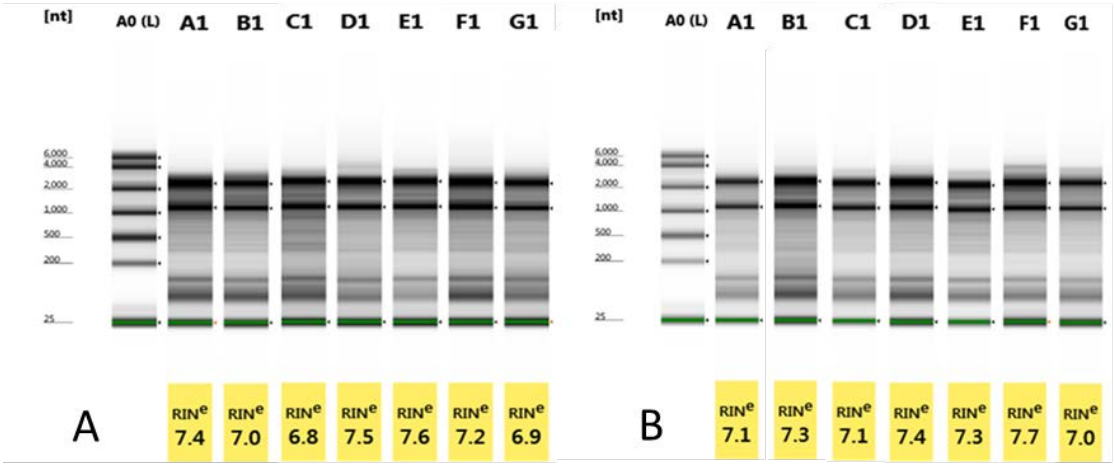
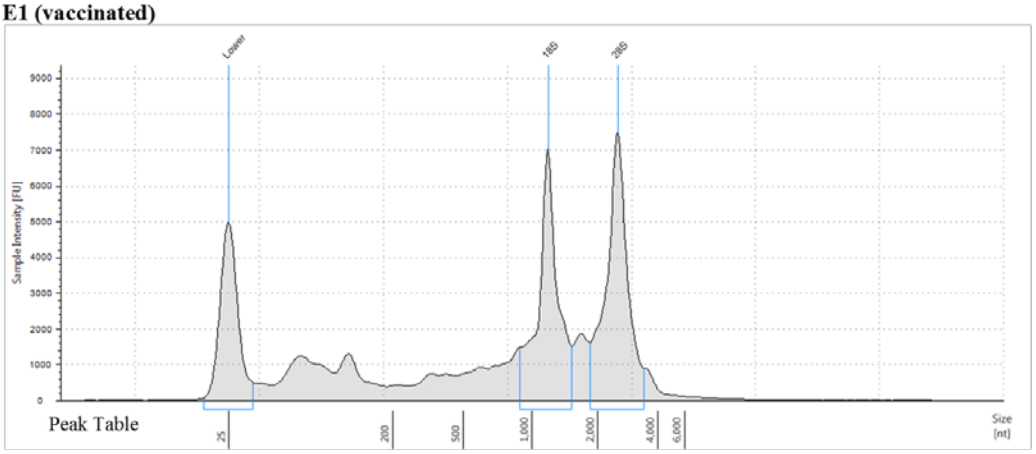


Figure 5.8 Gel images of tilapia spleen RNA analyses. **A** Vaccinated group: A0 ladder, A1 fish4, B1 fish29, C1 fish3, D1 fish8, E1 fish13, F1 fish17, G1 fish24. **B** Not vaccinated group: A0 ladder, A1 fish5, B1 fish8, C1 fish15, D1 fish17, E1 fish23, F1 fish27, G1 fish14. In yellow the RIN value of each sample.

The RNA concentrations in the vaccinated group were: fish4 272 ng/µl, fish29 217 ng/µl, fish3 239 ng/µl, fish8 205 ng/µl, fish13 183 ng/µl, fish17 247 ng/µl and fish24 185 ng/µl. The RNA concentrations in the not vaccinated group were: fish5 277 ng/µl, fish8 283 ng/µl, fish15 336, fish17 252 ng/µl, fish23 358ng/µl, fish27 209 ng/µl and fish14 171ng/µl.

The graphic representation of the results obtained in the Tape Station with the samples with the highest RIN values (fish13 vaccinated group and fish27 not vaccinated group) are presented in Figure 5.9.

E1 (vaccinated)

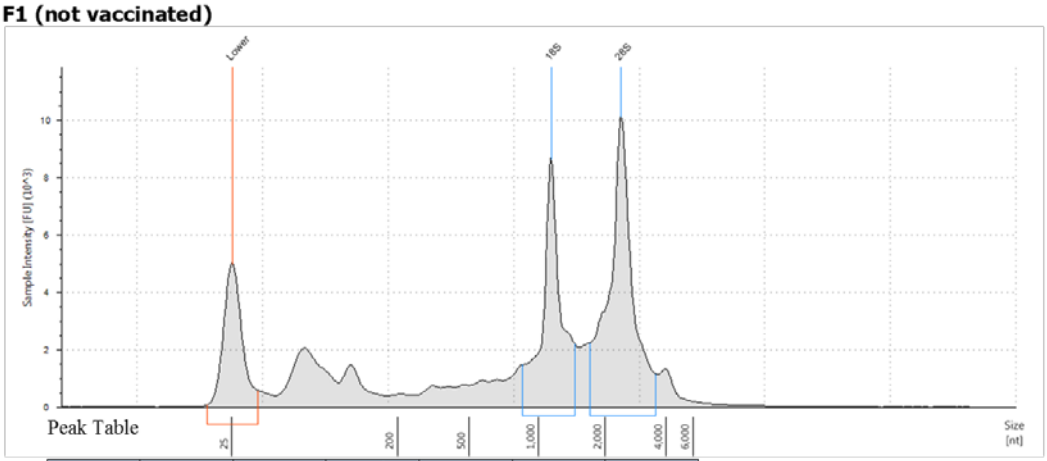


Size [nt]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Peak Comment	Observations
25	47.5	47.5	5590	-		Lower Marker
1,189	48.0	-	119	45.99		18S
2,516	56.3	-	65.9	54.01		28S

RNA Data Table

28S/18S (Area)	1.1
Total RNA Area	5.78
rRNA Area	2.20

F1 (not vaccinated)



Size [nt]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Peak Comment	Observations
25	47.5	47.5	5590	-		Lower Marker
1,135	50.7	-	131	40.12		18S
2,379	75.7	-	93.6	59.88		28S

RNA Data Table

28S/18S (Area)	1.2
Total RNA Area	6.97
rRNA Area	2.66

Figure 9 Graphic representation of the RNA integrity and concentration. **E1** fish13 from vaccinated group and **F1** fish27 from the not vaccinated group.

5.3.8.2 Libraries preparation (quality assessment and concentration)

The pool of the vaccinated individuals was integrated with fish 8, 13 and 17. The pool of the non-vaccinated fish was integrated with samples from fish 8, 17 and 27. The RIN value of the vaccinated pool was 7.3 and the RIN value of the non-vaccinated pool was 7.7. The original concentration of the pools indicated by Qubit was 180ng/µl for the vaccinated fish and 284 ng/µl for the non-vaccinated fish and it was adjusted to 20ng/µl in a volume of 50µl for library preparation. The initial step (purification of mRNA with magnetic beads) of the library preparation is illustrated in Figure 5.10

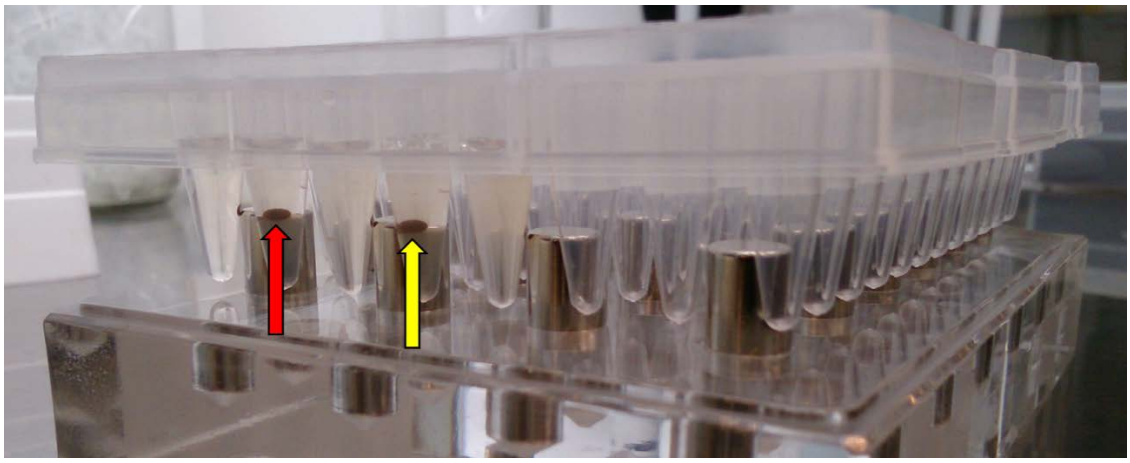


Figure 5.110 Library preparation for next generation RNA sequencing of the tilapia spleen transcriptomes. The red arrow points the purfied mRNA of the vaccinated fish and the yellow the purfied mRNA of the vaccinated fish.

The libraries did not yield the expected values in the Tape Station and therefore their quality was not considered as adequate to continue with the process. For this reason they were not loaded in the cartridge to be sequenced in the Illumina® MiSeq™ platform (Illumina, California, USA).

5.4 Discussion

Compared to terrestrial animals, controlling interaction between the aetiological agents, the hosts and environment is almost impossible in aquaculture as water is the perfect vehicle for bacterial transmission and disinfection of facilities is not always possible. The use of chemotherapeut*ic* agent creates a risk of antibiotic resistance and for these reasons, it has been proposed that disease control in modern aquaculture should be based firstly on good biosecurity measures, but also on stimulation of the immune system of the populations. (Austin, 2012; Toranzo *et al*., 2009; Van Muiswinkel, 2008)

Preparation of whole cell inactivated vaccines was the first approach for vaccine development in aquaculture, and it has proven to be an efficient and successful method of prevention and control of various diseases. The main advantage of inactivated vaccines over live attenuated vaccines relates to safety, because dead pathogens cannot cause infection, but they are able to generate an effective immune response (Austin, 2012).

The development of the present vaccine with whole inactivated cells of *Francisella orientalis* resulted in a stable emulsion without phase separation. The quality of the emulsion is of considerable importance because it has a direct effect on the efficacy and safety of the vaccine. The results of the quality controls performed were consistent with the manufacturer's recommendations indicating that the emulsion was suitable for use in live organisms. Although in this study the viscosity was not measured, its analysis is suggested by the adjuvant manufacturer in order to assess the syringeability of the final product and determine the intraperitoneal distribution of the vaccine formulation that could help avoid side effects.

Fluid water/oil W/O emulsions can be achieved having surfactants with an optimised hydrophilic-lipophilic balance (HLB) value called “required HLB”, which depends on the nature of the oil. W/O/W emulsions and O/W emulsions have a lower viscosity. The ratio between the oil phase and the aqueous phase influences the viscosity. A high dispersed phase leads to an increment of the viscosity on the final emulsion, because of a packed network between droplets, so the continuous phase must be increased to decrease viscosity. For these reason, in W/O emulsions with a ratio of 70/30, the viscosity will be ±50 mPa s, in contrast a similar preparation of 50/50 will have a 4 times higher viscosity (Aucouturier, 2001).

The microscopic inspection indicated that the component particles in the solution emulsion had an appropriate size (1µm) and distribution, normally emulsions having a small particle size and a homogeneous distribution are more stable. According to the adjuvant manufacturer an emulsion is not stable if the following features are presented, breakage, oil release, white layer and sedimentation.

Stability is the most important aspect after emulsification as it determines the durability and resilience of the vaccine preparation. It can be determined by various parameters: 1) the nature of the oil and if it was used alone or in combination; 2) the antigenic phase (its ratio in the emulsion and protein contained); and 3) if the emulsification process included using high mix homogenization. The commercial hand blender used in the present study was successful in mixing the vaccine components uniformly making a very stable vaccine.

The syringeabilty test showed that the gauge of the needles is an important factor in the vaccination process. The first practice attempts to perform the vaccination with insulin

syringes (30GX8mm) were not successful, the process was slow, complicated and some of the syringes broke while injecting the fish. For the vaccination experiment 22GX7mm and 21GX8mm hypodermic needles were used. This facilitated the process and enabled a smooth and seamless injection, reducing time and labour required to complete the experiment.

The bacterial inactivation process should kill the pathogen, but retain sufficient structural preservation to produce an immune response. This procedure can target extracellular components of the bacterial cell i.e. surface components (cell membrane) or intracellular component (nucleic acids) to impede their replication. The physical methods of inactivation include UV light, heat and sonication. UV light basically disables the ability of replication by dimerization of the nucleic acids. Heat has been shown to be a very effective method, but the downside is that even though it diminishes infectivity it may also reduce immunogenicity, therefore the ideal temperatures to destroy the first and maintain the latter are in the range of 50-60°C for 10-30 min. Sonication disrupts the bacterial morphological structure retaining the immunogenic compounds. The primary product for chemical inactivation is formaldehyde at low concentrations, essentially because it has the capacity of altering the extra and intracellular compounds while maintaining the immunogenic properties.

The safety test was carried out as suggested in the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines and the European Pharmacopoeia commission for vaccines for veterinary use. This test is performed to determine the performance and safety, with regard to toxicity and side effects. The majority of bacterial vaccines are inactivated with

0.5 % formalin with viability and sterility tests then carried out at 24 and/or 48 h, although there is some question associated with the concentration of formalin used and the effect it has on denaturing the vaccine antigens (Gudding *et al.*, 2014).

Toxicity is a very important aspect; there are reports of some vaccines e.g. *Yersinia* and *Aeromonas* that have been shown to be toxic to fish (Gudding *et al.*, 2014); using a double dose or "double-safe procedure" helps to highlight any problems with regard to vaccine safety. Toxicity problems may include a reduction in feeding behaviour, decreased swimming activity, pain, reduced gut function, energy-demanding immune response and anaphylactic shock (Gudding *et al.*, 2014).

The efficiency of a vaccine in terms of protection is difficult to assess, until today the only accepted method of determining the effectiveness of a vaccine is with the RPS values proposed by (Amend, 1981; Gudding *et al.*, 2014). There is a debate regarding ethical issues, involving deliberately infecting and killing experimental fish, but due to the fact that mechanisms of protective immune response given by a vaccine are unclear in fish, there is currently no alternative that can be considered reliable and/or successful enough to be used as a replacement by the aquaculture industry (Austin, 2012). Thus, to determine vaccine efficiency, evaluation of the level of protection is essential. Multiple factors including the virulence of the pathogen, dose standardization, fish susceptibility, optimization of experimental unit, as well as the general experimental design and statistical analysis must be taken into account to establish and standardise the challenge procedure. Keeping these variables (and the replicability of challenge) constant is difficult to achieve and that is why it is not easy to compare the efficiency between different vaccine batches of the same or different bacteria. To counteract this, an

alternative way to measure effectiveness can be to determine the mechanisms of vaccine protection or the mode of action, rather than evaluating mortality rates, especially in cases where the mortality rates ae unknown or the disease does not cause high mortality rates but has elevated morbidity (Gudding *et al.*, 1999; Gudding *et al.*, 2014).

In order to investigate the susceptibility of different genetic groups of tilapia a series of experiments was developed to estimate the medium lethal dose value (LD_{50}) of *F. orientalis* STIR-GUS-F2f7 in Mozambique (*Orecochromis mossambicus*) and red and wild type Nile tilapia (*O. niloticus*). The cumulative mortalities and the LD_{50} results clearly indicated a significant variation where the red Nile tilapia appears to be the most susceptible while the wild type is the most resistant.

The mortality rates of the pre-challenge generated a major query about the ideal dose (concentration of bacteria) to be used in the main experiment and this was due to the LD_{50}. Results indicated that with $1x10^3$ CFU/fish, the median lethal dose was achieved at day 23 post challenge, with 10^5 CFU/fish at day 6 and with 10^2 CFU/fish at day 35.

For the pre-challenge and vaccination challenge 10^4 CFU/fish seemed to be a very high dose (90% mortality in 21 days) and 10^3CFU/fish correlated with the findings of the previous trial where in 27 days, 58.3% of the population had died (60% in two tanks) compared to 60% at day 25 determined in the LD_{50}.

Soto *et al.* (2009b) used a different strain of Nile tilapia and a different isolate, and compared IP and immersion challenge, using the cumulative mortalities at 20 and 40 dpi, the LD_{50} for the IP challenged tilapia were $10^{-5.1}$ (1.8×10^4 CFU/fish), and $10^{-5.3}$ (1.2×10^4 CFU/fish), respectively.

The mortality curves in the vaccine trial in the present study showed a high level of protection (100%). The vaccine could perform differently under farm production conditions however, due to a range of factors such as stocking densities, husbandry, social behaviour, tank conditions, water quality or concomitant diseases or variations in the isolates present on the fish farm. It could be expected that the range of protection of an autogenous vaccine would differ when comparing lab conditions and the farm environment, but this can only be established if a vaccination trial is performed at the farms (Gudding *et al.*, 2014).

The difference in RPS values between the vaccinated, adjuvant and the control fish reflects the cellular and/or innate response given by the adjuvant (Schijns and Tangerås, 2005) and differences in the adaptive immune response observed in the vaccinated fish (Sarder *et al.*, 2001; Gudding *et al.*, 2014). The mode of protection of a vaccine can rely, in part, on the production of humoral antibodies in the host. If there is a disagreement between the presence of humoral antibodies to bacterial immunizations i.e. no correlation between protection and antibody production, this could be related to a lack of antibody assembly or low titres of antibodies indicating that these do not perform a key role in protection. Once antibody levels reach a level above a threshold considered to be protective, then they are said to have reached a signature for protective immunity (Pulendran *et al.*, 2010).

Besides the antibody response, there is the possibility that the mechanism of protection is centred on an innate or cellular response, for example stimulation of T cell, leukocyte proliferation, lysozyme activity and macrophage activating factor production (Gudding *et al.*, 2014). Also a joint response involving humoral, cellular and innate immunity can

be possible as well as the involvement of other mechanisms including the enhancement of nitric oxide response, mucosal immunity and immune gene expression (Gudding *et al.*, 2014; Schrøder *et al.*, 2009).

The main function of adjuvants is the antigen presentation process, distinguishing them in Signal 1 (actual antigenic presentation) and Signal 2 (secondary signal) facilitators; both involved in the activation of lymphocytes T and B (O'Hagan and Singh, 2003; Tafalla *et al.*, 2013). Signal 1 adjuvants depend on the status of the antigen in terms of time, injection site and concentration allowing immune cell aggregation. Unlike Signal 1, Signal 2 depends almost entirely on immune receptors; they are known to stimulate an increased innate response with discharge of cytokines and PAMPS/DAMPS; leading to receptor expression (PRRs) and T cell differentiation (T helper cells), involving the MHC peptides being recognized (co-stimulation signals during antigen recognition); the T helper (Th) cells will differentiate into Th1 and Th2 to activate the B cells that will differentiate to memory B cells (long lived antigen specific B cells) or plasma cells (effector cells that secrete antibodies). Th1 cells secrete IFN-y, which activates macrophages and induces production of opsonizing antibodies (pathogen is marked for ingestion and destruction by a phagocyte) by B cells, producing a cell mediated response protective against intracellular pathogens, with activation of cytotoxic T lymphocytes and natural killer cells. Meanwhile the Th2 cells secrete cytokines IL-4, IL-5 and IL-13 that induce B cells to produce neutralizing antibodies.

Further functions of adjuvants include enhancing and prolonging immune response, modulation of antibody response (spectrum and specificity), increase in immunogenicity of poor antigens and antibody production for weak responders, induction of mucosal

immunity and reducing dose of antigen as well as competition between antigens (polyvalent vaccines).

In the present study the adjuvant, Monatanide ^{TM} ISA 763 AVG, showed a functional behaviour towards general vaccine efficiency. Examples of Montanide emulsions for bacterial diseases include formalin killed *Pseudomonas plecoglossicida* (Ninomiya and Yamamoto, 2001; Sitjà-Bobadilla, 2008).

Studies of *Aeromonas hydrophila* vaccinations (in *Hippoglossus hippoglossus* and *Cyprinus carpio*) showed the comparison between Freund´s and Montanide ISA 711, evaluating antibody titres and intraperitoneal adhesions, in terms of humoral antibodies Freund´s adjuvant displayed an earlier and higher response compared to Montanide (Montanide 4 of 47 fish reached 1:1000 in 6 months; FCA 27 of 48 2 months), but Montanide produced less side effects (Bowden *et al.*, 2003).

Montanide ISA 763 was also tested in *Oncorhynchus mykiss* as adjuvants in *A. hydrophila* and *Lactococcus garvieae* vaccines and provided a high degree of protection, with 70% mortality obtained in control fish, and cumulative mortalities of 6.7 and 16.7% (30 and 90 days PV challenge) in vaccinated fish (Bastardo *et al.*, 2012) .

Another example in *O. mykiss* against *Yersinia ruckeri* using Montanide IMS 1312 VG (bath immersion). The control groups had a maximum survival of 6.6% and in vaccinated fish survival ranged from 93.3–100% and 80.1–100% in fish vaccinated with vaccine with and without Montanide, respectively (Soltani *et al.*, 2014).

A combination of RPS values and the specific antibody response strongly suggested that the vaccine tested in the current study was high efficacy.

The ELISA showed a clear detection of specific antibodies for *F. orientalis* in vaccinated fish and not in control nor adjuvant individuals, and this can be related to the reduction of mortalities (0%) in the challenge compared to 60% and 32% in the control and adjuvant treatments (Gudding *et al.*, 2014) .

Examples of the correlation between antibody levels and survival rates include work carried out in Atlantic salmon vaccinated with IPNV, showing that antibodies produced during the challenge reduced post-challenge mortalities (Gudding *et al*., 2014). In addition, a furunculosis vaccine in the same fish species showed correlation between increased humoral antibodies and decreased mortality (Gudding *et al*., 2014). Others examples include *Aeromonas salmonicida* (Lund *et al.*, 2008), *Streptococcus difficilis* (Bercovier *et al.*, 1997) and *Flavobacterium psychrophilum* (Crump *et al.*, 2005).

For the present study, there seems to be a direct relationship between protection and adaptive immunity. Although the bacterium is a facultative intracellular pathogen that can invade phagocytic cells and avoid detection, it is possible that the vaccine acts during the extracellular phase of the bacterial pathogenesis (Soto *et al.*, 2010a). In addition, as variability in individual response within vaccinated fish was detected at the three time points, it may be a matter of individual characteristics, determined by factors such as fish weight-size (variability of ±2 g), sex or even social hierarchy.

The importance of this result is based on the fact that the average the humoral response was over the threshold, indicating that it had reached a significant level of protective immunity (Gudding *et al.*, 2014), even falling within the concept of herd immunity that takes into account the protection provided to the whole population to prevent a disease outbreak including individuals with or without low immunity (Anderson and May, 1985;

Gudding *et al.*, 2014). This means that the proportion of high responders seen in the study would help to protect the poor responders.

The kinetics of the serum antibody response were unexpected, because very similar mean antibody titres (and ± SD) were observed between samples taken at the various time points dpv. This was highlighted when comparing the results to studies in Nile tilapia with heat-killed *Aeromonas hydrophila* vaccine and Montanide (no specifications) (Sarder *et al.*, 2001), where the kinetics showed a typical bell shaped curve, with the highest titre at 28 dpv and a decrement by 42dpv.

Many factors can influence the behaviour of the humoral response, in this case it is suggested that the vaccine stimulated a response by 15dpv, had its peak by 30dpv and remained elevated even at 45dpv, indicating that it is a continuous stimulation due to the properties of immunomodulation of the adjuvant, providing constant and long lasting release of the antigen.

In regarding the transcriptomic profiles, many factors could have influenced the low quality in the RNA. The fact that the samples were stop for 10 days at the customs in Santiago airport in Chile is thought to be the main reason for these results. Attempt to construct the libraries were performed despite the low RIN values obtained in the pools but as expected the quality of the libraries was not considered good enough to perform the sequencing. Further attempts to extract total RNA and construct new libraries are being performed with the samples left and other kits are being performed at the INCAR laboratory.

In tilapia there is a current patented vaccine developed by (Hawke and Soto, 2012; Soto *et al.*, 2011b) based on a genetically attenuated *Fo* strain. The efficacy studies for this vaccine consisted of testing the attenuated mutant and determining the protection acquired by injection and immersion with the wild type strain. The intraperitoneal injection of the mutant induced a strong humoral response in adults and enhanced the production of antibodies, with serum and mucus antibody response titres >52000. They also tested the functional ability of the antibodies by opsonophagocytosis and killing assays, and passive immunization trials. The tissues recovered were evaluated by mean number of granulomas found in a 10X microscopic field and their bacterial loads were measured by PCR.

After immersion immunisation with 10^7 CFU/ml of the mutant strain for 30-180 min, the RPS values obtained were 68.75 and 87.5% respectively, while with 10^3 CFU/ml RPS values were 56.25 and 62.5%. Although this vaccine was later patented in the USA for tilapia, it cannot be commercially used in Europe, as such vaccines must be authorized for the entire European Union via the European Medicines Agency due to a more strict legislation on genetically modified organisms (GMOs) prevailing in this continent.

In this context, the vaccine developed in the present study, represents not only the most efficient approach for prevention of piscine francisellosis, but also the most biologically safety and eco-friendly produced so far.

5.5 Conclusion

In conclusion, the vaccine here prepared against *F. orientalis* proved to be 100% effective in red Nile tilapia under laboratory conditions, when the fish was challenged at day 31dpv. What is more, the vaccinated fish developed an adaptive immune response that correlated directly with protection. It is imperative to consider the antigen dose in correlation to protective immunity, taking into account a threshold dose. Also it is important to consider the function of the adjuvants to assist with early immunity onset, long effector response (antibody titres; T cell activity) and diminishing requirement of boosters. The next step is to test the vaccine against other geographical isolates of *F. orientalis* under laboratory conditions as well as test its efficacy in the field.

5.6 Acknowledgments

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Chapter Six

General discussion and final conclusions

6.1 General discussion

Piscine francisellosis in an infectious emerging disease that continues expanding to new hosts and new geographical regions and for which no commercial treatment or vaccine exist. When the pathogen was first detected in the mid 90's using electron microscopy it was associated with the genus *Rickettsia* (due to its intracellular nature) and was referred to as a "rickettsia-like organism".

Another term used to refer the bacterium was *Piscirickettsia*-like organisms due to its similarity in ultrastructural morphology with *Piscirickettsia salmonis* which was first confirmed as the causative agent of granulomatous disease in farmed salmon in 1991 (Fryer *et al*., 1992).

It took more than a decade after the disease was first reported to molecularly identify members of the genus *Francisella* as the cause of the disease outbreaks and it was then when researchers started to use specially formulated synthetic media to successfully isolate it.

The use of this enriched medium allowed the recovery of several strains of *Francisella* spp. from diseased cod in northern Europe, but this was not always achieved in tropical aquaculture where there were many reports of francisellosis with no successful isolation of the pathogen despite having molecular identification of *Francisella*-like bacteria. Some reasons for this difficultly could be the fastidious nature of the bacterium (slow growth and exigent nutritional requirements) and its ability to enter into a viable but not culturable status. These properties have not only complicated the primary isolation of the

pathogen, but consequently prevented its adequate phenotypic and genetic characterisation.

At the start of this study there were only 3 reports of isolation of *Fno* and very little information was available on their phenotypic characteristics. In this context the selective tilapia blood agar proposed in Chapter 2 resulted in an excellent option for the primary isolation of the pathogen, especially where bovine products are not readily available or their use is not desirable in veterinary vaccine development. Using this agar a total 10 new isolates of *Fno* were recovered from tilapia for the first time in Europe, including STIR-GUS-F2f7.

After purification, the isolates were analysed for antimicrobial resistance. The MIC results indicated that the isolates were susceptible to neomycin, novobiocin, amikacin, ciprofloxacin, imipenem, gatifloxacin, meropenem, tobramycin, nitrofurantoin, and levofloxacin. The large inhibition zones in the disk diffusion method indicate that they are susceptible to enrofloxacin, kanamycin, gentamicin, tetracycline, oxytetracycline, florfenicol, oxolinic acid and streptomycin. Considering these results, florfenicol and oxytetracycline seemed to be the most adequate to treat the fish as these are authorised antibiotics for use in aquaculture in Europe and the farms were consequently medicated.

The isolation of the new strains emphasised the need for a suitable methodology to characterise them. Prior to this project researchers had limited their investigations to basic molecular identification i.e. PCR with universal eubacterial primers. Therefore in Chapter 2, the use of the Biolog GN2 plates to investigate the phenotypic and biochemical characteristics was proposed.

This was the first time that these plates were used with *Fno,* but the manufacture's protocol had to be optimised to cope with the fastidious nature of the bacterium. This optimisation included the development of a gradient of inoculum densities and the selection of an optimal concentration. Having made these modifications to the protocol more than 20 new metabolic features of STIR-GUS-F2f7 and other strains from Central America and Asia were discovered.

Additionally, to integrate a polyphasic approach a set of housekeeping genes was designed to genetically characterise the isolate STIR-GUS-F2f7. The selection of the genes was based on previous publications sequences from other *Francisella* spp. and they demonstrated a good level of resolution to classify the bacterium at the subspecies level. The primers to amplify and sequence the genes were originally designed based on the genome of *F. philomiragia*. While there were more than 30 fully sequenced genomes for other *Francisella* species, there were none from fish pathogenic strains at the beginning of this project.

In Chapter 3, next generation sequencing i.e. Illumina® Hi-Seq™ platform was used in collaboration with the Swedish Defence research agency to sequence and *de novo* assemble the complete genome of STIR-GUS-F2f7. A few weeks after the isolation of STIR-GUS-F2f7, Sridhar *et al.* (2012) released "Toba04" the first fully sequenced and assembled genomes from *Francisella noatunensis orientalis*. The primers for the house keeping genes were then redesigned using Toba04 as a backbone while STIR-GUS-F27 was still being sequenced.

The annotation of the genome presented in Chapter 3 represents an invaluable tool for future research on the metabolism and pathogenicity of *Fno*; not only could this be linked

to the phenotypic fingerprint obtained in Chapter 2, but it could also be useful to further investigate the OMVs-like structures observed in STIR-GUS-F2f7.

True OMVs have been recently described by Brudal *et.al* (2014b) in *Fnn* grown *in vitro* and *in vivo* in zebrafish. In that study the structures contained virulence factors like iglC, PdpD, PdpA, FopA and GroEL proteins. Interestingly when the OMVs were used as a vaccine they reduced proliferation of the bacterium and protected zebrafish embryos during experimental infections. If the structures here described in STIR-GUS-F2f7 are OMVs with similar functions to those presented by Brudal *et al*. (2014b) for *Fnn,* the use of proteomics and the annotated genome could contribute to the develop a new approach for vaccine development against this disease in tilapia and cod.

The genome sequence of STIR-GUS-F2f7 was also used in this study as a part of series of investigations that aimed to clarify its taxonomic status. In Chapter 4 sixteen *Fno* and sixteen *Fnn* strains (from a wide range of hosts and geographical regions) that had been collected and archived in different laboratories around the world during the last decade were phenotypically and genetically characterised to determine if the isolates belonging to the *taxon Fno* constitute an independent species.

Although in the last decade some “new” concepts of prokaryotic species have been proposed e.g. ecological species (Cohan, 2002), recombination species (Dykhuizen, 2005), phylo-phenetic species (Rosselló-Móra and Amann, 2001) and meta-population lineages (de Queiroz, 2005) no consensus has been reached yet to accept one or a group of them as the universal concept of “prokaryotic species”. Therefore, bacterial taxonomists continue to use the genomo-species concept (Wayne *et al.*, 1987) which indicates that two (pure) isolates that show 70% or greater DNA-DNA association with

5°C or less difference in ΔTm belong to the same species. Additionally the percent similarity in the 16S rRNA gene is also considered a standard species delineation. For this parameter the cut-off value is 98.65% and to use it the gene sequences must be complete i.e. ~1500bp.

More recently, with the boom of next generation sequencing technologies, bacterial taxonomists have had access to thousands of complete genome sequences to correlate them with crude DNA-DNA values. This has allowed them to establish an average nucleotide identity (ANI) value of 95-96% in a set of at least five (the longer the sequence the better) conserved genes e.g. housekeeping or core genes as a third "new" gold standard.

The use of the genomic sequence derived parameters from STIR-GUS-F2f7 and all the other sequenced strains indicated that according to the gold standards the *Fno* taxon does constitute a separate and independent species from *F. noatunensis*. Moreover, the endemic isolates from Chile represent a new true subspecies of *Francisella noatunensis* and one isolate from Ireland was classified as an "atypical strain" of the new *Fnn* subspecies. These results were supported by crude DNA-DNA values and other *in silico* analyses, which included phylogenetic networks and trees.

Additionally, the biochemical and phenotypical characterisation performed with the Biolog GN2 plates as per the protocol proposed in Chapter 2 correlated with the genetic observations. The modification to the manufactures protocol helped to discover more positive reactions that allowed even the classification of the atypical Irish strain. In 2007, Huber *et al.* (2010) had used the plates with one Norwegian *Fnn* from cod, however since they used the protocol as per the manufactures instruction they presented only 6 positive

reactions and missed the other eleven “false negatives” that were unveiled in this research. The phenotypic information presented here was used to amend the description of *F. noatunensis.*

While developing the gradient of densities a number of reactions appeared to be positive when OD_{600} over 1.0 were used to inoculate the plates in both, the cold water and warm water isolates. However, since the plates reached a point of saturation when this limit was passed, those reactions were not reported as positive. These carbon sources are for *Fnn* dextrin (A3), glycogen (A4), D-cellobiose (A12), glycyl-Lglutamic acid (F12), L-threonine (G12), glycerol (H9) and D-L-α-glycerol phosphate (H10). And for *Fno* D-cellobiose (A12), mono-methyl- succinate (C12), α-keto glutaric acid (E4), D,L-lactic acid (E6), succinic acid (E12), bromo succinic acid (F1), glycyl-Lglutamic acid (F12), L-ornithine (G4) and Thymidine (H4).

It is emphasised that more research is required, either screening the genomes or developing individual tests for each of these metabolites, to rule out whether the fish pathogenic *Francisella* cells have or don’t have the ability to metabolise those carbon sources before considering them as a part of their phenotypic description.

The nomenclature and taxonomic classification proposed here for the fish pathogenic *Francisella* strains is presented in Table 6.1. This is based on the phenotypic and genetic investigation performed in Chapter 4.

Table 6.1 Taxonomical rearrangement of fish pathogenic *Francisella* spp. proposed in this study and previous nomenclature

Genus	Species	Subspecies	Previous names	
Francisella	*noatunensis*		*F. philomiragia noatunensis*	*F. piscicida*
Francisella	*noatunensis*	*chilense*	*F. noatunensis noatunensis*	-
Francisella	*noatunensis*	*noatunensis*	*F. philomiragia noatunensis*	*F. piscicida*
Francisella	*orientalis*	-	*F. noatunensis orientalis*	*F. asiatica* ǂ; *F. victoria* ǂ

ǂ Never effectively or validly published

With the correct nomenclature established, an autologous vaccine against *F. orientalis* (*Fo*) was developed in Chapter five of the present study to protect tilapia. This vaccine was formulated with a commercial oil adjuvant emulsified with formalin killed *Fo* STIR-GUS-F2f7.

This is the first time that this approach is used for francisellosis in tilapia. The only previously attempt to develop a vaccine against this disease was the live attenuated vaccine reported by (Hawke and Soto, 2012; Soto *et al.*, 2011b).

Prior to the formulation of the vaccine the susceptibility or LD_{50} of 3 genetic groups of tilapia to *Fo* STIR-GUS-F2f7 bacterium was investigated. The results obtained in these experiments clearly indicated a significant variation, with the red Nile tilapia appearing to be more susceptible than Mozambique tilapia and wild type Nile tilapia, while the latter was found to be the most resistant to francisellosis. Further research is required to determine if this can be attributed to the families tested or is indeed characteristic of the species/populations. The mortality curves of the red Nile tilapia were not stable and consistent with the sigmoidal curves normally observed in dose dependent experiments.

There are no other reports in the literature where the susceptibility of different tilapia species against francisellosis had been compared, but the values obtained for Nile tilapia

wild type are in agreement with those reported by Soto *et al.* (2009b). In this sense the results presented here represent a starting point to look at resistance traits in different genetic groups of tilapia against francisellosis.

On the basis of these results and the relevance that the red tilapia market has in several countries, the red Nile tilapia was selected as the candidate to test the vaccine. In order to determine the best dose to challenge the vaccinated fish a pre-challenge with 20 naïve red Nile tilapia fingerlings took place. The dose used in this experiment was selected from the results of the LD_{50} experiments and was expected to cause 60% mortalities by around day 30. Unexpectedly the dose killed the 20 fish in only 15 days and the decision was taken to reduce it by one log for the final challenge. The dose finally used caused 63% of mortality in unvaccinated fish.

All the fish were vaccinated 30 days prior to the challenge, and the production of specific antibodies against *F. orientalis* was constantly monitored during that period. The results of the ELISAs confirmed that specific antibodies were being produced and the peak of protection was reached around day 30 post-immunisation.

These results are similar to those presented by Schrøder *et al.* (2009) who vaccinated cod with a whole cell inactivated vaccine but against *Fnn*. Soto *et al.* (2011b) also obtained a strong humoral response in adult tilapia and enhancement of antibodies production after intraperitoneal injection of the live attenuated vaccine.

The formulation of the vaccine consisted of formalin killed inactivated cells of STIR-GUS-F2f7 and the commercial oil adjuvant Montanide™ ISA763AVG. The inactivation of the bacteria was confirmed twice before emulsifying it with the adjuvant. Although

there were no reports of its use with *Francisella* it had been previously used with success in other fish pathogenic bacteria like *Yersinia ruckeri*, *Aeromonas hydrophila* and *Lactococcus garvieae* (Soltani *et al.*, 2014).

The adjuvant was considered adequate because the stability of the emulsion was maintained for weeks, no adherences were seen in the injected fish and no side effects were presented during the safety test experiments.

The final results of the experiment indicated that the vaccine was 100% effective in protecting the fish against the experimental infection. In contrast a significantly lower relative percent survival (42%) was observed in the fish immunised only with the adjuvant and 63.3% of mortality was seen in the control group.

6.2 Final conclusions

A new solid media (based on cysteine and tilapia blood) for the isolation and culture of fish pathogenic and other *Francisella* spp. was developed.

A polyphasic approach for the characterisation of fish pathogenic *Francisella* spp. is presented and its use to phenotypically and genetically characterise *Francisella* spp. from other sources is proposed.

This was the first documented report of the isolation and characterisation of *Fno* from tilapia in Europe.

The complete genome of the *Fno* (*Fo*) strain STIR-GUS-F2f7 was fully sequenced using next generation sequencing (Illumina™ Hi-Seq platform) technology.

The taxonomy of the genus *Francisella* was revised and the new bacterial species *F. orientalis* and the new subspecies *F. noatunensis chilense* are described. An emended description of *F. noatunensis noatunensis* is presented.

The susceptibility of three different genetic groups of tilapia (two species and two varieties) to STIR-GUS-F2f7 was investigated. The families from the red Nile tilapia group were the most susceptible while the wild Nile tilapia were the most resistant.

An inactivated (formalin killed/oil adjuvanted) vaccine against piscine francisellosis was developed.

The vaccine was tested in red Nile tilapia and it proved to be capable to stimulate strong humoral immune response in the experimental fish and 100% effective.

6.3 Recommendations for future research

The results obtained in this thesis, open new avenues for research that could be explored in the near future. In this section some of these ideas and other suggestions to give continuity to this study are presented.

- Control of piscine francisellosis by developing vaccines based on comparative proteomic analyses of several strains recovered from fish.

- Application of the polyphasic approach to clarify the taxonomic status of several strains *F. philomiragia* and other *Francisella* spp.

- Establishment of minimal standards for the description or delineation of species and subspecies within the genus *Francisella*.

- Performance of comparative genomic analyses of all the *Francisella* species that are causative agents of disease in aquaculture.

- Use the genomic information here obtained will contribute to develop highly sensitive, rapid detection methods for *Francisella orientalis* that could be used at the farm side such as loop-mediated isothermal amplification (LAMP) method.

- The challenge models here established could facilitate the study of the effect of different probiotic bacteria and bioactive algal compounds to prevent and mitigate piscine francisellosis in tilapia.

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