DEVELOPMENT OF CONTROL STRATEGIES FOR *Francisella noatunensis* subsp. *orientalis* in NILE TILAPIA, *Oreochromis niloticus*

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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 by any other degree.

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List of abbreviations

%	Percentage
~	Approximately
µl	Microliters
µm	Micrometres (microns)
µM	Micromolar (pmol/ml)
APCs	Antigen Presenting Cells
ARMS	Amplification-refractory mutation system
ATCC	American Type Culture Collection
BCP	1-bromo-3-chloropropane
BI	Bath immersion
BLASTN	Nucleotide-nucleotide basic local alignment search tool
bp	Base pair
CCUG	Culture Collection, University of Göteborg, Sweden
CFU	Colony forming units
CHAH	Cysteine Heart Agar with Haemoglobin
COGs	Cluster of orthologous groups
CPM	Cumulative percent mortality
Ct	Cycle threshold
dd	Degree days
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
dpc	Days post challenge
dpv	Days post vaccination
dsDNA	Double stranded Deoxy ribonucleic acid
DSMZ	German Culture Collection of Microorganisms and Cell Cultures
DW	Distilled water
EDTA	Ethylene-diamine-tetraacetic acid
EF1A	Elongation factor 1 alfa
ELISA	Enzyme-linked immunosorbent assay

List of abbreviations

et al.	*et alii* “and others”
FAO	Food and Agriculture Organization of the United Nations
Fe	Iron
FFPE	Formaline fixed parafine embedded tissues
fg	Femtogram
Fnn	*Francisella noatunensis* subsp. *noatunensis*
Fno	*Francisella noatunensis* subsp. *orientalis*
Fp	*Francisella philomiragia*
FPI	Francisella pathogenicity island
Ft	*Francisella tularensis*
FVGAL	Fish Vet Group Asia Limited
g	Gravities
gDNA	Genomic DNA
GE	Genome equivalent
h	Hours
H&E	Haematoxylin and eosin
HIS	Hyperimmune serum
HKDM	Head kidney derived macrophages
hpi	Hours post infection
hpv	Hours post vaccination
HRP	Horseradish peroxidase
HSWB	High slat wash buffer
i.e	*id est* “that is”
i.p.	Intraperitoneal
IgL	Intracellular growth locus
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IgZ	Immunoglobulin Z
IL	Interleukin
ITS	16S rRNA-23S rRNA intergenic spacer gene
KCl	Potassium chloride
kDa	Kilo Dalton

L	Litres
LAMP	Loop-mediated isothermal amplification
LC/ESI/MS/MS	Liquid chromatography electrospray ionization-tandem mass spectrometry
LD	Lethal dose
LFD	Lateral Flow Detection Device
LOD	Limits of detection
LOP	Lipo-oligo-polysaccharides
LPS	Lipopolysaccharides
LSWB	Low salt wash buffer
m	Metres
M	Molar
MAb	Monoclonal antibody
MALDI-TOF.MS	Matrix assisted laser desorption/ionization-time of flight mass spectrometry
$MgCl_2$	Magnesium chloride
mgf	Mascot generic format
MHCI	Major histocompatibility class I
MHCII	Major histocompatibility class II
MIC	Minimal inhibitory concentration
min	Minutes
mL	Millilitres
MLST	Multi-Locus Sequence Typing
MLVA	Multi-Locus Variable Number of Tandem Repeats
mM	Millimolar
MMHB	Modified Muller Hinton Broth
M.Q.	Milli-Q water
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
ng	Nanograms
NPV	Negative predictive value
NVI	Norwegian Veterinary Institute

List of abbreviations

^{o}C	Degrees Celsius
OD	Optical density
OMPs	Outer membrane proteins
OMVs	Outer membrane vesicles
OMTs	Outer membrane tubes
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate Buffer Saline
pc	Post-challenge
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PMNs	Polymorphonuclear cells
PMFs	Peptide mass fingerprintings
pmol	Picomole
PPV	Positive predictive value
PTMS	Post transitional modification
qPCR	Quantitative polymerase chain reaction
REST	Relative Expression Software Tool
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Recombinase Polymerase Amplification Assay
rpm	Round per minute
RPS	Relative Percent Survival
RT	Room temperature
RT-qPCR	Real-time quantitative PCR
s	Seconds
SC	Sequence coverage
SD	Standard deviation
SDS	Sodium Dodecyl sulfate
sp.	Species
spp.	Species plural
SSB	Single Stranded Binding Protein

ssp.	Subspecies plural
STE	Sodium chloride-Tris-EDTA buffer
subsp.	Subspecies
T	Type strain
T6SS	Type 6 secretion system
TAE	Tris Acetate EDTA
TE	Tris EDTA
TE Buffer	Tris-EDTA buffer
TGF	Transforming growth factor
Th	T-helper cell
THF	Tetra hydrofuran bridge
TLRs	Toll-Like receptors
TMB	3,3',5,5'-Tetramethylbenzidine
TNFA	Tumour necrosis factor alfa
Tris	Trisaminol or trisamine
Tris-HCl	Tris-hydrochloride
Tt	Time threshold
UK	United Kingdom
USA	United States of America
USD	United States Dollars
UV	Ultra violet
V/V	Volume per volume
VBNC	Viable but not culturable
VNTRs	Variable Number of Tandem Repeats
WC	Whole cell
Wt	Weight
W/V	Weight per volume

Abstract

Nile tilapia, *Oreochromis niloticus*, is one of the most important farmed fish globally. One of the most serious bacterial diseases constraining global tilapia production is Francisellosis caused by *Francisella noatunensis* subsp. *orientalis* (*Fno*). Although outbreaks of *Fno* are increasing worldwide, there are no licenced commercial vaccines to prevent the disease for use on tilapia farms. Thus, the current treatment of choice is the use of antibiotics combined with increasing water temperature up to 30°C. Studies investigating the diversity of circulating *Fno* isolates and the immune response of tilapia elicited by vaccination against piscine francisellosis are lacking. In addition, the current conventional and molecular tools used for detection of *Fno* have many drawbacks, making detection of *Fno* a challenging process.

In this study, five clinical isolates of *Fno* from diverse geographical locations (UK, Costa Rica, Mexico, Japan and Austria), previously characterised by morphology, genotype, antimicrobial susceptibility and virulence, were used in a proteomic study. The whole proteomic cell profile of the five isolates were homogenous by one-dimension sodium dodecyl polyacrylamide gel electrophoresis (1D-SDS-PAGE), while minor differences in the intensity of 15 proteins between the strains were observed by two-dimension SDS-PAGE (2DE), including some important virulence related proteins. The UK isolate was the most significantly different isolate when compared to the other *Fno* isolates in the current study. The *Fno* UK isolate had significantly higher abundance of 10/15 of the significantly expressed proteins including four of the essential pathogenicity and virulence related proteins (IglC, GroEL, DnaK, ClpB) compared to the other used *Fno* isolates. The antigenic profiles of the five *Fno* isolates were studied by 1D western blotting using tilapia hyper immune sera which recognised an immunodominant band of a molecular weight of ~ 17-28 kDa in all tested *Fno* isolates. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI/MS/MS) identified 47 proteins in this antigenic band. Some of the identified proteins are associated with *Fno* pathogenicity. 2D western blot analysis of the vaccine isolate (*Fno* UK) revealed differential antigen recognition between sera from vaccinated and non-vaccinated fish following experimental challenge (26 antigenic spots recognised by sera from vaccinated fish; 31 antigenic spots recognised by sera from vaccinated and challenged fish and 30 antigenic spots recognised by non-vaccinated and challenged fish). The identity of these

proteins was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and some of them are known *Francisella* virulence related proteins. Bioinformatics analyses revealed diverse categories of proteins with high biological functions, however the vast majority of these proteins are involved in energy production and metabolic pathways of the bacteria. This detailed analysis will facilitate the development of cross-strain protective subunit *Fno* vaccines and antigen-targeted *Fn*o diagnostics.

The outer membrane proteins (OMPs) of the same five *Fno* isolates were extracted using the ionic detergent sarkosyl. The OMP fraction of the different isolates were separated via 1D-SDS PAGE and the digested peptides of the UK isolate were analysed by LC/ESI/MS/MS. High degree of similarity was observed in the OMP profile of the five *Fno* isolates with an abundant protein band at 17-28 kDa, which was found to be antigenic by 1D western blot using convalescent tilapia sera. LC/ESI/MS/MS analysis of the OMPs of the *Fno* UK isolate identified 239 proteins, including 44 proteins in the antigenic band (17-28 kDa). Comparison between the proteins identified in the immunogenic band of whole cell lysate and OMP fraction of the *Fno* UK isolate showed 30 common proteins between the two preparations, 17 proteins were identified only in the whole cell extract and 14 were identified only in OMP fraction. Outer membrane proteins (e.g. Omp-A), virulence related proteins such (e.g. IglC) and other stress related proteins (e.g. AhpC/TSA family peroxiredoxin) were more abundant in the OMP fraction than the whole cell lysate. *In silico* analysis enabled prediction of the function and location of the OMPs identified by Mass-spectrometry. The findings of this study provide preliminary data on bacterial surface proteins that exist in direct contact with the host immune defence during infection and offering an insight into their potential role as novel targets for *Fno* diagnostics and vaccine development.

The efficacy of an injectable whole cell oil-adjuvanted vaccine was evaluated against challenge with heterologous *Fno* isolates in Nile tilapia, *Oreochromis niloticus*. Three duplicate groups of 130 healthy Nile tilapia (~15 g) were intraperitoneally (i.p.) injected with the vaccine, adjuvant-alone or PBS followed by an i.p. challenge with three *Fno* isolates from geographically distinct locations. The vaccine provided significant protection to all immunised tilapia groups with a significantly higher relative percent survival (RPS) of 82.3% against homologous challenge, compared to 69.8% and 65.9%

after heterologous challenge. Protection correlated with significantly elevated specific antibody responses and western blot analysis demonstrated cross-isolate antigenicity with sera from fish post-vaccination and post-challenge. Moreover, a significantly lower bacterial burden was detected by quantitative real-time polymerase chain reaction (qPCR) in conjunction with significantly greater expression of *IgM*, *IL-1β*, *TNF-α* and *MHCII* 72 hours post-vaccination (hpv) in spleen samples from vaccinated tilapia compared to those of adjuvant-alone and control fish. The latter results suggested stimulation of protective immune responses following vaccination.

In addition, a whole cell formalin killed autogenous immersion vaccine against *Fno* was developed using the same isolate used for the injectable vaccine. Duplicate tanks of 35 tilapia fry were immersed in the vaccine or in sterile Modified Muller Hinton broth (MMHB) diluted in tank water (1:10 dilution) for 30 s and at 30 days post-vaccination (dpv), all fish groups were immersion challenged with the homologous *Fno* isolate and monitored for 21 days. A moderate RPS of 43.7% was provided by the vaccine. Serum IgM levels were below the threshold in 30 % of the vaccinated fry 30 dpv. Also, the IgM levels of the vaccinated fry were not significantly different from control fry 21 days-post challenge.

A recombinase polymerase amplification (RPA) assay was developed and validated for rapid detection of *Fno*. The RPA reaction was performed at a constant temperature of 42°C for 20 min. The RPA assay was performed using a quantitative plasmid standard containing a unique *Fno* gene sequence. Validation of the assay was performed not only by using DNA from *Fno,* closely related *Francisella* species and other common bacterial pathogens in fish farms, but also by screening 78 Nile tilapia and 5 water samples collected from UK and Thailand. All results were compared with those obtained by previously established real-time qPCR. The developed RPA showed high specificity in detection of *Fno* with no cross-detection of either the closely related *Francisella* spp. or the other species of bacteria tested. The *Fno*-RPA performance was highly comparable to the published qPCR with detection limits at 15 and 11 DNA molecules detected, respectively. The *Fno*-RPA was rapid, giving results in approximately 6 min in contrast to the qPCR that required approximately 90 min to reach the same detection limits. Moreover, the RPA was more tolerant to reaction inhibitors than qPCR when tested with field samples. The fast reaction, simplicity, cost-effectiveness, sensitivity

and specificity make the RPA an attractive diagnostic tool that will contribute to control the infection through prompt on-site detection of *Fno*.

The overall results of this study indicated that *Fno* isolates from different origins share a high degree of homology in their proteomic and antigenic profile. Proteomic characterisation data of *Fno* isolates has contributed to understanding the diversity of *Fno* isolates and assisted in identifying suitable candidates for developing an effective *Fno* vaccine. Moreover, this study has proven the efficacy of a cross protective *Fno* injection vaccine in tilapia fingerlings, with further optimisation needed for immersion vaccination of fry, and given insights into the immune response of tilapia to vaccination against francisellosis. In addition, it provided a rapid, sensitive, specific and robust molecular tool for detection of *Fno* that can assist surveillance and control of piscine francisellosis on tilapia farms.

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Shahin, K., Ramirez-Paredes, J.G., Monaghan, S.J., Shinn, A.P., Metselaar, M., Thompson, K.D., Hoare, R. and Adams, A. Development of control strategies for *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia. The 5th PhD Research Conference, Institute of Aquaculture, University of Stirling, 17th April 2018, Stirling, UK.

Shahin, K., Ramirez-Paredes, J.G., Monaghan, S.J., Shinn, A.P., Metselaar, M., Thompson, K.D., Hoare, R. and Adams, A. Efficacy testing of an injectable whole cell vaccine against heterologous isolates of *Francisella noatunensis* subsp. *orientalis* in Nile tilapia. UK Veterinary Vaccinology Conference, University of Stirling, 18-19th January 2018.

Shahin, K., Harold, G., Lopez-Jimena, B., Ramirez-Paredes, J.G., Adams, A. and Weidmann, M. Development of a recombinase polymerase amplification assay for detection of *Francisella noatunensis* subsp. *orientalis*. Lunch time seminar, Institute of aquaculture, University of Stirling, 29th November 2017, Stirling, UK.

Shahin, K., Ramirez-Paredes, J.G., Monaghan, S.J., Metselaar, M., Thompson, K.D., Hoare, R. and Adams, A. Proteomic characterisation and functional analysis of five isolates of *Francisella noatunensis* subsp. *orientalis* from geographically distinct locations. The 18th international conference of diseases of fish and shellfish, Belfast, Northen Ireland, UK, 4-8th September 2017 (Student award granted for attendance).

Shahin, K., Harold, G., Lopez-Jimena, B., Ramirez-Paredes, J.G., Adams, A. and Weidmann, M. Recombinase polymerase amplification assay for molecular detection of *Francisella noatunensis* subsp. *orientalis*. The 10th symposium of diseases in Asian aquaculture (DAA10), Bali, Indonesia. 28th August-1st September 2017 (Poster presentation).

Shahin, K., Hoare, R., Adams, A. Francisellosis, an emergent disease in farmed Nile tilapia. GCUA summer school "Aquaculture – local solutions to global challenge"- Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, 22th June-1st July 2017 (Poster presentation).

Publications:

Shahin, K., Thompson, K.D., Inglis, N.F., Mclean, K., Ramirez-Paredes, J.G., Monaghan, S.J., Hoare, R., Fontaine, M., Metselaar, M., and Adams, A. (2018) Characterisation of the outer membrane proteins of *Francisella noatunensis* subsp. *orientalis*. *Journal of applied Microbiology.* https:// doi.org/10.1111/jam.13918

Shahin, K., Harold, G., Lopez-Jimena, B., Ramirez-Paredes, J.G., Adams, A. and Weidmann, M. (2018) Recombinase polymerase amplification assay for molecular detection of *Francisella noatunensis* subsp. *orientalis*. *PLOS ONE*. https://doi.org/10.1371/journal.pone.0192979

Ramirez-Paredes, J.G., Thompson, K.D., Metselaar, M., **Shahin, K.,** Soto, E., Richards, R.H., Penman, D.J., Colquhoun, D.J. and Adams, A. (2017). Apolyphasic approach for phenotypic and genetic characterization of the fastidious aquatic pathogen Francisella noatunensis subsp. orientalis. *Frontiers in Microbiology*. https://doi.org/ 10.3389/fmicb.2017.02324.

Soto, E., Ramirez-Paredes, J.G., Talhami, J, **Shahin, K.,** Griffin, M. and Adams, A. (2018) Isolation and characterization of *Francisella noatunensis* subsp. *orientalis* from Nile tilapia, *Oreochromis niloticus* farmed in Lake Yojoa, Honduras, *Diseases of Aquatic Organisms* (submitted manuscript).

Shahin, K. and Adams, S. (2018) Francisellosis in warm water fish. In F. Kibenge, C. Roger and B. Bernardo (Eds.) Aquaculture Pathophysiology and pharmacology, Elsevier (submitted manuscript).

Chapter One
General Introduction

Chapter One

1.1. Tilapia aquaculture

1.1.1. History of tilapia aquaculture

Tilapia is the common name for nearly a hundred species of freshwater and some brackish water fish belonging to the Cichilidae family. This large family of tropical fish is widely distributed throughout waters of Africa, the Middle East, coastal India, South East Asia, Central and South America. Although they are exotic to the USA, populations of tilapia are now established in Arizona, California, Hawaii, Florida, Nevada, North Carolina, and Texas. The tribe "Tilapiini", to which tilapias belong, emerged from Africa and Palestine (Jordan and coastal rivers), however, they have now been distributed worldwide. The most valuable commercial species are the Mozambique or Java tilapia (*Oreochromis mossambicus*), blue tilapia (*Oreochromis aureus*), Nile tilapia (*Oreochromis niloticus*), Zanzibar or Wami tilapia (*Oreochromis hornorum*), and the red-belly tilapia (*Oreochromis zilli*) (Sell, 1993; Chapman, 2009).

The culture of tilapia (especially Nile tilapia) can be traced back to ancient Egyptian times as depicted on bas-relief from Egyptian tombs dating over 4000 years ago, which showed the fish held in ornamental ponds. While significant worldwide distribution of tilapia, primarily *O. mossambicus*, occurred during the 1940s and 1950s, distribution of the more desirable Nile tilapia occurred during the 1960s up to the 1980s. Nile tilapia from Japan were introduced to Thailand in 1965, from where they were exported to the Philippines. Tilapia were introduced from the Ivory Coast to Brazil in 1971 from where they were exported to the United States in 1974 and in 1978 Nile tilapia were introduced to China (FAO, cultured aquatic species information programme, *Oreochromis niloticus,* (Linnaeus, 1758)). Currently, China is the top tilapia producer with production of 1.8 million tonnes, whilst production ranges from 57,000 – 1.1 million tonnes throughout Asia, South America and Africa. The top ten producers are shown in Figure 1.1.

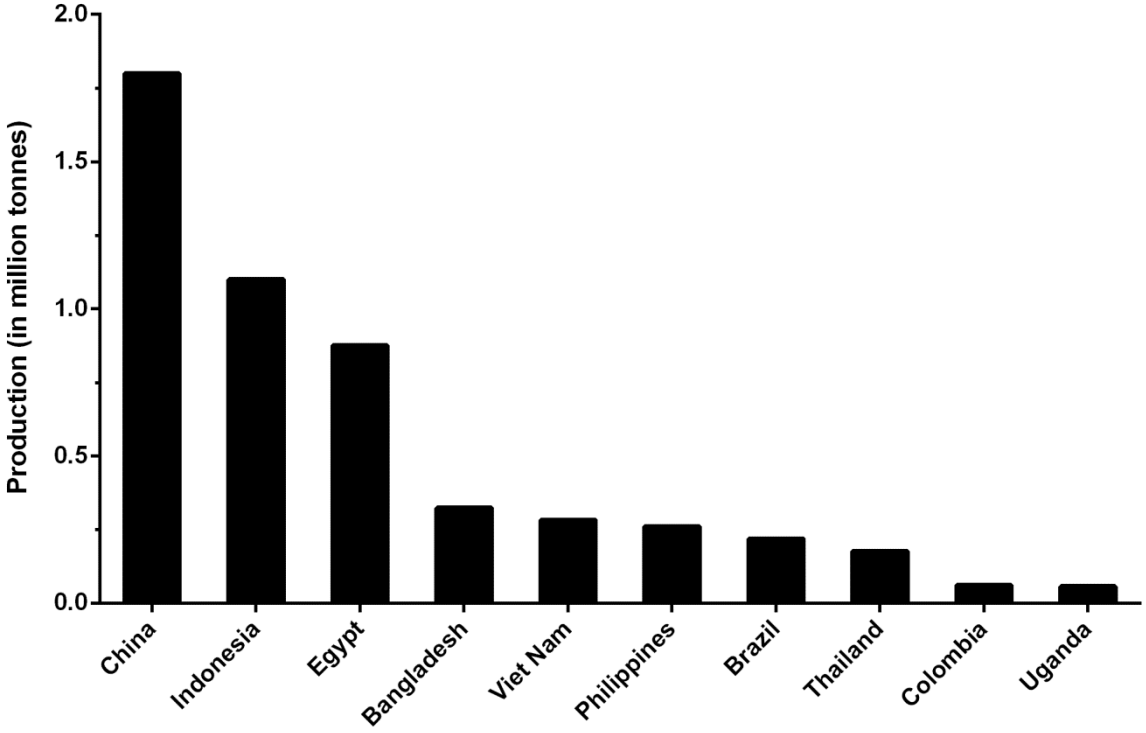


Figure 1.1 Top ten tilapia producing countries and their production in 2016 (FAO, 2017).

Nile tilapia were the major cultured species of tilapia between 1956-2002 representing 80% of total tilapia production, followed by Mozambique tilapia (3.6%). Besides the previously mentioned species, a minor contribution to tilapia production occurs in different countries for three spotted tilapia: blue tilapia, red breast tilapia and long fin tilapia (El-Sayed, 2006). Recently, Nile tilapia and hybrid red tilapia represent the most common species of tilapia used in aquaculture (Alston, 2009), where Nile tilapia ranks 6th among the most important cultured species, providing food, jobs and domestic and export industries for many people globally. The global production of tilapia was estimated to be 5.67 million tons with a value of USD ~ 7.5 billion in 2015 and is expected to reach 7.3 million tons by 2030 (FAO, 2017).

The wide use of tilapia in aquaculture can be attributed to many factors, including general hardiness, high tolerance to adverse environmental conditions, resistance to disease and their ability to withstand low oxygen conditions and a wide range of salinity (El-Sayed, 2006). Moreover, tilapia can grow and survive on a wide range of natural and artificial feeds, convert food efficiently, grow at a relatively fast rate and are attractive to a wide range of consumers due to their low cost and protein-rich meat (Ng and Romano, 2013). Furthermore, tilapia can be grown in a variety of culture systems ranging from simple systems with little infrastructure to more intensive and complex systems. Their uncomplicated biology, feeding and veterinary requirements have made them a favoured species for aquaculture (El-Sayed, 2006).

1.1.2. Production of tilapia on farms

The tilapia production cycle consists of three main stages, (i) spawning and hatchery stage: which includes spawning of the brood fish, fertilization by males and incubation of the fertilized eggs collected from the mouth of brood female till hatching new stock of fry, (ii) nursery stage: which includes production of fingerlings from the newly hatched fry, and (iii) grow-out stage: that includes rearing of fingerlings till reaching the desired marketable size (Little *et al.,* 2003). The production cycle of farmed tilapia in most of the tropical regions takes 4- 6 months (Liu *et al.,* 2016), however, it may be extended to 8- 9 months to produce larger marketable size fish (> 700 g). The production cycle of tilapia on farms is shown in Figure 1.2.

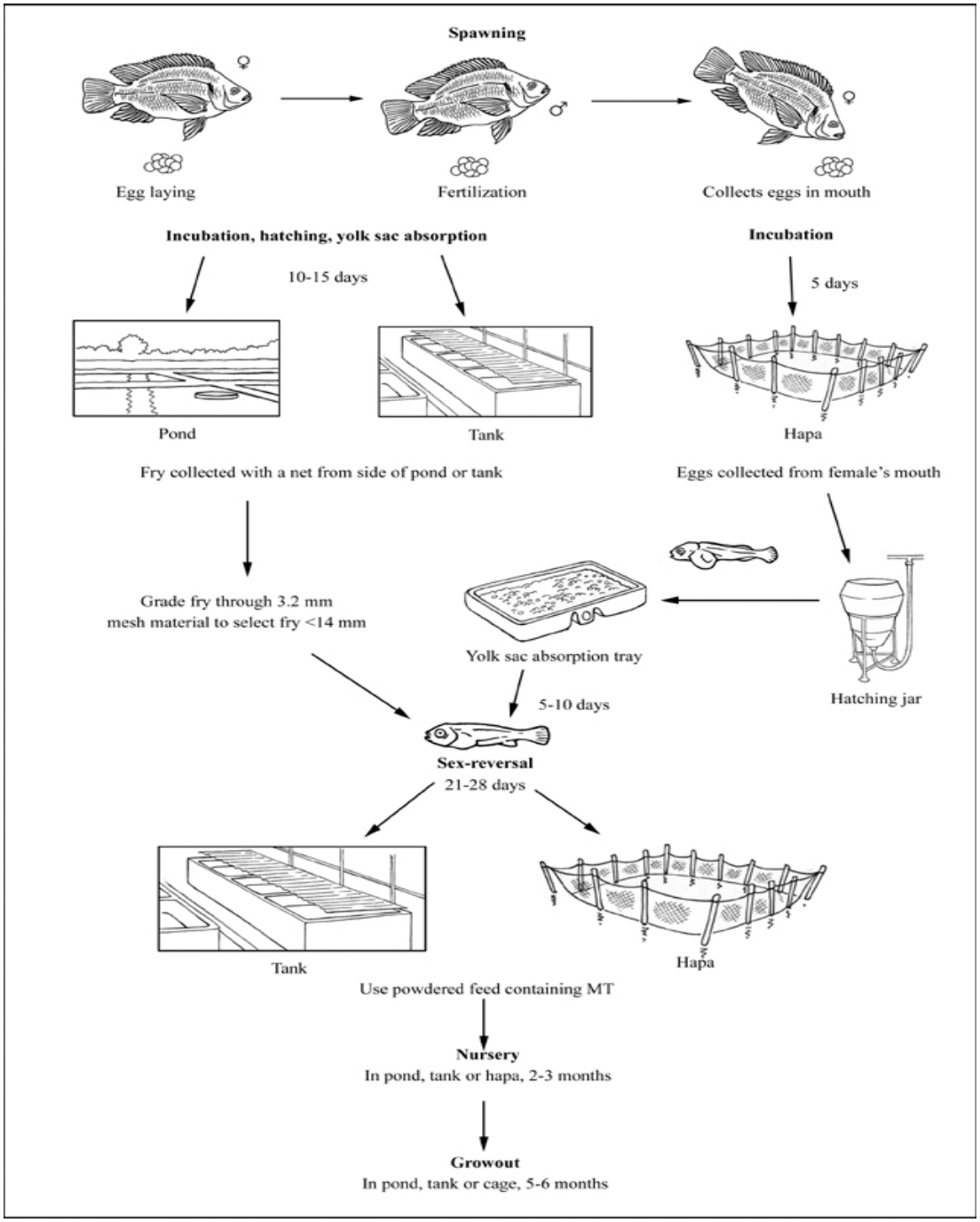


Figure 1.2. Production cycle of Nile tilapia (FAO, 2012). MT: Methyl testosterone

1.1.3. Diseases in tilapia aquaculture

Infectious diseases represent one of the major constraints to development of aquaculture world-wide. Diseases caused serious economic losses to finfish aquaculture globally that were estimated at US $ 1.05 to US $ 9.58 billion per year (Shinn *et al.,* 2015). Diseases in fish not only cause mortality and morbidity, but also reduce market value, growth performance and feed conversion in fish. In addition, costs of purchasing chemicals and medicines to treat these diseases represent a significant issue to the aquaculture industry (Paul, 2014).

With extensive tilapia farming, these fish have shown high resistance to infection when reared under properly controlled and managed conditions ensuring good water quality, suitable temperatures and proper husbandry practices. In contrast, as a result of intensification of culture systems (ponds, cages, raceways or recirculating systems), poor water quality and adverse environmental conditions, especially water temperature or improper handling, have exacerbated the impact of diseases (Plumb and Larry*,* 2010). Tilapia aquaculture has been affected by a diverse pool of diseases caused by viral, bacterial, parasitic and fungal pathogens (Shlapobersky *et al.*, 2010), of which the bacterial diseases constitute the most significant threat to the future growth and sustainability of tilapia farming. The most important tilapia pathogens are summarised in Table 1.1.

Table 1.1. Major pathogens in tilapia aquaculture

Disease category	Pathogen	Reference
Bacterial	1- *Aeromonas hydrophila*	Maluping *et al.,* (2005)
	2- *Edwardisella tarda*	Soto *et al.,* (2012b)
	3- *Flavobacterium columnare*	Eissa *et al.,* (2010)
	4- *Francisella noatunensis* subsp. *orientalis* (*Fno*)	Soto *et al.,* (2009a)
	5- *Lactococcus garvieae*	Evans *et al.,* (2009)
	6- *Plesiomonas* spp.	Maluping *et al.,* (2005)
	7- *Pseudomonas* spp.	Thomas *et al.,* 2014 Pereira *et al.,* (2010)
	8- *Streptococcus agalactiae, S. iniae*	Shoemaker *et al.,* 2001
	9- *Vibrio* spp.	Maluping *et al.,* (2005)
Viral	1-Birnavirus	Ariel and Owens, (1997)
	2-Iridovirus (Spinning tilapia syndrome)	ShaoWen *et al.,* (2003)
	3-Nodavirus (Viral Nervous Necrosis, VNN or viral encephalopathy and retinopathy (VER))	Shlapobersky *et al.,* (2010) Sinyakov *et al.,* (2011)
	4-Tilapia Lake virus (TiLV)	Nicholson *et al.,* (2017) Behera *et al.,* (2018) Mugimba *et al.,* (2018)
Parasitic	1- *Amyloodinium ocellatum*	El-Dien and Abdel-Gaber, (2009)
	2- *Cymothoa* spp.	
	3- *Diplostomum compactum*	Abd El-Galil and Aboelhadid, (2012)
	4- *Icthyophthirius multifilis*	
	5- *Piscinoodinium pillulare*	Supamattaya *et al.,* (2012)
	6- *Spironucleus* spp.	
	7- *Trichodina* spp.	Rameshkumar and Ravichandran (2010)
Fungal	*Saprolegnia* spp.	Zahran *et al.,* (2017)

1.1.4. Francisellosis in tilapia

1.1.4.1. Taxonomy of the aetiological agent

Francisella is an intracellular bacterium belonging to class *Gammaproteobacteria*, order *Thiotrichales* and it represents the family *Francisellaceae* with the newly classified *Allofrancisella* genus (Colquhoun and Duodu, 2011; Qu *et al.,* 2016). *Francisella* genus includes many species and subspecies of clinical importance to humans (Oyston *et al.,* 2005; Foley and Nieto, 2010), terrestrial animals (Cora *et al.,* 2010; Hotta *et al.,* 2016) and fish (Colquhoun *et al.,* 2014). In humans, the most important *Francisella* species are *F. tularensis* (*Ft)* that causes tularemia which is a fatal disease that has been categorised as a potential bioweapon due to high infectivity and multiple infection routes (Keim *et al.,* 2007), and *F. philomiragia* (*Fp)* that also affects other animals causing a serious granulomatous disease (Hollis *et al.,* 1989). *Francisella tularensis* includes four subspecies, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *mediasiatica*, *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *novicida* (Oyston *et al.,* 2005; Foley and Nieto, 2010). In aquatic organisms, francisellosis is mainly caused by *F. noatunensis* subsp. *noatunensis* (*Fnn*) in cold water fish species, *F. noatunensis* subsp. *orientalis* (*Fno*) in warm water fish species or *F. halioticida* (*Fh*) in mollusca (Colquhoun and Duodu, 2011). Recently, a novel strain of *Francisella* was isolated from cultured spotted rose snapper (*Lutjanus guttatus*) in Central America and recognised as *Francisella marina* sp. nov. based on its phenotypic and genotypic differences from other *Francisella* spp. (Soto *et al.,* 2018)

Fno is a Gram negative, encapsulated, pleomorphic or cocco-bacilli, non-motile bacterium ranging from 0.7 – 1.7 μm (0.57± 0.1 μm x 0.8 ± 0.2 μm) (Figure 1.3A) (Mauel *et al.,* 2005; Mikalsen and Colquhoun, 2009). It is a fastidious, slow growing aquatic pathogen that cannot be cultured on ordinary media commonly used in bacteriological diagnosis of fish pathogens but requires high levels of cysteine and glucose to grow (Colquhoun and Duodu, 2011). It is strictly aerobic, and the optimum incubation temperature is in the range of 22- 28°C (maximum 30°C), where it takes 3- 4 days for growth, but it cannot grow at 37°C (Soto *et al.,* 2009a). The colonies appear convex, smooth, semi-translucent mucoid grey, pale white, green or even bluish with a maximum diameter of 1mm (Figure 1.3B).

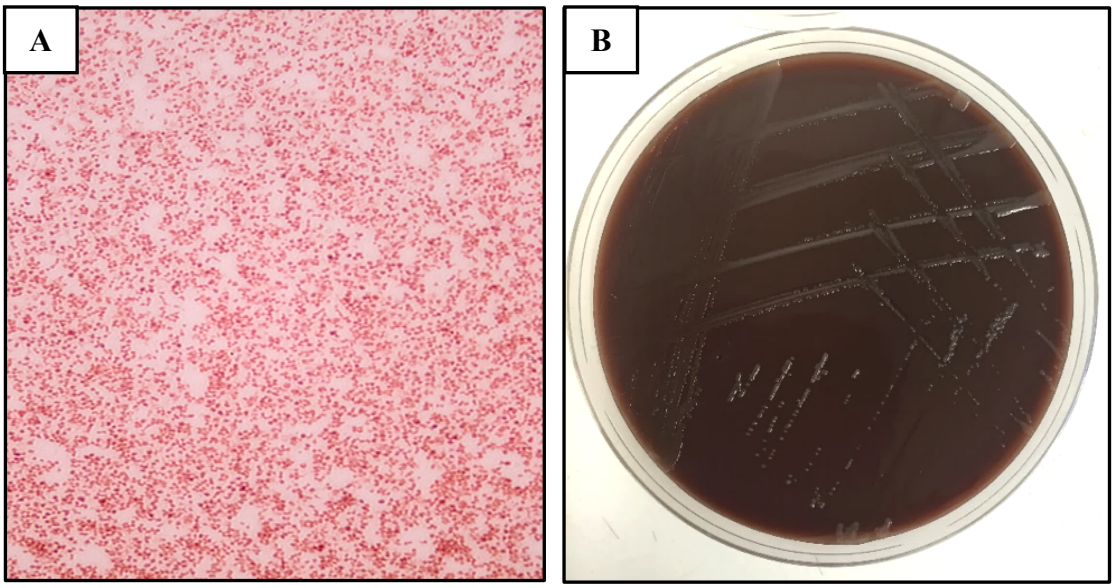


Figure 1.3. Morphological and cultural characters of *Fno*. (A) Gram-negative cocco-bacilli *Fno* isolated from infected Nile tilapia spleen, LM 100x. (B) colony morphology of *Fno* on CHAH. The colonies appear convex, smooth, semi-translucent mucoid grey after 72 h of incubation at 28 °C.

1.1.4.2. Pathogenesis

Studies on *Francisella* pathogenesis have revealed that these bacteria have various mechanisms that facilitate their ability to survive and replicate not only in the extracellualar environment but also inside eukaryotic cell, in particular those cells belonging to monocytes or macrophage lineage or other cells like neutrophils and B-cells (Soto *et al.,* 2010b; Furevik *et al.,* 2011; Vestik *et al.,* 2012 Celli and Zahrt, 2013; Brudal *et al.,* 2014; Lagos *et al.,* 2017). In tilapia and zebra fish (*Danio rerio*), head kidney derived macrophages (HKDM) represent the primary dwelling for *Fno*, where it can replicate within 72 hours post-infection (hpi), inducing inhibition of phagocytic activity, apoptosis, cytotoxicity and can also resist serum killing activity (Soto *et al.,* 2010a; Lagos *et al.,* 2017). The localisation of *Francisella* in phagosomes induces alteration in phagosomal maturation, causing arresting of the phagosome in the late maturation stage (Lai *et al.,* 2001; Clemens *et al.,* 2004). Once the bacteria are engulfed by host cell phagosomes, the phagosomal membrane is disrupted allowing escape of the bacteria into the cytoplasm and giving opportunity for massive multiplication (Golovliov *et al.,* 2003). Furthermore, *Francisella* decrease the acidity of the phagosome by recruitment of vacuolar ATPase that exacerbates weakness of the invaded cells that can then be easily disrupted and facilitate escape of the bacteria to adjacent cells or into the cytoplasm, where the bacteria can replicate to produce high numbers of bacterial cells (Golovliov *et al.,* 2003; Huynh and Grinstein, 2007). The process of recognition of the bacterial pathogen associated molecular patterns (PAMPs) by toll-like receptors (TLRs) orchestrates the caspase-1 activity that in turn triggers release of pro-inflammatory cytokines including *IL-1 β* and *IL-18* and consequently cell death (Celli and Zahrt, 2013).

The main promoter of *Francisella* virulence and intracellular survival machinery is the *iglABCD* operon which is found as part of the 30 Kb pathogenicity island (also known as *Francisella* pathogenicity island or FPI) that has been identified in all reported *Francisella* spp. genomes (Sjödin *et al.,* 2012; Sridhar *et al.,* 2012; Celli and Zahrt, 2013). This important locus in FPI with the help of other determinant factors (e.g. MglA and MglB) was reported to encode a type six secretion system (T6SS) that orchestrates entrance, survival and proliferation of *Francisella* within host cells (Brotcke *et al.,* 2006, Santic *e al,* 2005; 2006; de Bruin *et al.,* 2007; Nano and Schmerk, 2007; Chong *et al.,* 2008). Studies have revealed that any mutation in the core components of T6SS system

will adversely affect the ability of the bacterium to escape the phagosome and replicate intracellularly (Bröms *et al.,* 2012a;b). Nevertheless, comparison of the *iglABCD* operon DNA sequence revealed that *F. noatunensis* subsp. *orientalis* had percentage identities of 94% and 83% with *F. philomiragia* subsp. *philomiragia* and *F. tularensis* subsp. *novicida*, respectively (Soto *et al.,* 2009b).

The roles and functions of the genes of the FPI and their regulators in *Fno* are not fully understood and most of our understanding of these genes comes from studies on human parasitic *Francisella* including *F. novicida* and *F. tularensis* (Nano *et al.,*2004; Chou *et al.,* 2013). In addition, there is little information available about the effect of mutation of FPI components on virulence of *Fno*. The *iglC* gene is one of the highly expressed genes during the intracellular growth phase of *Francisella* (Nano and Schemark, 2007). It has been found that *iglC* is important in *Fno* for induction of disease and intramacrophage survival in tilapia (Soto *et al.,* 2009b; 2010b). Mutation of the *iglC* gene impeded *Fno* replication, cytotoxicity and apoptosis to tilapia HKDM, but did not affect complement lysis activity. Survival rates of 50 % and 100% were reported after immersion challenge with *Fno* wild type and *Fno-iglC* mutant strains, respectively (Soto *et al.,* 2009b, 2010a). *PdpA* is one of the genes that had been described in *Fno* FPI (Hansen *et al.,* 2013). In *F. tularensis* it is involved in intreacellular growth and survival (Schmerk *et al.,* 2009), however, its role in *Fno* is unknown. Mutation of pathogenicity determinant protein A gene (*pdpA*) opposed *Fno* virulence in a zebrafish model, *Dario rerio* (Hansen *et al.,* 2013). In addition, two *Fno-pdpA* mutant strains showed higher LD_{50} ($>2\times10^6$ CFU/mL) and lower resistance to oxidative stress using hydrogen peroxide (H_2O_2) compared to the wild type that displayed lower LD_{50} (891 CFU/mL) and more resistance to killing by H_2O_2 in an immersion challenge in hybrid red tilapia (Farrell, 2015). This study suggested that *pdpA* gene may contribute to the virulence of *Fno*, however, further studies are required to investigate this hypothesis.

Little is known of the immunopathological response of Francisellosis in fish. The lipo-oligo-polysaccharide residues (LOP-antigens) in the cell wall of *Francisella* play an important role in its interaction with innate immune elements by enabling the bacterial cells to impede recognition by host antibodies, complement or TLRs and dampen the pro-inflammatory responses (Chase *et al.,* 2009; Zarrella *et al.,* 2011). The lipo-polysaccharides (LPS) of *Fnn* have been shown to induce an immune response after

challenging of macrophages in cod. Upregulation of *IL-10* indicates stimulation of helper T-cell type 2 (Th2) response (Bakkemo *et al.,* 2011). One of the key factors for *Francisella* virulence is their ability to disrupt reactive oxygen species (ROS) generation following phagocytosis by inhibition of NADPH oxidase that limits the activity of phagocytes (e.g. polymorphonuclear cells (PMNs), monocytes) and fosters the survival of *Francisella* (Celli and Zahrt, 2013). Moreover, previous studies reported that *Francisella* requires iron (Fe) for maintenance and growth and consequently virulence. *Francisella* genome contains certain regions (fsL locus and fopA locus), which encode components for production, release and capture of polycarboxylate siderophores responsible for uptake of ferric ions (Sullivan *et al.,* 2006).

1.1.4.3. Epizootiology, risk factors and transmission

Francisellosis is a highly epizootic disease of tilapia species that can be found in all sizes and stages, ranging from small fingerlings to adult broodstock. Morbidity rates of 100% and mortality rates ranging from 5 to 95% were reported in cultured Nile tilapia reflecting the scale of the disease as a major threat to global tilapia farming (Colquhoun and Duodu, 2011; Ortega *et al.,* 2016; Sebastião *et al.,* 2017). The disease is characterised by high infectivity where as few as 23 colony forming units (CFU) of *Fno* could induce the disease by intraperitoneal injection in tilapia (Soto *et al.,* 2009b) and 32 CFU of *F. halioticida* caused 100% mortality by intramuscular injection in abalone (Kamaishi *et al.,* 2010).

There are many factors that favour the occurrence of francisellosis in tilapia and other warm water fish species. Water temperature is considered a determining factor in initiation of the clinical symptoms and provokes mortality rates in the affected fish (Mauel *et al.,* 2007). The disease frequently occurs in cooler months (October to April) in which the low temperature exacerbates stress and consequently increases the chance of disease incidence. The favourable temperature for occurrence of *Fno* is between 21.5°C to 26.5°C. (Mauel *et al.,* 2005; Colquhoun and Duodu, 2011). In an experimental challenge, the disease could be established either by i.p. injection or immersion at temperatures of 23-25°C (Soto *et al.,* 2009a; 2013b). Soto *et al.* (2012a) reported that fish maintained at 25°C showed considerably higher mortality rates (66.6 ± 11.8 %) and splenic bacterial burden (5.26 ± 0.6 log CFU/mg of spleen) after 2 weeks of immersion challenge with *Fno* in contrast with fish maintained at 30°C which showed lower mortality (46.6 ± 7.20 %) and

lower bacterial splenic concentration (3.15 ± 0.9 log CFU/mg of spleen). In addition, stocking density has an obvious effect on the incidence of *Fno,* where transmission of the disease was significant on tilapia farms with elevated biomass within the system, that consequently increases the level of waste and favours the horizontal transmission by greater contact between fish (Jeffery *et al.,* 2010, Ortega *et al.,* 2016). Fish stress by constant handling was also reported as a predisposing factor for the occurrence of *Fno* in farmed tilapia (Mauel *et al.,* 2003; 2007; Soto *et al.,* 2009a; Ortega *et al.,* 2016).

The horizontal mode of transmission of francisellosis in tilapia is considered the most common route of infection either by water borne transmission or direct contact between infected and/or dead fish and healthy fish (Mauel *et al.,* 2003; Soto *et al.,* 2009a; Jeffery *et al.,* 2010). Recently, it was found that vertical transmission of *Fno* through gametes can occur, where *Fno* was detected in 6/10 crossed families using Loop Mediated Isothermal Amplification (LAMP) in milt, roe, fertilised and non-fertilised eggs at different developmental stages of tilapia after *in vitro* fertilisation (Pradeep *et al.,* 2016). In addition, detection of *Fno* was reported in gonads of non-symptomatic tilapia, indicating its ability to transmit vertically from infected broodstock to their off-spring (Soto *et al.,* 2013b; Pradeep *et al.,* 2016). Asymptomatic carrier farmed, or ornamental fish have been suggested to play a role in transmission of *Francisella* species. This finding was exemplified in detection of *Fno* in farmed tilapia and Indo-Pacific reef fish such as fairy wrasses (*Cirrhilabrus* spp.) and blue-green damselfish (*Chromis viridis*) in USA, where importing of either farmed tilapia fry or other ornamental cichlids could establish the infection in these susceptible hosts (Soto *et al.,* 2011a; Camus *et al.,* 2013).

1.1.4.4. Host range and geographical distribution

Piscine francisellosis has emerged as a significant problem in a wide range of hosts (Colquhoun and Duodu 2011). Until now, there is no definite list of susceptible hosts or vectors for piscine francisellosis. The disease has so far been recorded in fresh and marine water, wild and cultured fish and vertebrate and non-vertebrate aquatic species. The current known distribution of francisellosis in fish and shellfish is shown in Figure 1.4.

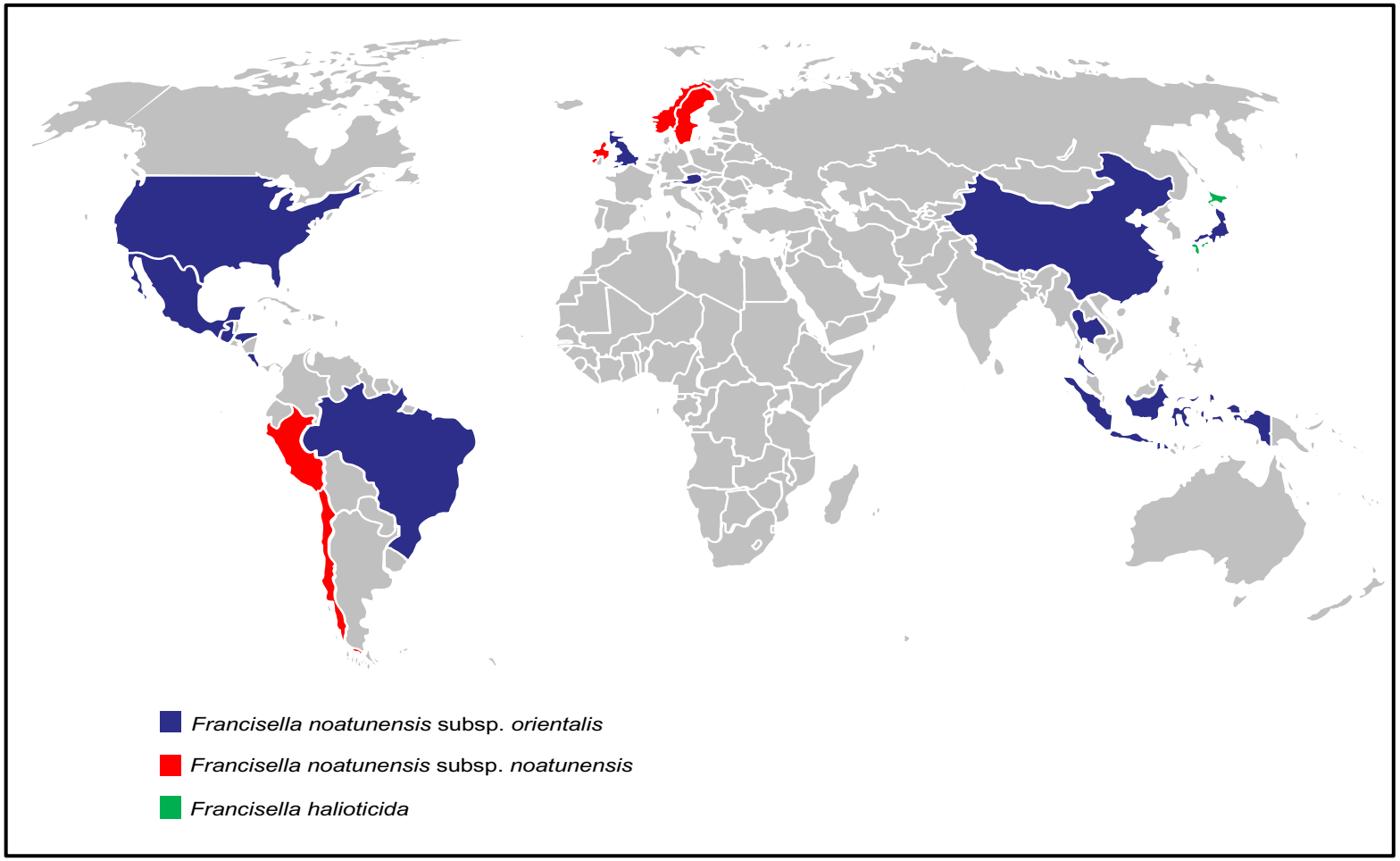


Figure 1.4. A schematic map of the world distribution of piscine francisellosis.

Previous studies have demonstrated that fish francisellosis can be attributed to either *Francisella noatunensis* subsp. *orientalis* (*Fno*) that affects mainly warm water fish or *Francisella noatunensis* subsp. *noatunensis* (*Fnn*) that affects mainly cold-water fish (Ostland *et al.,* 2006; Mikalsen and Colquhoun, 2009; Ottem *et al.,* 2009; Colquhoun *et al.,* 2014). *Fnn* had been identified in the North Sea and northern Atlantic area, where it was recovered from wild and farmed Atlantic Cod (*Gadus morhua*) in Norway (Nylund *et al.,* 2006; Olsen *et al.,* 2006; Mikalsen *et al.,* 2007; Ottem *et al.,* 2007, 2008) and in wild-caught adult Celtic sea Atlantic cod and juveniles reared in captivity in Ireland (Ruane *et al*., 2015). It was also identified in cultured Atlantic salmon (*Salmo salar*) in Chile (Birkbeck *et al.,* 2007; Bohle *et al.,* 2009). *Fno* has been identified in many warm water fish, however tilapia species are the most affected with outbreaks reported in many localities worldwide, such as Taiwan (Chen *et al.,* 1994; Hseih *et al.,* 2006), continental United States including Hawaii (Mauel *et al.,* 2003; Soto *et al.,* 2013a), Florida, California and south Carolina (Mauel *et al.,* 2005), Latin America (Mauel *et al.,* 2007), Costa Rica (Soto *et al.,* 2009a), U.K. (Jeffery *et al.,* 2010), Thailand (Nguyen *et al*., 2016), China (Qiang *et al.,* 2016), Brazil (Leal *et al.,* 2014; Sebastião *et al.,* 2017; Rodrigues *et al.,* 2017) and Mexico (Ortega *et al.,* 2016). Spotted rose snapper (*Lutijanus guttatus*) cultured in Pacific coast of Central Americas was recently found to be susceptible to a novel strain of *Francisella* spp. proposed as *Francisella marina* sp. nov. The diseased fish showed non-specific external signs, severe granulomatous inflammation and mortality was estimated at 3-8 % (Soto *et al.,* 2018).

Moreover, *Fno* has been transmitted through the ornamental fish trading between countries, where it was isolated from Indo-Pacific reef fish imported from Asia to the United States (Camus *et al.,* 2013), French grunt (*Haemulon flavolineatum*) and the Caesar grunt (*Haemulon carbonarium*) in Florida, USA (Soto *et al.,* 2014b) and Ornamental African cichlids in Austria (Lewisch *et al.,* 2014). Outbreaks of francisellosis were seldom detected in cultured hybrid striped bass (*Morone chrysops* x *M. saxatilis*) in USA (Ostland *et al.,* 2006) and three-line Grunt (*Parapristipoma trilineatum*) in Japan where it caused mass mortality among cultured stocks (Fukuda *et al.,* 2002; Kamaishi *et al.,* 2005). In shellfish, francisellosis was recorded in two farmed species of giant abalone *Haliotis gigantea* and *Haliotis discus* in Japan and the causative pathogen was classified as *Francisella halioticida* sp. nov. (Kamaishi *et al.,* 2010). Experimental injection of the

bacterial isolates resulted in a very high mortality (98.6%) confirming the suspected susceptibility of abalone and other bivalves to francisellosis (Brevik *et al.* 2011a).

Studies of the susceptibility of other fish species to francisellosis have been performed. Lewish *et al.* (2016) performed intraperitoneal (i.p.) challenge using *Fno* at doses of 2×10^6 CFU/mL, 2×10^7 CFU/mL and 2×10^8 CFU/mL in sun fish (*Lepomis giobosus*) and common carp (*Cyprinus carpio*). Interestingly, the sun fish were susceptible to the infection, where mild splenomegaly and renomegally were observed and the highest mortality reached 56.1 % after 96 hpi, while the carp were resistant. PCR analysis showed positive results in 63.8 % and 12.2 % in the challenged sun fish and carp, respectively. In a similar experiment, *Fno* was i.p. injected at a dose of 1.5×10^6 CFU/mL into striped catfish (*Pangasianodon hypophthalmus*) and carp. Both fishes were resistant with no mortalities or histopathological features of francisellosis. Paradoxically, 50 % and 100 % of the surviving striped catfish and carp were positive for *Fno* by PCR, respectively (Dong *et al.,* 2016b).

1.1.4.5. Genetic diversity of fish Francisella

Analysis of the 16S rRNA gene sequence of *Francisella* isolates retrieved from different fish species revealed that these isolates can fall into two distinct clades. Clade I represented by *Fnn*, which was found in conjunction with disease in Norwegian cod and Chilean salmon and Clade II represented by *Fno*, which induces the disease in tilapia spp., ornamental cichlids and three-line grunt. These studies showed that the fish-pathogenic *Francisella* members share a high degree of homology with *F. philomiragia* (*Fp*) which induces disease in immunocompromised humans and other animals (Birkbeck *et. al.,* 2011). The average nucleotide identity (ANI) of DNA sequence of *Fno* with that of *F. philomiragia* was found to be up to 98.6 % and for *Fnn* it was 99.3 % based on the 16S rRNA gene sequence, and 92.9-99 % based on similarities in other nucleotide sequences from 5 housekeeping genes including *groEL, pgm, shdA, rpoA and rpoB* (Mikalsen and Colquhoun, 2009; Mikalsen *et al.,* 2007). Furthermore, comparison between *Fnn* isolates from Norwegian cod and Chilean salmon revealed that the percentage identity of 16S rRNA gene sequences was about 99.8% (Birkbeck *et al.,* 2011) and the nucleotide percentage identity of six housekeeping genes was 99.5 %. Sequencing of the 16S rRNA gene and housekeeping genes of *Francisella* retrieved from Giant abalone in Japan and comparisons with genes of *F. philomiragia* and *F. noatunensis* subsp. *noatunensis* showed

that it was genetically different, with percentage identities of 49.2% with *F. philomiragia* and 61% with *F. noatunensis* subsp. *noatunensis,* respectively. Thus, it was established as a new species under the name *F. halioticida* sp. nov. (Brevik *et al.,* 2011a).

In addition, Brevik *et al.* (2011b) used multi-locus variable number of tandem repeats (VNTR) analysis (MLVA) to study the genetic diversity of *Francisella* sp. isolates including 22 isolates of *F. noatunensis* subsp. *noatunensis* retrieved from Norwegian farmed cod (*n*= 17), Norwegian wild cod (*n*= 4) and Chilean farmed salmon (*n*= 1), 4 isolates of *F. noatunensis* subsp. *orientalis* retrieved from Indonesian farmed Nile tilapia (*n*= 3) and Japanese three-line grunt, *P. trilineatum* (*n*= 1) and 7 isolates of *F. philomiragia* from human (*n*= 3), water (*n*= 1) and aquatic mammals (*n*= 3). A sequence-based system was performed on seven VNTR-loci and the results indicated a low allelic diversity in isolates retrieved from francisellosis outbreaks in cultured fish, and only two allelic profiles were seen in wild fish. Isolates from infected Chilean salmon showed differences in 6 out of 7 markers from those in infected Norwegian cod strains, which reflected the differences in their geographical and ecological origins and also in their host divergence. Moreover, the allelic profiles of *Fno* and *Fp* isolates were unique and easily separated. An improved simplified MLVA targetting five highly polymorphic VNTR loci in a single multiplex PCR was developed by Duodu *et al.* (2013) that resulted in identification of at least 13 allelic profiles of 91 isolates of *Fnn* from Atlantic cod in Norway. The data obtained from the MLVA showed that the source of francisellosis in cultured cod could be of human origin and provides a promising tool for typing of *Fnn* and studying francisellosis epizootics in fish.

In a similar study, the genetic fingerprinting of 62 locally isolated *Fno* isolates retrieved from natural outbreaks in Nile tilapia cage farms in Brazil between 2012-2013 were studied by repetitive element palindromic polymerase chain reaction (REP-PCR) and results showed that all the isolates were clonally related, despite being collected from different locations (Leal *et al.,* 2014). Recently, Ramirez-Paredes *et al*. (2017b) performed phylogenetic analyses of 5 *Fno* isolates from different locations using 11 house-keeping and core genes (16SrRNA,16SrRNA-23S rRNA intergenic spacer (ITS), 23S rRNA, *mdh*, *dnaA*, *mutS*, *prfB, putA*, *rpoA, rpoB, tpiA*), where they reported sequence percentage identities between 99-100 %. The newly identified *Francisella marina* sp. nov showed marked differences from the other known fish *Francisella* sp. using 16S rRNA sequence

comparison, multi-locus sequence typing analysis (MLSA) of selected house-keeping genes (*DnaK, gyrB, muts, pgm, pyrfB, rpoB,* sodB) and REP-PCR. The 16S rRNA sequence of the novel isolate showed 99% homology to a *Francisella* sp. (*Francisella* sp. isolate TX077308) isolated from sea water in Gulf of Mexico (Peterson *et al.,* 2009), while it showed < 99% similarity to the other validated fish, human and environmental *Francisella* spp. The REP-PCR confirmed the 16S rRNA sequence comparison and MLSA showed that the novel *Francisella* isolate retrieved from red snapper formed a discrete phyletic haplogroup close to *Fno, Fnn and Fp* cluster and far from the *Ft* subspecies cluster. The results of this analysis indicated that this novel isolate represents a distinctive divergent species with in the genus *Francisella* (Soto *et al.,* 2018).

1.1.4.6. Clinical and post-mortem findings of francisellosis in tilapia

Francisellosis in tilapia is a severe disease that can be present in all forms, acute, subacute and chronic, according to many factors including environmental conditions, size and susceptibility of the host. During an epizootic episode, mortality was reported up to 95%, especially with low water temperature and bad water quality (Mauel *et al.,* 2007). The disease is recognised in farmed tilapia in different developmental stages including larvae, juveniles or adults and the clinical and post-mortem signs are similar in most cases (Ortega *et al.,* 2016; Sebastião *et al.,* 2017). Infected fish show non-specific clinical signs including lethargy, erratic swimming - either vertical or circling with floating at the surface - and crowding towards the centre of the pond or the tank. Loss of appetite, exophthalmia and variable degrees of ascites also commonly occur, and skin appears ulcerated with petechial haemorrhages or scattered cutaneous lesions on the abdomen and in or around the fin with loss of scales in some cases. Upon necropsy, fish gills appear pale, patchy white or red and gills and/or skin parasites were also reported such as monogenean parasites or *Trichodina* spp. in association with *Fno* infection in tilapia. The characteristic lesions of francisellosis in fish include enlargement of most of the internal organs, especially head kidney where tissue size increases by approximately 5-50 fold and the appearance of focal or diffused whitish nodules of about 1-5 mm may appear that might have a shallow hole in their centre (ring shape foci) filled with clear serous fluid and appear as cyst-like lesions in other cases. The abdominal cavity often contains clear watery fluid and the gastro-intestinal tract is devoid of any feed. External muscles show black granulomatous lesions ranging from pin-point to large sized lesions (≥ 3cm). The presence

of nodules in the brain is only observed in exceptional cases of severe infection. Mesenteries are also irregularly thickened with multiple nodules and adhesion to the adjacent viscera (Mauel *et al.,* 2007; Soto *et al.,* 2009b, 2011a; Jeffery *et al.,* 2010; Camus *et al.,* 2013, Lewisch *et al.,* 2014). Clinical manifestations of francisellosis are shown in Figure 1.5.

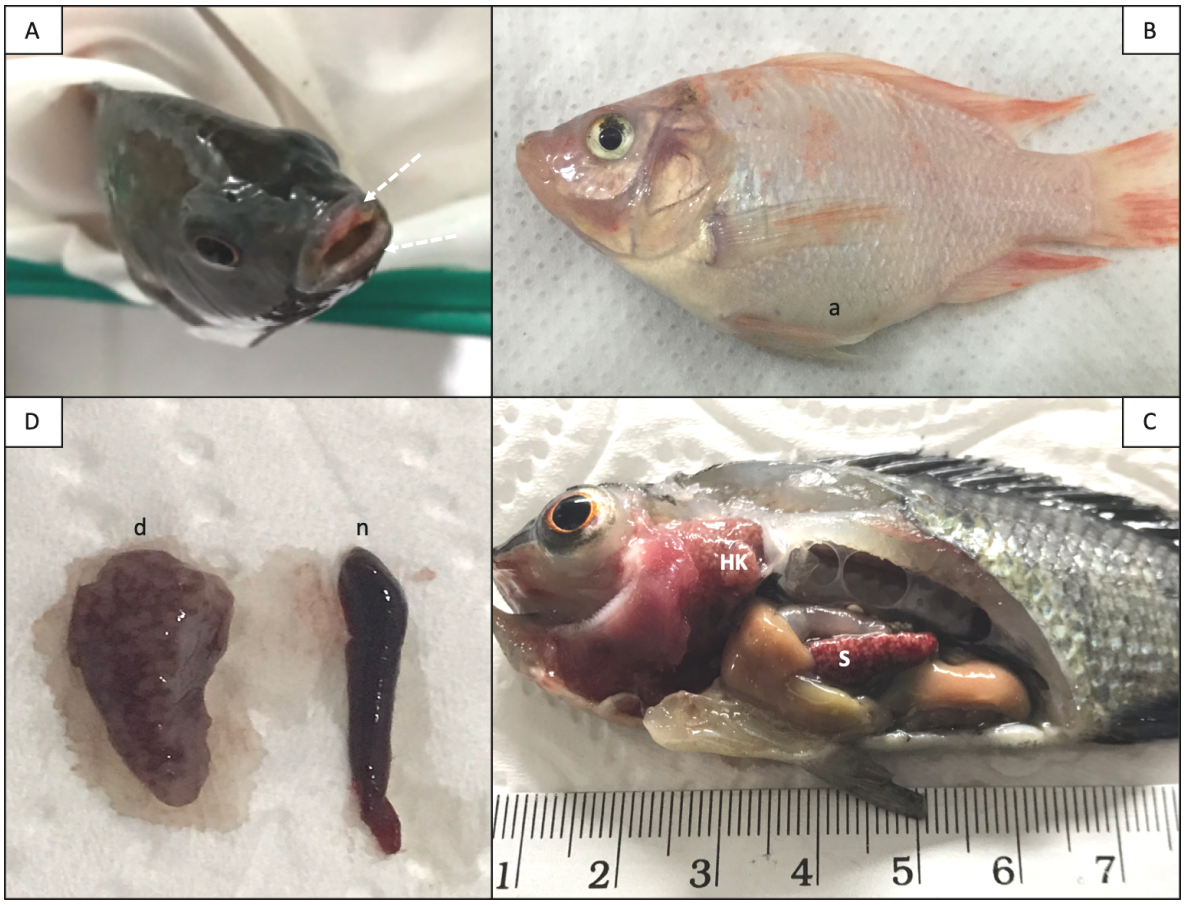


Figure 1.5. Clinical and post mortem signs of francisellosis in tilapia. [A] ulceration around mouth (dashed arrows), [B] abdominal distension as a result of ascites (a), [C] white nodules on spleen (S) and head kidney (HK) and [D] massive enlargement of infected spleen (d) compared to normal one (n).

1.1.4.7. Histopathological findings of francisellosis in tilapia

The most typical lesion of piscine francisellosis in fish is massive granulomatous reaction in internal organs with spleen and head kidney being the most affected tissues (Figure 1.6). The granuloma is characterised by central areas of necrosis and\or vacuolated foamy macrophages mixed with very few neutrophils and sometimes there are multifocal areas of eosinophilic, basophilic or mononuclear cell infiltration and the overall structures are surrounded by a fine fibrous capsule and small cuffs of lymphocytes. The vacuolated cells show variable numbers of small pleomorphic, Gram-negative coccobacilli and pyknosis was also observed. With the massive inflammatory response in the spleen, necrotic tissue and inflammatory cells replace the normal splenic parenchyma architecture (Colquhoun and Doudu, 2011).

Gills of *Fno*-infected fish reveal massive hyperplasia with lamellar fusion, loss of the inter-lamellar spaces and increased number of mucous cells. The heart shows a mild to severe epicarditis and endocarditis with endothelial hypertrophy and hyperplasia in the atrium and myocardial fibre fragmentation, while the stomach and intestine exhibit chronic inflammatory cell infiltration and necrosis in the lamina propria and submucosa. Necrotising vasculitis with the formation of fibrin thrombi and mild focal infiltration of inflammatory cells with cytoplasmic vacuolation are rarely seen in the brain, pancreas, ovary and testes. The lesions in the liver are mainly concentrated in the hepatic parenchyma that shows a granulomatous reaction surrounded by macrophages and necrotic tissue. Skin samples exhibit necrosis and chronic inflammatory cell infiltration in muscle fibers and epidermis that are lost in some infected fish. In severely infected tilapia, systemic infection extends to the brain and eyes which show macrophage infiltration in meninges and choroid gland, respectively (Mauel *et al.,* 2007; Soto *et al.,* 2009a, 2011a; Jeffery *et al.,* 2010; Camus *et al.,* 2013; Lewisch *et al.,* 2014).

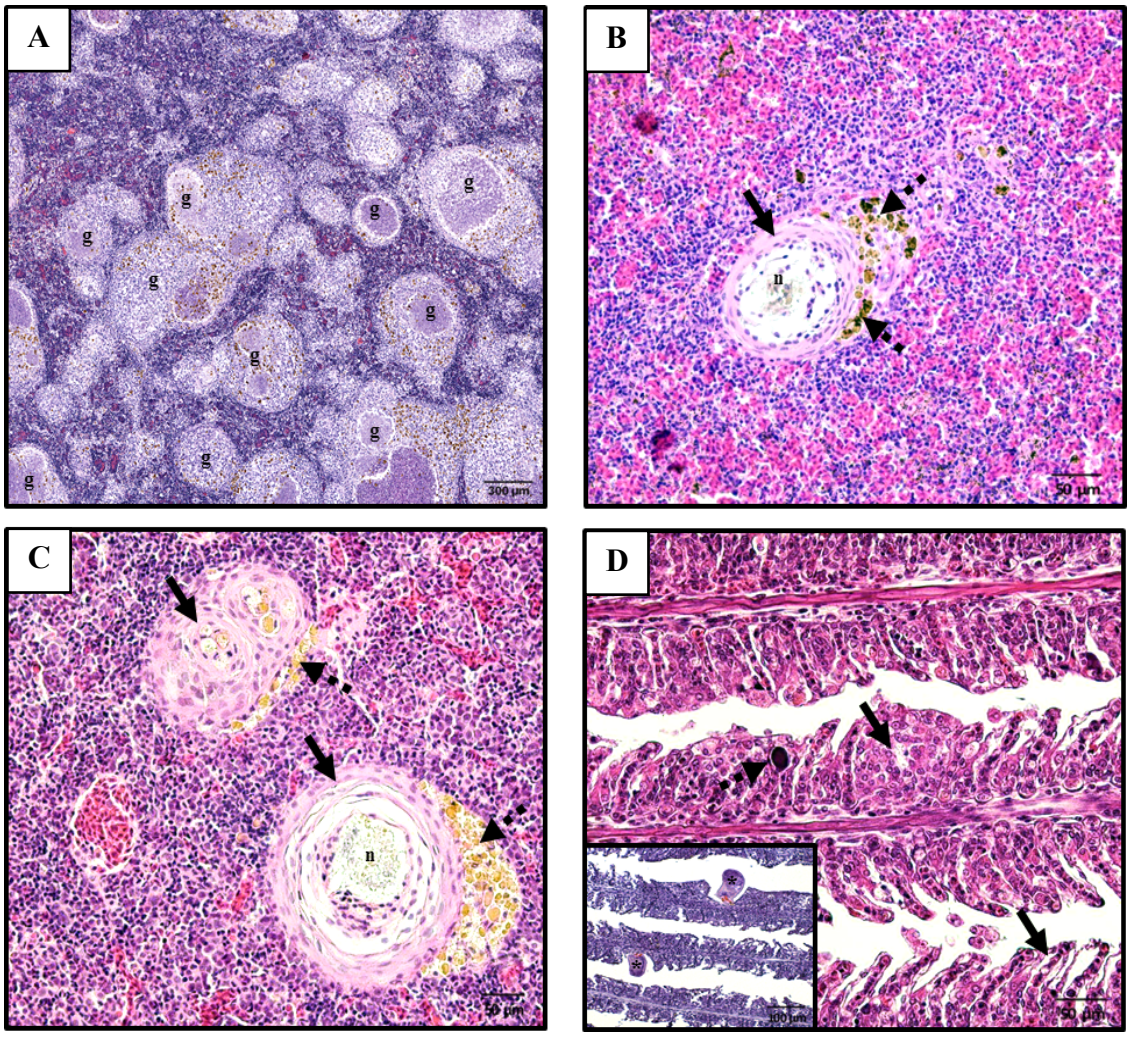


Figure 1.6. Pathological features of francisellosis in tilapia. Coalescent granulomas (g) replacing the majority of normal splenic parenchyma [Plate A] (H&E, 20x). Multifocal, round, well demarcated granulomas (solid arrows) with necrotic material in the centre (n) and epithelioid and melano macrophage infiltration of varying size (dotted arrows) in head kidney [Plate B] and spleen [Plate C], (H&E, 40x). Extensive hyperplasia and fusion of secondary lamellae (solid arrow) with mild infestation with epitheliocystis-like inclusions (dotted arrow) [Plate D] and cichlidogyrus (*) [Inset in Plate D] (H&E, 40x).

1.1.4.8. Clinical pathology of francisellosis in tilapia

Data on the clinical pathology of *Fno*-infected tilapia is scarce. Examination of blood samples from infected fishes revealed that there was a significant decrease in the haematocrit values with neutrophilia, low numbers of monocytes and total lipid values. Moreover, cytological examination of blood smears stained with Geimsa revealed variable numbers of pleomorphic bacteria in macrophages or free in serum (Chen *et al.,* 1994; Mauel *et al.,* 2003, 2005).

1.1.4.9. Fish immune response and interaction with Fno

The genus *Francisella* includes various members that all share characteristics of an intracellular life cycle in different hosts including mammals, fish and mollusca (Soto *et al.,* 2010b). Extensive research has been done on understanding the bacterial factors involved in survival and proliferation of *Francisella* spp. within their target host cell, however, the genetic basis of these mechanisms is still not fully understood (Celli and Zahrt, 2013).

An immune system within any organism is a combination of cellular and humoral components that are responsible for detection of various agents, including microorganisms (viruses, bacteria and parasites), toxins or malignant cells, protection against diseases and distinguish them from the host's normal tissues in response to endogenous or exogenous stimuli (Rauta *et al.,* 2012; Biller-Takahashi and Urbinati, 2014). As in other vertebrates, fish possess both innate and adaptive immune responses. The innate immune response represents the earliest basic immune strategy in all living organisms, where it shares common criteria of non-specificity and rapid response (Tort *et al.,* 2003). In fish, spleen and head kidney are integral constituents of the immune system. Unlike other vertebrates, including mammals and other terrestrial animals, the kidney in fish serves as a hemopoietic, immune-endocrine and secretory functioning organ (Zapata *et al.,* 1996; Castro and Taffala, 2015). In teleost fish, the kidney is anatomically differentiated into two compartments, head kidney (also known as anterior kidney or pronephros) and posterior kidney (also known as trunk kidney). The head kidney is the active part of the kidney which consists of hematopoietic, lymphoid and endocrine tissue and serves as the main factory for blood cells that are synthesized in its interlobular tissues (Castro and Taffala, 2015). In addition, it actively performs essential immune functions, including the production of antibodies via plasma cells and proliferated B-cells (Secombes and Wang, 2012), phagocytosis (Dannevig *et al.,* 1994) and is responsible for the formation of

immune memory via melano-macrophage centres (Tsuji *et al.,* 1990) and antigen processing (Kaattari and Irwin, 1985). Paradoxically, the trunk kidney performs an excretory function (Secombes and Wang, 2012).

The spleen in teleost fish follows the same anatomical and functional scheme as other terrestrial mammals. The white pulp and red pulp in the fish spleen are mainly involved in blood filtration, destruction of aged blood cells, antigen presentation and antibody production (Zapata *et al.,* 1996; Secombes and Wang, 2015; Castro and Taffala, 2015). Interestingly, the spleen and head kidney are the most targeted organs for pathogen invasion following *Fno* infection (Soto *et al.,* 2009a), where they exhibit the most significant lesions. These organs have been used as appropriate sample tissues for a number of applications including, detection and quantification of the pathogen in various infected fish species (Brudal *et al.,* 2014; Sebastião *et al.,* 2017; Rodrigues *et al.,* 2017), evaluation of antibiotics efficacy (Soto *et al.,* 2014b) and evaluation of vaccine efficacy (Brudal *et al.,* 2015; Lagos *et al.,* 2017) against fish pathogenic *Francisella* spp.

Cytokines play an essential role in the immediate and early immune response of fish to bacterial infection via various mechanisms such as lymphocytic activation and boosting phagocytosis and apoptosis (Reyes-Cerpa *et al.,* 2013). The expression of inflammatory cytokine related genes including interleukins, interferons and tumour necrosis factor after *Francisella* infection has been extensively investigated to elucidate the dynamics of response to the disease in various fish hosts.

Interleukins (*ILs*) represent a class of cytokines that are mainly synthesized by $CD4^+$ T-helper cells, macrophages, monocytes and endothelial cells (Secombes *et al.,* 2011). Interleukins are involved in controlling the differentiation and development of T cells (e.g. Th1, Th2, TREG and Th17 subsets) and B-cells (Brocker *et al.,* 2010; Holt *et al.,* 2010). *IL-1β* is one of the commonly expressed cytokines in various teleosts including salmonids, cyprinids, gadoids, periciforms and anguilliforms (Secombes *et al.,* 2011). It is a constitutive inflammatory mediator produced by macrophages in response to infection (Tort *et al.,* 2003) and Komatsu *et al.* (2009) reported that *IL-1β* is affiliated with bacterial invasion and colonisation. *IL-1β* was upregulated in splenic cells of Nile tilapia 24 and 96 hpi with 0.8×10^5 CFU/fish of *Fno* (Jantrakajorn and Wongtavatchai, 2016). *IL-1β* transcription level was also significantly increased in kidney cells of adult zebra fish vaccinated with *Fno*-derived outer membrane vesicles (OMVs) 1 day post-vaccination

(dpv) compared to control fish, with a slight increase at 7 dpv and 21 dpv, while no significant difference was observed in the expression levels between vaccinated and control fish 1 and 7 days post-challenge (dpc) with 1×10^6 CFU/fish of *Fno* (Lagos *et al.,* 2017). Brudal *et al.* (2014) reported increased transcription level of *IL-1β* in zebra fish embryos injected with fluorescently labelled *Fno, Fnn* and *F. tularensis* subsp. *novicida* at 22, 28 and 32 °C. In addition, up-regulation of *IL-1β* was reported in the spleen of Atlantic cod after 3 hpi with *Fnn* (Bakkemo *et al.,* 2011) and in zebra fish at 6 and 24 hpi with *Francisella* sp. (Vojtech *et al.,* 2009). Paradoxically, down regulation of *IL-1β* was reported after 2-24 hpi with *Fnn* and absence of expression by 48 hpi was also reported in Atlantic cod (Bakkemo *et al.,* 2011).

Interferons (*IFNs*) are known by their anti-viral activity in vertebrates, where they are divided into three families: IFN I, IFN II and IFN III, which were identified based on their specific receptors, cellular origin, genomic structure and their induced immune response. In teleost fish, two of the three recognised families are present and constitute the antiviral defence mechanism (Zou and Secombes, 2011). In addition, Type I IFN is further divided to two distinct sub classes based on cysteine residues and were reported to be released by any kind of cells as result of viral invasion (Boehm *et al.,* 1997; Zou and Secombes, 2011). Type II IFN, on the other hand is mainly advocated to cell-mediated immunity and produced by activated T-cells and natural killer cells (NK- cells) in response to intracellular pathogens (e.g. intracellular virus or bacteria). Two members were described in the latter type including the mammalian homologue *IFN-γ* and a fish specific member known as *IFN-γ* -related molecule (*IFN-γ* rel) (Boehm *et al.,* 1997; Zou and Secombes, 2011). In zebra fish injected with an *Fno*-derived OMV vaccine, only the *IFN-I* transcription level but not IFN-II was higher than control fish at 1 dpv and decreased by 7 dpv then interestingly elevated at 21 dpv (Lagos *et al.,* 2017).

Tumor necrosis factor (*TNF*) is a key factor in the body's immune response to tumor cells, bacteria and virus with a vital role in both acute reactions and systemic inflammation. In fish, *TNF-α* is a well-known pro-inflammatory mediator that has been cloned in various fish species (Hirono *et al.,* 2000; Bobe and Goetz, 2001; Laing *et al.,* 2001; Castillo *et al.,* 2002). It promotes cell proliferation, differentiation, necrosis, apoptosis and promotion of the other cytokines. In addition, it moderates effective anti-microbial responses in the form of induced apoptosis, infected cell killing, suppression of

intracellular pathogen replication and increased transcription of various immune-related genes (Reyes-Cerpa *et al.,* 2013). *TNF-α* was upregulated in spleen cells of Nile tilapia 6 hpi with *Fno* and was maintained at a high level also at 24-96 hpi (Jantrakajorn and Wongtavatchai, 2016). Furthermore, the transcription level of *TNF-α* was significantly higher in zebra fish larvae after 6 hpi with *Francisella* sp., however, lower levels were noted at 12 hpi before increasing again after 24 hpi. (Vojtech *et al.,* 2009). The kidney of zebra fish adults vaccinated with *Fno*-derived OMVs exhibited down-regulation of *TNF-α* 1-21 dpv, which then showed significant up-regulation 1 dpc of *Fno* (Lagos *et al.,* 2017). In contrast, zebra fish larvae showed upregulation of *TNF-α* 48 hpi with *Fno* and *F. tularensis* subsp. *novicida* and 7 dpc with *Fnn* (Brudal *et al.,* 2014).

Transforming growth factor (*TGF*) is described as a pleiotropic cytokine that performs multi-purpose immune-related functions including cell development, proliferation, differentiation and regulation of the survival of leukocytes of various origins such as lymphocytes, dendritic cells, natural killer cells, macrophages and granulocytes (Li and Flavell, 2006; Li *et al.,* 2008). In fish, there is little information about the function of *TGF-βl* compared to the mammalian homologues, which are well-described as immune-suppressive cytokines (Saxena *et al.,* 2008) that are mainly involved in harmonising immune tolerance and auto-immunity inhibition via their effect on T-cells (Lio and Flavell, 2008; Li *et al.,* 2006; Wan *et al.,* 2007). However, recent studies in gold fish (Haddad *et al.,* 2008), carp (Yang *et al.,* 2012) and red sea bream (Cai *et al.,* 2010) suggest that *TGF-βl* in teleosts exhibits the same immune-suppressive effect on leukocytes as seen in mammals. Jantrakajorn and Wongtavatchai (2016) reported tissue damage and increased granuloma formation in the spleen of *Fno*-infected tilapia accompanied with down regulation of *TGF-β.*

At a cellular level, *Francisella* spp. have shown high adaptatability to various host cell types including macrophages, dendritic cells, polymorpho-nuclear neutrophils in mammals (McCaffery and Allen, 2006; Hall *et al.,* 2008), as well as in tilapia (Soto *et al.,* 2010b), Atlantic cod (Gjessing *et al.,* 2011) and zebrafish (Vojtech *et al*., 2009; Brudal *et al.,* 2014, 2015). In fish, macrophages are the most involved immune cells in *Fno* infection, in which the bacterium can replicate, avoid phagolysosome activities and egress to the cytosol to infect more cells. This was exemplified by the ability of *Fno* to inhabit the cytosol of macrophage or macrophage-like cells in adult zebra fish without being degraded

and macrophage-promotor expressed gene (*mpeg1.1* promoter) was the most upregulated cell-marker following *Fno* infection (Lagos *et al.,* 2017). In a similar experiment, Brudal *et al.* (2014) showed the uptake of *Fno, Fnn* and *F. tularensis* subsp. *novicida* using enhanced green fluorescent protein-labelled macrophages or neutrophils in transgenic zebra fish cell lines after microinjection with these three pathogens, with macrophage uptake being more efficient leading to aggregation of more bacterial cells. In addition, wild type *Fno* maintained its ability to invade, survive and multiply within the milieu of tilapia head kidney-derived macrophages (HKDM) inducing apoptosis after activation of caspase 3 and 7, while a mutant strain of this bacteria lacking *iglC* gene was defective for all these activities (Soto *et al.,* 2010b).

The adaptive or acquired immune response is the second component of the immune system that performs a significant role in protection against pathogens (Ellis, 2001; Swain, 2006). Unlike the innate immune response, the acquired immune mechanism is very specific, highly complex and represents the key element of immune system that provides protection after vaccination. The teleost adaptive immune response is comparable to that of higher vertebrates. It has the ability to generate memory cells (cell mediated immunity) such as T and B lymphocytes and specific soluble and membrane-bound receptors (humoral immunity) including B and T-cell receptors (BCR and TCR) and immunoglobulins (Igs) which enable fast and efficient elimination of the specific fish pathogen upon re-encountering them (Thompson, 2017).

Antibody mediated immunity is a fundamental arm of the fish immune system. In teleost fish, the humoral immune response is relatively less advanced due to its limited immunoglobulin isotype diversity (Pilstorm and Bengten, 1996). To date, three major isotypes of immunoglobulin have been discovered in fish, IgM, IgD, IgT/IgZ, of which IgM was the first to be discovered and is the main antibody isotype in teleosts (Castro and Tafalla, 2015). More information is still needed relating to IgD and IgT/IgZ to be able to understand the role of their B-cell precursors (i.e. IgT positive and IgD positive B-cell population) within mucosal surfaces (Zhang *et al.,* 2010; Castro and Tafalla, 2015). Nevertheless, the synergistic effect of antibodies, cytokines and immune cells, including phagocytes, generally enables effective clearance of intracellular pathogens (Lagos *et al.,* 2017). The antibody-mediated immunity was shown to be critical against francisellosis in experimentally infected Nile tilapia fingerlings. Vaccination with live attenuated *Fno*

vaccine could produce specific antibodies that were detected in serum by ELISA and successfully protected the fish against a subsequent immersion challenge with a lethal dose of *F. asiatica* (Soto *et al.,* 2011b). In addition, IgM was upregulated in zebra fish post-immunisation with *Fno*-derived OMVs at 7 and 21 dpv and 1 dpc, suggesting activation of cell mediated responses by stimulation of B-cells. Moreover, a significantly high serum antibody response was reported in both vaccinated and non-vaccinated zebra fish at 21 dpc with *Fno*, suggesting the activation of humoral immune responses by increased production of antibodies against *Fno* infection following the decrease of IgM level at 21 dpv (Lagos *et al.,* 2017).

1.1.4.10. Diagnosis of francisellosis in tilapia

Detection of *Fno* in fish is a challenging process, due to its fastidious intracellular nature and the relatively low number of bacterial cells required to induce the disease (Soto *et al*., 2009a). With the increasing number of cases of francisellosis, various tools have been developed for isolation and identification of *Fno* from infected fish, including conventional culture, molecular assays, and antibody-based / immunological techniques (Soto *et al.,* 2010a).

A. Bacterial isolation on bacteriological media and cell culture

Diagnosis of *Fno* by bacterial isolation on culture media has been previously reported using various culture media. Cysteine heart agar medium supplemented with 1% bovine haemoglobin (CHAH) is the gold standard media used for isolation of piscine *Francisella* including *Fno* (Kamaichi *et al.,* 2005; Soto *et al.,* 2009a, 2011a, 2014; Nguyen *et al.,* 2016; Lewish *et al.,* 2016; Ortega *et al.,* 2016). Other media have also been used such as Muller Hinton base supplemented with 3% foetal bovine serum, glucose 1% and cysteine 0.1% (Soto *et al.,* 2009a), Cysteine heart agar medium supplemented with sheep blood (Mikalsen and Colquhoun, 2009) and Thayer-Martin agar (Hseih *et al.,* 2006). The optimum temperature for *in vitro* growth of *Fno* ranged from 25-28°C, with growth inhibited at 35-37°C (Soto *et al.,* 2009a). This temperature-dependent growth is a hallmark in the differential diagnosis of *Fno* from either closely related fish pathogenic *Francisella* spp. or human and environmental *Francisella* spp. In addition, *Fno* isolated from infected tilapia was successfully cultured in CHSE-214 cells resulting in cytopathic effect after 5-7 dpc (Hseih *et al.,* 2006), however this growth was not consistent as shown in other studies

(Ostland *et al.,* 2006; Jeffery *et al.,* 2010). The sensitivity of bacteriological culture for detection of *Fno* in carrier farmed tilapia has been tested, where the median sensitivity value was determined as 1.6% (Assis *et al.,* 2016). There are a number of issues using conventional culturing methods for the isolation of *Francisella* spp. from infected fish tissues including outcompeting overgrowth by other concomitant bacteria (Colquhoun and Duodu, 2011), the requirement for homogenisation of tissues and the possibility of false negative results (Soto *et al.,* 2010a).

B. Biochemical and phenotypic identification

a. Biochemical tests

Fish pathogenic *Francisella* spp. were reported to be biochemically non-reactive and there is a lack of reliable tests to differentiate between them, or even with the other members of the *Francisella* genus (Colquhoun and Duodu, 2011). There are some commercially available kits for identifying the phenotypic profile of piscine *Francisella* spp., however, they have resulted in weak reactions, which can be difficult to interpret (Mikalsen and Colquhoun, 2009). The bacteria show positive citrate, Voges-Proskauer and gelatinase reactions by API20 E, and only 8 out of 20 positive enzymatic reactions (acid phosphatase, naphthol-AS-BI-phosphohydrolase, esterase lipase, alkaline phosphatase, esterase, lipase, α-chymotrypsin and β-galactosidase) with APIzyme kit (Ramirez-Paredes *et al.,* 2017b).

b. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (MALDI-TOF-MS)

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has recently emerged as a potential tool for microbial identification and diagnosis. In MALDI-TOF-MS, the sample for analysis is prepared by mixing with a solution of an energy-absorbent, organic compound called matrix. When the matrix crystallizes on drying, the sample entrapped within the matrix also co-crystallizes. The sample within the matrix is ionized in an automated mode with a laser beam. Desorption and ionization with the laser beam generate singly protonated ions from analytes in the sample. The protonated ions are then accelerated at a fixed potential, where the are separated from each other on the basis of their mass-to-charge ratio (m/z). The charged analytes are then detected and measured using different types of mass analyzers including time of flight (TOF) analyser, that is commonly used for microbiological applications. During MALDI-TOF analysis, the

m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube. Identification of microbes by MALDI-TOF-MS is done by either peptide-mass fingerprintings (PMFs) matching via comparing the MS spectrum of the unknown microbial isolates with the MS spectra of known microbial isolates contained in the database or by matching the masses of biomarkers of the unknown organism with the proteome database (Singhal *et al.,* 2015). MALDI-TOF-MS was recently used to identify novel isolates of *Francisella* sp. retrieved from diseased red snapper cultured in Central America. The spectra obtained for the proteins of the novel isolate enabled its differentiation from other validated *Francisella* spp. including *Fno, Fp* and *Ft.* The spectra analysis showed that the new isolate was more similar to *Fno* than *Fp* and *Ft* (Soto *et al.,* 2018).

C. Histopathological examination

Histopathology is a powerful tool for diagnosis of many fish diseases by determination of tissue changes resulting from infectious or non-infectious etiologies. It was extensively used to describe the pathological features of francisellosis in formalin fixed paraffin embedded tissues, particularly in head kidney and spleen (Fukuda *et al.,* 2002, Soto *et al.,* 2009a, 2011a, 2013a, 2014b; Nguyen *et al.,* 2016; Qiang *et al.,* 2016; Ramirez-Paredes *et al.,* 2017b). The most common pathological feature in most cases is an extensive granulomatous inflammation with multi-organ granulomas (Colquhoun and Duodu, 2011). The granuloma is usually infiltrated by foamy macrophages, fibroblasts and leukocytes (Mauel *et al.,* 2007; Nylund *et al.,* 2006) and may show necrotic or liquefied centres (Soto *et al.,* 2014b) or bacterial colonisation in severe cases (Nylund *et al.,* 2006).

D. Molecular diagnosis

The use of molecular diagnostic tools for *Fno* offers an alternative to the problems associated with culture of this fastidious pathogen.

a. Conventional Francisella genus specific polymerase chain reaction (PCR)

The use of *Francisella* genus-specific PCR primers targeting the 16S rRNA gene sequence followed by sequencing of the PCR product has been successfully implemented to diagnose cases of *Fno* in various hosts including tilapia (Hseih *et al.,* 2006; Mauel *et al.,* 2007; Soto *et al.,* 2009a; Nguyen *et al.,* 2016; Qiang *et al.,* 2016; Ortega *et al.,* 2016; Ramirez-Paredes *et al.,* 2017b), three-lined grunt (Kamaishi *et al.,* 2005) and hybrid

striped-bass (Ostland *et al.,* 2006). However, the disadvantages of conventional PCR, including limited sensitivity and inability to differentiate between *Francisella* members on the species level or of subspecies, limits its usage in piscine francisellosis diagnosis.

b. Fno specific real-time PCR

Real-time PCR involves monitoring of PCR amplification by measuring fluorescence during the exponential phase of the reaction. Non-specific DNA binding dyes, which emit fluorescence upon binding to double-stranded DNA, such as SYBRGreen®, enable determination of the presence or absence of an amplicon, without giving any information on the precise nature of the product (Kim *et al.,* 2017). Improved accuracy of molecular tests for *Fno* detection and identification in tilapia and other susceptible species has been achieved using real-time PCR, alleviating the disadvantages of conventional PCR which depends on the results of agarose gel electrophoresis of endpoint reaction products. A TaqMan real-time PCR assay was developed by Soto *et al.* (2010a) targeting the *iglC* gene from *Francisella* strains isolated from Nile tilapia. The higher sensitivity of this assay (~25 genomic equivalents) could differentiate between *Francisella* isolates retrieved from infected tilapia and cod based on the sequence of the *iglC* gene as the sequence homology of that gene between *Fno* and *Fnn* was only ~ 90%. This assay has also successfully been used to diagnose *Fno* outbreaks in Brazil (Leal *et al.,* 2016; Sebastião *et al.,* 2017) and Mexico (Ortega *et al.,* 2016). With the recent availability of the whole genome sequences of fish *Francisella* isolates, a real-time PCR targeting specific genomic regions in *Fno* and *Fnn* could be used to identify and differentiate between both isolates with high specificity and sensitivity which represents a powerful tool for molecular diagnosis of the known fish *Francisella* strains (Duodu *et al.,* 2012).

c. Duplex-PCR

Multiplex PCR technology, including duplex PCR, is another type of molecular tools that was first developed by Chamberlain *et al.* (1988) and includes amplification of more than one target of interest in a PCR using multiple pairs of primers, resulting in specific amplicons of different sizes for the target organisms. Recently, a duplex PCR targeting 16S rRNA and a unique hypothetical gene sequence in *Fno* was developed that gave high sensitivity (~10-100 genomic equivalents) and specificity, which may be used for

identification of either clinically infected or latently infected fish from both farms or wild sources (Dong *et al.,* 2016a).

d. Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a simple, rapid, specific and cost-effective nucleic acid amplification method. This technique employs a Bst DNA polymerase (optimal activity at 66 ºC), that originates from *Bacillus stearothermophilus* and has 5′–3′ DNA polymerase and strand displacement activities but lacks 5′ –3′ exonuclease activity, a set of four primers (two outer and two inner) and two optional loop primers designed to recognize a total of six different sequences in the target gene. Amplification by LAMP resulting in generation of multiple products of predictable sizes. The amplified products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops, yielding > 500 mg/mL of PCR products (Nagamine *et al.*, 2001). A colorimetric LAMP assay was developed for screening milt, non-fertilized and fertilized eggs after crossing, different developmental stages of clinically healthy tilapia as well as water samples from rearing tanks to determine the presence of *Fno*. The assay could successfully detect the bacteria in all tested samples with detection limits as low as 1 fg of *Fno* gDNA (Pradeep *et al.,* 2016).

E. *In situ* hybridization (ISH)

In situ hybridization techniques involve detection of specific nucleic acid sequences in morphologically preserved chromosomes, tissues or cell preparations using a labeled complementary DNA, RNA or modified nucleic acids strand (i.e., probe) (Kim *et al.,* 2017). *In situ* hybridization was successfully applied for identification of *Fno* in tilapia and other ornamental cichlids, where the 16S rRNA gene was targeted by a dioxigenin probe (DIG) in different tissues. The hybridization signals appeared as a purple precipitate in the target tissues representing binding of the labelled probe to the target molecules. The ISH demonstrated higher performance than conventional PCR in detection of *Fno* in infected tissue samples containing damaged DNA, where it was able to identify and localise the target agent with high specificity and sensitivity (Hsieh et *al.,* 2006; 2007). The ISH was also useful for studying the developmental stages of granuloma formation within *Francisella*-infected tissues including kidney, spleen and liver and could also identify the

bacteria in the gills and intestinal epithelium suggesting their role as portals of entry (Dong *et al.,* 2016b).

F. Immunohistochemistry (IHC)

Immunohistochemistry is a powerful diagnostic technique based on antigen-antibody interactions that can be used to detect and localize specific antigens ranging from amino acids and proteins to infectious agents and specific cellular populations within cells or tissue sections from formaline fixed parafine embedded (FFPE) tissues (Kim *et al.,* 2017). A specific *Fno*-immunohistochemical (IHC) analysis was performed by Soto *et al.* (2012b) using formalin fixed paraffin embedded infected tissues collected from different fish from various locations, where the disease agent was initially diagnosed as Rickettsia-like organism. The test showed 45 positively stained tissues out of 68 after using mouse anti-*Fno* serum. The *Fno* antigen was detected in different tissues including gill, gastric epithelium, liver, meninges, pericardium, but the strongest positive reaction was seen in spleen and kidney granulomatous tissues.

1.1.4.11. Treatment of fish francisellosis with antibiotics

The use of antimicrobial agents for controlling piscine francisellosis often proves ineffective. This can be attributed in part to the intracellular nature of this aquatic micro-organism, its high infectivity, high transmissibility and the high morbidity of the disease. In addition, oral delivery of antimicrobials is not very practical as infected fish often exhibit poor feeding (Colquhoun and Duodu, 2011). The susceptibility of fish associated *Francisella* spp. to such antimicrobials has been extensively studied previously. The minimum inhibitory concentration (MIC) data obtained using *Fnn* isolated from Atlantic cod showed that there was high susceptibility to florfenicol (0.5 μg/mL), oxolinic acid (0.25 μg/mL), flumequine (0.25: 1 μg/mL) and rifampin (1 μg/mL) and low susceptible to oxytetracycline (64 μg/mL), trimethoprim/sulfadiazine (64: 128 μg/mL), ciprofloxacin (8 μg/mL), streptomycin (32: 128 μg/mL) and erythromycin (16 μg/mL) (Isachsen *et al.,* 2012). Ramirez-Paredes *et al.* (2017b) tested 39 antibiotics against *Fno*, and reported sensitivity to enrofloxacin (<0.12 μg/mL), gentamicin (<0.5 μg/mL), neomycine (<2 μg/mL) and streptomycine (<8 μg/mL) and low susceptible to sulphadimethoxine (128: 256 μg/mL), trimethoprim/sulfamethoxazole (38 μg/mL), penicillin (4: >8 μg/mL),

tylosine tartarate (20: >20 µg/mL) and clindamycine (>4 µg/mL). Florfenicol was effective against *Fno* in infected tilapia when applied at a dose of 15 mg/kg of fish body weight for a period of 10 days (Soto *et al.,* 2012d) and tetracycline was used at a concentration of 0.38gm per kg of feed for 10 days treatment of francisellosis in hybrid striped bass (Ostland *et al.,* 2006). In addition, oral administration of oxytetracycline medicated feed at a dose of 3 g/ 100 pounds of fish for 10 days resulted in a significant decrease in mortalities of *Fno*-infected grunts in Florida (Soto *et al.,* 2014b). Moreover, *F. halioticida* isolated from abalone showed sensitivity to cefetazidime, ciprofloxacin, gentamycin and tetracycline, but was resistant to ampicillin, erythromycin, cefuroxime and penicillin (Brevik *et al.,* 2011a).

Treatment of *Fno* by combinations of antibiotics with increased water temperature up to 30 ºC was reported to decrease mortalities in infected grunts. However, this approach did not give long lasting effects, as the mortality did not stop completely and screening of surviving fish by qPCR showed positive results for *Fno*, despite no growth of viable bacteria on CHAH (Soto *et al.,* 2014b). These results highlight the urgent need for other reliable control and/ or prevention strategies such as vaccination against *Fno.*

1.1.4.12. Vaccination against Fno in tilapia

A. History of fish vaccination

Vaccines are an integral tool in any health management strategy applicable to economically important reared fish. They can greatly reduce the need for drugs and chemicals as they act by enhancing immunity against pathogens (Klesius *et al.,* 2011). Vaccination plays an important role in combination with good management practices to increase resistance against subsequent infections by specific pathogens (Hill, 2005). The use of vaccines for the prevention of fish diseases remains an active area of study since the early findings of Snieszko *et al.* (1938) revealing increasing carp immunity after injection with killed *Aeromonas hydrophila* (Snieszko *et al.,* 1938, 1970). Prior to this, Duff (1942) showed the efficacy of using feed containing chloroform killed *Aeromonas salmonicida* for the protection of trout against furunculosis. Furthermore, Goncharove (1951, 1971) first reported the use of a vaccine for prevention of viral diseases following his studies on spring viremia of carp (SVC). These early novel experiments paved the way for a break-through in the fish vaccine industry that was continued later by Schaperclaus (1970; 1972) who discovered that carp can build-up strong immunity upon i.p. injection with killed

bacteria. The promising results achieved by the use of sulfa drug (e.g. sulphonamides) and antibiotics as potent therapeutic agents took the focus off vaccine development between the 1940s and 1950s, but this did not last due to the problems of antimicrobial resistance and the harmful impacts of such chemo-therapeutic agents (e.g. toxicity) on farmed animals and humans. This was a turning point for the revival of vaccine development in the 1960s (Ross *et. al.,* 1956; Klontz *et al.,* 1970). Since then, the field of successful fish vaccination has expanded and the process of producing safe, stable and protective vaccines against different fish pathogens remains an ongoing process.

B-Development of vaccines against *Fno*

Piscine francisellosis has been recognised as a major bacterial disease in fish in both aquaculture and in wild stocks from fresh, brackish and marine waters for more than 20 years (Colquhoun and Duodu, 2011). However, the pathogenesis of this disease is not fully understood. Additionally, the development of safe, efficient and broad-spectrum licensed vaccines against fish francisellosis has not been successful to date. Thus, thorough investigations of *Francisella* pathogenesis and development of vaccines are urgently required.

The first reported trial for vaccination against *Francisella* infection in tilapia was published by Soto *et al.* (2011b), where a mutant live attenuated vaccine was developed by a mutation in the *iglC* gene that is important for intracellular growth in macrophages. The newly developed vaccine was tested for its efficacy by immersion challenge with wild type *Fno* in tilapia fingerlings and adults. The vaccine was applied by immersion in two different administration times including 30 min and 180 min and the fish were challenged with high doses of the live bacteria (10^8 CFU/mL). The relative percents of survival (RPS) obtained from the vaccine were 68.75% and 87.5% after 30 min and 180 min immersion vaccination, respectively. Despite the significant serum and mucus antibody responses induced by this vaccine, it cannot currently be commercially used in tilapia farms because of safety issues, in particular, due to the concerns of reversion to virulence of the attenuated pathogen and the subsequent possibility of spreading the disease.

Ramirez-Paredes (2015) successfully developed an autogenous formalin whole cell killed vaccine applied by i.p. injection. After i.p. challenging the fish with 4×10^3 CFU/mL of the vaccine strain, the relative percent of survival (RPS) was 100% and 40% in the vaccinated and adjuvant injected fish groups, respectively. In addition, a significantly

higher titer of antibodies measured after 45 days post vaccination (dpv) was obtained in vaccinated fish compared to the adjuvant or un-vaccinated control group as well as a lower granuloma score in the vaccinated fish (13.3%) than the non-vaccinated survivors (90%). A recent trial was performed by Brudal *et al.* (2015) using OMVs for i.p. vaccination of zebra fish followed by i.p. challenge 32 dpv with 10^8 CFU/mL of *Fnn*. Their results showed an induced immunity represented by high survival rates, decreased bacterial burden in the spleen, heart and head kidney and reduced granulomatous response in the affected tissues 28 days post-challenge (dpc) in vaccinated fish compared with the unvaccinated fish. In a similar study, *Fno*-derived OMVs were successfully used for i.p. vaccination of zebra fish against a lethal dose of *Fno* (1×10^6 CFU/mL). It demonstrated a significantly higher survival rate of 65% associated with high levels of serum IgM, significant expression of immune related genes and reduced granuloma formation in the spleen 28 dpc (Lagos *et al.,* 2017). To-date, current vaccine developments for prevention of fish francisellosis in tilapia have not demonstrated the potential for cross-strain protection for *Fno*. Furthermore, the promising data achieved for the safe autogenous inactivated *Fno* vaccine reported by Ramirez-Paredes, (2015) requires further studies to ensure reproducible protection against experimental bacterial challenge. More studies need to be conducted for development of control strategies for such devastating diseases like francisellosis, in order to maintain the progress of global tilapia production.

1.2 Aims of the study

Fno is a serious pathogen that accounts for massive morbidities and mortalities in cultured tilapia spp. globally. Since its first discovery in 2006, the number of outbreaks has increased, however, studies contributing to the characterisation of *Fno* to identify the bacterial factors involved in pathogenicity and modulation of host immunity, development of rapid diagnostic tools and protective vaccines for this aquatic pathogen are limited. The overall aim of the current study was to establish effective control strategies for *Fno* infection in farmed tilapia based on rapid accurate diagnosis, development and application of protective vaccines and vaccination strategies.

The main objectives of the study were:

1- Comparison of the proteome and immunome of five clinical *Fno* isolates from diverse geographic origins.

2- Isolation, characterisation and identification of the antigenic profile of the *Fno* outer membrane proteins (OMPs).

3- Testing the efficacy of an injectable whole cell inactivated adjuvanted vaccine against heterologous *Fno* challenge in tilapia and investigation of immune-related genes expression post-immunisation.

4- Testing the efficacy of an autogenous whole cell inactivated *Fno* immersion vaccine in tilapia fry.

5- Development of a recombinase polymerase amplification assay (RPA) for rapid molecular detection of *Fno* on tilapia farms.

Chapter Two

Comparative analysis of the whole cell proteome and immunogenic profile of clinical isolates of *Francisella noatunensis* subsp. *orientalis* from different geographical locations

2.1. Introduction

Francisella noatunensis subsp. *orientalis* is the causative agent of warm water francisellosis, a highly infectious granulomatous bacterial infection of fish (Duncan and Duodu, 2011). The disease has a wide host range, with tilapia being the most affected species, and outbreaks reported in various geographical locations including Europe, Asia, North, Central and South America (Kamaishi *et al*., 2005; Soto *et al.,* 2009a; Lewish *et al*., 2014; Nguyen *et al*., 2016; Ortega *et al.,* 2016; Ramirez-Paredes, 2017b; Rodrigues *et al.,* 2017). The disease accounts for high economic losses and mortalities of up to 95% have been reported (Hsieh *et al.,* 2006; Ortega *et al.,* 2016). Previous genomics and proteomics studies have identified some potential *Fno*-pathogenicity determinants, including genes responsible for intracellular localization, survival and replication (Soto *et al.,* 2013b; Lagos *et al.,* 2017), however the functions of the conserved proteins derived from these genes are not fully-understood.

The recent rise in francisellosis cases has led to an increase in *Fno* surveillance. Studies focusing on *Fno* adaptation and genetic diversity using geographically distinct isolates have reported a high degree of homogeneity between isolates based on their whole genome sequence, that reached up to 99%. Thus, these studies suggested that the *Fno* isolates from diverse origins express a unique clonal-like behaviour (Figueiredo *et al*., 2016; Gonçalves *et al*., 2016; Ramirez-Paredes *et al.,* 2017b). The reported similarities in genetic composition may have important implications for conserved *Fno* protein expression, virulence, pathogenesis, and more importantly vaccine-induced immunity. To this end, studies ascertaining divergence of *Fno* strain populations at the level of protein expression rather than at the bacterial genomic level could provide more insights into the increased spread of francisellosis globally.

Traditional proteomic approaches have been implemented for characterisation of pathogenic micro-organisms, including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in combination with mass spectrometry for investigating total whole cell proteins or certain microbial protein fractions (West *et al.,* 2012; Liu *et al*., 2012; Watson *et al.,* 2014). Moreover, the application of immuno-proteomics is useful for understanding the pathogenesis and virulence mechanisms of immune evasion and development of diagnostics and vaccines against etiological pathogens, especially when taking advantage of recent post-genomic technologies (Khan *et al.,* 2006; Serruto and

Rappouli, 2006). These techniques were successfully applied for the discovery of novel immunogenic antigens in various clinically important pathogens, not only in human, such as *Francisella tularensis* (Janovska *et al.,* 2007a; Kasap *et al.,* 2017), *Francisella novicida* (Pierson *et al.,* 2011), *Francisella philomiragia* (Pierson *et al.,* 2011) and *Neisseria meningitidis* (Granoff, 2010), but also in fish including *Streptococcus iniae* (Shin *et al.,* 2007), *Flavobacterium psychrophilum* (Dumetz *et al.,* 2008; LaFrentz *et al.,* 2009), *Vibrio harveyi* (Pang *et al.,* 2010), *Aeromonas hydrophila* (Poobalane *et al.,* 2008), *Aeromonas caviae* , *Aeromonas veronii* and *Aeromonas jandaei* (Peepim *et al.,* 2016), *Edwardsiella tarda* (Kumar et *al.,* 2010) and *Francisella noatunensis* subsp. *noatunensis* (Brudal *et al.,* 2015).

In the current study, the whole cell protein profiles of 5 geographically distinct clinical *Fno* isolates, including 1 isolate that was previously used to produce an autogenous vaccine that gave 100% protection in Nile tilapia (*Oreochromis niloticus*, L) (Ramirez-Paredes, 2015), were comprehensively characterised using 1D and 2D PAGE. Additionally, investigation of the antigenic profiles of these diverse *Fno* isolates was performed using western blotting. The blots were screened with tilapia sera generated during an experimental *Fno* vaccine trial, using sera sampled post-vaccination and post-challenge to identify putative immuno-dominant antigens using liquid chromatography electrospray ionization-tandem mass spectrometry (LC/ESI/MS/MS) and Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The current study provides useful information regarding the diversity and virulence mechanisms of *Fno* and lays the groundwork for improving *Fno* vaccine design.

2.2. Materials and methods

2.2.1. Bacterial isolates, culture media and growth conditions

Five clinical *Fno* isolates were used in this study (Table 2.1). All the strains were stored as a master seed stock in Modified Muller Hinton broth (MMHB) (Difco, USA) with 2% IsoVitaleX (Becton Dickenson BBL, USA) and 0.1% glucose (Baker *et al.,* 1985) containing 20% sterile glycerol (Sigma-Aldrich, UK). A cryo-tube aliquot of the master seed stock was thawed and 50 µL was used to inoculate cysteine hear agar media (Difco, USA) with 1% bovine haemoglobin (Becton Dickenson BBL, USA) (CHAH) and incubated for 72 h at 28°C. A loopful of the bacteria was inoculated into 15 mL MMHB with the supplements and incubated at 28°C for 72 h with shaking at 140 rpm in a shaker

incubator (Kuhner, Germany). The incubation conditions were the same for all the strains to avoid any growth-related differences in their proteome profiles.

Table 2.1. Bacterial isolates used in the study

Isolate name	ID	Source	Origin and isolation year
Fno UK isolate	STIR-GUS-F2f7	Red Nile Tilapia (*Oreochromis niloticus*)	England (2012)
Fno Japanese isolate	DSMZ21254^T	Three-line grunt *Parapristipoma trilineatum*	Japan (2005)
Fno Mexican isolate	Fran-Cos1	Nile tilapia (*O. niloticus*)	Mexico (2013)
Fno Austrian isolate	NVI-9449	Malawi cichlids	Austria (2013)
Fno Costa Rican isolate	NVI-PQ1104	Nile tilapia (*O. niloticus*)	Costa Rica (2009)

DSMZ: The German collection of Microorganisms and Cell Cultures, NVI: Norwegian Veterinary Institute, Oslo, Norway. T: Type strain.

Following bacteria growth, cells were harvested by centrifugation at 5,000 × *g* at 4°C for 15 min. The pellets were washed twice with 15 mL sterile PBS (pH 7.0) then the optical density (OD_{600nm}) was adjusted to 0.4 (~1.2×10^9 CFU/mL) using 1X sterile PBS (pH 7.0) and suspension was aliquoted and kept in -70°C till used.

2.2.2. Preparation of whole cell protein extract

2.2.2.1. Extraction of the whole cell lysate

Extraction of whole-cell proteins from *Fno* isolates was performed according to Coelho *et al*. (2004), with some modifications. In brief, the bacterial cells were harvested by centrifugation of the bacterial suspension (OD_{600} = 0.4) at 5,000 × *g* at 4°C for 15 min. The pellets were resuspended in 1 mL lysis buffer (Appendix 1) containing 1% (v/v) of nuclease mix and 0.1% (v/v) of protease inhibitor mix (GE Healthcare, Uppsala, Sweden). The cell suspension was incubated on ice for 2 h, and insoluble cellular debris was

removed by centrifugation at 15,000 × *g* at 4°C for 60 min, and the clear supernatants were aliquoted and kept at - 70°C until use.

2.2.2.2. Cleaning, concentration, and quantification of proteins

The protein samples were cleaned using a Clean-up kit (GE Healthcare, USA) following the manufacturer's instructions. Briefly, 100 µL of each sample was transferred to 1.5 mL microcentrifuge tubes (Eppendorf, USA), 300 µL of precipitant solution was added then contents were quickly mixed on a vortex mixer (TopMix FB15024, Fisher scientific, UK) for 30 s. The tubes were incubated on ice for 15 min then 300 µL of co-precipitant solution was added and the tubes were inverted 5 times and mixed by vortexing for 30 s. The samples were then centrifuged at 15,000 × *g* at 4°C for 5 min after which the supernatant was removed and the pellet re-suspended in 40 µL of co-precipitant solution and incubated on ice for 5 min. Centrifugation was repeated at 15,000 × *g* at 4°C for 5 min and pellets were re-suspended in 25 µL milli-Q (MQ) water and mixed by vortexing for 30 s, before adding 1 mL of ice-cold wash buffer together with 5 µL of wash additive and mixed by vortexing for 1 min. Tubes were kept at -20°C for 30 min with quick vortexing for 30 s every 10 min. Tubes were centrifuged at 15,000 × g at 4°C for 5 min and the pellets were left to air dry for 4- 5 min. The pellets were re-suspended in a mix of 500 µL of lysis buffer (Appendix 1) containing 1% (v/v) of nuclease mix and 0.1% (v/v) of protease inhibitor mix, then vortexed for 30 s. After centrifugation at 15,000 × *g* at 4°C for 5 min, the cleared supernatant was transferred to fresh low protein binding microcentrifuge tubes and stored at -70°C.

The cleaned protein extracts were quantified using a 2-D Quant kit (GE Healthcare, USA) according to the manufacturer's instructions. In brief, a standard curve was prepared using 2 mg/mL of bovine serum albumin (BSA) standard solution at 6 concentrations: 0, 10, 20, 30, 40 and 50 µg. For the protein samples, 25 µL from each of the corresponding *Fno* protein samples were transferred to low protein binding 1.5 mL microcentrifuge tubes and 500 µL of precipitant solution were added to all the tubes, including the standard curve tubes. The tubes were mixed by vortexing for 30 s then incubated at room temperature (~ 22°C) for 3 min. 500 µL of co-precipitant was added and mixed by inverting tubes 10 times then by vortexing for 30 s. Tubes were then centrifuged for 15,000 × *g* at 4°C for 5 min and pellets were re-suspended in 100 µL of copper sulphate

and 400 µL of Milli-Q water before quickly mixing by vortexing until the protein pellet was dissolved. 1 mL of colour reagent mix was added to each tube and the tubes were then incubated for 20 min at room temperature. After incubation, 300 µL from each sample was transferred to a 96 well microtiter plate (ThermoFisher Scientific, UK), each sample was added in triplicate, then the absorbance was measured using a microtiter plate reader (Bioteck, Synergy HT, USA) at a wave length of 480 nm. The standard curve was generated by plotting the absorbance of the standards against the quantity of proteins in the respective wells. The concentrations of protein samples were obtained after comparing the values at OD_{480} to the standard curve values. The concentration of samples was optimised to 50 µg using Milli-Q water, aliquoted and kept at -70°C until further use.

2.2.3. 1D SDS-PAGE (1-DE)

The bacterial protein suspension was thawed on ice from -70°C and mixed with 2X protein sample buffer (National Diagnostics, USA) at a ratio 1:1, then incubated in a Grant digital heat block (Thomas Scientific, USA) at 90°C for 10 min. The samples were centrifuged for 3 min at 17,000 × *g*, then 10 µL of each sample and 5 µL from a precision protein dual colour standard (Bio-Rad, USA) were loaded into a 4-15%, 12-well comb, 20 µL Mini-PROTEAN® TGX™ precast gel (Bio-Rad, USA). Two technical replicates per sample were prepared to ensure reproducibility. The gels were electrophoresed in 1X Lamelli SDS electrophoresis buffer (Appendix 1) using a mini-PROTEAN® tetra cell (Bio-Rad, USA) at 100 v for 90 min and then stained with SimplyBlue™ Safe Coomassie stain (ThermoFisher Scientific, UK) and a ProteoSilver™ plus silver stain kit (Sigma-Aldrich, UK) following the manufacturer's instructions for each stain. For the Coomassie stain, the gels were washed three times with distilled water after electrophoresis, five min each, then stained with the safe blue stain for 1 h with continuous shaking on a Gyro-rocker (Stuart Scientific, UK). De-staining was performed using distilled water for 3 successive hours with water changes each 1 h. For silver staining, gels were fixed in 100 mL of fixing solution (Appendix 1) overnight on a rocking platform. The fixative was discarded, and gels were washed with 100 mL ethanol wash solution (Appendix 1) for 10 min. Gels were washed with 200 mL of ultrapure water for 10 min. Water was decanted and 100 mL of sensitizer solution (Appendix 1) was added with shaking on the rocker for 10 min. Washes were preformed twice as before then 100 mL of silver solution (Appendix 1) was added

with shaking for 10 min. Following washing for 1 min as mentioned before, 100 mL of developer solution (Appendix 1) were added with shaking for 4 min until protein bands were developed. The reaction was then stopped by adding 5 mL of proteosilver stop solution with shaking for 5 min. The stop solution was decanted, and the gels were washed with 200 mL of ultrapure water for 5 min. After staining, the gels were scanned using an Epson expression 1680 artist scanner (Epson, USA). The image obtained was evaluated using Irfanview software (http://www.irfanview.com).

For investigation of presence of polysaccharides and/or lipopolysaccharides within the proteome of the *Fno* whole cell lysate, proteinase-K digestion was performed following the method described by Kay *et al.* (2006) with some modifications. Briefly, *Fno* suspension (OD_{600} 0.4) was incubated with 2X sample buffer (1:1) as mentioned above, cooled at room temperature (RT) (22°C) for 5 min then mixed with 10% (v/v) proteinase-K (1mg/mL) (ThermoFisher Scientific, UK) and incubated at 60°C overnight followed by another incubation at 100°C for 10 min to inactivate the enzyme and stop digestion. The digested proteins were centrifuged for 10,000 × *g* at 4°C for 5 min and transferred to new set of tubes. 15 µL of the digested protein samples were resolved on SDS PAGE, stained with Coomassie blue and silver stain and gel images analysed as mentioned previously. Non-digested protein samples were included as controls.

2.2.4. 2D SDS-PAGE (2-DE)

Fifty µg of each cleaned and quantified protein sample were mixed with 125 µL of rehydration buffer (Appendix 1) containing 0.5% (v/v) IPG buffer pH 4-7 (GE Health care, Sweden) followed by a quick mix on vortex. The samples were used to passively rehydrate Immobilized pH gradient strips (IPG strips) (7 cm, pH 4-7, Bio-Rad, USA) in an IPG Box (GE Health care, Sweden) for 20 h at room temperature (22°C).

The first-dimensional isoelectric focusing (IEF) and second-dimensional SDS-gel electrophoresis were performed according to the Amersham Biosciences manual (Berkelman and Stenstedt, 1998). Separation of proteins in the first-dimension was performed using an IPGphor Ettan isoelectric focusing system (Amersham, Pharmacia, Biotech, USA) in an Ettan IPGphor cup loading manifold (GE Health care biosciences, AB). The manifold was placed on the focusing unit first then 9 mL/well of immobilline drystrip cover fluid (ThermoScientific, UK) was added. The strips were placed with gel side facing

up in each well then 2 wicks pre-wet with 150 µL of distilled water were placed overlapping the 2 ends of the strips. The electrodes were placed on the top of the wicks and their locks were closed. The IPG strips were run at a constant temperature of 20°C through a stepwise incremental voltage program consisted of 300 v for 30 min (step-on-hold), 1000 v for 30 min (gradient), 5000 v for 90 min (gradient) and 5000 v for 25 min (gradient) with constant electric current of 50 mA/strip. After the IEF, the strips were washed twice with ultrapure water then equilibrated by a two-step-equilibration on a rocking platform for 15 min. Initially, strips were equilibrated in equilibration buffer I (DDT-equilibration buffer) (Appendix 1) followed by a washing step with ultrapure water then a second equilibration was performed by equilibration buffer II (Iodacetamide-equilibration buffer) (Appendix 1). The second-dimension protein separation was done using 12% (non-gradient) IPG/prep-well Mini-Protean TGX™ Precast gels (Bio-Rad, USA). The gels were assembled in the same electrophoresis system used in the 1D PAGE step then 150 µL of agarose sealing solution (Appendix 1), pre-heated at 89°C in heat block, was placed on top of the gels followed by placement of IPG strips. A filter paper soaked with 5 µL of precision protein dual colour molecular weight standard (Bio-Rad, USA) was added on the anodic (positive) end of the strip. An extra 100 µL of the agarose sealing solution was added to fix the strip and the filter paper with the marker in tight contact with the gel. Electrophoresis and gel staining were performed as mentioned in Section 2.2.3. Following staining, gels were scanned with Image scanner III (GE Healthcare, Sweden) using lab scan software and the images were viewed using Irfan view image viewer software. Three technical replicate gels were used for analysis.

2.2.5. Image analysis and statistical significance

The gel images were analysed using ImageMaster™ 2D Platinum 7 (GE Healthcare, UK). The outer edges of the images were identically cropped using the automated crop tool. Stain background were filtered and the standardised areas of interest from all gels were matched and wrapped. Matching of gel images was performed using the software and by visual inspection as well. Density of each protein spot was normalised by linear regression model and statistical significance of protein spot expression was determined using ANOVA in the ImageMaster™ 2D Platinum 7 software package, where statistical significance level was set at $p < 0.05$. Gel spots showing highly significant different

abundance between isolates were excised from the gel using EXQuest Spot cutter (Bio-Rad, USA) to be used for protein identification.

2.2.6. Immunoblotting

2.2.6.1. Fish serum

Archived hyper immune serum samples collected from red Nile tilapia (12 ± 0.2 g), following an intraperitoneal (i.p.) vaccination and challenge were used in this experiment. The fish were stocked in triplicate static 15 L tanks with 20 fish each, i.p. injected with 0.1 mL of adjuvanted inactivated vaccine produced using *Fno* UK isolate (STIR-GUS-F2f7) at a concentration of 1×10^9 CFU/mL at 28 ± 2°C, then i.p. challenged at 23± 2°C with 0.1 mL of lethal dose (LD_{70}) of *Fno* UK (1×10^6 CFU/mL), *Fno* Mexican (3.1×10^4 CFU/mL), *Fno* Austrian (3.1×10^5 CFU/mL) or *Fno* Costa Rican (3.1×10^4 CFU/mL) isolates. The individual serum samples were obtained at 30 days post-vaccination (dpv) from vaccinated and mock vaccinated fish (PBS only) and at 15 days-post challenge (dpc) from survivor fish in all the challenged groups. Anti-*Fno* UK IgM were measured by indirect ELISA and serum samples with high levels were used for performing western blotting. Data of the tilapia vaccination trial is summarised in Table 2.2.

Table 2.2. Summary of tilapia vaccination experiment.

Fish serum	OD_{450} of Anti-*Fno* IgM measured by ELISA (at a serum dilution of 1:500)*					CPM (%)	RPS (%)
	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5		
Vaccinated (30 dpv)	0.690	0.557	0.643	0.0575	0.079	-------	--------
PBS control (30 dpv)	0.126	0.0614	0.201	0.128	0.215	-------	--------
Vaccinated and *Fno* UK challenged (15 dpc)	1.21	1.09	1.23	1.14	0.9035	50%	47%
Vaccinated and *Fno* Mexico challenged (15 dpc)	1.01	1.12	0.96	0.849	1.078	83.3%	11.7%
Vaccinated and *Fno* Austria challenged (15 dpc)	1.06	0.969	1.095	0.9545	1.15	83.3%	11.7%
Vaccinated and *Fno* Costa Rica challenged (15 dpc)	1	1.13	0.93	0.925	1.025	80%	12.6%
Non-vaccinated (PBS only) and *Fno* UK challenged (15 dpc)	1.06	0.89	0.98	0.993	1.13	94.4%	--------
Non-vaccinated (PBS only) and *Fno* Mexico challenged (15 dpc)	0.861	0.918	0.829	0.815	0.902	94.4%	--------
Non-vaccinated (PBS only) and *Fno* Austria challenged (15 dpc)	1.13	0.92	0.95	0.97	0.876	94.4%	--------
Non-vaccinated (PBS only) and *Fno* Costa Rica challenged (15 dpc)	0.752	0.90	0.892	0.532	0.91	91.6%	--------

* OD_{450} readings of serum IgM are average of 2 parallel wells. CPM: Cumulative percent mortality, RPS: Relative percent of survival.

2.2.6.2. 1D Western blotting

Immunoblotting was performed to detect the antigenic protein bands in the *Fno* whole cell lysate of the different isolates using pooled hyper-immune sera (*n*= 3) collected from survivor tilapia 15 dpc with 10^6 CFU/mL (LD_{60}) of *Fno* STIR-GUS-F2f7 isolate as described in Section 2.2.6.1. Individual sera used in the pool had high levels of specific anti-*Fno*-IgM, as measured by indirect ELISA (Table 2.2). Pooled sera (*n*=3) from mock vaccinated tilapia (PBS only) was used as a negative control.

The whole cell lysates of the five *Fno* isolates were resolved on a 12% NuPAGE™ Novex® Bis-Tris gel (NuPAGE™ , Invitrogen, USA) in NuPAGE™ MES-SDS running buffer (ThermoFisher Scientific, UK) at 200 V (constant) for 45 min. Folllwoing electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Invitrogen, USA) at 30 V (constant) for 45 min using 1X NuPAGE™ Transfer buffer (ThermoFisher Scientific, UK). Two separate membranes were used in this experiment, one for blotting with the *Fno* UK infected fish sera pool while the other was used for the control fish sera pool. Following transfer, the membranes were washed for 5 min in Tris-buffer saline (TBS) (Appendix 1) and blocked overnight at 4°C in TBS with 5% (w/v) dried skimmed milk (Marvel, Premier Foods Group Ltd, UK). After washing 3 times with TBST (Appendix 1) for 10 min on each wash, the membranes were incubated for 3 h at RT (~22ºC) with continuous agitation on a Gyro-rocker (Stuart Scientific, UK) with 5 mL of 1:50 diluted convalescent or control pooled fish sera in TBS with 1% (w/v) BSA (Sigma-Aldrich, UK), respectively. Washing was repeated as described before, then 5 ml of 1:50 mouse anti-tilapia IgM monoclonal antibody (Mab) (Fo4, Aquatic Diagnostics Ltd, UK) in TBS was added to each membrane and incubated with continuous shaking at RT for 1 h. Following washing, 5 mL of 1:200 goat anti-mouse HRP MAb (Sigma-Aldrich, UK) in TBS was added to each membrane with incubation for 1 h at RT. The membranes were then washed 3 times with TBST and once with TBS for 5 min, before the reaction was developed by adding 5 mL of ImmPACT DAB peroxidase substrate (Vector laboratories Ltd, USA) to the membranes with incubation for 2 min. The reaction was stopped by addition of 5 mL of distilled water. Membranes were left to dry then scanned using an Epson expression 1680 artist scanner (Epson, USA). Two technical replicate immuno-blots were prepared to ensure reproducibility.

2.2.6.3. 2D Western blotting

2D-immunoblotting was only preformed for the *Fno* UK isolate as this isolate was used previously for vaccine formulation (Ramirez-Paredes, 2015). 2D-PAGE of the *Fno* UK was done as described in Section 2.2.4 and western blot was performed as described in 1D blotting in Section 2.2.6.2. Four blots were prepared in this experiment, the first was blotted with pooled vaccinated fish sera 30 dpv (*n*= 3), the second was blotted with pooled control (PBS only) fish sera (*n*= 3), the third membrane was blotted with pooled sera from vaccinated fish which survived challenge with 10^6 CFU/mL (LD_{60}) of *Fno* UK isolate 15 dpc (*n*= 3) and the fourth membrane was blotted with pooled sera obtained from survivor non-vaccinated fish (*n*= 3) after challenge with 10^6 CFU/mL (LD_{60}) of *Fno* UK isolate. Membranes were left to dry, scanned as described in Section 2.2.6.2, then matched using the image analysis software (ImageMaster™ 2D Platinum 7) followed by a manual inspection for the spot detected by the software.

2.2.7. Identification of proteins of interest

2.2.7.1. In-gel tryptic digestion and identification of antigenic proteins on 1D-gel by liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS/MS)

Gel band on whole cell lysate corresponding to the immuno-dominant band on the 1D blot was excised and sliced horizontally from top to bottom to yield a series of equal slices of 2.5 mm deep. Each of the resulting gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis procedures as previously described by Shevchenko *et al.* (1996), with some modifications. Briefly, the gel slices were destained in 100 µL destaining solution (Appendix 1) at RT (~22°C) for 45 min on a KZ-variable speed vortex mixer (Cole-Parmer, UK) with removal of the destaining solution each 15 min. 100 µL of dehydration solution (Appendix 1) was added and mixing was done as before for 30 min with removal of solution every 10 min. Gel pieces were spun down at 2,000 × *g* for 30 s and all liquid was removed. Reduction was done by adding 100 µL of reduction solution (Appendix 1) followed by incubation at 50°C for 30 min then the liquid was removed by centrifugation as described above. 100 µL of alkylation solution (Appendix 1) was added followed by 30 min incubation at RT in the dark and liquid was removed by centrifugation at 2,000 × *g* for 20 min. Rehydration was repeated for 10 min and the gel pieces were dried in a SpeedVac centrifuge (Eppendorff, USA) for 30 min.

Digestion buffer (Appendix 1) at afinal concentration of 20 ng/ μL was added followed by incubation for 16 h at 37°C. The peptides were collected by centrifugation, dried in the SpeedVac centrifuge and resuspended in 0.1 % trifluoroacetic acid (Sigma-Aldrich, UK). The samples were transferred to HPLC sample vials and stored at 4°C until required for LC-ESI-MS/MS analysis. Liquid chromatography (LC) was performed following the protocol described by Batycka *et al.* (2006) using a Dionex Ultimate 3000 nano-HPLC system (ThermoFisher Scientific, Hemel Hempstead, UK) comprising a WPS-3000 well-plate micro auto sampler, a FLM- 3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micro-pump and an SRD- 3600 solvent rack controlled by Chromeleon chromatography software (www.thermoscientific.com/dionex). Briefly, a micro-pump flow rate of 246 μL/min was used in combination with a cap-flow splitter cartridge, affording a 1/82 flow split and a final flow rate of 3 μL/min through a 5 cm × 200 μm I.D. monolithic reversed phase column (ThermoFisher Scientific, UK) maintained at 50°C. Samples of 4 μL were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8% to 45% solvent (80% acetonitrile, 0.1% (v/v) formic acid) and directed through a 3 nL UV detector flow cell. LC system was interfaced directly with a 3-D high capacity ion trap mass spectrometer (Esquire HCTplus™, Bruker Daltonics, Bremen, Germany) via a low volume (50 μL/min maximum) stainless steel nebuliser (G1946-20260 Agilent Technologies, Wokingham, UK) and ESI. Parameters for tandem MS analysis were set as previously described (Batycka *et al.,* 2006) including the following conditions: spray voltage 4500 V; ion charge control 300–1700 (standard–enhanced); MS/MS scan (m/z) 200-2000 (ultrascan); precursor numbers = 4; active exlusions 0.5–0.8/ min; averaging 2–5 r and neublizer power 15 psi. Technical controls included BSA standard and a blank gel slice.

2.2.7.2. In-gel tryptic digestion and identification of antigenic protein spots on 2D gels by MALDI-TOF-MS.

In-gel-digestion of the spots of interest was done using the same protocol described in Section 2.2.7.1. The digested concentrated peptides were applied to a steel MALDI sample target plate in a solution of 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1 % trifluoro acetic acid and 50% acetonitrile (ACN). MS spectra of the targeted protein spots

were obtained using an Ultraflex II TOF/TOF instrument (Bruker Daltonics, USA) operated in the reflectron mode. The instrument was calibrated using known peptide standards (Bruker Daltonics PepMix 2, USA). Each spectrum was produced by accumulating data from 10 × 100 consecutive laser shots.

2.2.8. Data analysis and database mining

Deconvoluted MS/MS data in Mascot generic format (mgf) was imported into ProteinScape™ V3.1 (Bruker Daltonics, USA) for downstream database mining of the available annotated cognate chromosomal and plasmid *Fno* protein database derived from genomic sequences available at the National Centre for Biotechnology Information (Genbank), (http://www.ncbi.nlm.nih.gov) (Table 2.3.) and the NCBInr *Fno* sub-database, utilising the Mascot™ V2.5.1 (Matrix Science, London, UK) search algorithm (Perkins *et al.,* 1999). The protein content of the individual gel slices was established using the "Protein Search" and "Protein Compilation" features of the ProteinScape™ software. The separate compilation of the proteins contained in the gel slices of each of gel replicates was formed using the "protein extractor" feature of the software. Data was searched specifying Trypsin and Trypsin/P. Spectra used for protein identifications were re-searched against the available database to ensure accurate peptide assignments. Mascot search parameters were set in accordance with published guidelines (Taylor and Goodlett, 2005). To this end, fixed (carbamidomethyl "C") and variable (oxidation "M" and deamidation "N,Q") modifications were selected along with peptide (MS) and secondary fragmentation (MS/MS) tolerance values of 0.5 Da, whilst allowing for a single ^{13}C isotope. Molecular weight search (MOWSE) scores attained for individual protein identifications were inspected manually and considered significant only if two or more peptides were matched for each protein, and each matched peptide contained an unbroken "b" or "y" ion series represented by a minimum of four contiguous amino acid residues.

For the MALDI-TOF, peptides were identified by matching the measured monoisotopic masses to theoretical monoisotopic masses generated using MASCOT V 2.5.1 search engine, peptide mass fingerprinting (PMF). The search parameters were maximum of one missed cleavage by trypsin, variable modification of oxidation of methionine (M), modification of cysteine by propionamidation and carbamidomethylation (C), peptide tolerance of ± 50 ppm, fragment mass tolerance of ± 0.2 Da and peptide charge of +1. The available *Fno* database (Table 2.3.) was used for downstream mining

and Mascot scores greater than 43 were considered significant (P <0.05). A minimum of two peptide fragmentation spectra, peptide z-score higher than six and p-value lower than 10^6 were required for successful match achievement.

Table 2.3. *Fno* genomes used in this study

Bacteria ID	Source	Genbank accession no.	Genome status	Reference
Fno STIR-GUS-F2f7	Tilapia (UK)	LTD00000000.1	Complete	Ramirez-Paredes *et al.* (2017a)
FNO01	Tilapia (Brazil)	CP012153.2	Complete	Figueiredo *et al.* (2016)
FNO12	Tilapia (Brazil)	CP011921	Complete	Gonçalves *et al.* (2016)
FNO24	Tilapia (Brazil)	CP011922	Complete	Gonçalves *et al.* (2016)
FNO190	Tilapia (Brazil)	CP011923	Complete	Gonçalves *et al.* (2016)
Fno LADL--07-285A	Tilapia (Costa Rica)	CP006875.1	Complete	Un published
Fno Toba-04	Tilapia (Indonesia)	NC_017909	Complete	Sridhar *et al.* (2012)

2.2.9. Bioinformatics Analysis

The PSORTb algorithm (http://www.psort.org) was used to predict the subcellular location of identified proteins. The putative functional classification of the identified proteins was obtained by comparison of predicted proteins against clusters of orthologous groups of proteins (COGs) database using the EggNOG v4.5 server (http://eggnog.embl.de). Lipoproteins were identified using the LipoP 1.0 server (http://www.cbs.dtu.dk/services/LipoP-1.0/) and the presence of signal peptides sequence was searched using SignalP 2.0 server (http://www.cbc.dtu/services/Signal/).

2.3. Results

2.3.1. 1D PAGE, immunoblotting and identification of proteins by LC/ESI/MS/MS

The resolved 1D gels showed a homogenous protein pattern in all the whole cell lysates of the five *Fno* isolates with both Coomassie safe blue stain (Figure 2.1A) and silver stain (Figure 2.1B). Immunoblotting with the hyper immune serum revealed a strong immuno-dominant band corresponding to a protein band between 17-28 kDa and another faint band corresponding to a protein band between 49-62 kDa (Figure 2.2B). Blotting with the serum from control fish did not show any immunoreactivity with the whole cell lysate of any of the tested isolates (Figure 2.2C). The abundant immunoreactive protein band between 17-28 kDa observed on the 1D- PAGE of the whole cell lysate of *Fno* UK isolate was selected for identification by LC/ESI/MS/MS. Analysis of this band revealed a total number of 47 proteins, which were confidently identified and catalogued after downstream searching of the available *Fno* database. The identified proteins are listed in Table 2.4.

The protein extracts digested with proteinase-K showed no protein bands when stained with Coomassie blue stain, while an abundant band between 20-37 kDa and another faint band between 15-20 kDa were observed when stained with Silver stain in all the isolates. The digested and non-digested *Fno* whole cell proteins resolved in the 1D gel after staining with both stains are shown in Figure 2.3.

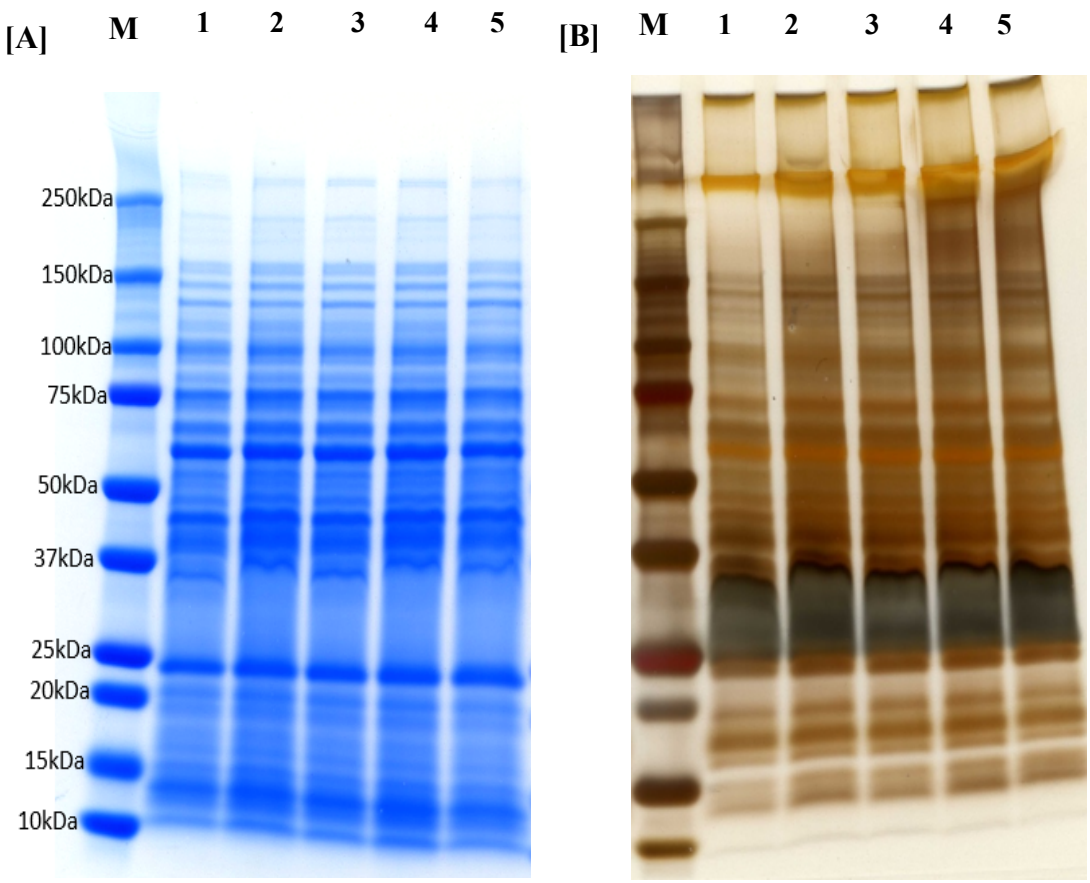


Figure 2.1. 4-15% gradient 1D-SDS PAGE showing the protein profile of the whole cell lysate of five *Fno* isolates. (A) 1D gel stained with SimplyBlue Safe Coomassie stain, (B) 1D gel stained with ProteoSilver™ plus silver stain. Lanes: M: protein standard; 1: *Fno* UK isolate; 2: *Fno* Austrian isolate; 3: *Fno* Mexican isolate; 4: *Fno* Costa Rican isolate; 5: *Fno* Japanese isolate. Each gel is a representative of duplicate gels analysed.

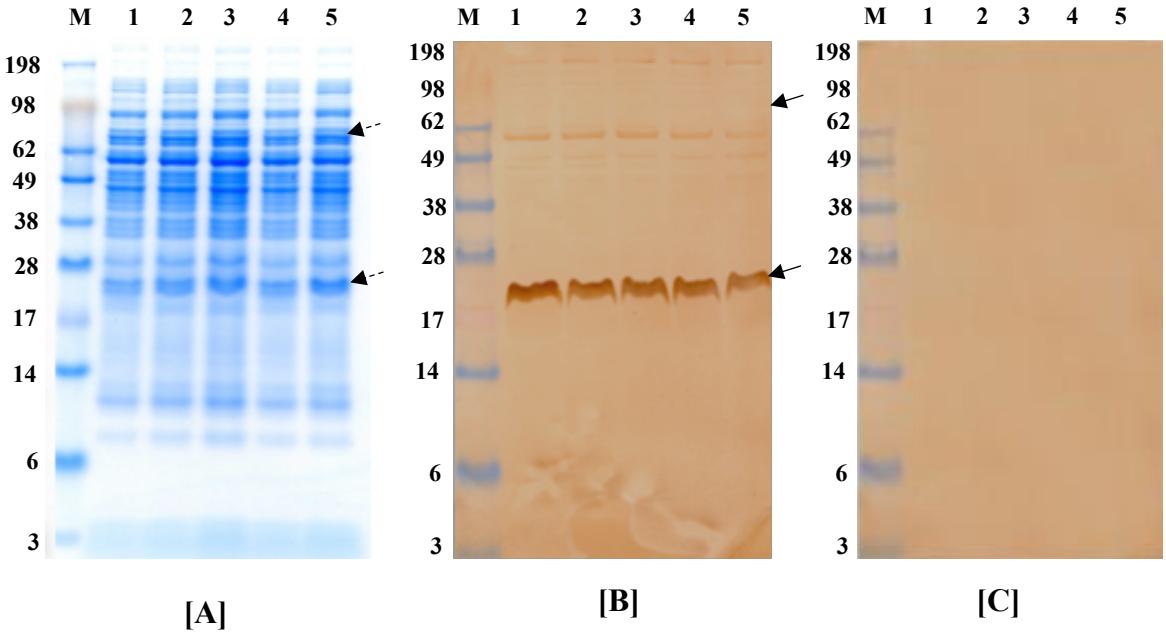


Figure 2.2. 1D Western blot of the different *Fno* isolates using tilapia hyper-immune serum. SimplyBlue Safe Coomassie stained 12% non-gradient 1D-SDS PAGE (Reference gel) (A) and representative immunoblots of whole cell lysate of 5 *Fno* isolates (B; C). A conserved strong immuno-dominant band between 17-28 kDa and faint antigenic band between 49-62 kDa were highlighted in all whole cell extracts when screened with hyperimmune serum (HIS) collected from surviving tilapia (B), while no immunoreactivity was observed with the control serum (C). Solid arrow refers to the immunogenic bands in the blots and dotted arrows refer to their corresponding protein bands in the reference 1D gel. M: protein standard; Lane 1: UK isolate; Lane 2: Austrian isolate; Lane 3: Mexican isolate; Lane 4: Costa Rican isolate; Lane 5: Japanese isolate. Each blot is a representative of duplicate blots.

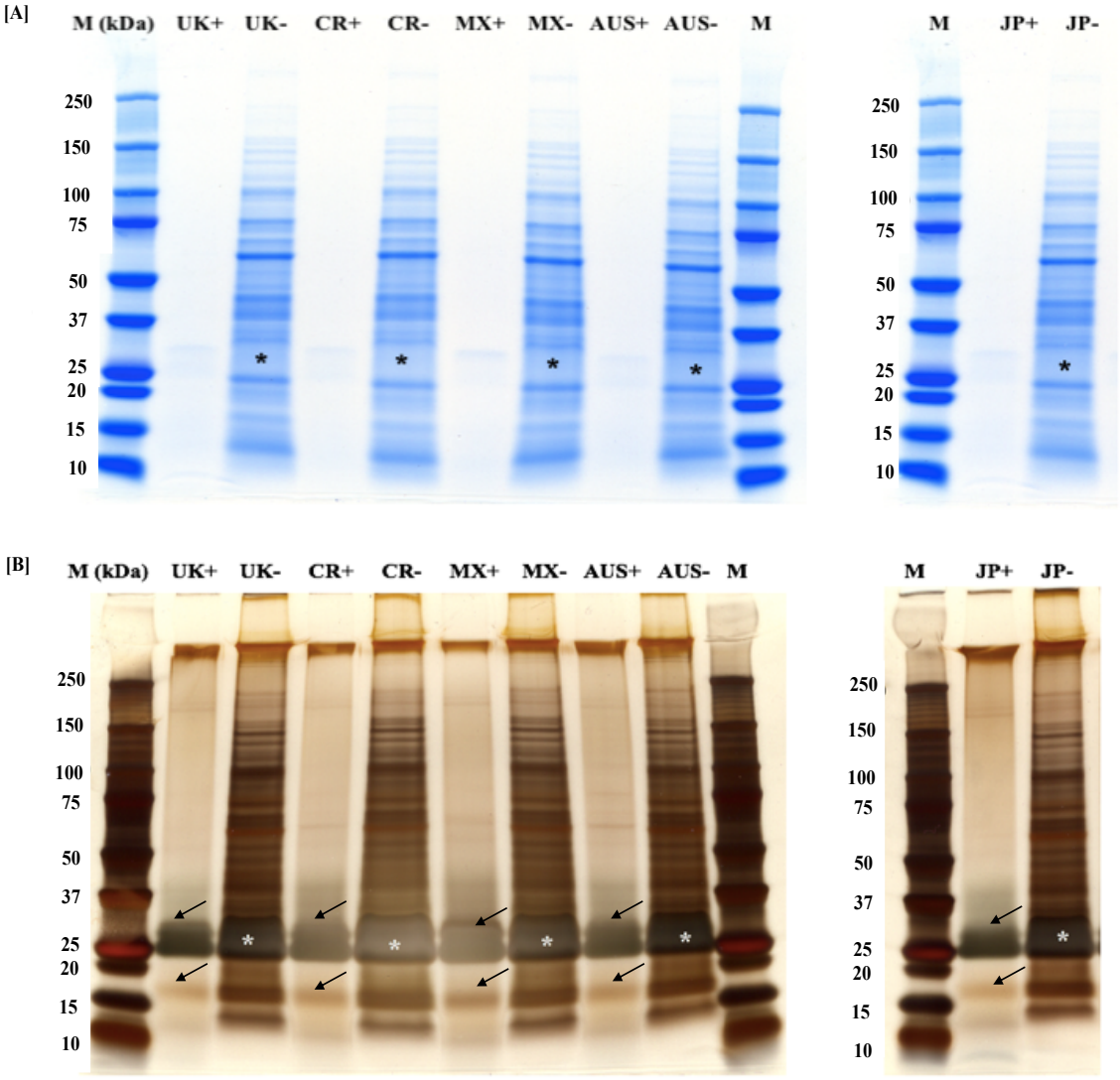


Figure 2.3. Proteinase-K digestion of the whole cell lysate of five *Fno* isolates. 4-15% gradient 1D PAGE of whole cell lysate of different isolates of *Fno* stained with SimplyBlue Safe Coomassie stain (A) and ProteoSilver™ plus silver stain (B). M: Protein marker, UK+: Protienase-K digested *Fno* UK isolate, UK-: non-treated *Fno* UK isolate, CR+: Protienase-K digested *Fno* Costa Rican isolate, CR-: non-treated *Fno* Costa Rican isolate, MX+: Protienase-K digested *Fno* Mexican isolate, MX-: non-treated *Fno* Mexican isolate, AUS+: Protienase-K digested *Fno* Austrian isolate, AUS-: non-treated *Fno* Austrian isolate, JP+: Protienase-K digested *Fno* Japanese isolate, JP-: non-treated *Fno* Japanese isolate. Asterisks (black and white) refer to the original abundant band before proteinase-K treatment. Arrows refer to the observed bands on the digested protein extracts of all *Fno* isolates.

Table 2.4. Identified proteins in the immunoreactive band (~17-28 kDa) of the whole cell lysate of *Fno* UK isolate

	Accession no.	Protein name	MW [kDa]	pI	Scores	Peptides	SC [%]
1	gi|300193842|	IglC	22.1	5.3	1304.4	13	74.7
3	gi|504528404|	enoyl-ACP reductase I	27.7	5.5	422.3	8	49
4	gi|564748870|	3-methyl-2-oxobutanoate hydroxymethyltransferase	28.1	5.6	789.7	14	60.0
5	gi|386871721|	Carbonate dehydratase	26.0	6.0	728.1	13	76.5
6	gi|504527238|	50S ribosomal protein L5	20.0	9.7	651.7	14	65.9
7	gi|505427577|	50S ribosomal protein L1	24.5	9.5	641.5	13	50.6
8	gi|504527248|	30S ribosomal protein S4	23.2	10.4	618.2	13	53.9
9	gi|386872019|	Oxidoreductase, short-chain dehydrogenase family protein	26.0	5.3	603.9	10	49.6
10	gi|386870855|	Septum formation inhibitor protein	24.7	6.3	541.6	10	51.6
11	gi|504527226|	50S ribosomal protein L3	22.1	9.5	498.8	8	48.6
12	gi|504527828|	IglA	20.4	8.6	494.2	9	47.2
13	gi|386871670|	Purine-nucleoside phosphorylase	26.9	5.2	486.7	9	43.8
2	gi|504527915|	AhpC/TSA family peroxiredoxin	21.8	5.0	472.4	15	65.9
14	gi|504527815|	Beta-ketoacyl-ACP reductase	26.3	9.6	460.0	7	48.2
15	gi|386871074|	Superoxide dismutase	22.0	5.7	453.5	8	51.0
16	gi|504527224|	30S ribosomal protein S7	17.8	10.1	793.8	13	55.4
17	gi|504527529|	Succinate dehydrogenase iron-sulfur subunit	26.5	8.8	415.7	8	38.6
18	gi|504527053|	Chorismate mutase	20.3	9.2	238.2	4	16.7
19	gi|504527242|	30S ribosomal protein S5	17.5	10.0	385.8	6	40.4
20	gi|504527683|	DNA-binding response regulator	25.5	6.2	368.4	5	32.0
21	gi|504527216|	30S ribosomal protein S2	26.5	8.8	368.1	6	24.7
22	gi|386871086|	Triose-phosphate isomerase	27.6	5.0	347.5	6	42.3

Table 2.4. continued

23	gi|504527227|	50S ribosomal protein L4	22.5	10.0	333.7	6	35.7
24	gi|752587925|	SAM-dependent methyltransferase	24.7	9.0	322.1	5	26.5
25	gi|855345305|	Transcription antitermination/antitermination protein NusG	20.0	6.8	312.7	7	41.2
26	gi|386870760|	Ribosome recycling factor	20.6	5.4	282.7	5	32.4
27	gi|504527082|	Hypothetical protein_OOM_0066	25.3	5.6	177.2	4	27.6
28	gi|504527834|	Hypothetical protein	24.3	5.6	274.5	5	33.5
29	gi|504528221|	Acyl dehydratase	19.7	6.6	170.3	3	26.8
30	gi|386871930|	Deoxycytidine triphosphate deaminase	21.1	6.6	239.9	5	36.7
31	gi|504527232|	30S ribosomal protein S3	24.5	10.0	411.1	8	44.4
32	gi|504527329|	OmpA family peptidoglycan-associated lipoprotein	23.4	4.8	233.4	5	32.5
33	gi|386872073|	Nucleoside-triphosphate-adenylate kinase	24.2	6.9	233.4	4	22.7
34	gi|504527240|	50S ribosomal protein L6	19.2	9.7	229.1	4	23.0
35	gi|386871705|	Rhodanese-like family protein	27.9	5.0	205.3	6	26.5
36	gi|386817600|	Protein-L-isoaspartate O-methyltransferase	23.6	5.6	160.7	4	30.1
37	gi|504527492|	NADH-quinone oxidoreductase subunit-C	25.3	6.5	160.4	4	29.3
38	gi|564748113|	Activator of osmoprotectant transporter	27.6	9.0	137.0	4	24.6
39	gi|386871417|	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase	20.1	5.6	125.8	3	24.0
40	gi|386870946|	Chromosome partition protein A, ATPase	23.7	5.3	123.0	2	13.2
41	gi|504527846|	CBC domain pair protein	22.9	6.8	114.7	2	18.0
42	gi|504527578|	50S ribosomal protein L10	18.7	9.1	110.8	2	20.3
43	gi|504527806|	Hypothetical protein OOM_0903	27.7	8.4	103.3	3	15.1
44	gi|386871751|	Isoprenoid biosynthesis protein	23.7	4.6	98.4	2	10.5
45	gi|504527599|	Hypothetical protein OOM_0658	23.7	6.1	96.8	2	10.4
46	gi|386871418|	Inorganic diphosphatase	19.5	4.8	71.6	2	15.6
47	gi|386871579|	Hypothetical protein OOM_1153	25.3	5.4	68.4	2	14.9

Mwt: Molecular weight; SC [%]: Sequence coverage percent; pI: Iso-electric focusing point.

2.3.2. 2D PAGE, immunoblotting and identification of proteins by MALDI-TOF/MS

Following staining of the whole cell proteins of the five isolates, well resolved and reproducible 2D gels were produced as shown in Figure 2.4. Following the automated spot detection in the image analysis software (ImageMaster™), high similarity in distribution of the protein spots between the isolates and an average of 73 matched protein spots were detected unambiguously in all gels, while 2 protein spots (defined as 57, 58) were only detected in the 2D gels of *Fno* Japanese, Mexican, Austrian and Costa Rican isolates and absent in all gel replicates of the *Fno* UK isolate (Figure 2.5). Confirmation of the spot detection was done using the class analysis feature in ImageMaster™. Variations in the spot intensities were also detected after comparing the gels, where the abundance of 15 protein spots were significantly different between isolates ($p < 0.05$) (Table 2.5). Three of these spots (56, 57 and 58) showed high significant difference in their intensities between the gels ($p < 0.0001$). Significantly higher abundance of 10/15 of these protein was observed in the *Fno* UK whole cell lysate, while significantly higher abundance of 2/15 was observed in both the Japanese and Mexican *Fno* isolates and 1/15 was significantly higher in the *Fno* Costa Rican isolate. In addition, significantly lower abundance of two proteins were observed in the Austrian strain only compared to the other *Fno* isolates. Protein spots that were missing in the UK isolate or those showed highly significant differences in their intensity/abundance between the different isolates (spots 56; 57; 58) were subjected to identification by MALDI-TOF-MS. Analysis showed that all the three selected proteins were identified as "Aconitate hydrates" that also displayed multiple charge variants (Table 2.6).

Table 2.5. Abundance and statistical analyses of matched spots in 2-DE of 5 *Fno* isolates

Fno isolates		Japanese isolate	UK isolate	Mexican isolate	Austrian isolate	Costa Rican isolate	
Spot	Match count	Density of spots					Stats (ANOVA)
1	5	0.094748	0.09716	0.124118	0.073305	0.090195	NS
2	**5**	**1.730390**	**1.99387↑**	**1.835790**	**1.815881**	**1.720011**	**
3	5	1.844110	1.72557	1.423520	1.473521	1.748050	NS
4	5	0.147536	0.12490	0.171677	0.179181	0.126230	NS
5	5	0.085782	0.07208	0.089223	0.054544	0.054112	NS
6	5	0.175736	0.15078	0.169914	0.123109	0.141955	NS
7	5	0.181725	0.20085	0.176306	0.194875	0.150568	NS
8	5	8.259901	7.18432	6.267650	6.016310	7.423491	NS
9	5	0.071566	0.05167	0.068959	0.054316	0.043227	NS
10	**5**	**2.705111**	**3.05892↑**	**2.513631**	**2.418775**	**2.727912**	*
11	5	0.105820	0.09210	0.103413	0.079054	0.077779	NS
12	5	0.303575	0.37762	0.303154	0.293768	0.294687	NS
13	5	0.575145	0.52014	0.463341	0.394732	0.501969	NS
14	5	2.008650	1.80723	1.948122	2.132020	1.858382	NS
15	5	0.898633	1.04252	0.935625	1.005761	1.042792	NS
16	5	1.614191	1.46542	1.484113	1.734991	1.523023	NS
17	5	1.172660	1.11321	1.366261	1.243210	1.335304	NS
18	5	0.078258	0.09485	0.077931	0.069849	0.075794	NS
19	5	0.894911	1.00091	0.906052	1.020090	0.960755	NS
20	5	1.107091	1.07909	0.997563	0.978928	1.018751	NS
21	5	1.090322	1.19339	1.248711	1.152131	1.262891	NS
22	5	1.542191	1.31269	1.320961	1.592010	1.371970	NS
23	5	0.150691	0.14423	0.143639	0.115394	0.121683	NS
24	5	0.047792	0.06237	0.055938	0.048826	0.052949	NS
25	5	2.320920	2.78374	2.358032	2.967091	2.624230	NS
26	**5**	**0.078137**	**0.18604↑**	**0.119553**	**0.099245**	**0.066148**	*
27	**5**	**0.137551**	**0.24101↑**	**0.165299**	**0.151702**	**0.177732**	*
28	5	0.997432	1.04121	1.002712	0.990866	1.072699	NS
29	5	0.033204	0.01661	0.020673	0.013254	0.027708	NS
30	5	9.504591	8.28893	9.112441	10.24430	9.515443	NS
31	**5**	**0.391853**	**0.49900↑**	**0.414952**	**0.400841**	**0.382592**	*
32	5	0.810006	0.90209	0.879067	0.884858	0.928525	NS
33	5	1.293972	1.16037	1.203241	1.266871	1.251276	NS
34	5	0.686557	0.67391	0.800310	0.788019	0.862129	NS
35	5	0.204072	0.21842	0.187659	0.207349	0.186918	NS
36	**5**	**0.791596**	**1.36584↑**	**0.888610**	**0.490198**	**0.542919**	**
37	**5**	**0.054599**	**0.10512↑**	**0.046862**	**0.039047**	**0.053033**	*
38	5	0.083494	0.11328	0.101476	0.120410	0.099811	NS
39	5	0.071243	0.07283	0.116764	0.091799	0.102298	NS
40	5	0.653177	0.86085	0.692062	0.726569	0.737331	NS
41	5	24.98730	23.9513	24.36141	25.24160	25.40736	NS

Table 2.5. continued

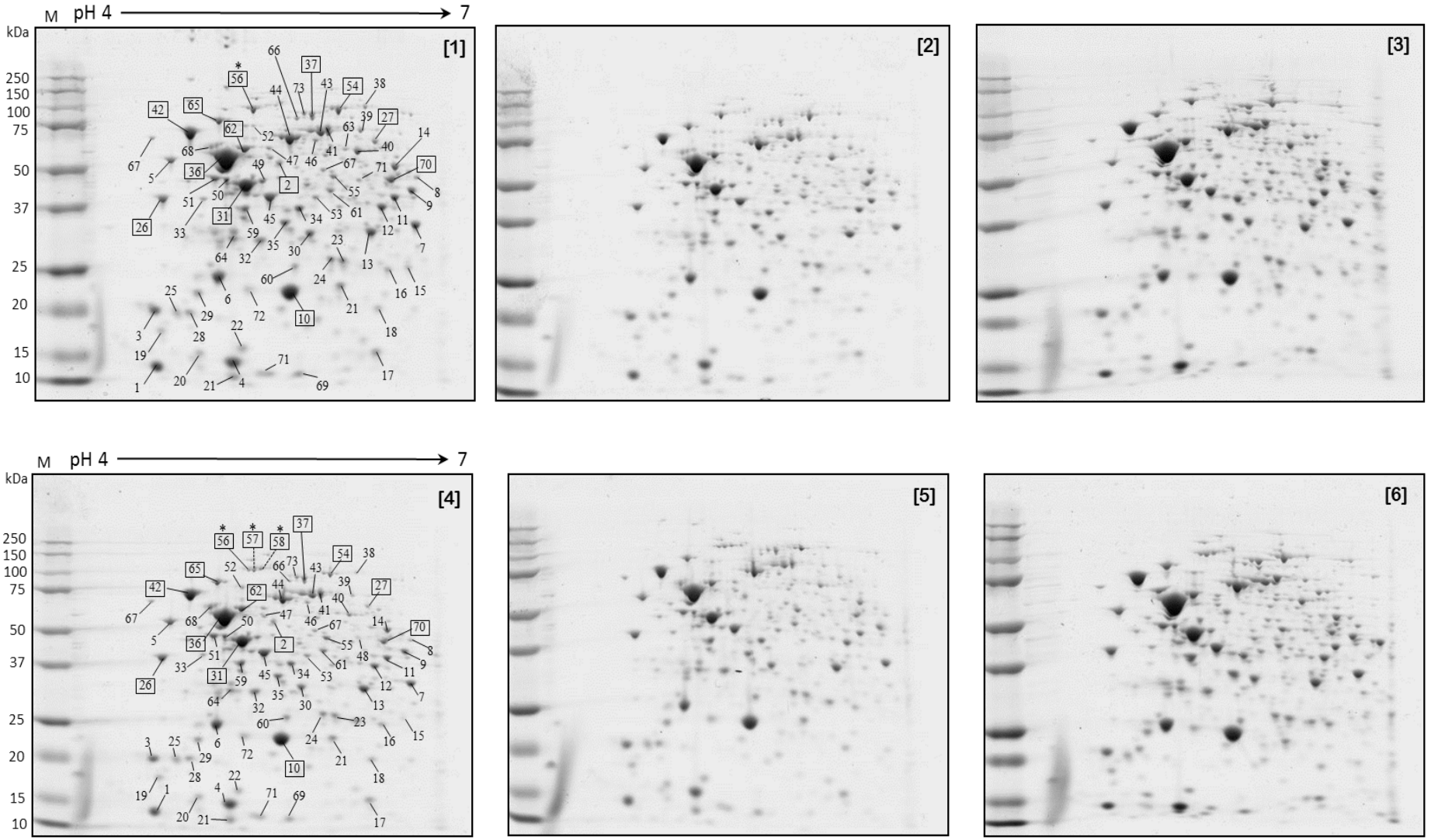
42	**5**	**1.953331**	**2.29020↑**	**2.063331**	**2.100101**	**2.023337**	*
43	5	0.146421	0.12379	0.160965	0.148257	0.142921	NS
44	5	0.115059	0.14466	0.128464	0.126525	0.129655	NS
45	5	4.562882	4.38837	4.583931	4.547931	4.958848	NS
46	5	0.520711	0.54139	0.541453	0.447709	0.513428	NS
47	5	1.732472	1.90985	1.867571	1.899760	1.786410	NS
48	5	1.220853	1.34472	1.325412	1.309510	1.240378	NS
49	5	0.511506	0.42174	0.598469	0.531120	0.448811	NS
50	5	7.209882	6.72228	6.302523	7.007221	6.818084	NS
51	5	1.099221	1.07984	1.210241	1.208811	1.166955	NS
52	5	0.462441	0.40849	0.505604	0.418394	0.404338	NS
53	5	0.148147	0.12517	0.138391	0.130669	0.140828	NS
54	**5**	**0.705584**	**1.251801**	**1.12895↑**	**1.204057**	**0.906619**	*
55	5	0.388511	0.35147	0.248408	0.370275	0.336576	NS
56[a,b]	**5**	**0.140264**	**1.06201↑**	**0.110433**	**0.050706↓**	**0.106453**	****
57[a]	**4**	**0.290101↑**	**----------**	**0.210617**	**0.117562**	**0.248577**	***
58[a]	**4**	**0.192966↑**	**----------**	**0.120511**	**0.051151↓**	**0.147096**	***
59	5	0.125754	0.139611	0.135714	0.11459	0.122522	NS
60	5	0.229151	0.213641	0.211082	0.237551	0.203430	NS
61	5	0.040560	0.022835	0.029111	0.044085	0.025373	NS
62	**5**	**0.807514**	**1.11421↑**	**0.923391**	**0.812479**	**0.925435**	*
63	5	0.147684	0.167319	0.169524	0.160156	0.167939	NS
64	5	0.556329	0.776350	0.732561	0.737944	0.709743	NS
65	**5**	**0.281874**	**0.376215**	**0.306974**	**0.214808**	**0.449110↑**	*
66	5	0.911887	0.895211	0.880191	0.936491	0.950006	NS
67	5	0.531266	0.521372	0.544602	0.545505	0.567681	NS
68	5	0.141887	0.125542	0.118363	0.131818	0.112381	NS
69	5	1.803991	1.887670	1.990812	1.997391	1.865739	NS
70	**5**	**0.350011**	**0.371010**	**0.40660↑**	**0.362112**	**0.350201**	**
71	5	0.215786	0.245720	0.244075	0.237604	0.210836	NS
72	5	0.072622	0.070275	0.072901	0.090270	0.073948	NS
73	5	0.063032	0.066752	0.059972	0.091471	0.064793	NS

[a] Protein spots that were picked for analysis by MALDI-TOF-MS due to absence in some isolates. [b] Protein spots that were picked for analysis by MALDI-TOF-MS due to strong significantly greater abundance in spot intensity. Proteins spots with significantly different abundance are in bold. ↑: High abundance, ↓: Low abundance. Asterisks denote significance difference. NS: not significant, significant difference is represented by stars (NS: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$). All values are average of triplicate readings/spot.

Table 2.6. MALDI-TOF-MS analysis of selected spots in *Fno* isolates with highly significant different intensities between isolates.

Fno isolate	Spot no.	Accession no.	Protein ID.	Mw [kDa]	pI	Scores	SC [%]
UK	56	gi|855345336|	Aconitate hydratase	102.4	5.2	280.0	43.9
Japanese	56	gi|855345336|	Aconitate hydratase	102.4	5.2	119.0	20.0
Japanese	57	gi|855345336|	Aconitate hydratase	102.4	5.2	124.0	23.3
Japanese	58	gi|855345336|	Aconitate hydratase	102.4	5.2	86.8	21.9
Austrian	56	gi|855345336|	Aconitate hydratase	102.4	5.2	68.0	14.3
Austrian	57	gi|855345336|	Aconitate hydratase	102.4	5.2	100.0	26
Austrian	58	gi|855345336|	Aconitate hydratase	102.4	5.2	56.0	13.2
Mexican	56	gi|855345336|	Aconitate hydratase	102.4	5.2	110.0	25.1
Mexican	57	gi|855345336|	Aconitate hydratase	102.4	5.2	118.0	24.6
Mexican	58	gi|855345336|	Aconitate hydratase	102.4	5.2	81.0	22.5
Costa Rican	56	gi|855345336|	Aconitate hydratase	102.4	5.2	95.0	34
Costa Rican	57	gi|855345336|	Aconitate hydratase	102.4	5.2	115.0	28.6
Costa Rican	58	gi|855345336|	Aconitate hydratase	102.4	5.2	84.0	26.4

Mw: Molecular weight, pI: Isoelectric focusing point, SC: Sequence coverage percent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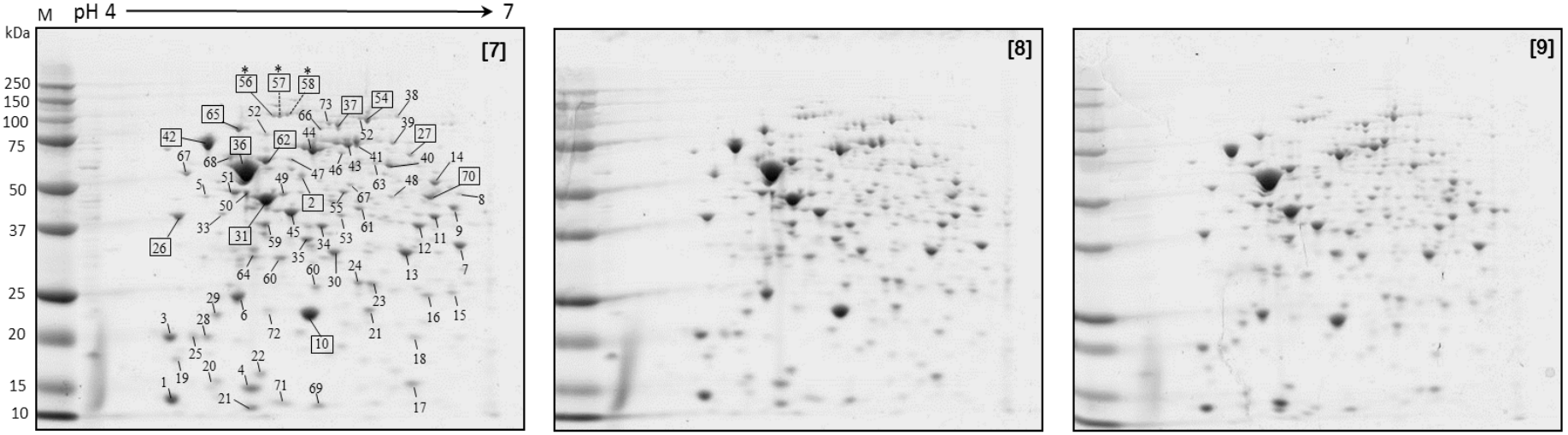
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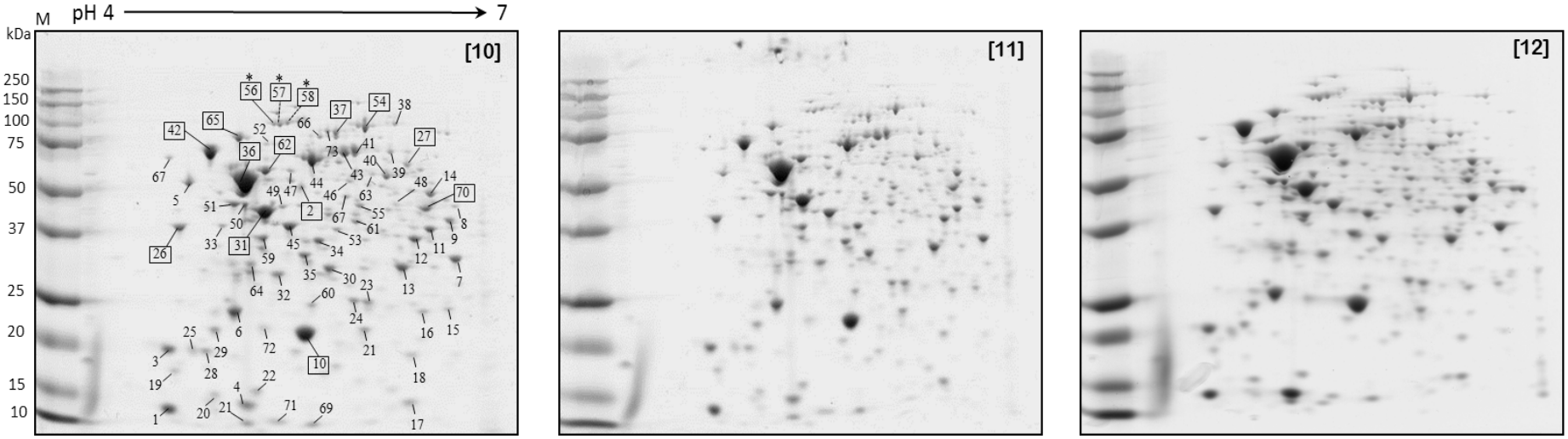
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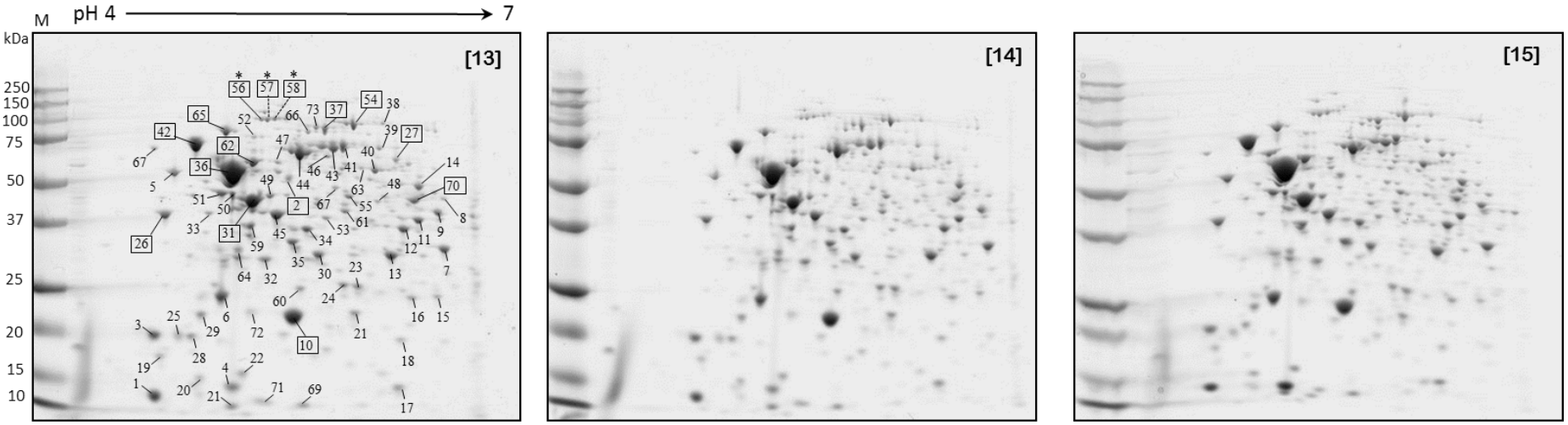


Figure 2.4. 2-DE of whole cell proteins of five different *Fno* isolates after focusing in 7-cm IPG strips, separation on 12% SDS-PAGE gels and staining with SimplyBlue™ Safe Coomassie stain. Molecular mass sizes (in kDa) are noted on the left side of each gel. Plates 1-3: replicates of *Fno* UK isolate, plates 4-6: replicates of *Fno* Japanese isolate, plates 7-9: replicates of *Fno* Mexican isolate, plates 10-12: replicates of *Fno* Austrian isolate, plates 13-15: replicates of *Fno* Costa Rican isolate. Labels in squares refer to spots with significantly different abundance between isolates. Stars mark the spots with the highest significantly different abundance between isolates. Dashed arrows indicate protein spots which are missing in the UK isolate.

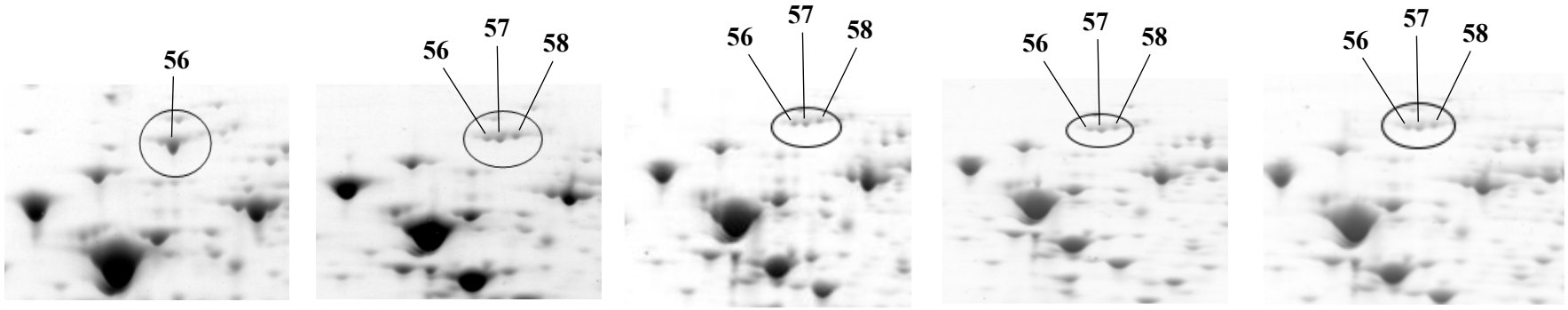
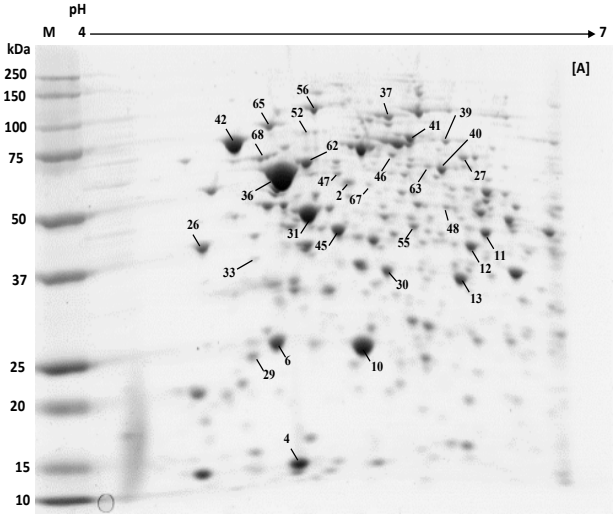


Figure 2.5. Close-up image of the protein spots with highest significant intensity difference between *Fno* isolates (56; 57; 58). These spots were further identified by MALDI-TOF. A: *Fno* UK isolate, B: *Fno* Japanese isolate, C: *Fno* Mexican isolate, D: *Fno* Austrian isolate, E: *Fno* Costa Rican isolate.

The 2D immunoblotting was performed with the resolved whole cell extract of the *Fno* UK isolate only. A total no. of 26, 31 and 30 immunoreactive spots were highlighted by pooled serum samples from vaccinated (Figure 2.6B), vaccinated and challenged (Figure 2.6C) and non-vaccinated challenged tilapia (Figure 2.6D), respectively. No specific spots were recognised by the control fish serum pool (Figure 2.6E). The protein spots in the reference gel corresponding to the immuno-reactive proteins in the different blots were excised by spot cutter and subjected to MALDI-TOF-MS analysis.

The application of MALDI-TOF-MS resulted in successful identification of 28 proteins corresponding to 31 immunoreactive spots, as some of the proteins (n=3) occurred in multiple charge variants. The IDs of these protein spots are listed in Table 2.7 and their positions are marked in the reference 2D gel (*Fno* UK) (Figure 2.6A). Analysis showed that the five proteins, which were not recognised by vaccinated non-challenged sera (26 spots/ 23 proteins) were identified as chaperone protein-DnaK (spot 42), dihydrolipoyllsine acetyle transferase (spot 48), outer membrane associated protein (spot 45), chaperone Hsp-90 Heat shock protein (spot 46) and AhpC/TSA family peroxiredoxin (spot 29) (Figure 2.6B). One protein (spot 30) was not recognised by non-vaccinated challenged sera (30 spots/ 27 proteins) and was identified as glycerophosphoryl diesterase (Figure 2.6D). A summary of immunoreactivity by the serum pools from different treatments is shown in Table 2.8 and Figure 2.7.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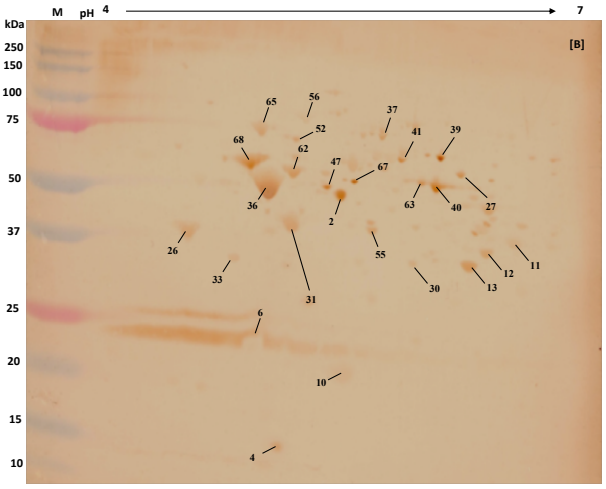
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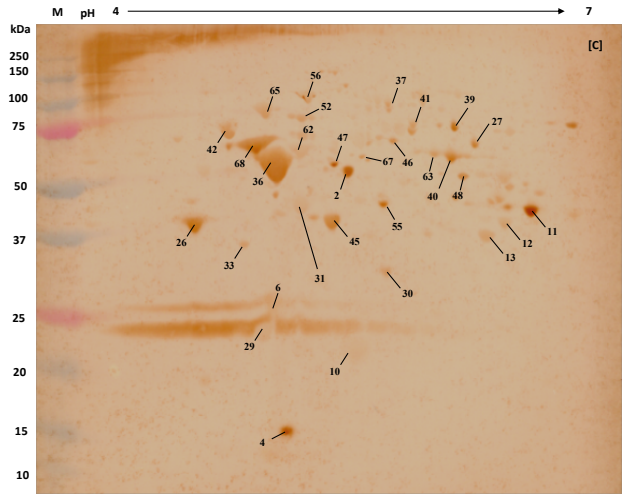
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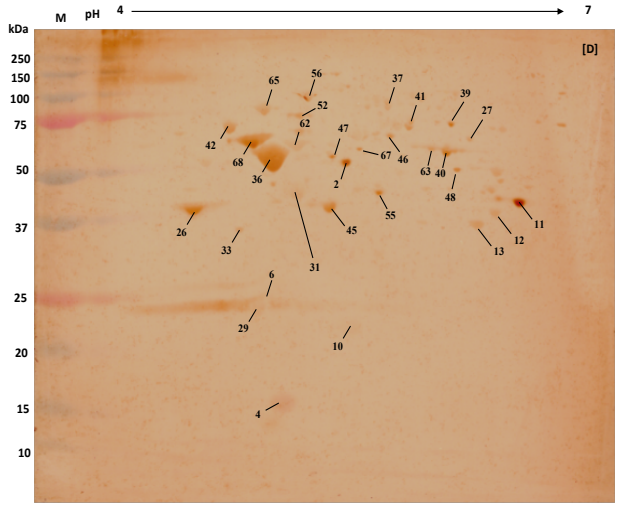
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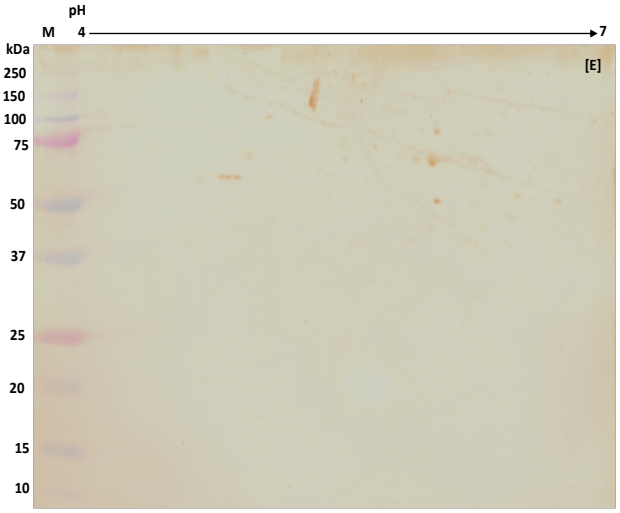
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Figure 2.6. Screening of the *Fno* UK whole cell lysate by vaccinated, control, vaccinated challenged and non-vaccinated challenged sera. (A) 2D PAGE reference map of *Fno* UK proteins. (B-E) representative 2D blots showing immunoreactive proteins of *Fno* UK isolate in pH range of 4-7 recognised by serum from vaccinated fish 30 dpv (B), vaccinated challenged fish 15 dpc (C), non-vaccinated challenged fish 15 dpc (D) and PBS control fish (E). Identified immunoreactive protein spots are designated by numbers that refer to Table 2.7.

Table 2.7. Identified proteins in the reference 2-DE gel of *Fno* UK whole cell lysate using MALDI-TOF-MS corresponding to the immunoreactive spots on the 2D blots.

spot no.	Accession	Protein ID	Mw [kDa]	pI	Scores	Peptides	SC [%]
2	gi|855345175|	Hypothetical protein	51.8	5.7	145.0	23	54.2
4	gi|300193833|	Hypothetical lipoprotein	14.24	5.52	43.7	9	48.4
6	gi|504527329|	OmpA family peptidoglycan associated lipoprotein	23.4	4.8	150.0	20	44.2
10	gi|855345037|	Intracellular growth locus protein C	22.1	5.3	165.0	16	71.8
11	gi|386870866|	OmpA family protein	47.2	6.0	183.0	19	54.5
12	gi|504527821|	Type I glyceraldehyde-3-phosphate dehydrogenase	35.4	5.9	162.0	19	71.5
13	gi|386870616|	Universal stress protein	30.1	5.4	146.0	21	80.0
26	gi|855344042|	Cell division protein	39.3	4.6	119.0	17	63.4
27	gi|504527123|	aspartate--tRNA ligase	66.7	6.0	313.0	30	50.4
29	gi|386871478|	AhpC/TSA family peroxiredoxin	21.8	5.0	135.0	13	61.8
30	gi|855361144|	Glycerophosphoryl diester phosphodiestrase	35.1	5.4	126.0	11	53.2
31	gi|855345307|	Elongation factor Tu	43.3	5.0	254.0	28	74.1
33	gi|855345110|	DNA-directed RNA polymerase alpha subunit	35.1	4.9	49.9	8	22.7
36	gi|855345251|	Chaperonin protein, GroEL	57.1	4.9	294.0	38	60.8
37	gi|504528561|	ATP-dependent chaperone ClpB *	96.0	5.4	170.0	32	42.7
39	gi|386871625|	Catalse-peroxidase	82	5.7	64.8	12	21.1
40	gi|855345348|	Succinate dehydrogenase *	65.7	5.9	258.0	29	49.2
41	gi|504528561|	ATP-dependent chaperone ClpB *	96.0	5.4	155.0	33	45.2
42	gi|855345003|	Chaperone protein – DnaK	69.0	4.8	242.0	27	45.5
45	gi|386871696|	Outer membrane associated protein	41.3	5.2	144.0	22	53.2

Table 2.7. continued

46	gi|386870797|	Chaperone Hsp90, Heat shock protein HtpG	72.2	5.3	165.0	16	29.9
47	gi|855344195|	Fumarate hydratase, class I	54.8	5.1	130.0	23	49.0
48	gi|504527658|	Dihydrolipoyl lysine-residue acetyltransferase	49.0	5.9	48.0	11	22.3
52	gi|855345336|	Aconitate hydratase *	102.4	5.2	76.9	15	17.7
55	gi|855344149|	Hypothetical protein	45.4	5.7	143.0	17	44.8
56	gi|855345336|	Aconitate hydratase *	102.4	5.2	145.0	24	24.2
62	gi|855344037|	30S ribosomal protein S1	61.5	5.2	349.0	37	72.3
63	gi|855345348|	Succinate dehydrogenase *	65.7	5.9	222.0	22	47.7
65	gi|855350520|	Pyruvate dehydrogenase complex, E2 component	80.6	5.3	320.0	39	59.4
67	gi|504527659|	Dihydrolipoamide dehydrogenase	50.3	5.9	60.3	12	32.8
68	gi|855345345|	Dihydrolipoamide succinyle transferase component of 2-oxoglutarate dehydrogenase complex, E2 component	52.5	5.0	140.0	17	40.0

Mw: Molecular weight, pI: Isoelectric focusing point, SC: Sequence coverage percent, * Proteins with multiple charge variant.

Table 2.8. Comparative immunoreactivity of *Fno* UK whole cell protein lysate with serum pools from vaccinated, control, vaccinated challenged and non-vaccinated challenged tilapia

Spot No.	Spot ID	Detection by tilapia antibodies			
		PBS control sera (30 dpv)	Vaccinated fish sera (30 dpv)	Vaccinated and F*no*-UK challenged fish sera (15 dpc)	Non-vaccinated and F*no*-UK challenged fish sera (15 dpc)
2	Hypothetical protein [**, §]	-	+	+	+
4	Hypothetical lipoprotein [**]	-	+	+	+
6	OmpA family PAL[**]	-	+	+	+
10	Intracellular growth locus protein C [**, §]	-	+	+	+
11	OmpA family protein [**]	-	+	+	+
12	Type I glyceraldehyde-3-phosphate dehydrogenase	-	+	+	+
13	Universal stress protein	-	+	+	+
26	Cell division protein [**, §]	-	+	+	+
27	Aspartate--tRNA ligase [§]	-	+	+	+
29	AhpC/TSA family peroxiredoxin [**]	-	-	+	+
30	Glycerophosphoryl diester phosphodiesterase [**]	-	+	+	-
31	Elongation factor Tu [**, §]	-	+	+	+
33	DNA-directed RNA polymerase alpha subunit	-	+	+	+
36	Chaperonin protein- GroEL [**, §]	-	+	+	+
37	ATP-dependent chaperone ClpB [**, §]	-	+	+	+
39	Catalase-peroxidase [**]	-	+	+	+

Table 2.8. continued

40	Succinate dehydrogenase **	-	+	+	+
41	ATP-dependent chaperone ClpB **, §	-	+	+	+
42	Chaperone protein – DnaK **, §	-	-	+	+
45	Outer membrane associated protein **	-	-	+	+
46	Chaperone Hsp90, Heat shock protein HtpG **	-	-	+	+
47	Fumarate hydratase, class I	-	+	+	+
48	Dihydrolipoyl lysine-residue acetyltransferase	-	-	+	+
52	Aconitate hydratase **	-	+	+	+
55	Hypothetical protein **	-	+	+	+
56	Aconitate hydratase **, §	-	+	+	+
62	30S ribosomal protein S1, §	-	+	+	+
63	Succinate dehydrogenase **	-	+	+	+
65	Pyruvate dehydrogenase complex, E2 component **, §	-	+	+	+
67	Dihydrolipoamide dehydrogenase **	-	+	+	+
68	Dihydrolipoamide succinyle transferase component of 2-oxoglutarate dehydrogenase complex, E2 component **	-	+	+	+

(+) recognised by serum, (-) not recognised by serum, (**) immunoreactive proteins previously reported in *F. tularensis* LVS using serum from tularemic patients (Janovska *et al.,* 2007a), (§) proteins with significantly different expressions between the 5 *Fno* isolates as shown in Figure 2.4, dpv: days post vaccination, dpc: days post challenge.

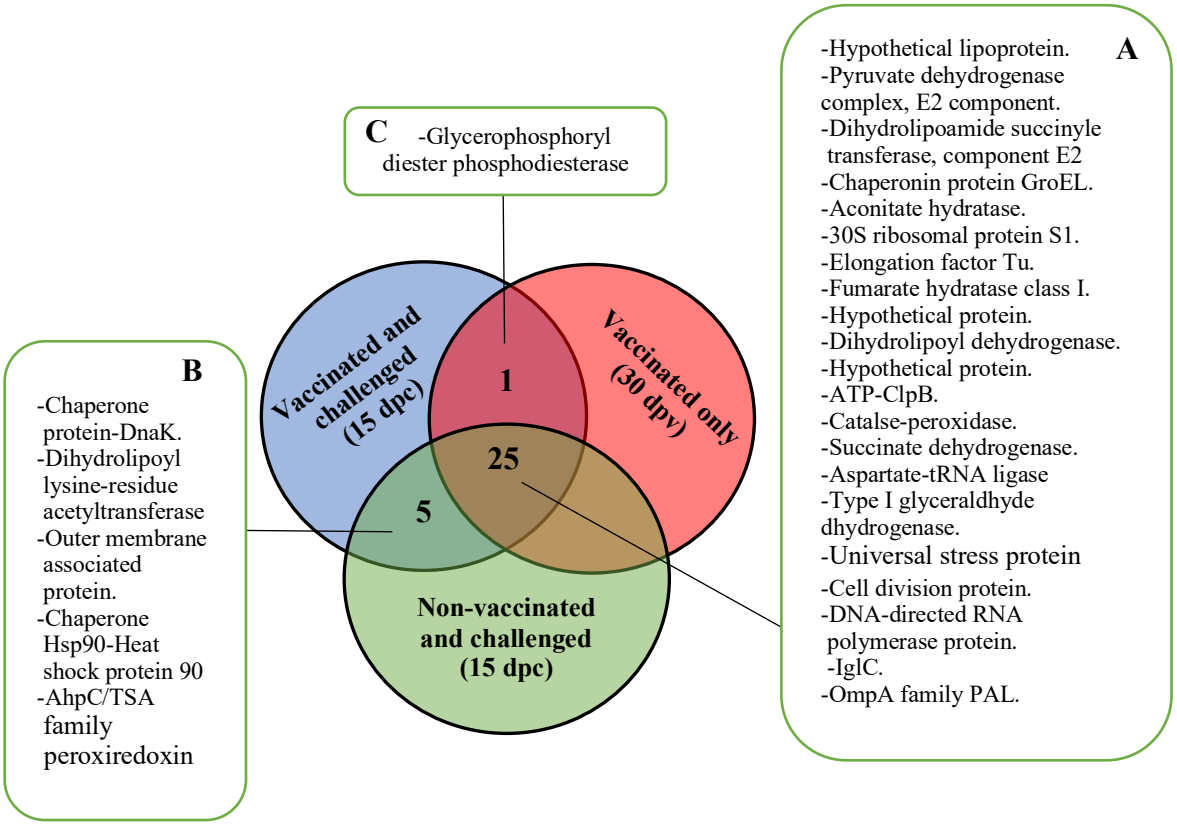


Figure 2.7. Venn diagram showing the immunoreactive proteins identified in the *Fno* UK whole cell lysate using vaccinated, vaccinated-challenged and non-vaccinated challenged sera. Numbers in circles refer to number of immuno-reactive proteins, (A) proteins highlighted by vaccinated (30 dpv), vaccinated challenged (15 dpc) and non-vaccinated challenged (15 dpc) sera, (B) proteins recognised by fish after challenge (vaccinated and non-vaccinated challenged fish sera), (C) proteins recognised by vaccinated non-challenged and vaccinated-challenged tilapia sera only.

2.3.3. Functional analysis of the identified proteins

All *Fno* immunoreactive proteins were sorted into 9 functional categories using bioinformatics analysis. Prediction of functions, locations and cleavage by signal peptidase I or II are shown in Figure 2.8. The majority of the proteins were cytoplasmic (74%), however, 6% were cytoplasmic membrane proteins and 8% outer membrane proteins. Only 3% were peri-plasmic proteins, 6% were cytoplasmic membrane proteins, while the other 9% were with unknown localization (Figure 2.8A). Almost 1/3 of the total immunogenic proteins identified were found to be involved in energy production and conversion (33%) whilst 18% were involved in post-translational modification and protein turnover and 17% were associated with translation, ribosomal structure and biogenesis (Figure 2.8 B). Only 14 % of the identified proteins were lipoproteins of which 6% were found to be cleaved by signal peptidase enzyme I (SpI) and 8% were cleaved by signal peptidase II (SpII) (Figure 2.8C).

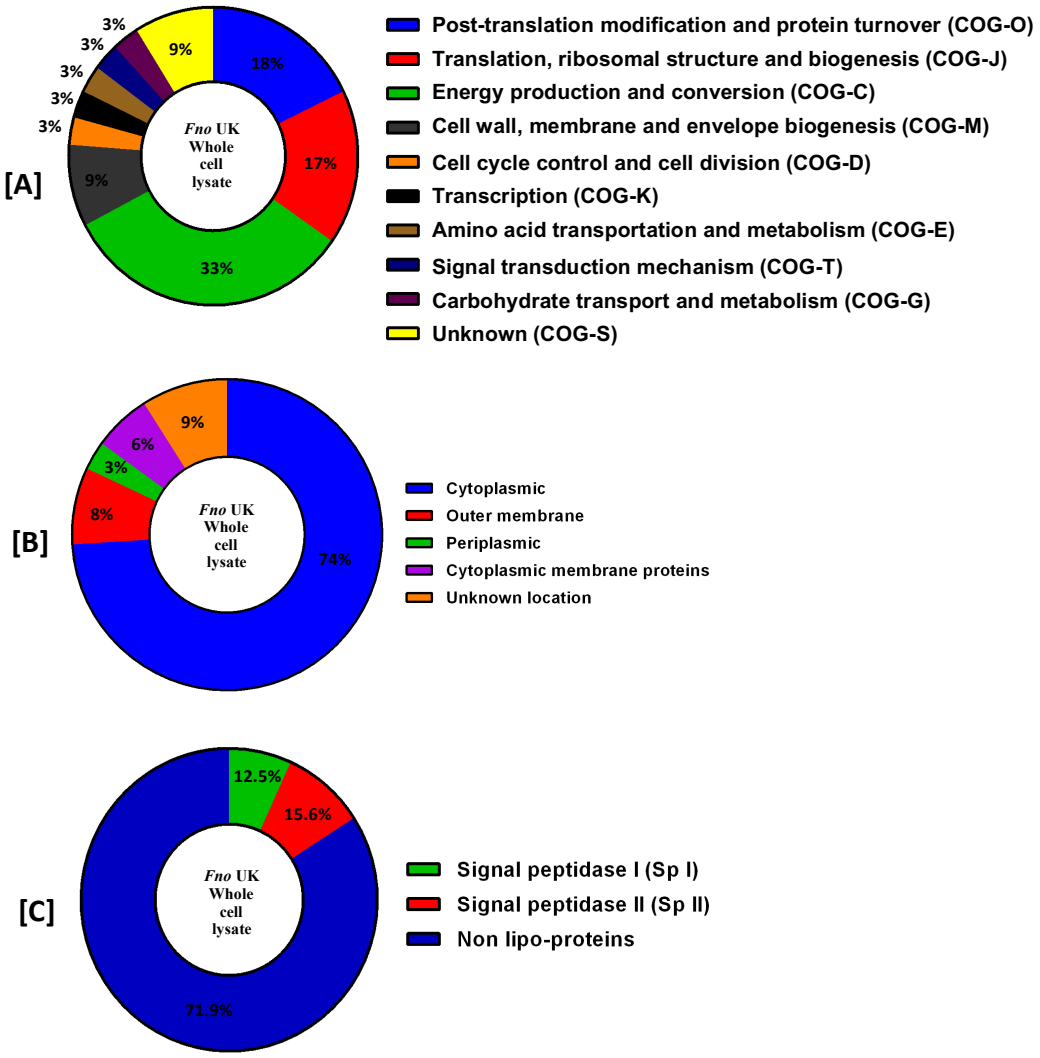


Figure 2.8. Bioinformatics analysis of identified antigenic proteins in the *Fno* UK whole cell lysate. Prediction of functions [A], location [B] and lipoproteins cleavage [C].

2.4. Discussion

Francisella noatunensis subsp. *orientalis* is one of the most threatening pathogens to the tilapia industry, where as few as 23 CFU are capable of causing mortalities (Soto *et al.,* 2009b). Understanding the mechanisms involved in pathogenesis of *Fno* requires comprehensive knowledge of the proteins involved in the infection process. As with other pathogenic bacteria, *Fno* can employ different invasion mechanisms that may involve expression and post-transitional modification of their proteins, especially during host-pathogen interaction (Ravikumar *et al.,* 2015). Since *Fno* was first associated with extensive granulomatous inflammatory disease (Kamaishi *et al.,* 2005), various studies have been conducted to understand the pathogenicity basis of this important aquatic pathogen. While genes for some potential pathogenicity factors had been previously identified in *Fno* including *igl A, B, C, D* (Soto *et al.,* 2009b), the full array of pathogenicity determinants remain unknown. Understanding such information will improve development of therapeutics, diagnostics, and control strategies against *Fno* infection.

In the current study, a comprehensive proteomic comparison between the whole cell lysate of five clinical isolates of *Fno* from distinct geographical locations was performed. The comparison using 1D PAGE showed a homogenous protein pattern for all tested isolates. The inability to detect a clear visual difference in the proteomic patterns between the tested *Fno* isolates may be attributed to the limitation of 1DE to separate single proteins during electrophoresis. Thus, 2D electrophoresis (2-DE) was performed to investigate any difference between these isolates. The 2-DE analysis provided higher resolution of the protein profiles of the clinical *Fno* isolates, where minor differences in the proteome patterns were observed between the isolates using image analyser. This was represented by 97% matching of the gel replicates with differences noted in comparison to the UK isolate, which was missing 2 spots in three gel replicates. This finding was similar to a recent proteomic study on three isolates of *F. tularensis* from human patients and the environment where they reported 96% matching of the resolved whole cell protein profiles of those strains despite their different sources and locations (Kasap *et al.,* 2017). The similarity reported between the *Fno* isolates may be due to adaptation of the *Fno* isolates to the culture conditions *in vitro,* resulting in a similar milieu for protein expression. This might mask other variations in proteomic patterns. Different protein expressions have been

reported in various bacteria including *F. tularensis* when the bacteria are grown under a variety of culture conditions (e.g. *in vivo* vs. *in vitro*) (Hazlett *et al.,* 2008) or after exposing to stressors like hydrogen peroxide (Janovska *et. al.,* 2007a) or iron deprived medium (Lenco *et al.,* 2007). Thus, further studies comparing the protein profiles of *Fno* isolates prepared under different culture conditions will give a clear idea about the proteome patterns of the different isolates. This may contribute to identification of the metabolic networks used by the bacteria in response to the various growth conditions or stressors and discovery of the identity and function of previously unidentified proteins.

Fifteen differentially expressed proteins were identified by cluster analysis and there was a highly significant difference in abundance of 3 of these spots between isolates. Interestingly, identification of the three spots from the different *Fno* gels by MALDI-TOF/MS revealed a similar protein identity which was "Aconitate hydratase". Aconitate hydratase was previously identified as an abundant immunogenic protein in the *F. tularensis* live vaccine strain (LVS) and found to play a role in energy production (Janovska *et al.,* 2007a). It was of note that this protein was expressed as 1 highly abundant spot (56) in the UK isolate, while it displayed 3 variably expressed spots (56; 57; 58) in the other isolates (i.e. Japanese, Costa Rican, Mexican and Austrian *Fno* isolates). The reason for this difference is not clear, but this may be due to the occurrence of these proteins as multi charge variants. This finding was previously reported with different proteins in 2-DE of *F. tularensis* whole cell preparations, where proteins displayed multi charge and mass variants (Hubalek *et al.,* 2004; Janovska *et al.,* 2007a). In addition, the *Fno* UK isolate proteome profile displayed a higher number of these significantly abundant proteins compared to the other isolates examined in this study. Matching these differentially expressed proteins with the identified proteins in the 2D reference gel map of *Fno* UK isolate used for immunoblotting (Figure 2.6A) revealed that the majority of the aforementioned proteins have been involved in important functions including virulence and pathogenicity of *Francisella* such as IglC, GroEL, DnaK and ClpB proteins. Taken together with the results of the virulence study performed by Djainal, (2018), who reported that the *Fno* UK isolate was the most virulent isolate either in a Wax moth larva (*Galleria mellonella*) model or in tilapia when compared with the Japanese, Mexican, Austrian and Costa Rican isolates, may explain the reason for differences in the distribution and expression of some proteins in the *Fno* UK isolate. This finding was also in agreement

with the proteomic comparisons carried out between high and low virulent isolates of *F. tularensis*, where identification of more proteins and /or the abundance of the proteins with virulence-related functions (e.g. ClpB; Heat-shock protein-20; AcpA, PilP) were reported in the virulent strain *F. tularensis* subsp. *tularensis* compared to the less virulent species *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediaasiatica* (Hubaleck *et al.,* 2004; Konecna *et al.,* 2010). The minor differences in protein abundance pattern between the *Fno* isolates used in the current study might explain the variation in profile and virulence between these isolates. However, more studies are required to investigate any post-translational modification (PTMs) that may take place and include more isolates of *Fno* with comprehensive identification of the differentially expressed proteins for better understanding of this finding. Passage history of the used isolates may have an effect on their virulence and possibly their protein profile. Thus, performing virulence and proteomic studies using recent *Fno* isolates may contribute to our understanding of the basis of variation between *Fno* isolates.

In this study, the immunoproteomic analysis in association with mass spectrophotometry led to confident identification of immunoreactive proteins of *Fno* UK isolate after 1-DE and 2-DE immunoblotting, respectively. Twenty-two of the proteins identified, highlighted in Table 2.8, have also been reported as immunogenic proteins in *F. tularensis* LVS (Janovska *et al.,* 2007a). Bioinformatics analysis of the immunoreactive proteins revealed different categories of proteins with different functions, including chaperones, cell division proteins, outer membrane proteins, chromosome partitioning proteins, peroxidase/catalase proteins and transporters. Interestingly, the vast majority of the immunoreactive proteins are involved in essential functions, and notably 33% of these proteins are responsible for energy production and conversion including pyruvate dehydrogenase complex-component E2, dihydrolipoamide succinyle transferase component of 2-oxoglutarate dehydrogenase complex- E2 component, aconitate hydratase, fumarate hydratase I, dihydrolipoyl lysine residue acetyle transferase, dihydrolipoyl dehydrogenase, glycerophosphoryle diester phospho diestrase, succinate dehydrogenase and malate dehydrogenase. The abundance of energy production-related proteins was also reported in whole cell extracts of various human and environmental *Francisella* spp. including *F. tularensis* subsp. *holarctica* LVS (Janovska *et al.,* 2007a; Kasap *et al.,* 2017) and *F. tularensis* subsp. *tularensis strain* SCHU S4 (Hubalek *et al.,* 2004). Moreover, the

immunoreactivity of these proteins against murine or human specific anti-*Francisella tularensis* LVS sera was previously described (Twine *et al*. 2006, 2010; Havalsova *et al*., 2005; Eyles *et al*., 2007; Janovska *et al*. 2007a, b; Sundaresh *et al*., 2007). Glycerophosphoryl diester phosphodiestrase, that was identified only with vaccinated and vaccinated-challenged serum, is a secreted protein previously reported as an immunogenic antigen in different pathogenic bacteria including *Staphylococcus aureus* (Nakano *et al.,* 2002), *F. tularensis* LVS (Janovska *et al.,* 2007a), *Corynebacterium glutamicum* (Ishige *et al.,* 2003) and *Bacillus subtilis* (Antelmann *et al.,* 2000). It is worth mentioning that a wide range of secerted proteins are involved in mediating host-pathogen interactions and have been identified as potential targets for development of novel vaccines, antimicrobial chemotherapy and diagnostic tools (Jungblut *et al.,* 1999; Bumann *et al.,* 2002; Bonin-Debs *et al.,* 2004). Thus, identification of this protein category as immunoreactive antigens in the *Fno* proteome may indicate a role in *Fno* pathogenicity.

A number of chaperone proteins (ClpB; DnaK; HtpG; groEL) and stress proteins (AhpC/TSA protein; universal stress protein) were identified as antigenic in *Fno* whole cell extracts. Generally, bacterial pathogens produce a number of chaperone proteins to promote their survival in hostile environments or during changing environments and periods of stress (Neckers *et al.,* 2008). In addition, some chaperone proteins have also been involved in bacterial virulence (Takaya *et al.,* 2004). DnaK chaperone assists protein folding and interacts with ClpB in re-stimulation of aggregated proteins after heat shock processes (Lund, 2001). DnaK chaperone and ClpB have been shown to be involved in the invasion of epithelial cells and host intra-macrophage survival, promoting successful systemic infection in various pathogenic bacteria including *F. tularensis* (Meibom *et al.,* 2008), *Listeria monocytogens* (Chastanet *et al.,* 2004) and *Salmonella enterica* (Lund, 2001). Interestingly, DnaK was not detected by the non-challenged tilapia serum and was only detected after challenge with *Fno* by vaccinates and control tilapia sera, which may indicate its role in host-pathogenic interaction. LaFrentz *et al.* (2009) reported significant expression of ClpB, HtpG and universal stress protein in *Flavobacterium psychrophilum* during growth of the bacterium *in vivo* in fish and in iron-depleted media *in vitro,* and they suggested that those proteins may be important for *in vivo* survival and pathogenesis of *F. psychrophilum*. Moreover, GroEL and other relevant heat shock proteins (e.g. Hsp90/HtpG) identified in the *Fno* whole cell lysate in the current study were previously

reported as potential targets for the anti-tularemic antibodies and could induce significant cellular immune response, represented by stimulation of $CD4^+$ and $CD8^+$ α and β T-cells (Lee *et al.,* 2006). Also, the surface-associated-GroEL was reported to be crucial for *Legionella pneumophila* to be able to invade HeLa cells (Garduno *et al.,* 1998). AhpC/TSA assists host-pathogen interaction and upregulated expression of this protein was reported in *F. tularensis* LVS in association with conditions that mimic the hostile environment such as oxidative stress (Lenco *et al.,* 2005) and iron depletion (Lenco *et al.,* 2007).

The immunoreactive intracellular growth locus-C (IglC) protein, is one of the key elements of the pathogenicity-life style of different members of *Francisella* spp. required with the help of others factors including ClpB and transcriptional regulator -MglA proteins to allow the bacteria to grow in the hostile milieu (Gray *et al.,* 2002; Bröms *et al*., 2010). It was commonly reported as a prominent immunogenic antigen in *F. tularensis* LVS (Havlasova *et al.,* 2002, 2005; Janovska *et al.,* 2007a), *Fnn* (Brudal *et al.,* 2015) and *Fno* (Soto *et al.,* 2009b; Lagos *et al.,* 2017). Mutation of *IglC* gene resulted in a lack of growth of *F. tularensis* and *Fno* in mice and tilapia models, respectively (Santic *et al.,* 2005; Soto *et al.,* 2009b). It is worth mentioning that other virulence related proteins were highlighted by human tularemic serum in *F. tularensis* LVS, including intracellular growth locus A (IglA), which was also co-stimulated by MglA (Janovska *et al.,* 2007a) and was reported to be crucial for intracellular growth and survival of *F. tularensis* and *F. novicida* in human and murine macrophages, and amoebae, respectively (Barons *et al.,* 1998; Lauriano *et al.,* 2004). It is of note that IglA was not detected in the current study. This may be attributed to using narrow range pH IPG strips (4-7) in the current study, while IglA (pI 8.7) was detected in *F. tularensis* LVS when IPG strips of pH range of 6-11 were employed (Janovska *et al.,* 2007a). Moreover, the whole cell of *Fno* used in this study is in essence a soluble protein lysate so, some of the proteins present may have lost their native conformational structure during sample preparation, leading to a lack of recognition of the epitopes by the serum antibodies and consequently, lower numbers of immunoreactive proteins may be recognised. In addition, cell division and chromosome partitioning proteins (cell division protein) identified in the current *Fno,* have been described as potential drug targets against clinically important bacterial pathogens as *Mycobacterium tuberculosis* (Nisa *et al.,* 2010). Proteins involved in transcription, translation, synthesis

and metabolism, which were successfully identified in *Fno* whole cell lysate (peroxidase/catalase; elongation factor TU; 30s ribosomal protein S1, proline t-RNA ligase; Aspartate tRNA ligase, NADP-specific glutamate dehydrogenase; type I glyceraldhyde-3-phosphate dehydrogenase), are also well-known targets for inhibition of bacterial pathogens (Hong *et al.,* 2014). Therefore, those proteins may serve as new drug targets for *Fno*, however, more studies are required to investigate their immunogenicity in depth.

The outer membrane proteins (outer membrane protein A, OmpA family protein; outer membrane associated proteins), outer membrane lipo-proteins (outer membrane protein peptidoglycan associated lipoproteins (PALs)) and lipoproteins (hypothetical lipoproteins; hypothetical protein *FNO*12_1528; hypothetical protein *FNO*12_0263; glycerolphosphoryl diester phosphophodiesterase) were found to be immunoreactive in the current study. This result is consistent with the reported immunogenicity of four peptidase-II cleaved proteins (conserved hypothetical lipoproteins; OmpA family proteins; hypothetical membrane protein FTT1676; lipoprotein) and 2 peptidase-I cleaved proteins (hypothetical protein FTT1402; glycerolphosphoryl diester phosphophodiesterase protein) in resolved 2-DE whole cell extract of *F. tularensis* LVS (Janovska *et al.,* 2007a). OMPs are integral constituent of Gram-negative bacteria that play an important role in the host-pathogen interactions, pathogenicity and resisting host defence mechanisms (Lin *et al.,* 2002). In addition, OMPs have been shown to provide significant protection against various fish pathogens (Xiong *et al.,* 2011). Moreover, OmpA was reported to be an excellent vaccine candidate, as it has abundant distribution on the bacterial cell surface, giving it the advantage of being easily accessible to various antibodies (Huntley *et al*., 2007). The efficacy of OmpA as a protective vaccine candidate was tested by producing specific monoclonal antibodies that induced 40-50% and 80 % survival of mice infected with *F. tularensis* LVS (Savitt *et al.,* 2009; Hickey *et al.,* 2011). Also, the affinity of a recombinant OmpA protein (FopA) of *F. tularensis* to anti-tularemia serum antibodies was tested on an ELISA assay, which showed acceptable binding in comparison with the wild type *F. tularensis* LVS (Hickey *et al.,* 2011). This may contribute to the development of a new generation of diagnostic and prophylactic assays against *Fno*. Interestingly, three of the proteins that were not recognised by the sera from the non-challenged vaccinated fish, were recognised by challenged vaccinated and non-vaccinated fish and were identified as

OmpA family protein and Omp PALs. This may reflect the role of OMP in the interaction of *Fno* with their host and indicate a potential role in their virulence. There is still little information on the OMP of *Fno*. Characterisation and cataloguing of proteins of the *Fno* OMP fraction, and more importanely, identification of its immunogenic antigens, will enhance our understanding of the importance of this group of proteins and may give insight to future applications in *Fno* diagnostics and/or prophylaxis.

Proteinase-K treatment of the *Fno* whole cell lysates revealed that the corresponding antigenic band in the 1D-immunoblot (20-37 kDa) is rich in glycoproteins or polysaccharides as determined by silver stain, which is more sensitive than Coomassie blue, especially for glycoprotein and polysaccharide staining. In a previous report by Kay *et al.* (2006), a heavily stained band of ~ 20 kDa and another low molecular band of ~ 10 kDa were highlighted in a proteinase-K digested whole cell protein lysate of an isolate of *Fno* from tilapia, named as *Francisella victoria*, after immunoblotting with polyclonal antisera. Chemical and spectroscopy analyses identified these bands as lipo-oligosaccharides and lipoprotein bands, respectively (Kay *et al.,* 2006). In addition, screening whole cell lysate of *Fnn* with rabbit anti-*Francisella* serum or serum from Atlantic cod (*Gadus morhua,* L.) vaccinated with a monovalent *Fnn* vaccine or a multivalent *Fnn* and *Vibrio anguillarum* vaccine revealed an immunodominant band of ~20 kDa (Schrøder *et al.,* 2009). The LPS of *Francisella* spp. are characterised by unusually low biological activity and have been targeted as a potential constituent of tularemia vaccines (Fulop *et al.,* 2001; Sjostedt, 2003; Isherwood *et al.,* 2005). Also, LPS have been reported as protective antigens in many fish pathogens such *Aeromonas hydrophila* (Fernandez *et al.,* 2014), *Vibrio salmonicida, V. anguillarum* (Bøgwald *et al.,* 1992) and *V. alginolyticus* (Li *et al.,* 2016). This finding may help to explain the immunogenicity of the dominant protein band (~ 17-28 kDa) recognised by the anti-*Fno* hyperimmune sera, provide more insight to the importance of the proteins identified in this band in *Fno* virulence and highlight their potential use as drug or vaccine candidates against *Francisella* infection in tilapia or other susceptible species. Future study is required to test the anti-*Fno* tilapia immune sera against the proteinase-K digested *Fno* to see if the antibodies will recognise the carbohydrate moieties as opposed to the protein components shown in the current study.

2.5. Conclusions

The present study is one of the first to compare the proteome and antigenic patterns of different clinical isolates of *Fno*. The study demonstrated considerable degrees of protein profile homogeneity between the geographically distinct *Fno* isolates, however, variable expressions of proteins between the isolates did exist. The limited heterogeneity of the antigenic profile of the diverse *Fno* isolates shown by the anti-*Fno* UK hyperimmune sera and the significant abundance of the majority of the highly expressed proteins in *Fno* UK isolate, including some of the biologically important proteins, may provide a new biological basis for immune protection by this isolate. This will have a direct impact on improving vaccine design for francisellosis in tilapia. The identified immunoreactive antigens in *Fno* UK isolate will facilitate a better selection of protein candidates for the development of new generation of diagnostic or prophylactic tools. In addition, the current proteomic study highlights the importance of mass spectrometric analysis for identification of variable expressed proteins with virulence or immune related functions. Taken together, the methods used in this study could support currently available diagnostic methods for rapid strain characterisation and differentiation, diagnosis, treatment and/or prevention of *Fno* infection. It is worth noting that, analysis of the sub-immunoproteome of insoluble *Fno* protein preparations like outer membrane enriched fraction (OMPs) may decrease the complexity of the sample compared to the whole cell lysate, thus may facilitate better protein separation and yield significant differences between isolates that were poorly seen by the *Fno* whole cell proteome analysis. Therefore, a future study is needed for separation and characterisation of *Fno* outer membrane proteome with special consideration to highlight its immunogenic components.

Chapter Three

Characterisation of the outer membrane proteome of *Francisella noatunensis* subsp. *orientalis*

3.1. Introduction

Outer membrane proteins (OMPs) are specific highly conserved components of Gram-negative bacterial cells that including individual proteins associated with bacterial pathogenicity (Seltman and Holst, 2002), nutrient uptake (*e.g.* iron), antimicrobial peptide resistance and survival in the host environment (Koebnik *et al.,* 2000). Their location on the surface of the bacteria facilitates interaction with the host immune system and thus antibodies raised against these proteins are likely to result in host generated neutralising activity against target microorganisms (Lin *et al.,* 2002). The OMPs of a variety of fish-pathogenic bacteria have previously been characterised, including those of *Flavobacterium columnare* (Liu *et al.,* 2008; Luo *et al.,* 2016), *Streptococcus iniae* (Cheng *et al.,* 2010), *Edwardisella tarda* (Kumar *et al.,* 2009; Sun *et al.,* 2011), *Edwarsiela ictaluri* (Dumpala *et al.,* 2009), *Aeromonas hydrophila* (Wang *et al.,* 2013), *Aeromonas salmonicida* (Ebanks *et al.,* 2005), *Vibrio harveyi* (Yu *et al.,* 2013) and *Vibrio alginolyticus* (Qian *et al.,* 2008). A more comprehensive characterisation of this vital group of proteins facilitated development of new generation of diagnostic and prophylactic tools for various bacterial diseases of economic importance to farmed and ornamental fish species such as *A. hydrophila* (Maji *et al.,* 2006; Thangaviji *et al.,* 2012; Divya *et al.,* 2015), *V. parahaemolyticus* (Mao *et al.,* 2007), *V. harveyi* (Li *et al.,* 2008; Yu *et al.,* 2013), *V. alginolyticus* (Qian *et al.,* 2007) and *E. tarda* (Maiti *et al.,* 2011).

Sub-immunoproteome analysis can reduce the sample complexity compared to whole cell preparation via better separation of proteins (Janovska *et al.,* 2007a) and has enabled comprehensive identification of specific compartments in bacterial proteomes (Watson *et al.,* 2014). In chapter 2, a proteomic study using the soluble whole cell proteome of *Fno* revealed minor differences between the whole cell lysate profile of 5 clinical *Fno* isolates from diverse origins. Thus, investigation of more simple insoluble protein preparations, such as OMP, with a potential protective antigenicity may be a useful alternative approach. This may provide a deeper understanding of the pathogenicity of *Fno* and help identify new immuno-relevant antigens that would be useful for the development of novel drug targets, diagnostics or vaccines for warm water francisellosis, as with other pathogens (Hickey *et al.,* 2011; Qian *et al.,* 2008; Lu *et al.,* 2016). To this end, the OMP profiles of the previously studied isolate panel were investigated. The OMP fraction of the vaccine strain (UK isolate / STIR-GUS-F2f7) were selected, catalogued and proteins

recognised by pooled hyperimmune sera collected from infected tilapia were identified using LC-ESI-MS/MS.

3.2. Materials and methods

3.2.1. Bacterial isolates and growth conditions

The five *Fno* isolates previously described in Chapter 2 were used. Cultivation of the bacteria was performed as previously described in Section 2.2.1, Chapter 2.

3.2.2. Extraction of OMPs

OMPs were obtained by the method of Gauthier *et al.* (2003), with slight modifications. Briefly, bacterial cells of each of the 5 *Fno* isolates in 20 mL of liquid culture were harvested by centrifugation at 5,000 *g* for 15 min at 4°C, supernatant was discarded, and the cell pellet was washed three times with 10 mL of chilled 50 mM Tris HCl (pH 7.0) at 3,000 × *g* for 10 min. The wash buffer was discarded, and the pellet re-suspended in 1 mL of 50 mM Tris HCl (pH 7.0) containing 20% (w/v) sucrose, 10 mM Na-EDTA, 10 μg/mL lysozyme (Sigma-Aldrich, UK) and 10 μL of protease inhibitor cocktail (Sigma-Aldrich, UK). The cell suspension was then transferred to 1.5 mL micro-centrifuge tubes (Eppendorf, Germany) containing 0.1 mL Zirconium silica beads (Thistle scientific, UK), and cells were disrupted in a FastPrep homogeniser B101011 (MP Biomedicals, USA) for 6 ×30 s, with cooling on ice for 5 min between each cycle. The lysate was then transferred to a fresh 1.5 mL micro-centrifuge tube and cell debris was removed by centrifugation at 16,000 × *g* for 2 min at 4°C. The insoluble material containing the membrane proteins was obtained by ultracentrifugation at 100,000 × *g* for 40 min at 4°C, the supernatant was discarded, and the pellet washed by addition of 1 mL of 10 mM Tris HCl (pH 7.0), without re-suspension, and incubation on ice for 1 min prior to discarding the supernatant once more. The cell pellet was then re-suspended in 1 mL of 10 mM Tris HCl (pH 7.0) containing 0.5% (w/v) N-lauryl-sarcosine (Sigma-Aldrich, UK) and centrifuged at 100,000 × *g* for 40 min at 4°C. The supernatant was discarded, and the cell pellet was washed in 1 mL of 10 mM Tris HCl (pH 7.0), as previously described, before the supernatant was discarded, and pellets were air dried at RT (22°C) for 30 s. Finally, the pellet was re-suspended in 1 mL of 10 mM Tris HCl (pH 7.0) containing 0.5% (w/v) N-lauroylsarcosine and 0.1% (w/v) SDS (Sigma-Aldrich, UK) on a variable speed vortex mixer (Cole-parmer,

UK) for 30 s. The protein concentration of the OMP preparations was determined by BCA assay (Pierce BCA protein assay Kit; Thermo, USA) according to the manufacturer's instructions. Briefly, a series of 6 proteins standard dilutions (250, 125, 50, 25, 5, 0 µg/mL) using 2 mg/mL bovine serum albumin (BSA) was prepared in duplicate 1.5 mL centrifuge tubes (Eppendorff, Germany). 100 µL of the crude OMPs of each isolate extract was added to 2 mL of working reagent in 1.5 mL centrifuge tubes in duplicates followed by a quick mix on a vortex mixer (Cole-parmer, UK) for 30 s. Tubes were covered by aluminium foil and incubated in a heat block (Iso-temp 125D, Fisher scientific, UK) at 60°C for 30 min followed by 5 min cooling at RT (~ 22°C). The absorbance of all samples and standards replicates was measured using a spectrophotometer (Cecil 2301, Buck scientific, USA) at OD_{562}. The protein concentration of the OMPs samples was calculated from the prepared standard curve and standardised to 50 µg/µL using Milli-Q water (Thermo, UK). Aliquots of 20 µL were prepared and stored at -80°C until use.

3.2.3 1D SDS-PAGE

One hundred µg sample of the OMPs was resolved on a 12% NuPAGE™ Novex® Bis-Tris Gel (NuPAGE ™, Invitrogen, USA) in 20X NuPAGE™ MES-SDS running buffer (ThermoFisher scientific, UK) at 200 V (constant voltage) for 45 min. After electrophoresis, the separated proteins were stained and scanned using same conditions previously described in Section 2.2.3, Chapter 2. Two technical replicates of SDS-PAGE were performed to ensure reproducibility.

3.2.4 Western immunoblotting

The immunoblotting was done following the protocol described in Section 2.2.6.2, Chapter 2. Two technical replicates of immunoblots were prepared to ensure reproducibility. Hyper immune serum (HIS) and control serum samples pool (*n*= 5) previously used in immunoblotting of the whole cell lysate in Chapter 2 were also used to blot the OMPs derived from the *Fno* bacterial panel resolved in Section 3.2.3.

3.2.5. LC-ESI-MS/MS identification, data interpretation and functional analyses

The resolved OMPs of the *Fno* UK isolate (STIR-GUS-F2f7) was selected for LC-ESI-MS/MS analysis. The entire gel lane was excised and sliced horizontally to 6 slices of ~ 2.5 mm depth. Destaining, reduction, alkylation and trypsinolysis of the resulting gel

slices, data analysis and interpretation and functional analyses of the identified proteins were performed as described in Section 2.2.7.1 in Chapter 2.

3.3. Results

3.3.1. 1D PAGE and immunoblotting

Following electrophoretic separation of OMPs of the five *Fno* isolates (Figure 3.1A), immunoblotting was performed using either convalescent immune sera from *Fno*-infected tilapia or control tilapia sera. The protein profile of the five OMP extracts appeared homogenous with identical distribution of bands on the gel. A conserved abundant protein band was observed between 17-28 kDa on the stained gel, and similarly, the pooled immune sera reacted with an equivalent size band on the western blot of the OMPs extracts of the different *Fno* isolates (Figure 3.1B). No immuno-reactivity was detected with the control fish pooled sera (Figure 3.1C).

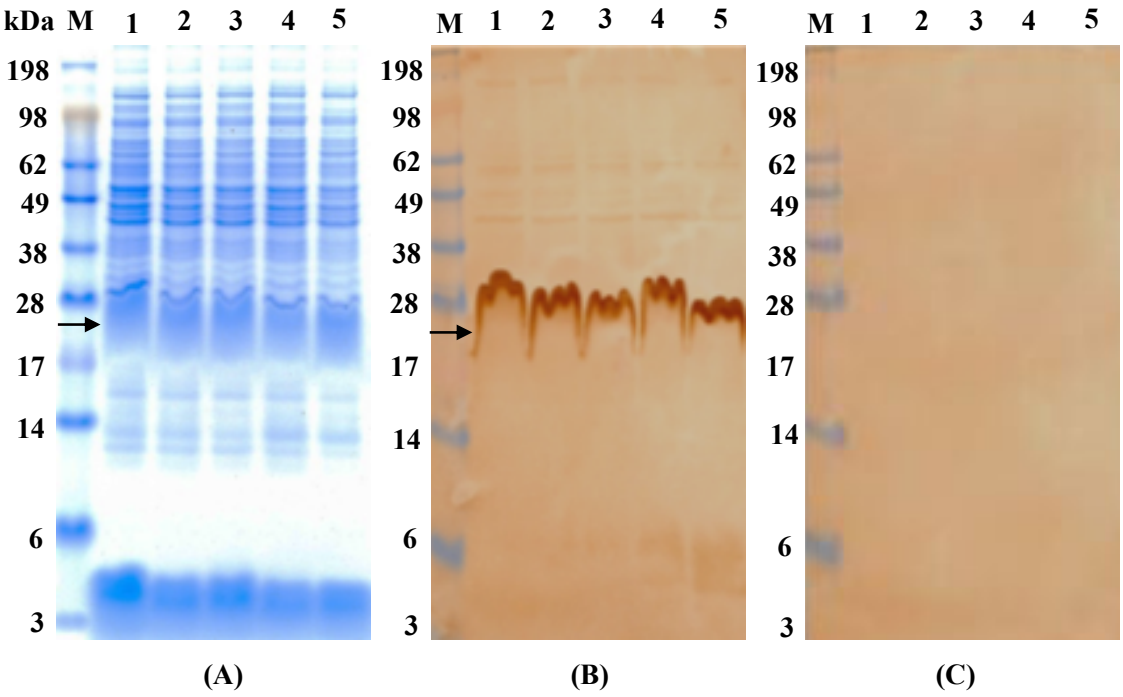


Figure 3.1. Safe blue stained 12% non-gradient 1D-PAGE showing outer membrane proteins (OMPs) profile of five clinical *Francisella noatunensis* subsp. *orientalis* isolates (A) and 2 representative immunoblots (B) and (C). Immunoreactivity was seen between the pooled anti-*Fno* UK sera from tilapia surviving infection and the OMPs of the five *Fno* isolates (B), while no reaction was observed with naïve tilapia sera (C). M: Protein standard, 1: *Fno* UK isolate, 2: *Fno* Costa Rican isolate, 3: *Fno* Mexican isolate, 4: *Fno* Austrian isolate, 5: *Fno* Japanese isolate. Black arrows refer to the immunoreactive band on blot and its corresponding protein band on the 1-DE gel. Gel and blots are representatives of two replicates.

3.3.2. Protein identification by LC/ESI/MS/MS

Mass spectrometric analysis facilitated the confident identification of a total of 239 proteins in the OMP fraction, including 44 proteins in the immunoreactive band (17-28 kDa) highlighted by the immune tilapia serum pool. The top 20 protein IDs of the *Fno* UK OMPs fraction and proteins identified in the immunodominant band (17-28 kDa) are listed in Tables 3.1 and 3.2. The full protein lists are presented in Tables S1 and S2 in Appendix 2.

Comparison between the proteins identified in the immunogenic band (17-28 kDa) of the OMP proteome of *Fno* UK isolate in the current experiment and those identified in the corresponding band in the whole cell lysate in Chapter 2, revealed 30 common proteins between both preparations, 17 unique proteins in whole cell lysate only and 14 unique proteins in the OMP fraction only (Tables 3.3- 3.5). The score of 17/30 proteins in the OMP was higher than their homologues in whole cell lysate, from which OmpA peptidoglycan-associated lipoprotein (PAL), IglC, IglA and AhpC were the most important identified proteins due to their role in *Francisella* virulence.

3.3.3. Prediction of function, subcellular localisation and lipoproteins of the identified proteins

EggNOG v4.5, PSORTb® v2.0, LipoP v1.0 servers were used to predict the function, subcellular location and lipo-protein nature of the 239 proteins identified in the OMPs of *Fno* UK isolate. Proteins associated with translation, ribosomal structure and biogenesis were the most abundant (42%), followed by those involved in energy production and conversion (31%), and those associated with cell wall biogenesis and post-translational modification (20%). The sub-cellular localisation of 82.8% of the *Fno* OMPs was putatively identified, where the majority of them were cytoplasmic proteins (62%). These were followed by cytoplasmic membrane proteins (8.8%), outer membrane proteins (5.8%), periplasmic proteins (2.5%) and extracellular proteins (0.8%). Lipoproteins represented 16.7% of *Fno* OMPs. Results of bioinformatics analysis of the top 20 protein IDs in the *Fno* UK OMPs are listed in Table 3.6 and Figure 3.2. The full data are shown in Table S3 in Appendix 2.

Table 3.1. List of top 20 proteins identified in the outer membrane proteome of the *Fno* UK isolate

No.	Accession no.	Protein name	Mw[kDa]	pI	Scores	Peptides	SC [%]
1	gi|300193845|	PdpD	139.9	6.2	4477.2	72	61.4
2	gi|386872131|	Chaperone ClpB	96.0	5.4	2588.9	49	57.7
3	gi|169589436|	PdpD	139.6	6.1	2478.8	39	52.1
4	gi|300193842|	IglC	22.1	5.3	2388.4	14	84.2
5	gi|386871181|	Chaperonin GroEL	57.1	4.9	1857.1	33	63.7
6	gi|103012949|	Ribosomal L29e protein family	126.8	8.9	1839.5	42	46.6
7	gi|386870877|	OmpA family peptidoglycan-associated Lipoprotein	23.4	4.8	1585.1	29	75.6
8	gi|386872079|	Bifunctional proline dehydrogenase/pyrroline-5-carboxylate	149.5	7.8	1539.6	33	34.6
9	gi|300193843|	IglB	57.5	4.7	1526.5	30	59.9
10	gi|386870689|	30S ribosomal protein S1	61.5	5.2	1389.2	26	58.1
11	gi|386870694|	Cell division protein FtsZ	39.3	4.6	1354.0	24	85.4

Table 3.1. continued.

12	gi|386871696|	Outer membrane associated protein	41.3	5.2	1307.4	22	42.8
13	gi|300193831|	PdpA	94.9	8.9	1244.6	15	47.8
14	gi|386870866|	OmpA family protein (FopA)	47.2	6.0	1090.3	30	62.1
15	gi|169589422|	PdpB	126.5	9.2	1046.8	24	44.6
16	gi|386871950|	Ribonuclease E	101.4	8.3	1036.3	19	27.4
17	gi|300193844|	IglA	20.4	8.6	1010.3	32	57.2
18	gi|386871082|	Alpha-ketoglutarate decarboxylase	105.5	6.1	1009.2	21	27.9
19	gi|386870797|	Heat shock protein 90	72.2	5.3	992.4	21	36.9
20	gi|386871083|	2-oxoglutarate dehydrogenase complex, E2 component	52.5	5.0	991.1	18	45.6

MW: Molecular weight; pI: Isoelectric point; SC %: Sequence coverage percent.

Table 3.2. List of the top 20 proteins identified in the immunoreactive band (17-28 kDa) of the OMPs of *Fno* UK isolate.

No	Accession	Protein name	MW [kDa]	pI	Scores	Peptides	SC [%]
1	gi|300193842|	IglC	22.1	5.3	2388.4	14	84.2
2	gi|504527828|	IglA	20.4	8.6	754.5	12	74.2
3	gi|504527329|	OmpA family peptidoglycan-associated lipoprotein	23.4	4.8	753.9	9	64.9
4	gi|504527815|	Beta-ketoacyl-ACP reductase	26.3	9.6	601.2	10	55.9
5	gi|504527529|	succinate dehydrogenase iron-sulfur subunit	26.5	8.8	540.4	11	54.9
6	gi|504527238|	50S ribosomal protein L5	20.0	9.7	526.4	11	61.5
7	gi|504527915|	AhpC/TSA family peroxiredoxin	21.8	5.0	510.0	9	59.8
8	gi|504528404|	enoyl-ACP reductase	27.7	5.5	449	11	55.4
9	gi|386871251|	Hypothetical protein OOM-0776	22.5	9.8	388.6	9	41.3
10	gi|504527834|	Hypothetical protein	24.3	5.6	376.8	5	33.5
11	gi|504527577|	50S ribosomal protein L1	24.5	9.5	374.1	9	39.4
12	gi|504527226|	50S ribosomal protein L3	22.1	9.5	359.4	6	40.5
13	gi|504527216|	30S ribosomal protein S2	26.5	8.8	333.7	5	21.3

Table 3.2. continued

14	gi|855345305|	Transcription termination/antitermination protein nusG	20.0	6.8	333.1	7	41.2
15	gi|855345177|	Hypothetical protein	27.9	9.4	330.4	6	27.2
16	gi|504527578|	50S ribosomal protein L10	18.7	9.1	274.5	8	45.3
17	gi|504527053|	Chorismiteate mutase	20.3	9.2	267.1	5	40.6
18	gi|504527683|	DNA-binding response regulator	25.5	6.2	261.9	5	32.9
19	gi|504527248|	30S ribosomal protein S4	23.2	10.4	245.0	4	24.8
20	gi|504527363|	LemA-like protein	21.9	6.0	235.4	6	37.2

Table 3.3. Common proteins identified in immunoreactive band (~17-28 kDa) of outer membrane protein fraction (OMPs) and whole cel lysate (WC) of *Fno* UK isolate.

No	Protein name	Accession no.	Meta-score		Peptide no.		SC (%)	
			OMPs	WC	OMPs	WC	OMPs	WC
1	Hypothetical protein OM_0066 **	gi|504527082|	226.3	177.2	6	4	31.2	27.6
2	AhpC/TSA family peroxiredoxin **	gi|386871478|	510	472.4	9	15	59.8	65.9
3	IglA **	gi|300193844|	754.5	494.2	12	9	47.2	47.2
4	50S ribosomal protein L1	gi|504527577|	374.1	641.5	9	13	39.4	50.6
5	Beta-ketoacyl-ACP reductase **	gi|504527815|	601.2	460.0	10	7	55.9	48.2
6	50S ribosomal protein L3	gi|386870769|	359.4	498.8	6	8	40.5	48.6
7	50S ribosomal protein L5	gi|504527238|	526.4	651.7	11	14	61.5	65.9
8	30S ribosomal protein S7	gi|504527224|	220.9	793.8	4	13	31.8	55.4
9	Protein-L-isoaspartate O-methyltransferase **	gi|386871600|	179.2	160.7	3	4	23.3	30.1
10	50S ribosomal protein L10	gi|386871131|	274.5	110.8	8	2	45.3	20.3
11	30S ribosomal protein S4	gi|386870793|	245.0	618.2	4	13	24.8	53.9
12	30S ribosomal protein S3	gi|386870775|	174.4	411.1	3	8	14.9	44.4
13	50S ribosomal protein L4	gi|504527227|	225.7	333.7	5	6	31.9	35.7
14	IglC **	gi|300193842|	2388.4	1304.4	25	13	89.9	74.7

Table 3.3. continued

	OmpA family peptidoglycan-associated							
15	Lipoprotein **	gi|504527329|	753.9	233.4	9	5	64.9	32.5
	Transcription termination/antitermination							
16	Protein nusG **	gi|752587918|	333.1	312.7	7	7	41.2	41.2
17	Chorismate mutase **	gi|386870594|	267.1	238.2	5	4	40.6	16.7
18	Hypothetical protein OOM_0903 **	gi|386871365|	212.1	103.3	5	3	22.7	15.1
19	Acyl dehydratase **	gi|386871786|	197.2	170.3	5	3	35.1	26.8
20	30S ribosomal protein S5	gi|504527242|	208.6	385.8	4	6	24.1	40.4
21	Enoyl-ACP reductase I **	gi|504528404|	449	422.3	11	8	46.9	49
22	Succinate dehydrogenase iron-sulfur subunit **	gi|504527529|	540.4	415.7	11	8	54.9	38.6
23	DNA-binding response regulator	gi|504527683|	261.9	368.4	5	5	32.9	32.0
24	50S ribosomal protein L6	gi|504527240|	197.0	229.1	4	4	51.1	23.0
25	CBC domain pair protein **	gi|504527846|	138.7	114.7	3	2	28.0	18.0
26	Hypothetical protein OOM_0658 **	gi|504527599|	193.9	96.8	4	2	22.2	10.4
27	NADH-quinone oxidoreductase subunit-C **	gi|504527492|	226.1	160.4	5	4	34.9	29.3
28	Hypothetical protein **	gi|504527834|	376.8	274.5	5	5	33.5	33.5
29	30S ribosomal protein S2	gi|504527216|	333.7	368.1	5	6	21.3	24.7
30	Septum formation inhibitor protein	gi|386870855|	65.3	541.6	2	10	18.0	51.6

WC: *Fno* whole cell lysate, OMPs: *Fno* outer membrane protein extract, ** Proteins with higher score in OMPs than in whoe cell lysate

Table 3.4. Proteins identified only in the immunoreactive band (~17-28 kDa) of the whole cell lysate of *Fno* UK isolate.

No	Protein name	Accession no.	Meta-score		Peptide no.		SC %	
			OMPs	WC	OMPs	WC	OMPs	WC
1	Chromosome partition protein A, ATPase	gi|386870946|	0.0	123.0	0	2	0.0	13.2
2	Nucleoside-triphosph—e--adenylate kinase	gi|386872073|	0.0	233.4	0	4	0.0	22.7
3	Rhodanese-like family protein	gi|386871705|	0.0	205.3	0	6	0.0	26.5
4	3-methyl-2-oxobutanoate hydroxymethyltransferase	gi|564748870|	0.0	789.7	0	14	0.0	60.0
5	Carbonate dehydratase	gi|386871721|	0.0	728.1	0	13	0.0	76.5
6	Deoxycytidine triphosphate deaminase	gi|386871930|	0.0	239.9	0	5	0.0	36.7
7	Isoprenoid biosynthesis protein with amidotransferase-like domain	gi|386871751|	0.0	98.4	0	2	0.0	10.5
8	Ribosome recycling factor	gi|386870760|	0.0	282.7	0	5	0.0	32.4
9	SAM-dependent methyltransferase	gi|752587925|	0.0	322.1	0	5	0.0	26.5
10	Purine-nucleoside phosphorylase	gi|386871670|	0.0	486.7	0	9	0.0	43.8
11	Inorganic diphosphatase	gi|386871418|	0.0	71.6	0	2	0.0	15.6
12	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase	gi|386871417|	0.0	125.8	0	3	0.0	24.0
13	Hypothetical protein OOM_1153	gi|386871579|	0.0	68.4	0	2	0.0	14.9
14	Oxidoreductase, short-chain dehydrogenase	gi|386871579|	0.0	603.9	0	10	0.0	49.6
15	Triose-phosphate isomerase	gi|386871086|	0.0	347.5	0	6	0.0	42.3
16	Activator of osmoprotectant transporter	gi|386878113|	0.0	137.0	0	4	0.0	24.6
17	Superoxide dismutase	gi|386871074|	0.0	453.5	0	8	0.0	51.0

WC: *Fno* whole cell extract, OMP: *Fno* outer membrane protein extract, SC: Sequence coverage

Table 3.5. Proteins identified only in immunoreactive band (~17-28 kDa) of the OMP fraction of *Fno* UK isolate.

No.	Protein name	Accession no.	Meta-score		Peptide no.		SC %	
			OMPs	WC	OMPs	WC	OMPs	WC
1	Ubiquinone/menaquinone biosynthesis methyltransferase	gi|386870968|	126.8	0.0	2	0	11.6	0.0
2	Glutamine amidotransferase subunit PdxT	gi|504528036|	179.6	0.0	3	0	26.8	0.0
3	Hypothetical protein OOM_0776	gi|386871251|	388.6	0.0	9	0	41.3	0.0
4	Hypothetical protein	gi|504528150|	118.6	0.0	3	0	12.4	0.0
5	LemA-like protein	gi|504527363|	235.4	0.0	6	0	37.2	0.0
6	Hypothetical protein OOM_1699	gi|386872052|	129.5	0.0	5	0	22.1	0.0
7	Hypothetical protein	gi|504528047|	117.3	0.0	3	0	18.7	0.0
8	Hypothetical protein	gi|855345177|	330.4	0.0	6	0	27.2	0.0
9	Acid phosphatase	gi|504528393|	118.4	0.0	3	0	16.7	0.0
10	Shikimate kinase I	gi|504528505|	145.7	0.0	3	0	18.8	0.0
11	Hypothetical protein	gi|504528150|	218.9	0.0	5	0	40.7	0.0
12	Hypothetical protein OOM_0748	gi|504527673|	150.4	0.0	3	0	23.2	0.0
13	Membrane protein	gi|504527668|	71.7	0.0	2	0	20.5	0.0
14	ABC transporter ATP-binding protein	gi|504527852|	175.7	0.0	4	0	28.4	0.0

WC: *Fno* whole cell extract, OMP: *Fno* outer membrane protein extract, SC: Sequence coverage

Table 3.6. Bioinformatics analysis of the top 20 proteins identified in the OMP fraction of *Fno* UK isolate.

N.	Protein	PSORTb[a]	COGs[b]	LipoP[c]	SignalP[d]
1	PdpD	Outer membrane	S	N	N
2	Chaperone ClpB	Cytoplasmic	O	N	N
3	PdpD	Outer membrane	S	N	N
4	IglC *	Unknown	S	N	N
5	Chaperonin GroEL	Cytoplasmic	O	N	N
6	Ribosomal L29e protein family	Outer membrane	S	N	N
7	OmpA family peptidoglycan-associated lipoprotein *	Outer membrane	M	Y	Y (SpII)
8	Bifunctional proline dehydrogenase	Cytoplasmic	C	N	N
9	IglB	Cytoplasmic	S	N	N
10	30S ribosomal protein S1	Cytoplasmic	J	N	N
11	Cell division protein FtsZ	Cytoplasmic	D	N	N
12	Outer membrane associated protein	Outer membrane	M	Y	Y (SpI)
13	PdpA	Unknown/multiple localization	S	N	N
14	OmpA family protein	Outer membrane	M	Y	Y (SpII)
15	PdpB	Outer membrane	M	N	N

Table 3.6. continued

16	Ribonuclease E	Cytoplasmic	E	N	N
17	IglA *	Cytoplasmic	S	N	N
18	Alpha-ketoglutarate decarboxylase	Cytoplasmic	G	N	N
19	Heat shock protein 90	Cytoplasmic	O	N	N
20	2-oxoglutarate dehydrogenase complex, E2 component	Cytoplasmic	C	N	N

[a] Subcellular localization as predicted by PSORTb v. 2.0 (https://psort.org/).

[b] Functional classification of the tentative proteins as predicted by EggNOG v. 4.5 server (https://eggnog.embl.de). The COGs functional categories are: C, energy production and conversion; D, Cell cycle control, cell division and chromosome partitioning; E, amino acid transport and metabolism; F, Nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, Coenzyme transport and metabolism; I, lipid transport and metabolism; J, Translation, ribosomal structure and biogenesis; K, Transcription; L, replication, recombination and repair; M, cell wall/membrane biogenesis; O, Post-translational modification, protein turnover, and chaperones; P, Inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport, and catabolism; S, Unknown function (includes Category R with general function and category N not in known COGs); T, Signal transduction mechanisms; U, Intracellular trafficking, secretion, and vesicular transport; V, Defence mechanisms.

[c] Lipoproteins prediction by LipoP v. 1.0 (https://www.cbs.dtu.dk/services/Lipo/), Y: Yes, N: No

[d] Signal peptide sequence prediction by SignalP v. 2.0 (https://cbs,dtu.dk/services/Signal P/), Y: Yes, N: No, SpI: Signal peptides cleave by signal peptidase I, SpII: Signal peptides cleaved by signal peptidase II. * Immuno-reactive proteins in the *Fno*-OMPs.

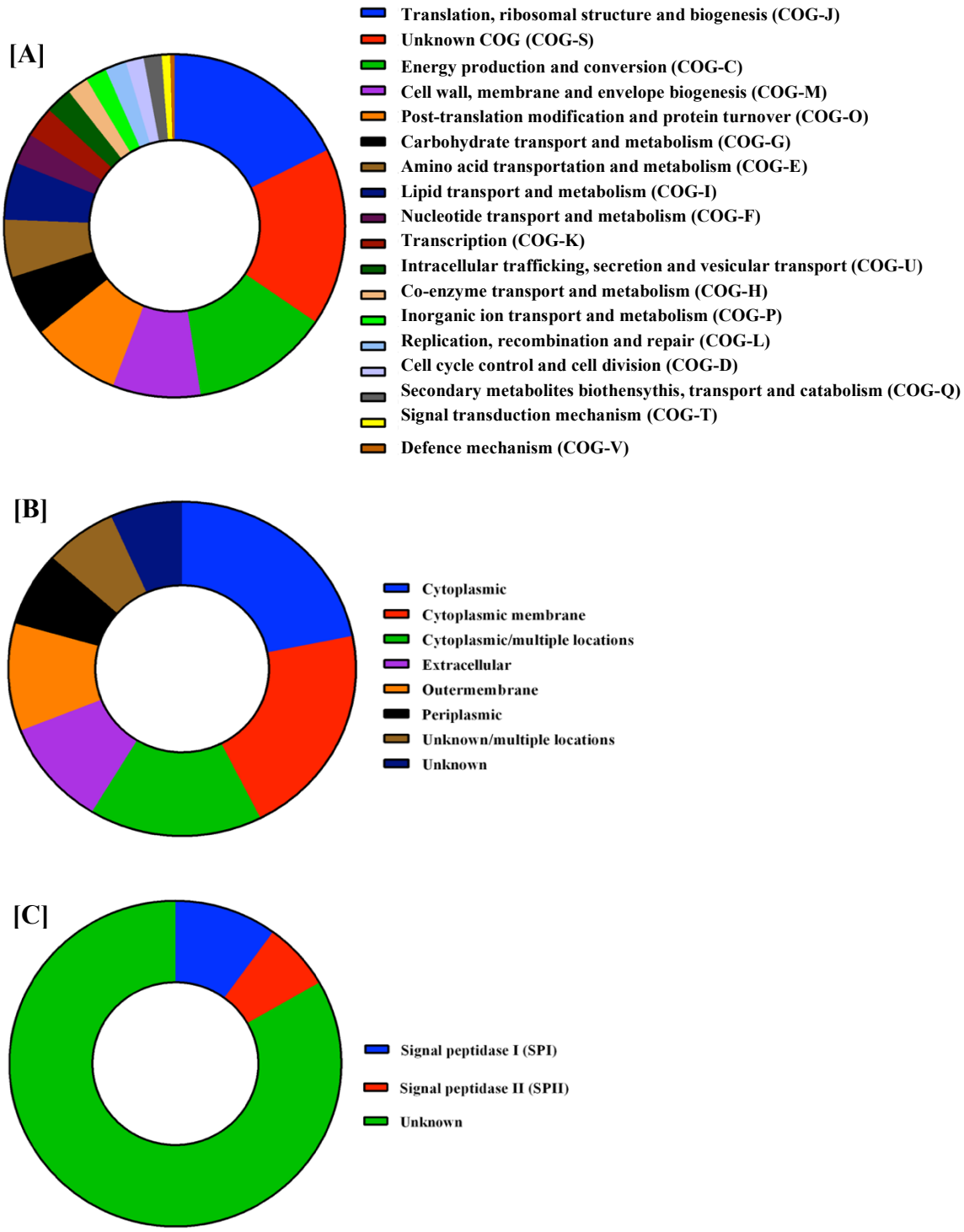


Figure 3.2. Summary of bioinformatics analysis of proteins identified in the OMP fraction of *Fno* UK isolate. Prediction of biological functions (A), subcellular localisation (B) and cleavage by signal peptidase (I; II) (C).

3.4. Discussion

Outer membrane proteins (OMPs) play an important role in the pathobiology of various bacteria by facilitating their adaptation to a wide range of different environments. Due to their prominence at the host-pathogen interface, the OMPs represent antigens with the potential to induce protective humoral and cellular immune responses in the host capable of inactivating the bacteria (Lin *et al.,* 2002; Mukhopadhaya *et al.,* 2006). Despite their potential importance, to date, no studies have been conducted on outer membrane proteins of *Fno*. In the current study, comparative analysis of the proteome of OMPs derived from five geographically distinct *Fno* isolates showed homogenous proteomic and antigenic profiles represented by a uniform distribution of protein bands by 1-DE and a conserved antigenic band on western blot of the different OMP preparations. The LC-ESI/MS/MS confidently identified a total of 239 *Fno* OMPs. Interestingly, many of these were observed to share similarities with proteins found in *Fno*-derived outer membrane vesicle (OMV) described by Lagos *et al.* (2017), where 52 % of the OMV proteins identified were predicted to be cytoplasmic, while the outer membrane and extracellular proteins were 5% and 1%, respectively, compared to 5.8% and 0.8% in the current study.

The presence of cytoplasmic, periplasmic or inner membrane proteins in the current *Fno*-OMP preparation can be attributed to the fact that most bacterial outer membranes are involved in the transportation of substances between the intracellular and extracellular membranes. This may allow contact between the OMPs and other inner membrane proteins or the periplasmic proteins as an essential component of the membrane associated-enzyme complex (Vipond *et al.,* 2006). Identification of different classes of non-outer membrane proteins in OMP preparations has been previously reported (Liu *et al.,* 2008; Kumar *et al.,* 2009; Watson *et al.,* 2014). The reason for this is unknown, but as previously shown for other bacteria such as *A. hydrophila, A. salmonicida, F. tularensis and S. agalctiae* (Henderson and Martin, 2011), *Fno* may express non-classically associated outer membrane proteins known as “moonlighting proteins” on its surface, which are known to have more than one function both within the cytoplasm and extracellularly, and have been reported to perform various biological functions associated with bacterial virulence (Henderson and Martin, 2011). Definitive assignment of OMPs to specific subcellular locations within Gram-negative bacteria remains unclear. This may be due to OMPs spanning the three layers of the bacterial cell membrane as “β-barrel trans-

membrane proteins" for transportation of ions and other micro-molecules (Wimely, 2003; Pavkova *et al.,* 2005). Alternatively, post-translational modification (PTMs) may enable the OMPs to associate with other proteins including lipoproteins and glycoproteins (Santoni *et al.,* 2000). This may explain the high percentage of non-OMPs in the extracted *Fno*-OMPs preparation. Further studies are needed to confirm the identity and biological functions of these non-OMPs. In addition, the extraction method may have an effect on existence of other non-OMPs within the OMP fraction following bacterial cell lysis, as there may be some carry-over proteins that stick to the OMP extract and thus will be resolved together by electrophoresis. Therefore, optimisation of the extraction protocol for maximum recovery of OMP and reduce other proteins left-over is needed.

Functional analysis of the proteins identified using EggNOG v. 4.5 revealed that most of the abundant proteins were involved in vital biological functions including energy production, cell wall/cell membrane formation, post-translational modification, and metabolism of various cellular components (including protein, carbohydrate and lipid metabolism), transcription and transport activities. It has already been reported that proteins responsible for translation/transcription, catalytic activity, and transporting activity are the most abundant proteins found in *Fno*-derived OMVs (Lagos *et al.,* 2017). This highlights the diversity of biological functions associated with OMPs and may reflect the importance of the OMPs in the pathobiology of *Fno*.

The presence of lipoproteins in the current *Fno*-OMPs was predicted using LipoP server v0.2 from which 24 were predicted to be cleaved by signal peptidase I and 16 were cleaved by signal peptidase II. In addition to their role in the acquisition of nutrients, it has been suggested that lipoproteins have the ability to switch-on the host's immune response by interacting with Toll-like receptor 2 (Nguyen and Götz, 2016). Moreover, 31 ribosomal proteins, mainly 30s and 50s, were detected in the OMPs of *Fno*. The presence of ribosomal proteins has also been reported in OMPs preparations of other bacteria as *F. tularensis* (Janovska *et al.,* 2007a), *Flavobacterium columnare* (Liu *et al.,* 2008) and *Pasteurella multocida* (Boyce *et al.,* 2006) and it was found that ribosomal proteins play an important role in the biogenesis and translocation of integral membrane proteins (Herskovits *et al.,* 2002). Thus, they may be involved in bacterial pathogenesis.

In this study, PdpD, IglA, IglB and IglC, outer membrane-A family protein (FopA), peptidoglycan associated lipoprotein (PAL), GroEl and ClpB displayed high

scores in comparison to the other proteins identified in the OMPs of *Fno* UK isolate. Interestingly, all of these proteins have already been detected in various protein preparations, including OMPs and OMVs, from different *Francisella* spp., including *Fno* (Lagos *et al.,* 2017)*, F. novicida* (Pierson *et al.,* 2011) and *Francisella tularensis* (*Ft*) (Melillo *et al.,* 2006; Huntley *et al.,* 2007; Hickey *et al.,* 2011). Homologues of some of the *Fno* OMPs identified in this study have previously been described as immunogenic in the *F. tularensis* live vaccine strain of (LVS), as demonstrated by immunoblotting using sera from tularemic patients (Janovska *et al.,* 2007 a,b). Nevertheless, identification of the previously mentioned virulence-determinant proteins in our study suggests a possible role for OMPs in *Fno* virulence and its interaction with the fish immune system.

The PdpA, PdpB, PdpD, IglA, IglB and IglC proteins represent the core elements of the *Francisella* pathogenicity island (FPI), which itself constitutes the major determinants associated with *Francisella* virulence and intracellular replication within host macrophages (Nano and Schmerk, 2007; Bröms *et al*., 2010). PdpA suppresses cell signalling by macrophages including growth factors, cytokines and adhesion ligands, thus suppressing the macrophages ability to recruit and stimulate other immune cells (Nano *et al.,* 2004). Ludu *et al.* (2008) reported that PdpD protein is localised to the outer membrane of *Francisella novicida* and is involved in the extracellular virulence of the bacterium by affecting the localisation of other FPI proteins including IglA, IglB, IglC and T6SS. IglA and IglB are two cytoplasmic proteins that constitute an essential part of the type VI secretion system in *F. novicida* and both are required for intra-macrophage growth through stimulating secretion of effector molecules, that affect host cell processes (Barker *et al.,* 2009). It has also been demonstrated that IglA is required for virulence and supporting the growth of the bacterium inside macrophages (de Bruin et *et al.,* 2007).

IglC protein, which was associated with the immunogenic band (17-28 kDa) of *Fno* OMP in the current study, is one of the important proteins upregulated during intracellular growth of *Francisella* spp. in macrophages (Golovliov *et al.,* 1997, 2003). Earlier studies reported that IglC, with its regulator MglA, assist the ability of *F. tularensis* to modulate biogenesis of the phagosome, preventing the formation of the phagolysosome, and thus facilitating escape of the bacteria into the cytoplasm of the host cell following replication (Clemens *et al.,* 2004; Santic *et al.,* 2005). Furthermore, IglC has been reported to play a role in production of inflammatory cytokines (Telepnev *et al.,* 2003) and

subsequent induction of cell apoptosis (Lai *et al.,* 2004). Mutations of this protein alter bacterial virulence and impair intracellular growth in human derived macrophages (Santic *et al.,* 2005) as well as tilapia macrophages (Soto *et al.,* 2009b).

The *Francisella* outer membrane-A family protein (FopA), identified within the immunogenic band of the OMPs of *Fno* UK, is the predominant outer membrane protein that is highly expressed on the cell surface and has been found to be highly immunogenic in *F. tularensis* (Fulop *et al.,* 1996; Huntley *et al.,* 2007). Readily accessible to different antibodies, it provided good protection when tested as a candidate subunit vaccine antigen against human tularemia in mice exposed to lethal intradermal and intranasal *F. tularensis* SchuS4 challenge (Hickey *et al.,* 2011). The GroEL chaperone protein, is a heat shock protein which was found to be up-regulated in a mutant of *F. tularensis* LVS deficient in iron superoxide dismutase ($sodB_{Ft}$) that used for vaccinaction of mice against respiratory tularemia (Bakshi *et al.,* 2008). It is also thought to induce long-lasting recall of $CD4^+$ and $CD8^+$ T cells in association with other heat shock proteins like DnaK and GroES by stimulating specific anti-tularemic antibodies (Havlasova *et al.,* 2002; Lee *et al.,* 2006). Peptido-glycan-associated lipoproteins (PALs) detected in OMPs of *Fno* in the current study, are ubiquitous proteins, found in many pathogenic Gram-negative bacteria including *Escherichia coli* (Hellman *et al.,* 2002), *Vibrio cholerae* (Heilpern and Waldor, 2000) and *F. novicida* (McCaig *et al.,* 2013). The PALs are thought to perform virulence-related functions and assist survival of pathogenic bacteria by modulating the host immune response and initiating the release of pro-inflammatory cytokines (Buwitt-Beckmann *et al.,* 2006; Oscarsson *et al.,* 2008; Godlewska *et al.,* 2009). The efficacy of PALs as potential subunit vaccine candidates has previously been tested for non-typeable *Haemophilus influenza* (Murphey *et al.* 2006), *Campylobacter jeuni* (Wyszynska *et al.,* 2002) and *Legionella pneumophilia* (Yoon *et al.,* 2002). Some of the proteins identified in the OMPs of *Fno* and other *Francisella* spp., such as IglB, IglC and PdpD have been tested as vaccine candidates in either mice (Tempel *et al.,* 2006) or fish (Soto *et al.,* 2011b). Mutations in the *IglC* gene of *F. novicida* and *Fno* have been used to develop live attenuated vaccines, resulting in survival levels (RPS) of 50% and 87.5% in mouse and tilapia, respectively (Pammit *et al.,* 2006; Soto *et al.,* 2011b). Also, vaccination of zebra fish (*Danio rerio*) with *Fno*-derived OMVs, that were shown to be rich with various

immunogenic proteins, gave an RPS of 65.5% after 28 days post-infection with *Fno* (Lagos *et al.,* 2017).

A previous genomic study performed by Sridhar *et al.,* (2012) revealed major differences between human pathogenic *F. tularensis* (*Ft*) and fish pathogenic *Fno* genomes, especially in their pathogenicity island (FPI), where *F. tularensis* possess two copies of FPI, but *Fno* contains only one copy. More importantly, the number of protein coding genes are lower in *Fno* (*n*= 1595) than *Ft* (*n*= 1664) where *pdpC*, encoding one of the FPI proteins, was one of the most important genes missing in *Fno* and it was reported to be crucial for growing of *Francisella* spp. in mammalian cells (Hazlett and Cirillo, 2009). Interestingly, the current proteomic study confirms the latter findings, where the PdpC protein was not detected. This highlights the importance of proteomic approaches in complementing genomic studies for establishing valid and definitive information about the microbial phenotype, especially in selection of candidates for therapeutic or diagnostic applications.

When the OMP profile of the different *Fno* isolates were examined by immunoblotting using the hyper immune sera from convalescent tilapia, an immunoreactive region was observed between 17-28 kDa, while no immunoreactivity was seen with the control sera. A similar antigenic pattern of the current *Fno* OMP was obtained in the whole cell proteome analysis of *Fno* isolates described in Chapter two when screened with the same sera. In addition, this result was in agreement with that of Schrøder *et al.,* (2009) who reported an immunoreactive band of 20 kDa after probing *Fnn* whole cell protein extract with serum from Atlantic cod (*Gadus morhua, L*) immunised with either a monovalent *Fnn* vaccine or a multivalent vaccine containing *Fnn* and *V. anguillarum* and the same immunoreactive band was also observed with a polyclonal rabbit antiserum raised against *Francisella* sp. In a separate study by Kay *et al.,* (2006), polyclonal antisera raised against *Francisella victoria*, isolated from tilapia, recognised an immuno-dominant band of approximately 20 kDa in the large lipo-oligosaccharides fraction (LOS) of the proteinase-K treated whole cell protein lysate. Thus, presence of the immunoreactive band (~17-28 kDa) in the OMP fraction in the current study may support the results obtained in the previous studies. Enrichment of such immunodominant proteins by using a simple subcellular fraction as opposed to the complex whole cell makes it easier to identify immunogenic proteins previously reported in whole cell preparations of other

fish pathogenic *Francisella* spp. More importantly, establishing the proteins present in the *Fno* OMPs fraction may enable a greater understanding of which proteins are involved in stimulating the fish immune system in response to *Fno* infection.

Equally important, variation was observed in the protein profile of the antigenic band (17-28 kDa) between the *Fno* outer membrane proteome and whole cell (WC) lysate, where a set of unique proteins were identified in each preparation, including 14 in OMPs and 17 in the WC lysate. This was in agreement with results reported by McCaig *et al.* (2013), where they compared the proteome of whole cell extract, OMP, periplasmic proteins, secreted proteins, outer membrane vesicles (OMVs) and outer membrane tubes (OMTs) of *F. novicida* and found that each of these has a unique profile. Interestingly, the OMPs of *Fno* UK isolate showed a higher score of a group of important proteins compared to their analogous in the whole cell lysate. This included some of the well-known virulence related proteins, such as IglC and IglA (Soto *et al.,* 2009b; Janovska *et al.,* 2007a), OmpA which was previously identified as immunoreactive antigen in other bacteria (Dabo *et al.,* 2003; Janovska *et al.,* 2007a) and the oxidative-stress related protein AhpC (Lenco *et al.,* 2005; 2007). This finding highlights the importance of the insoluble fraction of *Fno* OMP with regards to better separation and /or concentration of proteins. These proteins, once identified and validated, could potentially serve as candidates for improved *Fno* vaccines.

One of the key features of OMPs is that they are highly conserved among pathogens (Koebink *et al.,* 2000). For instance, a 28 kDa OMP is a common antigen of *V. anguillarum* and *V. parahaemolyticus* (Dong *et al.,* 2004); 36 kDa OMPs are conserved antigens between *V. parahaemolyticous*, *V. anguillarum, V. icthyoenteri, V. alginolyticus, V. harveyi and V. vulnficus* (Tang *et al.,* 2009); the amino acid sequence of the 48 kDa OMP of *A. caviae* was reported to be similar to that of *Aeromonas salmonicida, V. cholera, V. parahaemolyticuos, Salmonella enterica* (Vázquez-Juárez *et al.,* 2004), and four iron regulating OMPs (IR-OMPs) with molecular weights of 70, 77, 77 and 82 kDa were expressed in 18 strains of *A. salmonicida* (Hirst and Ellis, 1994). Thus, outer membrane conserved antigens in different strains of the same or different serotypes are most likely to induce cross-protection against infection by either heterologous strains or even related strains with similar or different serotypes. Kawai *et al.* (2004), reported that a conserved OMP of 37 kDa resulted in 50%, 65.5% and 70% protection in Japanese

flounder (*Paralichthys olivaceus*) against three *E. tarda* strains of different serotypes. A 40 kDa major OMP (OM porin II) gave 70 % and 80 % protection against i.p challenge with lethal doses of *A. salmonicida* and *A. hydrophila* in rainbow trout (*Oncorhynchus mykiss*), respectively (Merino *et al.,* 2005). In addition, a 43 kDA recombinant OMP induced 87.5%, 75% and 44.4% protection in Three-spot Gourami (*Trichogaster trichopterus*) challenged with two strains with similar serotypes and one strain with a different serotype of *A. hydrophila* (Fang *et al.,* 2004). Visualising the cross-reaction of the sera raised against *Fno* UK isolate with the OMP extract of the other isolates (Costa Rican, Japanese, Mexican and Austrian *Fno* isolates) in the current study, may suggest that these geographically distinct *Fno* isolates share a conserved OMP structure. Further proteomic and genomic analysis of the OMP of these isolates needs to be performed to reveal the protein distribution within this bacterial panel and highlight the conserved antigens that could be tested as candidates for a broad spectrum *Fno*-vaccine.

To the best of knowledge, this is the first report describing identification of proteins comprising the OMP fraction of *Fno*. Interestingly, most of these proteins were previously reported to be immunogenic in *F. tularensis* (Havlasova *et al.,* 2002; Lee *et al.,* 2006). When taken together, these results give more insight into the importance of *Fno* OMPs and highlight their potential use in future diagnostic and control of *Fno* infection in farmed tilapia. It is worth mentioning that, some of the pathogenicity-related proteins that showed high scores in the current *Fno*-OMP preparation, such as PdpD and FopA were not recognised by the sera from challenged tilapia. However, they were previously described as immunogenic antigens in *F. tularensis* using sera from tularemic patients (Hickey *et al.,* 2011; Huntly *et al.*, 2007; Janovska *et al.,* 2007 a). This anomaly may be attributed to the limited resolution offered by the 1D-SDS-PAGE and its inability to separate complex mixtures of proteins that co-migrate as a single band. To this end, the use of higher resolution 2-D gel electrophoresis together with immunoblotting and downstream mass spectrometry may facilitate a more precise characterisation of the protein complement of the *Fno* OMPs fraction. In summary, the data presented here offers a first insight into OMPs of *Fno*, which helps build our understanding of how this organism is able to cause disease.

3.5. Conclusions

The current study provides the first describtion of the proteomic profile of *Fno* outer membrane proteins of isolates from geographically distinct origins. It has been demonstrated that these isolates share a homogenous proteomic and antigenic pattern. Further, OMPs of *Fno* UK isolate were successfully catalogued and characterised using immunoproteomics and LC-ESI/MS/MS. The analysis showed that OMPs of *Fno* contained known *Francisella* virulence-associated proteins that were more abundant than their corresponding proteins in the whole cell lysates. This finding broadly supports the advantage of using the insoluble OMP enriched fraction as a simple sub-immunoproteome for proteomic analysis rather than the complex whole cell lysate. Identification of such antigenic proteins in the *Fno* OMP fraction may provide preliminary data on *Fno* surface proteins that have potentiality to interact with the host immunity during infection and may offer an insight into their role in *Fno* pathogenesis. To follow up on the present study, future work will focus on the separation and identification of individual immuno-reactive proteins that co-migrate as single bands in low resolution 1-D gels. In addition, further study should be carried out to examine the possible vaccine candidate molecules in *Fno* OMP and to understand in depth the adaptive and innate immune responses of fish post-vaccination and post-challenge. As differences between the proteome of geographically diverse *Fno* isolates were highlighted in Chapter 2 and abundance of antigenic proteins recognised by tilapia anti-sera have been identified in Chapter 2 and 3, the protective antibody-mediated immune response should be investigated following challenge with multiple *Fno* isolate in vaccine efficacy trials.

Chapter 4

Efficacy testing of an inactivated whole cell injection vaccine in Nile tilapia, *Oreochromis niloticus* (L), against multiple isolates of *Francisella noatunensis* subsp. *orientalis* from diverse geographical origins

4.1. Introduction

Francisella noatunensis subsp. *orientalis* (*Fno*) is a serious emerging bacterial pathogen affecting a wide range of ornamental and farm-raised cichlids globally (Colquhoun and Duodu, 2011). Due to its fastidious nature, high infectivity (~ 23 CFU can induce clinical disease), wide host range, transmission by both horizontal and vertical routes, capacity to survive in multiple environments and co-existence with other pathogens, it has been highlighted as one of the major threats to the tilapia industry where mortalities of >90% have been reported (Soto *et al.,* 2012a). On tilapia farms, several strategies have been adopted to control francisellosis. Conventional practices of increasing the water temperature from 25 to 30°C have been used to inhibit the development of francisellosis in infected tilapia and other susceptible species of ornamental fish (Soto *et al.,* 2012a; 2014b). Moreover, treatment of *Fno* in fish with approved antibiotics like oxytetracycline (Terramycin®) and florfenicol (Aquaflor®) has also been reported to be effective (Mauel *et al.,* 2003; Soto *et al.,* 2012d; Soto *et al.,* 2014b). The latter can potentially improve fish health by significantly decreasing mortalities due to the disease (Soto *et al.,* 2014b), however, using of antibiotics is not ideal as sick fish will not eat medicated feed and there is a risk of developing antibiotic resistance in the bacteria (Bruun *et al.,* 2000; Soto and Hawke, 2017). In addition, *Fno* is an intracellular pathogen which limits the efficacy of antibiotic to completely clear-out of the bacteria from the infected fish.

Currently no commercial therapeutics or prophylactic treatments are available for use against *Fno* in farmed fish. The emergence of *Fno* outbreaks in different countries has raised concerns of a potential francisellosis pandemic, thus efforts to develop protective vaccines against *Fno* are increasing. Ideally, the vaccine should confer high level of protection against all *Fno* isolates, be cost effective and easy to administer (Barnes, 2017). In a previous vaccination study in tilapia, the highest Relative Percent Survival (RPS) obtained was 87.5 % using a live attenuated immersion vaccine (Soto *et al.,* 2011b). However, use of live attenuated mutants in vaccines is not acceptable in all countries due to the different regulations related to safety. Recently, Ramirez-Paredes (2015) developed an injectable whole cell adjuvanted bacterin vaccine against *Fno* in Nile tilapia. The vaccine in this study was developed using a virulent *Fno* isolate (UK isolate/ STIR-GUS-F2f7) obtained from a diseased tilapia farmed in England (Ramirez-Paredes *et al.,* 2017b). Following intraperitoneal (i.p.) administration and challenge with the homologous vaccine

isolate, the vaccine stimulated protective antibodies and resulted in high protection (RPS of 100%); however, cross protection against heterologous isolates still needs to be determined.

An adequate understanding of the innate and adaptive immune response of tilapia to *Fno* infection is a constraint to the development of an efficacious vaccine against *Fno* (Soto and Hawke, 2017). Jantrakajorn and Wongtavatchai (2016) reported significant expression of the pro-inflammatory genes, interleukin-1β (*IL-1β*) and tumor necrosis factor-α (*TNF-α*) within 24 h post-injection (hpi) till up to 96 hpi and down-regulation of the anti-inflammatory gene, transforming growth factor-β (*TGF-β*) 24 hpi in spleenic cells of juvenile Nile tilapia i.p. injected with 0.8×10^5 CFU/Fish of *Fno*. Recently, the immune response of adult zebra fish immunised with injectable *Fno* OMV-based vaccine was investigated, however similar studies on vaccinated tilapia, especially using inactivated whole cell vaccine, are lacking. Genotype and biotype diversity of the target pathogen represents another major challenge for vaccine development in fish (Munan'andu *et al.,* 2016). Recent studies of *Fno* genetic diversity using PCR-based typing or sequencing methods, revealed no discrimination among *Fno* iolates from different countries, thus demonstrating a clonal behavior pattern among these isolates (Leal *et al.,* 2014; Figueiredo *et al*., 2016; Gonçalves *et al*., 2016; Ramirez-Paredes *et al.,* 2017b). In addition, cross-reactivity between anti-serum raised against *Fno* UK isolate and *Fno* isolates from distinct geographical regions, with minor antigenic differences between isolates has been highlighted in the proteomic study presented in Chapter 2. Hence, the similarity in antigenic profile of these isolates might suggest cross-protection between heterologous *Fno* isolates with the *Fno* vaccine described above. Based on the results of the proteomic studies in Chapter 2 and 3, a whole cell formaline killed adjuvanted vaccine was developed using *Fno* UK isolate. The present chapter aimed to investigate the efficacy of this formalin-killed vaccine to induce protection in Nile tilapia, *Oreochromis* niloticus, against i.p. challenge with *Fno* isolates obtained from different geographical regions. The specific IgM response in fish serum post-vaccination and post-challenge and the expression levels of four immune related genes (*IgM*, *TNF-α*, *MHCIIβ* and *IL-1β*) in response to vaccination were examined. In addition, cross-reactivity of serum raised against the vaccine isolate when screened against heterologous isolates of *Fno* using

Western blotting was investigated and *Fno* load in surviving fish post-challenge was quantified using real-time qPCR.

4.2. Materials and methods

4.2.1. Fish

4.2.1.1 Source and acclimatisation of fish

Nile tilapia, *O. niloticus* (L.) with a mean weight 13 ± 0.8 g and an average length of 10 ± 0.13 cm were obtained from a commercial tilapia farm in Petchaburi province, Thailand, a source with no previous history of *Fno* infection, and transported to the wet laboratory of Fish Vet Group Asia Ltd. (FVGAL), Chonburi, Thailand. Upon arrival, fish were acclimated for 2 weeks in 100 L tanks in a recirculation system (Fleuren and Nooijen, Netherlands) with dechlorinated water, aerated with air stones. The water quality parameters were as the following: temperature ~28°C, dissolved oxygen (DO) 6.5-7 mg/L, pH 7-7.5, free ammonia ≤0.1mg/L, nitrite ≤0.25 mg/L, nitrate ≤0.2 mg/L. The parameters were monitored throughout the day using a water quality sensor (Senseye® Reef V2, UK) which sends out alerts when any of the water parameters are outwith the specified range and the photoperiod was maintained at 12 h light :12 h dark. Fish were fed a commercial tilapia feed containing 30 % crude protein (CPF, Thailand) at a rate of 3% body weight per day. All experimental procedures with live fish were performed in accordance with the UK animals (Scientific Procedures) Act 1986 and associated guidelines (EU Directive 2010/63/EU for animal experiments) and were approved by the Animal Welfare and Ethical Review Body (AWERB) of the Institute of Aquaculture, University of Stirling, UK.

4.2.1.2. Confirmation of Fno free status

Francisella genus specific PCR (Forsman *et al*. 1994) was used to confirm the *Fno*-free status of the fish proposed for the experiment. Six fish were euthanized by overdose of benzocaine (500 mg/L) (Sigma-Aldrich, UK) and head kidney and spleen were sampled and preserved in 95% ethanol (Sinopharm, China) for screening by PCR. Total DNA was extracted from pooled spleen and head kidney samples using DNeasy Blood and Tissues kit (QIAGEN, Germany) following the extraction protocol for tissues. Briefly, 20-30 mg of the tissue samples were lysed in lysis solution (180 µL of buffer ALT and 20 µL of 20 µg/µL of proteinase-K). The tissues were homogenised and mixed thoroughly with the

lysis buffer on a vortex mixer (Clifton Cm-1, UK) for 30 s then incubated at 56°C overnight. After lysis, the samples were mixed for 15 s, then 200 µL of buffer AL in the kit and 200 µL of 100 % ethanol (Sigma, UK) were added and samples were mixed again for 10 s. The mixture was pipetted into DNeasy mini columns placed in 2 mL collection tubes and centrifugated at 6,000 × *g* for 1 min at 10°C in a refrigerated SIGMA 2-16K centrifuge (Sigma squib, UK). The collection tubes containing the flow-through were discarded. The membrane was washed using 500 µL of buffer AW1, tubes were centrifuged at 6,000 x *g* for 1 min and flow-through was discarded. Tubes were washed again with 500 µL of buffer AW2 and centrifugated at 20,000 × *g* for 3 min to dry the DNeasy membrane. The DNA was eluted by adding 200 µL of Buffer AE to each tube followed by centrifugation at 6,000 × *g* for 1 min. The concentration of the eluted DNA was measured using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK). DNA samples were standardised to 100 ng/ µL with Milli-Q (M.Q.) water.

PCR was performed in 25 µL reaction formed of 1X ReddyMix PCR Master Mix (Thermo Scientific, UK), 0.2 µM of each primer pair (F5: 5'-CCTTTTTGAGTTTCGCTCC-3', F11: 5'-TACCAGTTGGAAACGACTGT-3') (MWG Eurofins, UK), 100 ng/ µL of DNA template and up to 25 µL M.Q. water. Cycling conditions consisted of an initial denaturation step of 2 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 65°C and 1 min at 72°C, and a final extension step of 5 min at 72°C in a Light Cycler™ 96 (Roche, Germany). Five µL of the amplification products were visualized on 1% (w/v) agarose gel containing 0.1 µg/mL EcoDye ™ DNA staining solution (SolGent, Bio fact Co. Ltd, Korea) in Tris-Acetate-EDTA (TAE) buffer (ThermoFisher Scientific, UK) after electrophoresis for 40 min at 80 volts. A 100 pb DNA ladder (GeneRuler Express DNA ladder, Fisher Scientific, UK) was used to confirm the presence of the *Fno* 16S rRNA gene amplicon of 1140 pb and 100 ng of *Fno* DNA and Milli-Q water were used as positive and non-template controls, respectively. The gel was visualized under a UV illuminator (Bio-Imaging, Syngene, UK).

4.2.2. Preparation of the vaccine

The vaccine was prepared as described by Ramirez-Paredes (2015). Briefly, an aliquot of the stock culture of *Fno* UK isolate (STIR-GUS-F2f7) maintained at -70°C in modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (Becton

Dickenson BBL, USA) and 0.1% glucose (MMHB) with 20% glycerol (Fisher Scientific, UK) was defrosted and 30 µL was streaked onto cysteine heart agar supplemented with 1% bovine hemoglobin (CHAH) (Becton Dickenson BBL, USA) for 72 h at 28°C. A single colony from the agar plate was inoculated into each of three 50 mL centrifuge tubes containing 15 mL MMHB with 2% IsoVitaleX and 0.1% glucose (Baker *et al*., 1985). Bacteria were grown as a primary culture overnight at 28°C in a shaker incubator (Biosan, Korea) at 175 rpm. After incubation, 50 µL from each bacterial culture was streaked onto CHAH to check purity and the remaining culture was added to each of three 500 mL screw capped bottles (Fisher, UK) containing 250 mL of MMHB with IsoVitaleX and glucose. Bottles were incubated in a shaker incubator at 28°C at 150 rpm for 18 h. Each bottle was divided into 6 × 50 mL centrifuge tubes. The tubes were centrifuged in an Eppendorf 5417C centrifuge (Eppendorf, UK) at 4000 × *g* for 15 min at 4°C and the supernatants were discarded. Filtered sterile PBS (20 mL) was added to each tube and vortexed until the pellets were completely resuspended. Tubes were centrifuged as described above, and the supernatant was discarded. Washing was repeated 3 times and 5 mL of filtered sterile PBS was added to each tube and vortexed to re-suspend the bacterial pellets. All 6 tubes were combined into a sterile 250 mL bottle and the optical density (OD_{600}) was adjusted to 0.4 (~1.2 $\times 10^9$ CFU/mL) using sterile PBS. The number of viable bacteria (CFU/mL) in the vaccine was confirmed by performing drop count. In brief, the bacterial suspension was adjusted to OD_{600} 0.4 followed by ten-fold serial dilutions, and 20 µL from each dilution was pipetted onto five sections of a CHAH plate, which was incubated for 72 h at 28°C. The number of bacteria (CFU/mL) was calculated by multiplying the average number of colonies per drop by 50 and the dilution factor. The bacteria were then inactivated with 0.5 % (v/v) of formalin 40 % (Formaldehyde 37 %, Sigma, UK) with gentle stirring on a magnetic stirrer (VWN, Korea) at 4°C for 96 h, then 1 % (v/v) of 15 % sodium metabisulfite (Sigma, UK) was added to neutralise the formalin with gentle stirring at 4°C for 48 h. The inactivated bacteria were aliquoted into 50 mL centrifuge tubes (40 mL each) and centrifuged at 3500 × *g* for 20 min, supernatants were discarded, and the pellets were washed 3 times for 20 min using 1X sterile PBS. The inactivated bacterial suspensions in all tubes were combined into one sterile 100 mL bottle and OD_{600} was adjusted using sterile PBS to 0.4 (1.2 $\times 10^9$ CFU/mL). To confirm purity and inactivity, 30 µL of the bacterial suspension was streaked onto CHAH and incubated at 28°C for 72 h. The vaccine

was formulated by adding 33 mL of the concentrated killed bacteria at OD_{600} 0.4 to 77 mL of a commercial adjuvant (Montanide ISA 763 A VG, Seppic, France) and emulsified using variable speed blender in 4 homogenisation steps including 4000 rpm for 3 min, 5000 rpm for 30 s, 4000 rpm for 1 min and 5000 rpm for 30 sec, respectively. The vaccine was then stored at 4°C until used. The physical characteristics of the vaccine were evaluated as follows:

A- Homogeneity and droplet size: 1 drop of the vaccine was put on a clean glass slide then gently covered by cover slip and examined under a light microscope using 40x lens.

B- Stability: the vaccine was stored at 4°C for 24 h, 72 h and 1 week, respectively and the stability of the emulsion was checked by holding the bottle toward a light source and a drop test performed by transferring one drop of the vaccine to a beaker filled with water and observing the stability of the drop on the surface of the water.

C- Syringability: 1 mL tuberculin syringe connected with 27G x1/2″ hypodermic needle (NIPRO Europe N.V., Belgium) was filled with the vaccine. The ease and time required for releasing the vaccine from the syringe under a standard force was assessed.

D- Safety: 10 fish (~14 g) were injected i.p. with 0.2 ml (i.e. twice the recommended dose) of the vaccine, held at 28°C, fed 3% body weight/day for 10 days and checked for any abnormalities, signs of toxicity or mortalities post-vaccination.

4.2.3. Pre-challenge of un-vaccinated fish with multiple *Fno* isolates

A pre-challenge was performed using three pathogenic *Fno* isolates from clinically infected tilapia from different geographical locations including 1 homologous isolate and 2 heterologous isolates (Table 4.1). Bacterial isolates were injected i.p. to identify the bacterial dose resulting in 60-70 % mortalities (LD_{60-70}) in naïve tilapia. One hundred and fifty fish (mean weight 18 ± 0.3 g) divided into 3 groups each consisting of 5 tanks were used in the trial. The tanks contained 20 L of recirculating chlorine-free water with 10 fish each.

Table 4.1. *Fno* isolates used for the pre-challenge experiment.

Isolate name	ID	Source	Origin
Fno UK isolate	STIR-GUS-F2f7	Red Nile Tilapia	England
Fno Mexican isolate	Fran-Cos1	Nile tilapia	Mexico
Fno Costa Rican isolate	NVI-PQ1104	Nile tilapia	Costa Rica

The bacterial isolates were grown on CHAH and incubated at 28°C for 72 h in a 74 L incubator with ventilation (Memmert IN 75, Germany). The cultures were transferred into 15 mL of MMHB (BD diagnostics) with 0.1% glucose (Sigma Aldrich) and 2% IsoVitaleX (BD diagnostics) then incubated in an orbital shaking incubator (ES-20 Biosan, Korea) at 150 rpm for 18 h. After incubation, the tubes were centrifuged at 4000 × *g* at 4°C for 15 min. The pellets were washed twice with 15 mL of sterile PBS. The OD_{600} of the bacterial suspension was adjusted to 0.4 (~1.2×10^9 CFU/mL) and serially diluted to achieve five doses for performing the pre-challenge (Table 4.2). The doses used for injection were confirmed by performing drop counts as described in Section 4.2.2. After dose confirmation, fish were anaesthetized with benzocaine (Sigma-Aldrich, UK) (10% in 100% ethanol) and each group was i.p. injected with 0.1 mL of each isolate of *Fno* at the different doses shown in Table 4.2, using 27 G ×1/2″ hypodermic needle.

Table 4.2. Doses of *Fno* isolates used in the pre-challenge experiment.

Fno isolate	Challenge dose (CFU/mL)				
	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5
UK	1.1×10^8	1.3×10^7	1.25×10^6	1.4×10^5	1.2×10^4
Mexico	1.15×10^8	1.19×10^7	1.2×10^6	1.1×10^5	1.35×10^4
Costa Rica	1.25×10^8	1.15×10^7	1.3×10^6	1.25×10^5	1.3×10^4

Fish were examined four times per day for 15 days, fed three times per day to satiation and the water temperature was maintained at 23 ± 2°C to mimic the natural conditions for the occurrence of francisellosis. Dead fish were removed, and moribund fish were euthanized, necropsied and examined by bacteriology after streaking spleen homogenates on CHAH and incubating at 28°C for 72 h. Molecular identification of *Fno*

was performed using the PCR protocol described in Section 4.2.1.2. After 15 days, the survivors were euthanized by an overdose of benzocaine to terminate the experiment.

4.2.4. Fish vaccination

Following acclimation for 2 weeks, fish (mean weight 15 ± 0.2 g) were divided into four groups, group 1: vaccination group (*n*= 260), group 2: adjuvant-alone group (*n*= 260), group 3: PBS control group (*n*= 260) and group 4: naïve group (*n*= 20). The fish were stocked in 100 L recirculation tanks filled with chlorine-free water. The vaccinated, adjuvant-alone and PBS control groups consisted of duplicate tanks (130 fish/tank), while the naïve group consisted of a single tank of 20 fish.

Fish were starved for 24 h, anaesthetized with benzocaine and injected i.p. with 0.1 mL of adjuvanted vaccine, adjuvant (30% sterile PBS (Sigma-Aldrich, UK) emulsified with 70% of adjuvant) or sterile PBS using a vaccination gun connected with multi-fit bottle, counter and adjustable self-refilling 23G x 4 mm short bevel needles (Kaycee for veterinary products, UK). The naïve group did not receive any treatments during the experiment. Fish were fed 3% of their body weight 3 times/day 24 h after injection, and water quality was monitored throughout the trial as described above. Fish were maintained at 28 ± 2°C for 30 days (840 degree days (dd)) and checked 3 times/day for any abnormalities.

4.2.5. Fish challenge

At 30 days post-vaccination (30 dpv) (840 dd), each of the three main groups (i.e. vaccinated, adjuvant-alone and PBS control), were subdivided into 3 sub-groups, each one consisted of 2 tanks holding 30 fish/tank. Fish were anaesthetized as described above and each two parallel tanks received i.p. injection of the LD_{60} (obtained from pre-challenge) of one of the three *Fno* isolates as shown in Table 4.3. (i.e. 2 tanks for *Fno* UK, 2 tanks for *Fno* Costa Rica and 2 tanks for *Fno* Mexico). Fish were maintained for 15 days at 23 ± 2°C, examined 4 times per day and water quality monitored as described above. Fish received feed *add libitum*, mortalities were removed, and moribund fish were euthanized, necropsied and specific mortalities determined by bacteriology and PCR. The experimental design is shown in Figure 4.1.

Table 4.3. Doses of *Fno* isolates used in the challenge experiment.

Fno isolate	Dilution
UK	1.25 ×10^6 CFU/mL (1.25 ×10^5 CFU/fish)
Costa Rica	1.2 ×10^6 CFU/mL (1. 2 ×10^5 CFU/fish)
Mexico	1.3 ×10^6 CFU/mL (1.3 ×10^5 CFU/fish)

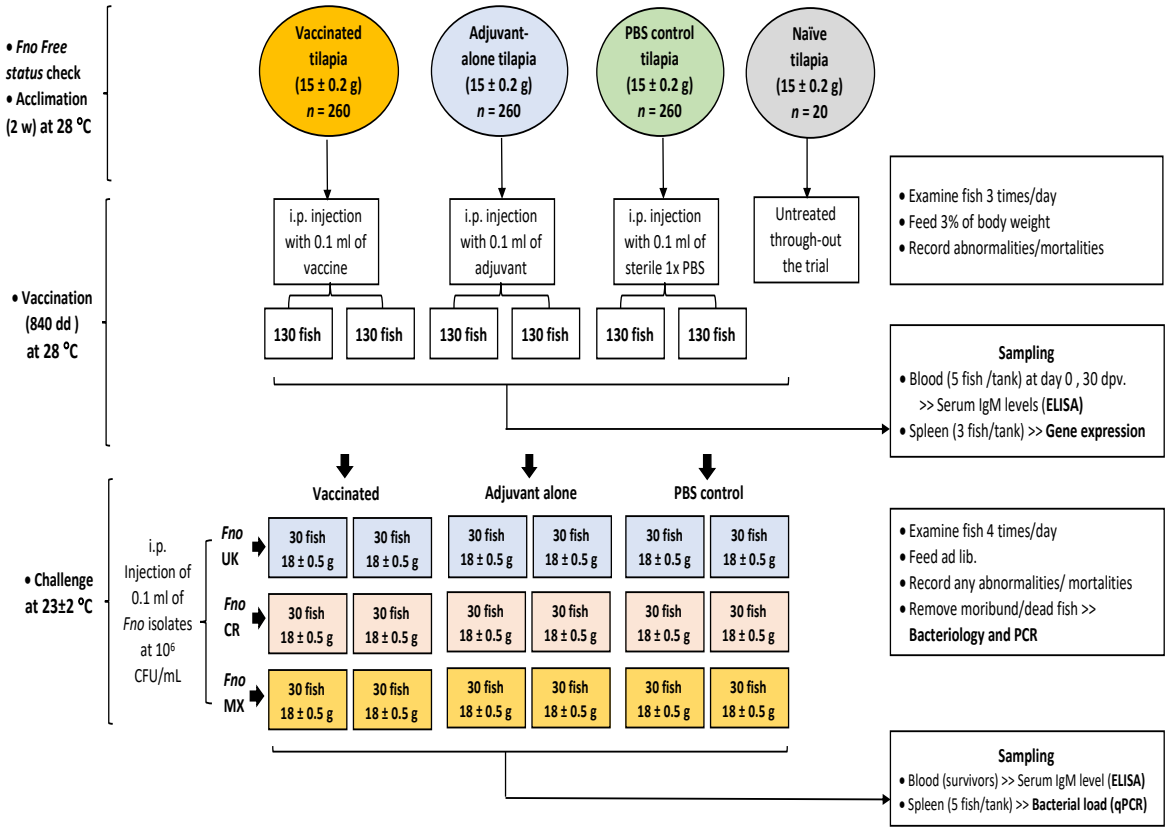


Figure 4.1. Design of *Fno* vaccine efficacy experiment. UK: *Fno* UK isolate, CR: *Fno* Costa Rica isolate, Mx: *Fno* Mexico isolate. dd: degree days.

4.2.6. Sampling

Fish were euthanized with an over dose of benzocaine (terminal anaesthia; 5 mL/L) at 6, 24 and 72 h postvaccination (hpv) and spleens were sampled for analysis of immune gene expression (*n*= 6). Spleen samples were stored in 1 mL of RNA later (Sigma, UK) at 4°C overnight, then the RNA later was removed, and the tissues were stored at -80°C until used. Blood samples were collected by caudal vein puncture from five fish per tank under terminal anaesthia at day zero (D_0), 30 days post-vaccination (30 dpv) and 15 days post-challenge (15 dpc) from the surviving fish using a sterile ½ cc insulin syringe (Nipro®, Japan) with 27G needle (Termu®, Germany). Blood was transferred to microcentrifuge tubes, kept at 4°C overnight then centrifuged at 3000 × *g* for 10 min. Serum was collected, aliquoted into new tubes and kept at -20°C until used. The relative percent survival (RPS) in this experiment was calculated according to the following formula (Amend, 1981):

$$\text{RPS} = (1 - \frac{\%\ of\ mortality\ in\ vaccinated\ fish}{\%\ of\ mortality\ in\ non-vaccinated\ fish}) \times 100.$$

4.2.7. Indirect enzyme-linked immunosorbent assay (ELISA) for determination of specific IgM in serum of tilapia

An indirect ELISA was used to measure the specific antibody response in tilapia sera at 30 dpv and 15 dpc to the three different *Fno* isolates. Briefly, 96-well ELISA plates (Immulon®4 HBX-USA) were coated with 100 µL of 1% (w/v) poly - lysine in carbonate-bicarbonate buffer (Sigma-Aldrich, UK) and incubated at room temperature (RT) (~22°C) for 1 h. The plates were then washed three times by 1X low salt wash buffer (LSWB, Appendix 1). One hundred µL of *Fno* UK isolate in PBS at OD_{600} 0.4 (~1.2×10^9 CFU/mL) was added to each well and plates were incubated at 4°C overnight. The bacterial suspension was prepared as described in Section 2.2.2. The plates were washed three times with LSWB and bacteria were fixed by adding 100 µL/well of 0.05% (v/v) glutaraldehyde (Sigma-Aldrich, UK) in LSWB and incubated for 20 min at RT. Plates were washed as before. Endogenous peroxidase activity was prevented by adding 100 µL/well of 3% stock solution of hydrogen peroxide (Sigma-Aldrich, UK) and plates were incubated for 1 h at RT. Plates were washed three times as before and non-specific

antibody binding sites were blocked by adding 250 µL/well of 5% (w/v) dried skimmed milk (Marvel, Premier Foods Group Ltd, UK) in distilled water for 3 h at RT. After washing plates three times by LSWB, 100 µL/well of serum from 10 fish from different groups (Day 0, 30 dpv and 15 dpc) at a dilution of 1:500 in LSWB with 1% bovine serum albumin (BSA) (Fisher scientific, UK) was added and plates were incubated overnight at 4°C. *Fno* positive and *Fno* negative sera were included on each plate as assay controls at the same dilutions used in the tested serum samples. After incubation, the plates were washed 5 times by 1X high salt wash buffer (HSWB, Appendix 1) with a five min soak on the last wash to ensure removal of unbound antibodies. 100 µL/well of anti-tilapia IgM monoclonal antibody at dilution of 1/75 in PBS (Fo4, Aquatic Diagnostic Ltd, UK) was added and plates were incubated at RT for 1 h. Washing was repeated using HSWB as before and 100 µL/well of goat anti-mouse IgG labelled with horse radish peroxidase (HRP) (Sigma-Aldrich, UK) diluted to 1:3000 in LSWB with 1% BSA was added and plates incubated for 1 h at RT. Plates were washed once with HSWB to remove excess HRP and 100 µL/well of chromogen in substrate buffer (Appendix 1) was added and plates were incubated for up to 5 min at RT. The reaction was stopped by adding 50 µL/well of 2M sulphuric acid (Sigma, UK) and the absorbance was measured at OD_{450} using a micro-plate reader (Biotek Synergy HT, USA).

4.2.8. Serum cross-reactivity

The whole cell protein lysate of *Fno* UK (homologous vaccine isolate), Costa Rica and Mexican isolates (heterologous isolates) were resolved on 1D SDS-PAGE as previously described in Section 2.2.3, Chapter 2. Serum samples collected from fish at day zero (D0), 30 dpv and 15 dpc from the different treatments were used to perform western blot. After transfer of the whole cell lysate of the different isolates, the nitrocellulose membrane was cut into strips containing the individual transferred protein of each *Fno* isolate and placed into an octaline disposable polypropylene tray (Pateof ApS, Denmark). Western blotting was carried out following the protocol described in Section 2.2.6.2. in Chapter 2. *Fno* positive and *Fno* negative sera were included as assay controls.

4.2.9. Immune-genes expression

4.2.9.1. RNA extraction

RNA was extracted from 40-50 mg of spleen samples of three fish per tank, (*n* = 6/group) at 6, 24, 72 h post vaccination (hpv) using TRI Reagent (Sigma, UK) following the manufacturer's protocol. Briefly, tissues were transferred into a 1.5 mL skirted screw capped tube (Thermo, UK) containing 0.5 mL of TRI Reagent, followed by incubation for 15 min on ice then homogenized for 60 s by a mini-beadbeater (BioSpec products, USA) until full disruption. Tubes were incubated at RT (~22°C) for 5 min then 50 µL of 1-bromo-3-chloropropane (BCP) (Sigma, UK) was added into the disrupted tissues followed by a vigorous shaking for 15 s and incubation at RT for 15 min. The aqueous phase was separated by centrifugation at 20,000 × *g* for 15 min at 4°C in a refrigerated SIGMA 2-16K Centrifuge (Sigma, UK) and transferred to a new 1.5 Eppendorf tube (Eppendorf, UK). RNA was precipitated by adding 75 µL of RNA precipitation solution (ThermoFisher Scientific, UK) then inverted gently 4-6 times, incubated at RT for 10 min and centrifugation was repeated for 10 min at 20,000 × *g*. The supernatant was removed, and the RNA pellet was washed for 15 min in 1 mL of 75% ethanol (Fluka, UK) at RT and centrifuged at 20,000 × *g* for 5 min. The resulting RNA pellet was air dried at RT for 5 min to enable evaporation of any traces of ethanol, resuspended in 50 µL of RNase free water (ThermoFisher Scientific, UK) and incubated at RT for 30 min to enable full resuspension. The RNA samples were then stored at -70°C until use.

The quality and quantity of RNA samples were evaluated using a Nanodrop ND-1000 Spectrophotometer (ThermoFisher Scientific, UK). 1 % (v/v) agarose gel (Bioline, UK) containing 0.1 µg/mL ethidium bromide (Sigma, UK) in Tris-Acetate-EDTA (TAE) buffer (Sigma-Aldrich, UK) was used to check the integrity of the RNA. An aliquot of RNA (300-500 ng/µL) mixed with 6X loading dye (Thermo Fisher, UK) was incubated at 65°C for 2 min in a thermoblock (Biometra T1, Germany) and incubated on ice for 1 min then run on an agarose gel. RNA was visualized after electrophoresis under a UV illuminator (Bio-Imaging, Syngene, UK).

4.2.9.2. DNase treatment

The DNA in the RNA samples was removed using DNA-*free*™ kit (Ambion, UK) following the manufacturer's instructions. Firstly, the RNA concentration was adjusted to

2 µg using nuclease free water (ThermoFisher Scientific, UK). A DNA digestion mix was prepared from 1 µL of 1X DNase I buffer, 0.25 µL of 2 U rDNase I and 10 µL of 2 µg RNA sample. Incubation of the mix was performed at 37°C for 30 min then 1 µL of DNase inactivation reagent was added, and the tubes incubated at RT (~ 22°C) for 2 min. Following a centrifugation at 10,000 × *g* for 90 s, the treated RNA was collected and transferred to new 1.5 mL Eppendorf tubes. The quantity and quality of the DNase treated-RNA samples were checked as described in Section 4.2.9.1, the concentration of the DNAse-treated RNA samples was adjusted to 0.5 µg using Milli-Q water and stored at -20°C till used.

4.2.9.3. cDNA synthesis

cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA) following the manufacture's protocol. In brief, 20 µL of reverse transcription (RT) master mix was formulated using 2 µL of 1X RT buffer, 0.8 µL of 4 mM dNTPs, 2 µL of primer mix (1.5 µL of 1X RT random primers and 0.5 µL of 0.5 µM oligo dT (MWG Eurofins genomics, UK)), 1 µL of 50 U of MultiScribe^{TM} Reverse Transcriptase, 1 µL of 20 U of RNase inhibitor (Applied Biosystem, UK), 10 µL of 0.5 µg DNAse-treated RNA sample and 3.2 µL Milli-Q water. Reverse transcription-PCR (RT-PCR) amplification was performed in a thermocycler gradient (Biometra, Germany) using the following cycling conditions: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. A negative RT (RT^-) control was prepared using a sample without MultiScribe^{TM} Reverse Transcriptase in the RT master mix to check any residual contamination with genomic DNA in the prepared RNA samples. Following amplification, the resulting cDNA was diluted 10 times by nuclease free water (ThermoFisher Scientific, UK), aliquoted and kept at -20°C until further use.

4.2.9.4. Reverse transcription quantitative real-time PCR (RT-qPCR)

RT- qPCR reactions were performed using SYBR Green I master mix (ThermoFisher Scientific, UK) and primers (MWG Eurofins genomics, UK) listed in Table 4.4, in white 96-well plates using an Eppendorf® Real plex-2 Mastercycler gradient-S (Eppendorf, UK).

Table 4.4. Primers used in the RT-qPCR.

Gene	Oligo sequence (5' - 3')	Genbank accession no.	Product Size (bp)	Annealing temperature (°C)	Reference
β-actin	F: CCACACAGTGCCCATACTACGA	XM_003443127	144	60°C	Liu *et al.* (2011)
	R: CCACGCTCTGTCAGGATCTTCA				
EF-1α	F: GCACGCTCTGCTGGCCTTT	NM_001279647	250	57°C	Wang *et al.* (2015)
	R: GCGCTCAATCTTCCATCCC				
IgM	F: GGGAAGATGAGGAAGGAAATGA	KC708223	120	57°C	Wang *et al.* (2015)
	R: GTTTTACCCCCCTGGTCCAT				
TNF-α	F: CTTCCCATAGACTCTGAGTAGCG	NM_001279533	161	60°C	Liu *et al.* (2011)
	R: GAGGCCAACAAAATCATCATCCC				
IL-1β	F: TGCACTGTCACTGACAGCCAA	XM_019365844	113	57°C	Liu *et al.* (2011)
	R: ATGTTCAGGTGCACTTTGCGG				
MHC-II	F: ACTGACTGGGACCCGTCCAT	XM_003459253	204	57°C	Pang *et al.* (2013)
	R: ACAGGAAGCAGCCGCTTTTA				

The RT-qPCR was performed in 20 µL reaction mix consisted of 10 µL 1X SYBR® Green I buffer, 0.6 µL of 0.3 µM forward and reverse primers, 5 µL of ten-fold diluted cDNA and 3.8 µL Milli-Q water. The cycling conditions were 94°C for 15 s, 40 cycles of denaturation at 95°C for 30 s, annealing at the optimal temperature of each primer as indicated in Table 4.4. for 30 s and a final extension at 72°C for 1 min. Melting curve analysis included amplification at 60°C to 90°C with 0.1°C increments per second to evaluate the qPCR products specificity. Samples were run in duplicate and each qPCR run included RT negative (RT^-) and non-template controls (NTC) (Milli-Q water only). Serial dilutions of a pool of all cDNA samples were prepared in nuclease free water (ThermoFisher Scientific, UK) including seven dilutions at 1:10, 1:20, 1:50, 1:100, 1:500, 1:1000 and 1:10000. The threshold cycle (Ct) values of these dilutions were plotted versus log concentration to generate a standard curve in the realplex software V2.2 (Eppendorf, UK). The quality of the generated standard curve was evaluated using the slope curve and the correlation co-efficient (R^2). The efficiency of the amplification of the qPCR targets was judged by the line slope following the equation, $E= (10^{-1/slope}) -1$. All the primers used in this study were analysed for self-annealing using NCBI Blast sequence analyser (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

4.2.10. Quantification of *Fno* load in survivor fish by quantitative real-time PCR (qPCR)

Ten spleen samples were randomly collected from surviving fish in the different treatments 15 dpc and preserved in 95% ethanol (Sinopharm, China) for quantification of *Fno* load using quantitative real-time PCR (qPCR). DNA was extracted from 20 mg of spleen tissues using DNeasy blood and tissue kit (Qiagen, UK) as described in Section 4.2.1.2. After extraction, the DNA concentration was measured using a nanodrop ND-1000 Spectrophotometer (ThermoFisher scientific, UK) and standardised to 100 ng/µL by Milli-Q water (Thermo, UK). 1 µL of each DNA sample was visualised by UV illuminator (Bio Imaging, Syngene) after electrophoresis on 1.0 % (w/v) agarose gel containing 0.1 µg/mL ethidium bromide (Sigma, UK) in Tris-acetate EDTA (TAE) buffer (ThermoFisher Scientific, UK). Real time qPCR was performed to quantify the *Fno* genomic load in copy numbers using a dilution range of 10^7: 10^1 copies/ µL of DNA plasmid standard containing

a unique gene (Hypothetical protein gene, Genbank accession no. JQ780323) previously identified in *Fno* strains only (Duodu *et al.,* 2012). The assay was performed using a LightCycler® 2.0 (ROCHE) in a 20 µL reaction volume consists of 0.6 µL of 0.3 µM primers (F: 5'-CAT GGG AAA CAA ATT CAA AAG GA-3' and R: 5'- GGA GAG ATT TCT TTT TTA GAG GAG CT-3') (MWG Eurofins genomics, UK), 10 µL of 1X Luminaris color HiGreen™ qPCR master mix (ThermoFisher Scientific, UK), 1 µL DNA template and nuclease free water (ThermoFisher Scientific, UK) up to 20 µL. The qPCR cycling conditions were adopted from Duodu *et al.* (2012) as following: 50°C for 2 min for uracil-DNA glycosylase enzyme activity, 95°C for 10 min to start denaturing the UNG enzyme and activate the DNA polymerase enzyme then 45 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve analysis formed of 1 cycle at 95°C for 30 s, 55°C for 30 s and 95°C for 30 s. All samples were run in triplicate. After the run, analysis was performed using the default calculation of the threshold fluorescence (Ct value).

4.2.11. Statistical Analysis

Data processing was performed using Microsoft Excel 2013, while GraphPad prism version 7 (GraphPad software Inc., Sand Diego, CA, USA) was used to conduct pairwise Kaplan-Meier survival analyses with subsequent Mantel-Cox log-rank test applied to the mortality data to calculate the survival probabilities and to compare the survival distributions of fish in each experimental group (Kaplan and Meier, 1958). Normality of the data from serum antibody responses and *Fno* load in spleen samples was tested using Komogorov-Smirnov test. One-way ANOVA with a Tukey post hoc test was performed to analyse the differences in optical density (OD_{450}) values representing antibody responses in serum samples and *Fno* load between the different treatments (vaccinated, adjuvant and PBS control groups). In all cases a *p*-value of < 0.05 was considered significant.

The expression of the target genes in this study was normalised to the expression of *β-actin* and *EF-1α*. The fold change in the expression of the target genes in spleen samples of vaccinated and adjuvanted fish compared to the unvaccinated-control samples was calculated following the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001) using the Relative Expression (REST©) Software (Pfaffl *et al.,* 2002). The expression of the target genes in both vaccinated and adjuvanted samples was considered significantly different from that of the control samples when a *p*-value <0.05 was obtained.

4.3. Results

4.3.1. Confirmation of *Fno* free status of fish

PCR results showed that spleen and head kidney samples of the tested naïve fish (*n*= 6) were all negative for *Fno* (Figure 4.2).

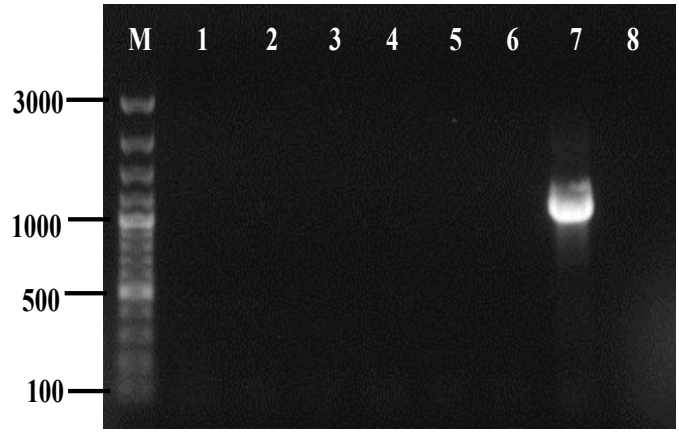


Figure 4.2. 16S rRNA PCR for screening tilapia for the presence of *Fno* before vaccination. 1% agarose gel showing negative results for *Fno* in tested fish. M: 100bp Molecular marker, Lane 1-6 head kidney and spleen pool of 6 naïve tilapia, Lane 7: Positive control, Lane 8: negative control (Milli-Q water only).

4.3.2. Assessment of the physical characteristics and safety of the vaccine

4.3.2.1. Homogeneity and droplet size

A homogenous distribution of the continuous and dispersed phases of the mixture (vaccine-adjuvant) and thin emulsion with particles size of ~ 1-2 µm were observed after microscopical examination of a drop of the produced vaccine.

4.3.2.2. Stability and drop test

The stability of the emulsion was tested after 24 h, 48 h and 1 week after the emulsification. Observations indicated that there was no separation of the liquid phases of the solution, no creaming, breakage, sedimentation or releasing of oil on the surface. Thus, the emulsification of the vaccine had resulted in a stable solution that could be used for fish vaccination. The drop test showed that the vaccine droplet stayed on the surface. This reflects the stability of the vaccine creating a linear drop where the oily component stayed in the superficial layer and a major portion of the drop stayed on the surface.

4.3.2.3. Syringability

By applying of a standard force, the vaccine drops diffused smoothly from the needle and no obstruction was observed. Also, during i.p. injection of the fish, no pressure was required to release the vaccine into their abdominal cavity.

4.3.2.4. Safety

No mortalities were recorded in any of the injected fish. The fish appeared normal and upon dissection, deposition of vaccine was observed in the abdominal cavity either below the swim bladder or distributed through the cavity.

4.3.3. Pre-challenge of un-vaccinated tilapia

Necropsy of either recently dead or moribund fish showed typical signs of francisellosis including enlarged spleen and kidney with multiple white nodules on the surface and massively enlarged gall bladder (Figures 4.3 and 4.4). The pre-challenge with the different bacterial doses revealed that doses of 10^4 and 10^5 CFU/mL caused low mortalities (0%; 20-30%, respectively), while 10^7 and 10^8 CFU/mL induced higher mortalities (>90%) with all isolates used for the challenge. A dose of 10^6 CFU/mL of the three *Fno* isolates used was able to induce ~ 60% mortality, therefore this dose was chosen to challenge the fish at 30 dpv. The cumulative mortality in the pre-challenged fish is shown in Figure 4.5.

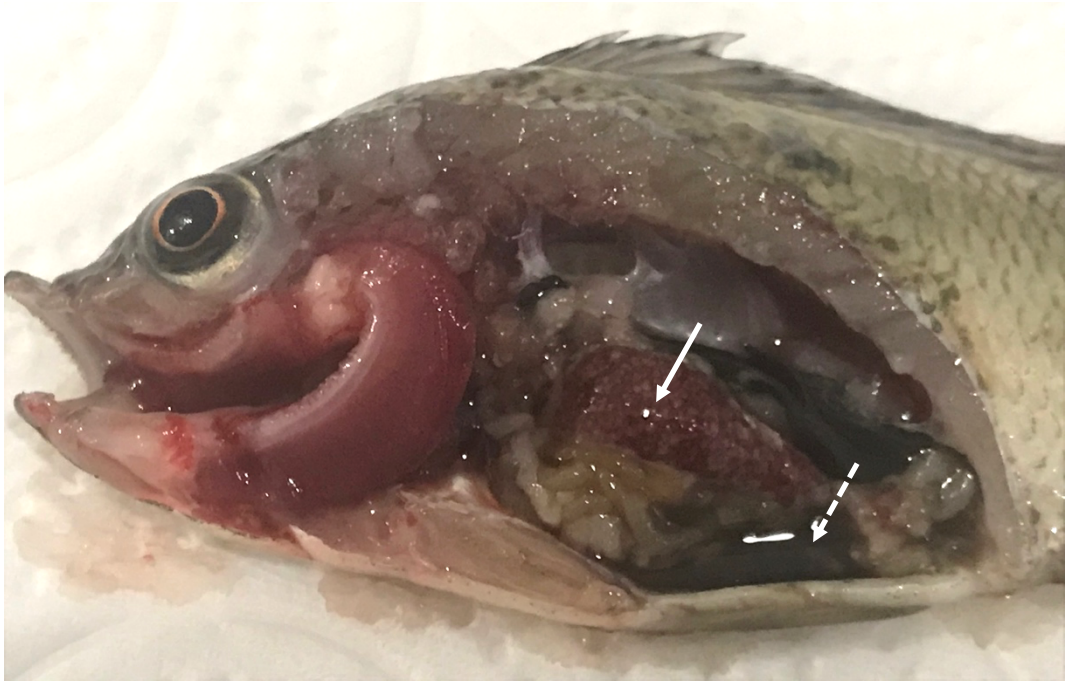
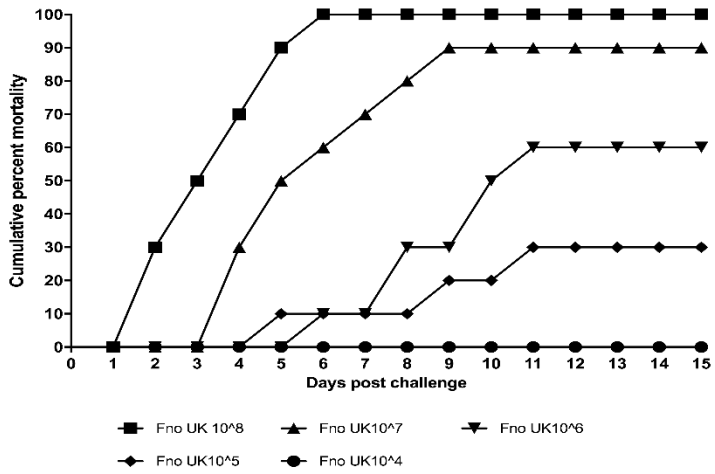


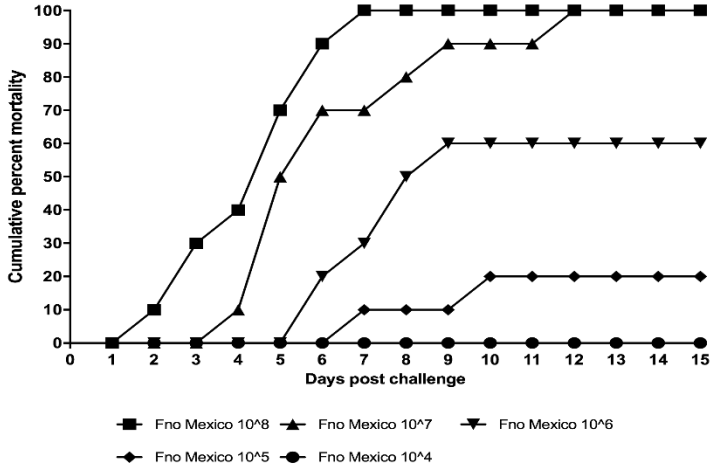
Figure 4.3. Splenomegaly (solid arrow) and enlarged gall bladder (dashed arrow) after *Fno* i.p. injection.

Figure 4.4. *Fno*-infected Nile tilapia showing enlargement of head kidney (dashed arrow) with appearance of white nodules and adhesions in viscera (solid arrow).

A



B



C

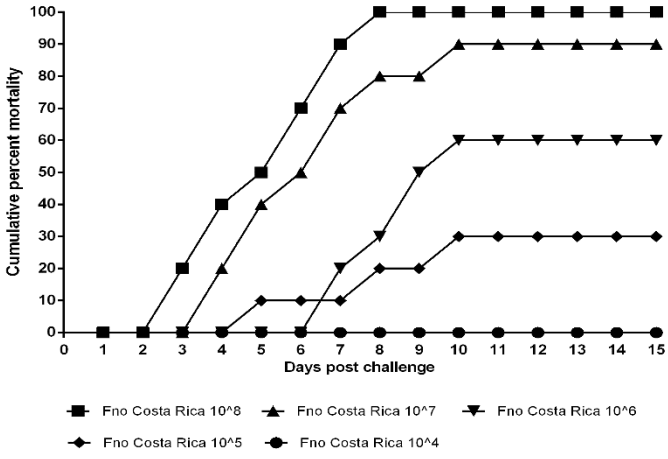


Figure 4.5. Percentage mortality following i.p. injection of five doses of *Fno* UK (A), Mexico (B) and Costa Rica (C) isolates, respectively.

4.3.4. Fish vaccination and challenge

Observation of fish in vaccinated, adjuvant-alone or PBS control groups showed slight differences in their behaviour after the challenge. The challenged vaccinated fish appeared more active, had good appetite and were well distributed in the tanks. The other groups of fish appeared lethargic, gathered toward the sides of the tanks and had reduced appetite. The moribund fish showed abnormal swimming with a distended abdomen. Mortalities started at day 6-8 post-challenge (pc) in the vaccinated group and at day 3- 4 pc in both adjuvant-alone and the PBS groups. Upon necropsy of the mortalities and moribund fish, signs of *Fno* infection were evident, including the presence of ascites and enlargement of the spleen and head kidney, with white or creamy nodules covering their surfaces (Figure 4.6). Detection of *Fno* was confirmed by bacteriology from swab taken from the spleen of moribund fish and recent mortalities (Figure 4.7) and by conventional PCR (Figure 4.8).

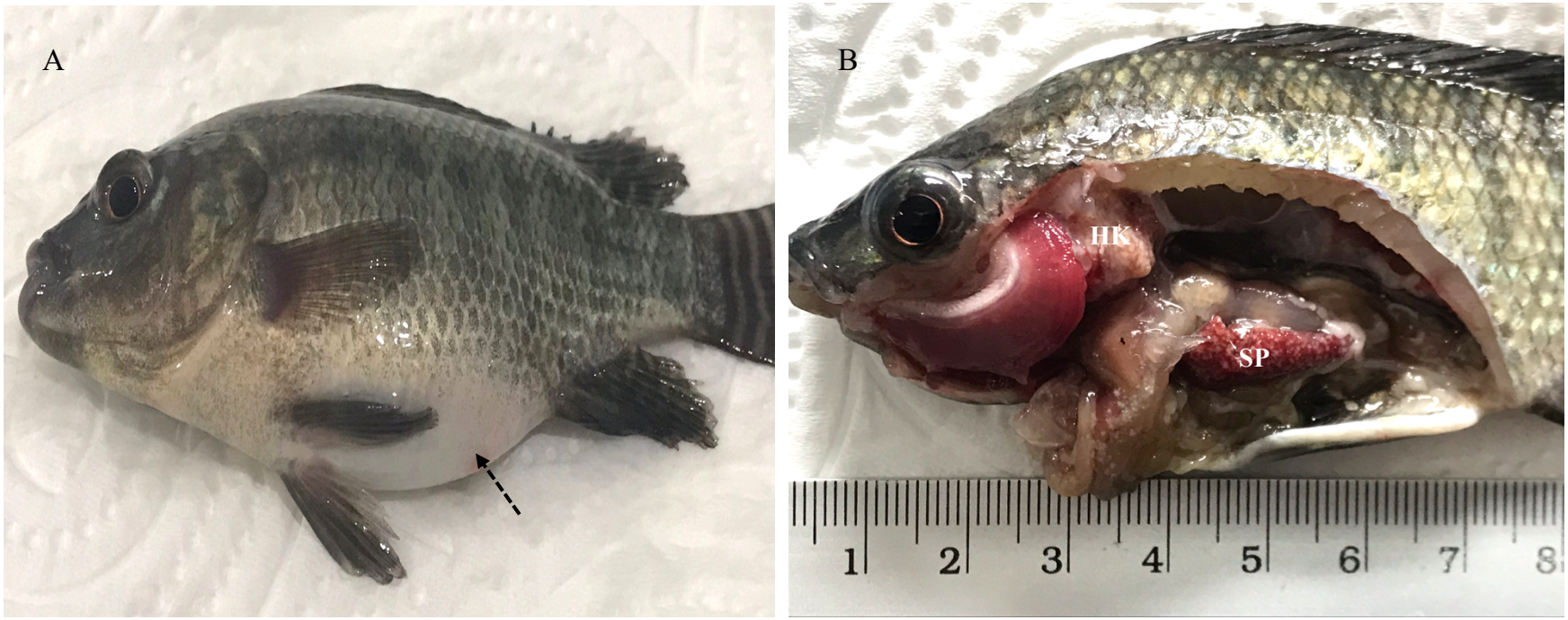


Figure 4.6. Clinical signs of francisellosis in moribund (A) and recently dead (B) tilapia after heterologous i.p. challenge with three *Fno* isolates. (A) Ascites (dashed arrow), (B) enlargement of spleen (SP) and head kidney (HK) with appearance of white nodules on their surfaces.

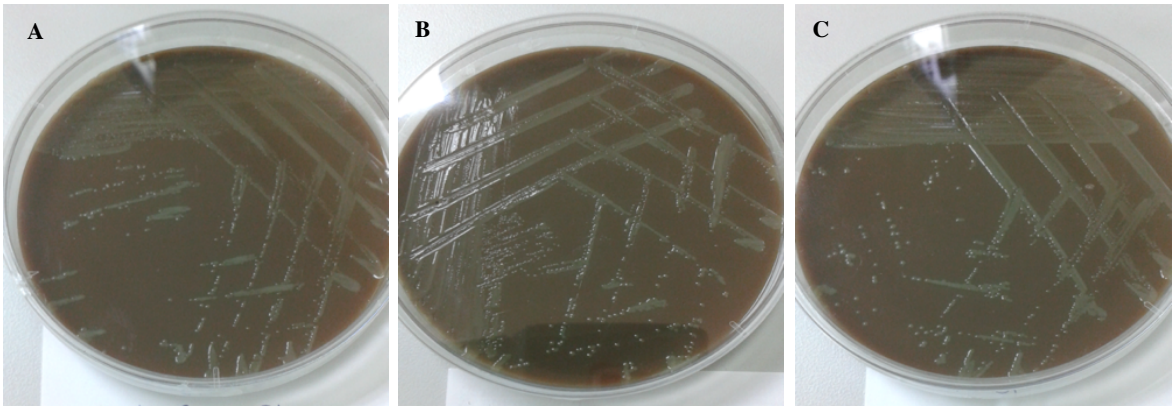


Figure 4.7. Grey, semi translucent and mucoid *Fno* colonies retrieved from spleen homogenate of moribund tilapia after i.p. challenge with *Fno* UK (A), Costa Rica (B) and Mexico (C) isolates on CHAH.

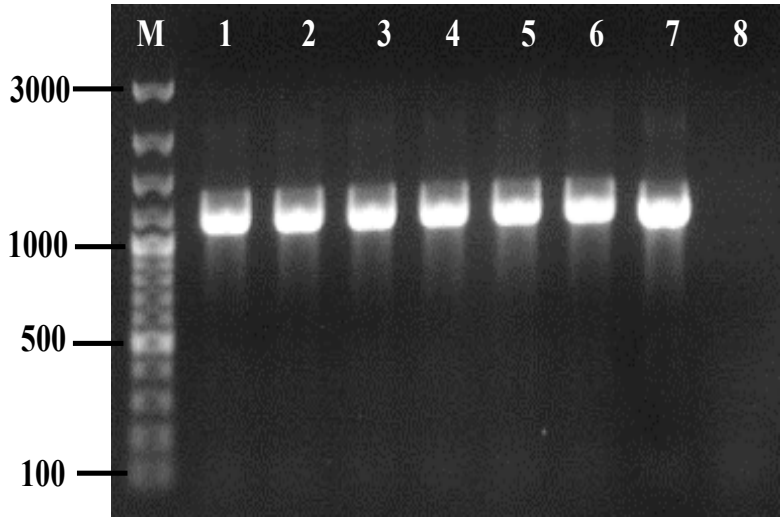


Figure 4.8. *Francisella* genus specific PCR (16S rRNA) for detection of *Fno* in moribund fish and recent mortalities post-challenge with three different *Fno* isolates. 1% agarose gel showed amplicon of 1140 bp. M: DNA ladder, Lanes 1 – 6: spleen of representative moribund fish (1-3) and recent mortalities (4-6) post the heterologous challenge with *Fno* UK (Lanes 1,4), Costa Rica (Lanes 2,5) and Mexico (Lanes 3,6). Lane 7: positive control, Lane 8: Negative control (Milli-Q water only).

The non-vaccinated fish (PBS group) showed the highest mortality at 15 dpc with the different *Fno* isolates. Fish injected with the adjuvant alone had higher survival rates than the PBS control, however these were not significant ($p>0.05$). The vaccinated fish demonstrated the lowest mortalities following challenge and survival was significantly higher than adjuvant-alone and non-vaccinated tilapia post-challenge ($p< 0.001$) (Table 4.5 and Figure 4.9).

Table 4.5. Accumulated mortality in the different groups of fish after challenge (Average mortality % ± SD of 2 parallel tanks holding 30 fish/tank/challenge group)

Fno isolate	Cumulative mortality in vaccinated fish 15 dpc (*n*= 60)	Cumulative mortality in adjuvant-alone fish 15 dpc (*n*= 60)	Cumulative mortality in PBS control fish 15 dpc (*n*= 60)
UK	13.3% (± 0.49)	63.3% (± 1.33)	75% (± 1.8)
Mexico	25 % (± 0.82)	60% (± 1.68)	73.3% (± 1.26)
Costa Rica	21.7 % (± 0.56)	56.7% (± 1.5)	71.7 % (± 1.74)

n: number of fish per challenge group

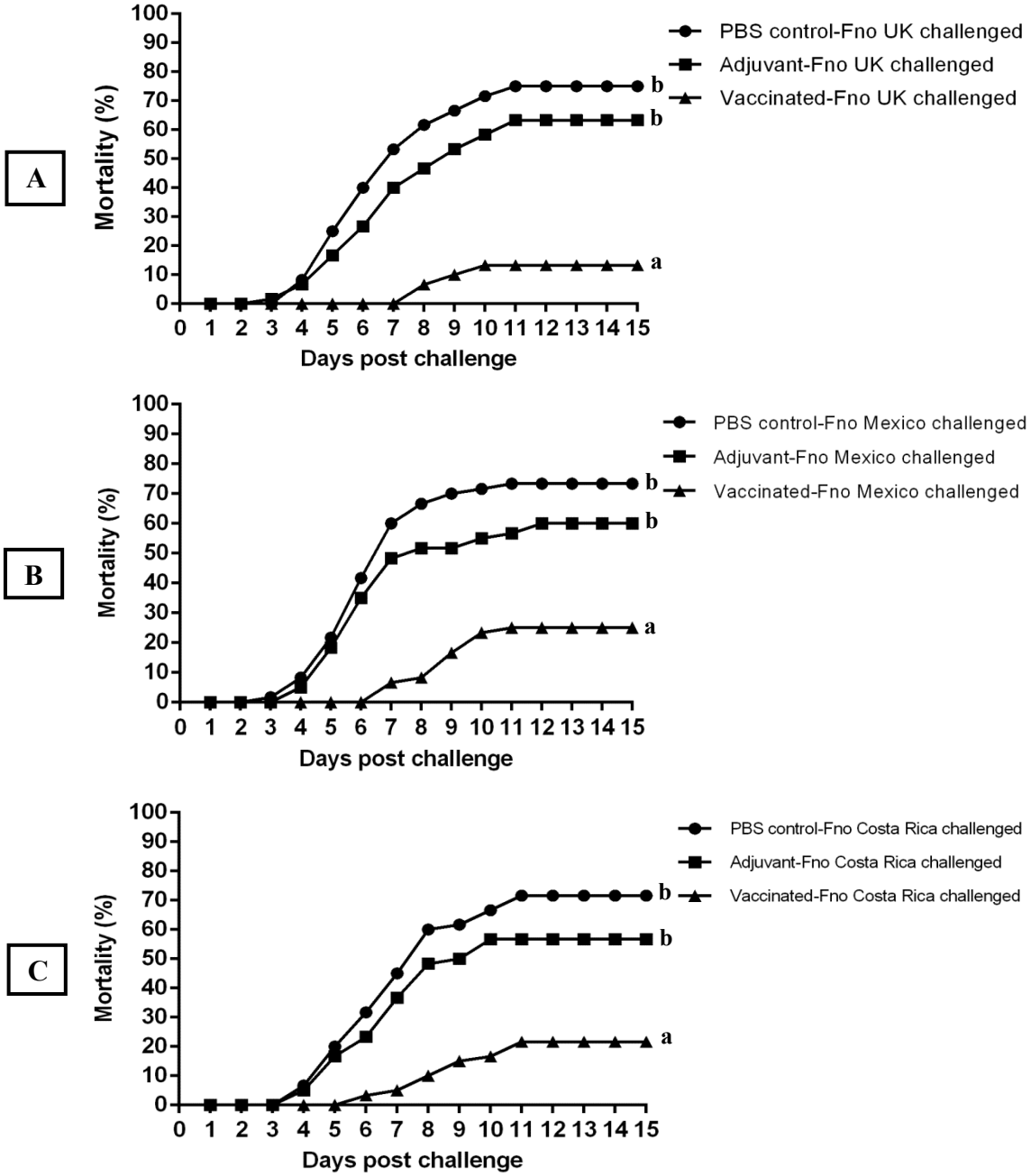


Figure 4.9. Mortalities in vaccinated, adjuvant-alone and control fish after i.p. challenge with *Fno* UK (A), Mexico (B) and Costa Rica (C) isolates. Each line displays the average mortality of 2 parallel tanks with 30 fish /tank/challenge group. Groups that do not share similar symbols are significantly different ($p<0.05$).

The vaccinated fish had RPS levels of 82.3%, 69.8% and 65.9%, while the adjuvant-alone groups had RPS levels of 15.6%, 20.9% and 18.2% after challenge with *Fno* UK, Mexico and Costa Rica *Fno* isolates, respectively. Significantly higher survival ($p< 0.05$) was observed in all vaccinated groups after challenge with the three different *Fno* isolates compared to the adjuvant-alone and unvaccinated control fish. No significant difference ($p> 0.05$) was observed in survival of fish after challenge with the homologous isolate (UK *Fno*) or the heterologous isolates (Mexico and Costa Rican *Fnos*) in the adjuvant-alone or non-vaccinated groups. However, in the vaccinated group, fish challenged with the homologous isolate displayed significantly higher survival ($p< 0.05$) than fish challenged with heterologous isolates which showed non-significant difference between their survival rates ($p> 0.05$). In addition, in the adjuvant-only treatment, fish challenged with Costa Rican isolate was the only group that showed significantly higher survival ($p< 0.05$) to non-vaccinated fish challenged with either the UK or the Mexican isolates (Figure 4.10).

4.3.5. Antibody response after vaccination and challenge

Serum IgM response measured by ELISA showed that vaccinated fish produced significantly higher *Fno* specific IgM levels (OD_{450} at 1:500 dilution) than the adjuvant injected and unvaccinated control fish at 840 dd after immunization. No specific immune response was detected at day 0 in any of the treatment groups. Analysing the IgM levels after vaccination and challenge with the three different *Fno* isolates (15 dpc) showed that the vaccinated fish in the different treatment groups had significantly higher antibody responses ($p<0.05$) compared to the adjuvant-alone and unvaccinated control fish (Figure 4.11). In addition, the IgM level in the *Fno* UK challenged fish was significantly higher ($p<0.05$) than that of fish challenged with either the Mexican or Costa Rican isolates.

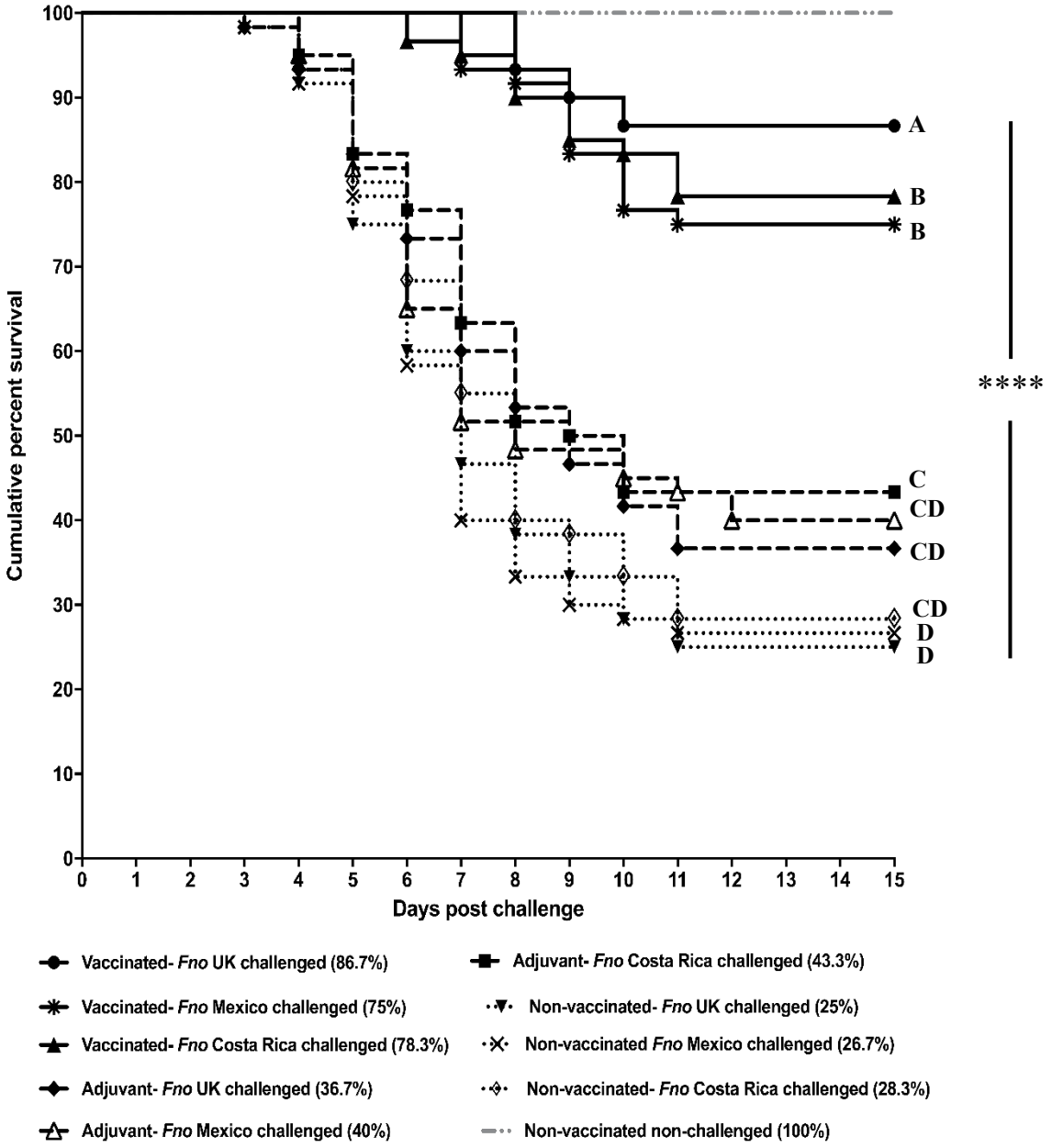


Figure 4.10. Kaplan-Meir (Log-rank Mantel Cox) representation of cumulative survival of tilapia fingerlings 15 dpc with 10^6 CFU/mL of *Fno* UK, Mexico and Costa Rica isolates. Each curve represents the average results of 2 parallel tanks holding 30 fish/tank/challenge group. The non-vaccinated, non-challenged curve represents data from 1 tank with 20 fish. Asterisks refer to the overall significant differences in survival rates between the different treatments ($p < 0.0001$). Groups that do not share similar symbols are significantly different ($p<0.05$).

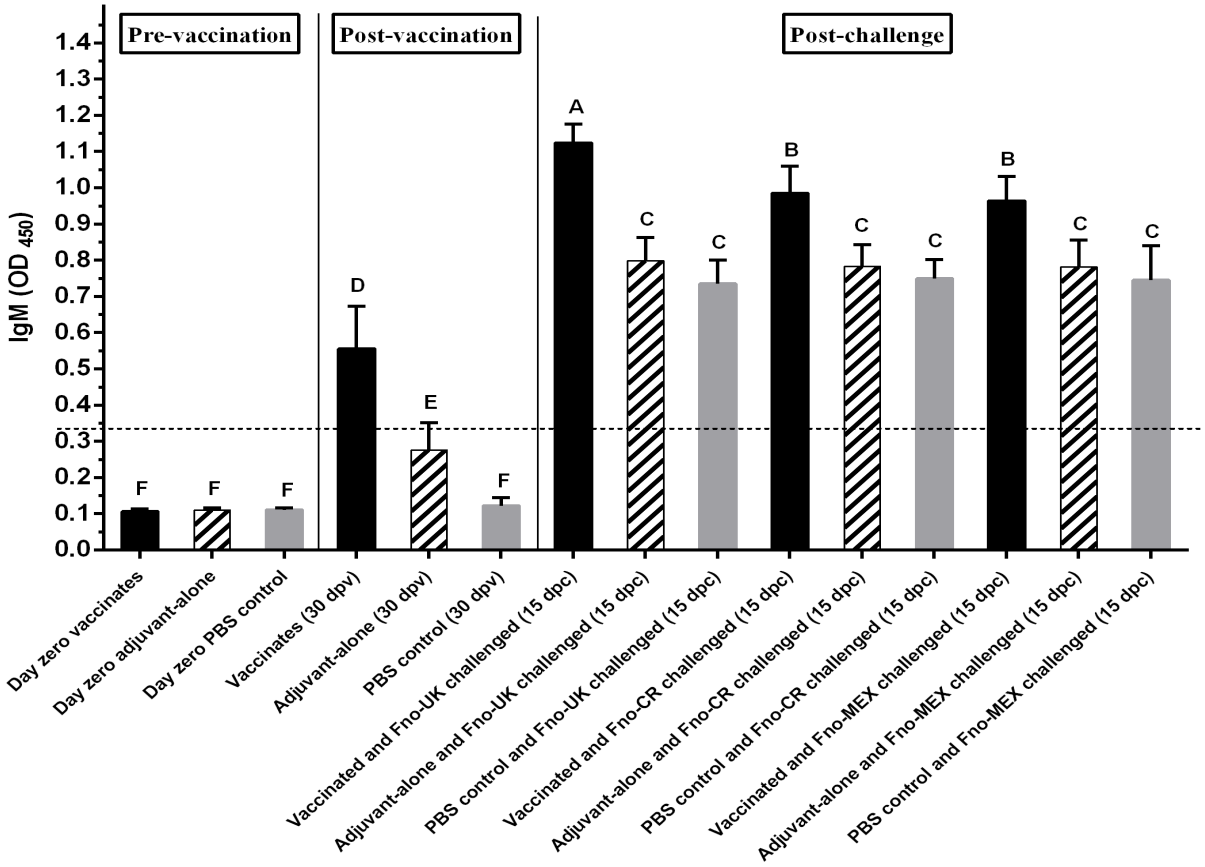


Figure 4.11. Serum IgM response (Mean of OD_{450} ± SD) of tilapia following i.p. injection of vaccine, adjuvant-alone and PBS at 30 dpv (840 dd) and 15 dpc with three *Fno* isolates. UK: *Fno* UK isolate, CR: *Fno* Costa Rica isolate, MEX: *Fno* Mexico isolate, dpv: days post vaccination, dpc: days post challenge. Each bar represents average of IgM at OD_{450} of 10 fish/ treatment. The dashed line represents the cut-off (three times the absorbance of the negative control (PBS)). Groups that do not share letters are significantly different (p<0.05). Serum dilution was 1:500.

4.3.6. Serum cross-reactivity

When subjected to 1D SDS-PAGE, the whole cell lysate of the *Fno* isolates from the three geographical regions showed a similar protein banding pattern as seen in Figure 4.12A. Coomassie Blue and Silver staining revealed a conserved abundant band between 20-37 kDa. This band showed immunoreactivity whith sera obtained from pre-challenged vaccinated fish at 30 dpv (Figure 4.12A). Considerable variability was observed in intensity of the immunoreactive band in the blots between the different *Fno* isolates, where the UK isolate showed the highest intensity. No immunoreactivity was seen with the day zero (D_0) fish sera, pre-challenged adjuvant-alone or non-vaccinated control fish serum samples 30 dpv. The same band was also observed with a stronger intensity when the whole cell lysate of the vaccine isolate (*Fno* UK) was blotted with sera of surviving fish from the different challenge groups 15 dpc (Figure 4.12B).

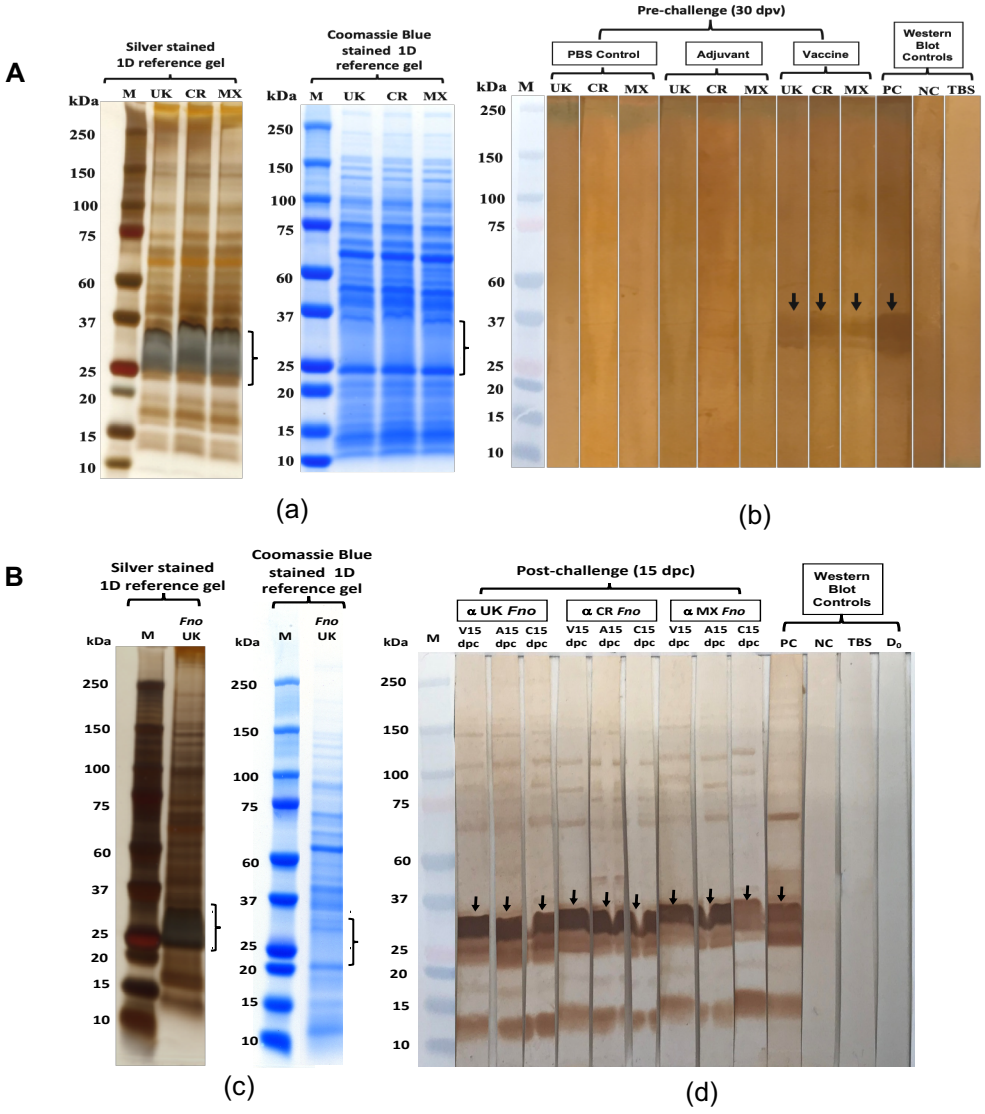


Figure 4.12. Western blot showing immunoreactivity of serum of vaccinated, adjuvant-alone and control tilapia 30 dpv against homologous and heterologous *Fno* isolates (A) and immunoreactivity of serum of survivor tilapia 15 dpc with the different *Fno* isolates in vaccinated, adjuvant-alone and control groups against *Fno* UK (vaccine isolate) (B). The antigenic band on the blots (b, d) is indicated by black arrows and its corresponding protein band on the reference gels (a, c) is indicated by brackets. M: marker, UK: *Fno* UK whole cell lysate, CR: Costa Rica isolate whole cell lysate, MX: *Fno* Mexico isolate whole cell lysate. α *Fno* UK: anti-serum raised against *Fno* UK isolate, α *Fno* CR: anti-serum raised against *Fno* Costa Rican isolate, α *Fno* MX: anti-serum raised against *Fno* Mexican isolate, PC: *Fno*-positive tilapia serum, NC: *Fno*-negative tilapia serum, TBS: Internal control (Tris-buffer saline). Serum dilution was 1:50.

4.3.7. Analysis of immune genes expression by RT-qPCR

The integrity of the RNA extracted from spleen samples was checked by electrophoresis on a 1.0% agarose gel as shown on Figure 4.13, before performing DNase treatment. The relative expression of the target genes *IgM, IL-1β, TNF-α, MHCII* was normalized against *β-actin* and *EF-1α*. The relative fold change in expression of these genes in spleen samples from vaccinated and adjuvant-alone groups of tilapia was compared to the expression in control fish and is summarized in Table 4.6. At 6 hpv, a significant up-regulation of *IL-1β* was seen in both treatments with significantly higher expression in vaccinated groups ($p < 0.001$) than adjuvant-alone ($p < 0.01$). In addition, a significant up-regulation of *TNF-α* ($p < 0.001$) and *MHCII* ($p < 0.01$) was seen in the vaccinated group only. At 24 hpv a significantly higher expression of *IL-1β* and *TNF-α* was observed in vaccinated fish ($p < 0.001$) when compared to the fish given adjuvant-alone ($p < 0.01$ and $p < 0.05$, respectively). *MHCII* was significantly upregulated in vaccinated fish ($p < 0.01$), while it was significantly down-regulated in the adjuvant-alone group ($p < 0.01$). At 72 hpv, a significant up-regulation of *IgM, IL-1β, TNF-α and MHCII* ($p < 0.01$) was observed in the vaccinated group with a significant down-regulation of *MHCII* ($p < 0.01$) in the adjuvant-alone group.

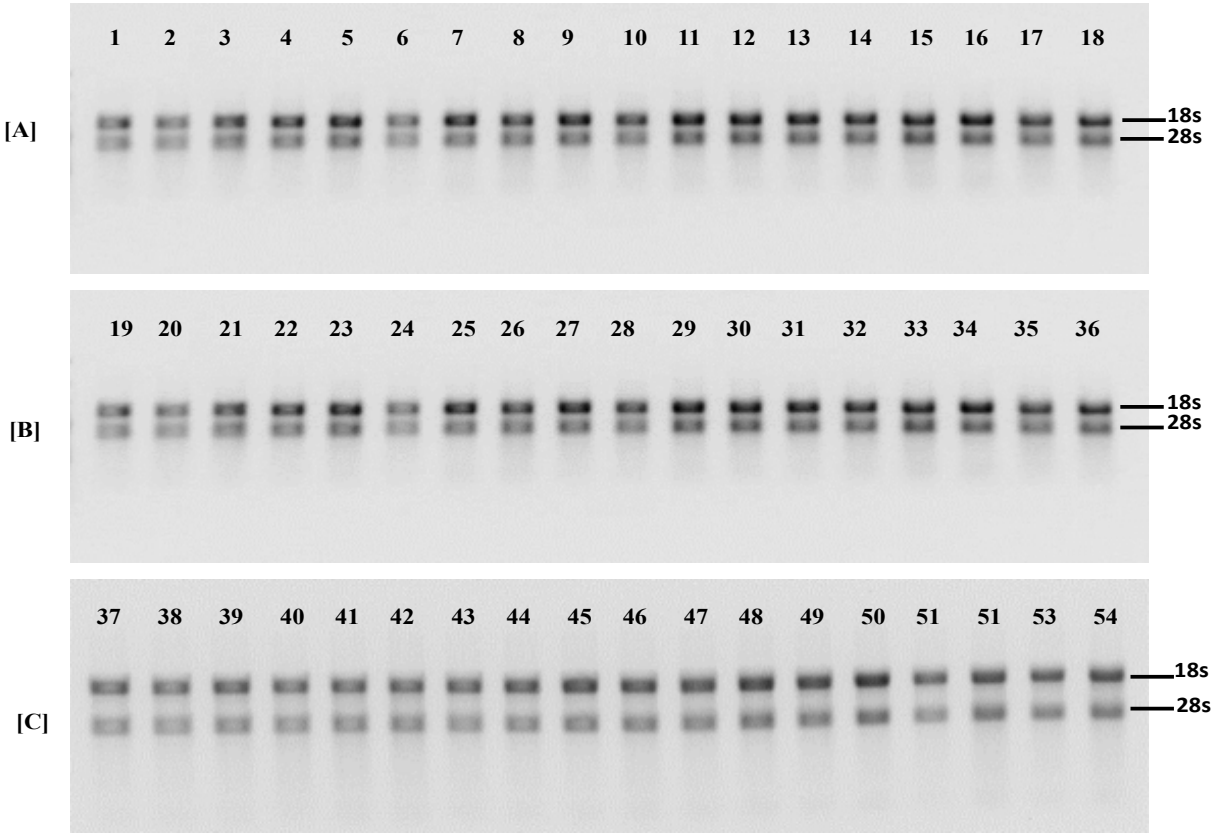


Figure 4.13. Agarose gel electrophoresis showing integrity of RNA in 1% agarose gel. (A) RNA from spleen samples in adjuvant-alone treatment (Lanes 1-18), (B) RNA from spleen samples in PBS control treatment (Lanes 19-36), (C) RNA from spleen samples in vaccine treatment (Lanes 37-54).

Table 4.6. Relative expression of pro-inflammatory and immune related genes in spleen samples of vaccinated and adjuvant-alone groups of tilapia at 6 h, 24 h and 72 h post-vaccination compared to the non-vaccinated control group. (↑ or ↓), (↑↑ or ↓↓) and (↑↑↑ or ↓↓↓) indicates significant up or down regulation relative to controls at ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$), respectively.

Gene	Treatment	**6 hpv**		**24 hpv**		**72 hpv**	
		Expression	SE	Expression	SE	Expression	SE
IgM	Vaccinated	2.306	0.261-22.013	3.077	1.248-7.406	4.956 ↑↑	2.384 - 11.362
	Adjuvanted	1.443	0.403-4.624	2.800	0.694 - 14.70	0.777	0.274 - 3.188
IL-1β	Vaccinated	7.884 ↑↑↑	3.685 - 12.028	5.811 ↑↑↑	1.911 - 11.435	4.977 ↑↑	3.319 - 9.852
	Adjuvanted	5.761 ↑↑	1.728 - 37.970	4.404 ↑↑	1.713 - 11.199	4.269 ↑	0.951 - 28.387
TNF-α	Vaccinated	2.467 ↑↑↑	1.949 - 3.108	2.991 ↑↑↑	2.164 - 3.998	4.539 ↑↑	2.543 - 12.118
	Adjuvanted	1.188	0.876 - 1.659	1.473 ↑	1.112 - 2.199	1.692	0.895 - 4.483
MHCII	Vaccinated	3.409 ↑↑	1.854 - 4.927	4.190 ↑↑	2.048 - 7.672	4.506 ↑↑	2.815 - 6.063
	Adjuvanted	0.770	0.414 - 1.428	0.627 ↓↓	0.435 - 0.861	0.395 ↓↓	0.267 - 0.587

4.3.8. *Fno* load in the spleen of survivor fish post-challenge

Quantification of the bacterial burden (copies/µL) in the spleen samples from the different fish treatments showed significantly higher *Fno* loads (p<0.05) in the un-vaccinated control and the adjuvant-alone groups than vaccinated group after challenge with *Fno* UK, Costa Rica and Mexico isolates, respectively (Figure 4.14). No significant difference was seen in the bacterial burden measured in spleen samples from fish challenged with different *Fno* isolates within the same treatment.

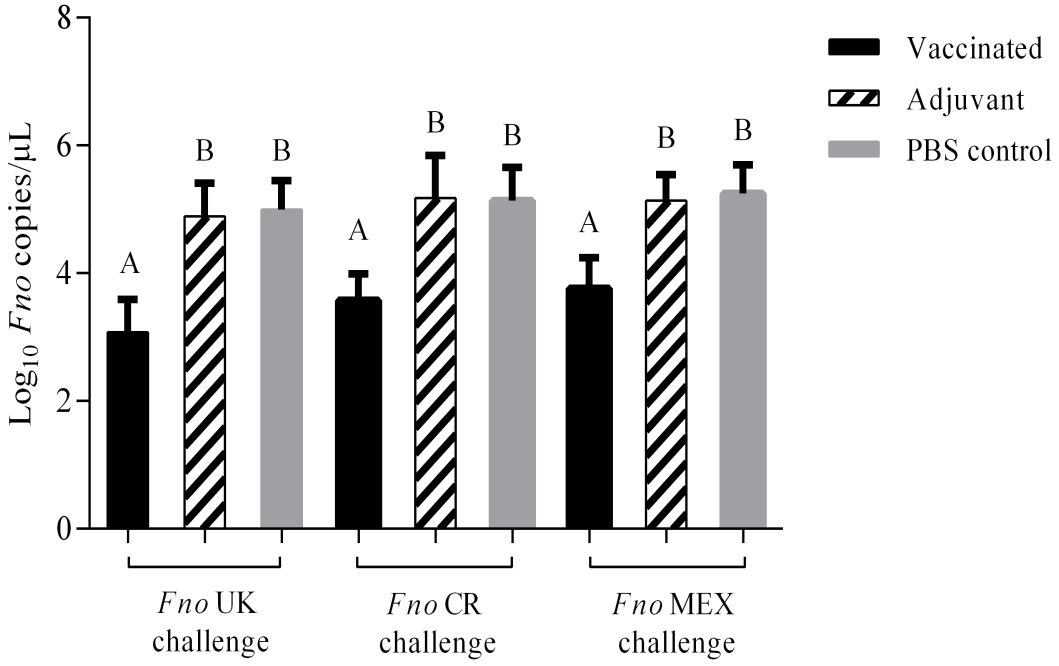


Figure 4.14. *Fno* load (Log_{10} of mean copies/µL±SD) quantified by qPCR in spleen of survivors after i.p. challenge with *Fno* UK, Costa Rica (CR) and Mexico (MEX) isolates in the different treatment groups. Each bar represents average of *Fno* load of 10 spleen samples/treatment. Groups that do not share letters are significantly different (p < 0.05).

4.4. Discussion

Vaccination is the most widely accepted and effective strategy for prevention of infectious diseases in aquaculture. Tilapia farming has been established in many countries due to the high demand of the fish amongst consumers (Pretto-Giordano *et al.,* 2010). With the emergence of piscine francisellosis outbreaks in different countries around the world, high mortality and serious economic losses are being reported in farm-raised tilapia due to infection with *Fno*. Therefore, efforts to develop potent, safe, cost-effective vaccines against *Fno* have become a priority.

In the current study, the efficacy against three clinical *Fno* isolates including one homologous and two heterologous isolates with distinct geographical origins was demonstrated in Nile tilapia fingerlings following immunisation with an inactivated injectable whole cell oil-based vaccine. In addition, the fish immune response was examined by serology post-vaccination and post-challenge and analysis of expression of pro-inflammatory and immune related genes post-vaccination. The vaccine tested in this study conferred significant protection against challenge with a homologous as well as two heterologous *Fno* isolates in tilapia fingerlings (~15 g), compared to fish that received the adjuvant-alone or were mock vaccinated with PBS (Figure 4.10, $p < 0.05$). The significantly higher RPS values ($p< 0.05$) in the vaccinated fish in the present study (82.3 %; 69.8 %; 65.9 %) as opposed to adjuvant injected fish (15.6 %; 20.9 %; 18.2 %), post-challenge with the homologous isolate (UK *Fno*) and the two heterologous isolates (Mexican and Costa Rican *Fno* isolates, respectively) are in agreement with results reported by Ramirez-Paredes, (2015) using the same vaccine, which induced significant protection in tilapia, demonstrated by RPS value of 100% compared to 46.6% in the adjuvant-alone group post-challenge with the same vaccine isolate. Furthermore, the RPS values in the current vaccinated tilapia were similar to those obtained in tilapia fingerlings vaccinated with a live-attenuated *Fno* vaccine after immersion challenge with a self-genotype *Fno* isolate that resulted in RPS of 68.75%: 87.5% (Soto *et al.,* 2011b). Interestingly, the survival rates in the current study were higher than that obtained in zebrafish, *Danio rerio*, i.p. immunised with a *Fno*-outer membrane vesicle (OMV)-derived vaccine and i.p. challenged with the homologous *Fno* vaccine isolate, where a survival rate of 65.5 % was reported 28 dpc (Lagos *et al.,* 2017). This suggests a weaker stimulation of the zebrafish immune system by OMVs when compared with the adjuvanted whole cell

vaccine developed in the current study, although differential susceptibility to *Fno* between tilapia and zebrafish is likely to be a major influential factor.

Analysing different vaccine efficacy experiments, it could be observed that several factors can affect the efficacy of these vaccines, including, antigenicity of the master seed, vaccine composition and use of adjuvants, concentration of the vaccine, route of administration, fish size, temperature of the water, fish species, stocking densities, husbandry, rearing system (recirculation vs flow through) or difference in the bacterial batch used for vaccine production and/or challenges (Pridgen and Klesius, 2013; Evans *et al.,* 2004, Liu *et al.,* 2016; Munang'andu *et al.,* 2016). The difference in the level of protection observed against the homologous isolate (i.e. *Fno* UK isolate) obtained in the current study (RPS 82.3 %) and the study of Ramirez-Paredes, (2015) mentioned above may be attributed to the variability in the genetic make-up and susceptibility of the fish that were used in the experiment. The fish used in the current study were of the wild type Nile tilapia obtained from a commercial farm and therefore may have been exposed to other stressors commonly present in the farm environment, while the autogenous vaccine experiment of Ramirez-Paredes, (2015) included indoor raised-hybrid red tilapia obtained from the in-house aquatic research facility that maintains its own tilapia seed without using fish from any external sources. Moreover, different concentrations of inoculum were used for the fish challenges in the two experiments to achieve specific mortalities. A bacterial dose of 10^5 CFU/Fish used to achieve 70 % mortality (LD_{70}) in the current study, while 10^3 CFU/Fish and 10^5 CFU/Fish were required to achieve the same mortality level in tilapia and zebrafish, respectively (Ramirez-Paredes, 2015; Lagos *et al,* 2017) which may have influenced the RPS observed following vaccination in the different studies.

Immersion vaccines target the natural routes of pathogen entry including fish gills and skin (Soto *et al.,* 2011b). However, immersion vaccines are mainly used for smaller fish (<10 g) and have been found to stimulate the mucosal immune system of the fish leading to enhanced production of specific B-cells located in gills and skin epithelium with a minor effect on the systemic immunity of the fish (Barnes, 2017). Therefore, protection may be short lived and vaccinated fish are likely to require a booster vaccination. Moreover, live vaccines are well known to induce a strong cell-mediated response in addition to humoral immune memory, which is the commonly stimulated immune arm post injection immunisation (Barnes, 2017). Thus, a higher level of protection is frequently

obtained by live attenuated vaccines. Whilst the live attenuated vaccine may seem more effective than the inactivated vaccines, there are concerns related to potential reversion to virulence in fish under stress in the field and release of the live genetically modified organisms (GMOs) into the environment (Muanang'andu *et al.,* 2016), costs and complexity of production (Barnes. 2017), which limit their marketability and regulatory acceptability in fish farms in some countries.

Adjuvants play an important role in modulating the intrinsic immunogenicity of the vaccine antigens and stimulation of non-specific defence mechanisms which can induce protection against a wide range of pathogens (Guy, 2007). Generally, adjuvants can promote induction of both cellular and humoral immune responses in fish mediated by downstream activation of natural killer cells (NKc), phagocytes including macrophages and a distinct subset of T-helper cells (Th cells) (Schijns and Tangerås, 2005; Jiao *et al.,* 2010; Tafalla *et al.,* 2013). Montanide or oil based-adjuvants have been widely used in the formulation of fish vaccines aiming to improve their efficacy and stability (Tafalla *et al.,* 2013). The slow release of the antigen incorporated in the adjuvant (W/O emulsion) can contribute to a highly effective and long-lasting protection against the target pathogen (Brudeseth *et al.,* 2013). An oil-based adjuvant (Montanide™ ISA 763 A VG) has been demonstrated to improve the efficacy of the developed *Fno* vaccine in the current study. When mixed with the inactivated *Fno* and i.p. injected, the vaccine conferred the fish with a high degree of resistance to *Fno* infection. This result was in agreement with that reported by Ramirez-Paredes, (2015), where RPS of 46.6 % was obtained in fish given the adjuvant-alone and challanged with the vaccine isolate. The non-mineral oil adjuvant Montanide ISA 763 A VG used in the current study was successfully used in other commercial and lab-based vaccines for tilapia including a whole cell-inactivated vaccine (AQUAVAC® Strep Sa) and a recombinant vaccine against *S. agalactiae* (Liu *et al.,* 2016). Comparison of the efficacy of tilapia vaccines with and without adjuvant was also reported. Firdaus-Nawi *et al.* (2013) observed that a survival rate of 100% was obtained using a feed-based vaccine containing Freund's complete adjuvant in tilapia post-challenge with *S. agalactiae*, compared to a lower survival rate of 57% when the vaccine was administrated alone without the adjuvant. In rainbow trout, *Oncorhynchus mykiss,* immunisation against *Yersinia ruckeri* resulted in survival rates of 97.5%, 87% and 60% post-injection of adjuvanted vaccine, vaccine alone and adjuvant alone (Montanide ISA

763 A VG), respectively, compared to 100 % mortality in the control fish (Jaafar *et al.,* 2015).

A widely used parameter to predict vaccine efficacy in fish and other higher vertebrates is assessment of the resulting antibody responses and its correlation with protection (Plotkin, 2008; 2010; Munang'andu *et al.,* 2016). A strong correlation between specific antibody production and the level of protection was observed in the current study. At 30 dpv, a relatively weak antibody response was observed in vaccinates, although it was significantly higher ($p< 0.05$) than both adjuvant-alone and PBS control group fish. This corroborates previous results with this vaccine (Ramirez-Paredes, 2015), but was in contrast to weak and non-significant mucosal or serum antibody responses to a non-adjuvanted live attenuated *Fno* immersion vaccine 4 weeks post-vaccination (wpv) in tilapia and an OMV-derived *Fno* injectable vaccine trialled in zebrafish 3 wpv, respectively (Soto *et al.,* 2011b; Lagos *et al.,* 2017). The reason for the lower antibody responses in these studies may be attributed to the difference in the vaccine composition (i.e. addition of adjuvant), the method of vaccine delivery, the vaccine inoculum dose, or the fish species used in the experiment. Elevated specific serum IgM levels were detected in all treatments 15 dpc with the different isolates with significantly higher IgM values in the vaccinated fish. More importantly, significantly higher IgM responses were detected, which correlated with higher survival rates (RPS) in the vaccinated fish challenged with the *Fno* UK (homologous/vaccine isolate) than fish challenged with other *Fno* isolates (e.g. *Fno* Costa Rican and Mexican isolates).

The protective mechanism of immunity against piscine francisellosis is yet to be determned. However, previous results of correlation between the survival in vaccinated fish and the antibody levels in serum or mucus post-challenge in tilapia (Soto *et al.,* 2011b) and zebrafish (Lagos *et al.,* 2017) can corroborate the importance of the antibody-mediated immunity in protection against *Fno* infection. A similar pattern was reported with other bacterial pathogens of tilapia including *Streptococcus iniae* (Shelby *et al.,* 2002), *S. agalactiae* (Pasnik *et al.,* 2005) and *Flavobacterium columnare* (Grabowski *et al.,* 2004), where the humoral immune response has been paramount for protection against these pathogens. Moreover, the specific antibody produced in response to vaccination/or infection with the majority of Gram-negative bacteria act synergistically with the

complement system leading to a direct bactericidal effect on the invading bacteria or can assist phagocytic cell activity. Stimulation of phagocytosis is mainly facilitated by the effect of antibodies on Fc receptor bearing macrophage-like cells and NK cells to destroy the engulfed bacterial cells including intracellular bacteria (Soto *et al*., 2011b). This was previously demonstrated by the ability of antibodies in the serum obtained from tilapia immunised with live attenuated *Fno* vaccine to co-stimulate phagocytosis of *Fno* by head kidney derived macrophages (HKDM) while neither the heat inactivated, nor normal sera obtained from adult tilapia damage the bacteria *in vitro* (Soto *et al*., 2011b). Thus, further *in vitro* studies analyzing the role of the antibodies produced in serum obtained from vaccinated or adjuvant-alone fish post-challenge with the multiple *Fno* isolates to mediate phagocytic uptake of *Fno* may enhance our understanding of the role of the antibody-mediated response in protection against piscine francisellosis in tilapia.

There is a lack of information regarding the role of cellular immunity against piscine francisellosis and most present understanding is based on results from vaccine experiments with *Francisella tularensis*. It was reported that *F. tularensis* has the ability to trigger T-cell mediated immune responses, mainly antigen-specific *IFN-γ* recall responses, besides humoral mediated immunity (Huntely *et al*., 2008; Cong *et al*., 2009) and a strong cell-mediated immune response was suggested to prevent *Francisella* spp. infection in other vertebrates (Kirimanjeswara *et al*., 2008; Chou *et al*., 2013). *IFN-γ*, is a well-known Th1 marker that plays an important role in response to microbial infection via triggering effective innate and adaptive immune response (Vilcek, 2003). *IFN-γ* has been cloned in teleost fish and was reported to be a powerful stimulator of macrophages and can boost a strong *MHC-I* and *II* expression, suggesting a role in antigen presentation (Zou *et al*., 2005), beside modulation of genes responsible for induction of microbicidal activity (Martin *et al*., 2007). In a recent study by Lagos *et al*. (2017) a significant up-regulation of *IFN-γ-1* transcription was reported in zebrafish 24 h post-immunization with *Fno*-derived OMVs vaccine that continued until 21 dpv. These authors suggested a similar role for *IFN-γ* in zebrafish by preventing the escape of *Fno* from the phagosome. Taking into account the complexity of the *IFN-γ* machinery in teleosts as opposed to the mammalian analogue in terms of types, levels of ligands, receptors and the variability in the recruited cell populations, we can expect diverse expression ranges of this gene in different teleost

species. In addition, high expression of the p40 subunit of *IL-12* and *IL-17* in Atlantic cod indicated that *Fnn* infection could drive a stronger immune response towards effective T cell proliferation (Bakkemo *et al.,* 2011). Thus, more studies are required to investigate the role of cell-mediated immunity in protection of tilapia against *Fno* infection at the molecular level (i.e. genes associated with immune cell stimulation).

The antigenicity of the vaccine master seed may constitute a major factor in the efficacy of the vaccine against heterologous bacterial isolates (Pretto-Giordano *et al.*, 2010). The proteomic and antigenic pattern of the five isolates from diverse geographical locations, including the isolates used in the current study, were successfully characterised in Chapter 2 and 3. The proteomic study showed only minor differences in their antigenic profile and the previous molecular-genotyping studies suggested that these *Fno* isolates are clonally-related (Leal *et al.,* 2014; Figueiredo *et al.*, 2016; Gonçalves *et al.*, 2016; Ramirez-Paredes *et al.,* 2017b). However, it is unknown whether the inclusion of one isolate in the current vaccine would cross protect against isolates from different origins. Immunoblotting with sera obtained from vaccinated tilapia before the challenge or sera from tilapia post-challenge with either the homologous or the heterologous *Fno* isolates revealed a cross-isolate antigenicity. Taken together with the induced high survival rates (i.e RPS) post-challenge with the different *Fno* isolates, cross-protection ability of the developed vaccine against challenge with heterologous *Fno* isolates may be highlighted. Further studies using other geographically distinct *Fno* isolates will give us more insights into the efficacy, and in particular, establishment of the cross-protectivity of the developed vaccine. Future work could also include development and efficacy testing of a vaccine containing more than one *Fno* isolate (i.e. bivalent or polyvalent vaccine).

In order to investigate the effect of the developed vaccine on tilapia immune response at the molecular level, expression of some genes associated with innate and adaptive immunity were analysed. Efficient antigen recognition and presentation by the host immune cells is a paramount step in the success of vaccination, which primarily involves Major Histocompatibility Complex (MHC) molecules (MHC-I and MHC-II) located on the surface of the antigen presenting cells (APCs) (Secombes and Belmonte, 2017). MHC-II is restricted to the so-called "professional APCs" which are the immune cells able to internalize extracellular pathogens or proteins including macrophages, dendritic cells and B cells (Secombes and Belmonte, 2017). MHC-II plays an important

role in modulation of the adaptive immune response following vaccination, where interaction of the vaccine antigen-MHC-II complex with the T-cell receptor (TCR) will mount a T-helper cell activation. The later step is crucial in adaptive immunity (Secombes and Belmonte, 2017). In the current study, the transcription of *MHCII* was significantly up-regulated in the spleen of vaccinated fish 6 hpv and at 24 - 72 hpv represented by ~ 4.5 times fold change, while a significant down-regulation was observed in the adjuvant-alone group. This finding suggests the successful recognition of *Fno* antigens in the vaccine and presentation of these antigens by the fish APCs, which is a key step in triggering of a subsequent adaptive immune response.

The rapid activation of pro-inflammatory cytokines in response to the vaccine in the current study was evidenced by an early (6 hpv) significant up-regulation of *IL-1β* and *TNF-α* , which is produced by the activated macrophages in spleen tissue (Zhu *et al.,* 2013). Interleukin 1β (*IL-1β*) is one of the earliest pro-inflammatory cytokines to be expressed in a prompt response to infection via triggering of a cascade of reactions, including up or down-regulation of expression of other cytokines and chemokines. It is mainly involved in lymphocyte activation, leukocyte migration, phagocytosis and diverse bactericidal activities (Ryes-Cerpa *et al.,* 2013). The finding in the current study is in agreement with a previous study by Lagos *et al.* (2017), where a significantly higher *IL-1β* expression was seen in kidney of adult zebra fish vaccinated with *Fno*-derived OMVs at 1 dpv compared to control fish. Moreover, *IL-1β* expression was up-regulated in splenic tissue of Nile tilapia 24-96 h post-challenge with *Fno* (Jantrakajorn and Wongtavatchai, 2016). TNF-α is a well-known pro-inflammatory cytokine that has been identified in various fish species and is expressed in multiple isoforms (Zou *et al.,* 2002; Saeji *et al.,* 2003). It is mainly involved in killing of infected cells, inhibiting intracellular pathogen replication, apoptosis, up-regulation of transcription of various immune-related genes and recruiting leukocytes to the site of inflammation (Reyes-Cerpa *et al.,* 2013). In contrast to the significant up-regulation of *TNF-α* 6-72 hpv in the current study, *TNF-α* transcription in the head kidney of zebra fish adults vaccinated with *Fno*-derived OMVs exhibited down-regulation 1-21 dpv (Lagos *et al.,* 2017). While challenge with *Fno* successfully induced up-regulation of *TNF-α* at 6-96 hpc in tilapia and 1 dpc and 48 hpc in zebra fish adults and larvae, respectively (Brudal *et al.,* 2014; Jantrakajorn and Wongtavatchai, 2016; Lagos *et al.,* 2017).

Despite an observed upregulation of the aforementioned cytokines in the adjuvant alone group, the fold change of their transcription was lower and of shorter duration than that obtained by the vaccine at 6, 24 and 72 hpv. Notably, the *TNF-α* upregulation started earlier at 6 hpv in the vaccinated samples than in the adjuvant-alone group in which *TNF-α* was significantly up-regulated only at 24 hpv. The ability to enhance expression of genes associated with innate and adaptive immune responses was previously reported with other adjuvants, where injection of flagellin in Atlantic salmon triggered up-regulation of *TNF-α, IL-1β*, *IL-6*, *IL-8*, however, this immune response is primarily "non-specific" and lacks the ability to produce specific and or protective antibodies against the target antigen (Hynes *et al.,* 2001). This finding was corroborated by the ELISA and western blot analyses in the current study, where sera obtained from the adjuvant-alone groups 30 dpv demonstrated significantly lower anti-*Fno* IgM compared to the vaccinated group and these sera did not recognize any specific bands on western blots.

Generally, fish vaccination aims to trigger a specific antibody response that is paramount for protection when subsequent infections occur (LaFrentz, *et al*., 2002). This protective adaptive immune response is mainly based upon a massive proliferation of multiple classes of T and B-lymphocytes, where the later constitutes the main repertoire of antibodies in fish. In teleost fish, it was thought, until recently, that IgM was the only well characterised immunoglobulin, but recent researches has demonstrated the presence of other classes including IgT and IgD (Zhang *et al*., 2010; Xu *et al*., 2013; Castro and Tafalla, 2015). However, IgM is the dominant circulating antibody that can be detected in blood, skin, gut, gill mucus and bile (Morrison and Nowak, 2002) suggesting a potential role in both systemic and mucosal immunity with a direct correlation to protection against pathogens and/or immunogens of various origins (Solem and Stenvik, 2006; Castro and Tafalla, 2015). In the present study, a significant up-regulation of *IgM* transcription was noted in the spleen of immunised tilapia at 72 hpv. This indicates the activation of B cells in response to the vaccine antigen that can be also correlated to the increased serum IgM detected at 30 dpv. These results are consistent with the finding of Lagos *et al*. (2017) who reported an up-regulation of *IgM* at 7 dpv that was maintained to 21 dpv following i.p. immunisation of zebra fish adults by *Fno*-derived OMVs. It is worth mentioning that, in the current study only spleen samples post-vaccination were analysed, which were also

collected at early time points following initial immunisation (6, 24, 72 hpv). Hence, further analyses of other tissue samples (e.g. kidney, gills, liver), collected at different time points (i.e. early and late sampling) either post-vaccination or post-challenge would give more insights into the immune responses triggered by the vaccine. More importantly, investigation of the innate/non-specific immune-related factors including antimicrobial peptides, alternative complement and lysozymes, lectins, hemolysins, agglutinins, proteolytic enzymes and phagocytosis activity in the serum of vaccinated fish would improve our understanding of the protective mechanisms induced by injection vaccination against *Fno* in tilapia.

Furthermore, study of the bacterial burden showed that vaccinated fish did become infected, albeit with fewer bacteria than adjuvant-alone and control fish. However, this bacterial load was significantly lower ($p< 0.05$) in comparisons to that detected in adjuvant and the control groups 15 dpc with the different *Fno* isolates. Thus, the protection provided by the developed vaccine may be associated with the ability to enhance clearance and/or limit dissemination of the infection, probably by recruiting activated phagocytes that were found to be the most abundant cell type post *Francisella* infection and identified as the favorable niche for both piscine and mammalian *Francisella* spp. (Clemens *et al*., 2004; Bakkemo *et al*., 2011; Soto *et al*., 2011b; Celli and Zahrt, 2013; Lagos *et al*., 2017). This finding may support the advantage of using bacterial load quantification as one of the vaccine efficacy assessment approaches. Future histological studies investigating the inflammatory and tissue-associated damage post-challenge between vaccinated and control tilapia would allow greater insights into the protection mechanisms of the developed vaccine at the tissue level. Synergism between antibodies, immune-related cytokines, phagocytes and activated complement system has been suggested to be crucial in clearance of intracellular pathogens (Soto *et al*., 2011b). Interestingly, stimulation of the majority of the aforementioned components was observed in this study that may contribute to the efficacy of the produced vaccine against *Fno* infection.

4.5. Conclusions

The current study represents the first report of the efficacy of an oil-based inactivated injectable vaccine against different isolates of *Fno* from diverse geographical origins in Nile tilapia fingerlings. The developed vaccine successfully induced a

significantly high RPS against the different *Fno* isolates which was correlated with significantly higher specific antibody responses in vaccinated tilapia than adjuvant-injected or control fish suggesting the importance of antibody-mediated immune responses in the control of *Fno* infection in tilapia. In addition, the potential cross-protection of the vaccine was highlighted by inter-isolate cross-reactivity shown by immunoblotting that was also supported with a significantly lower *Fno* burden detected in spleen samples from vaccinated tilapia compared to other treatments following challenge with isolates from different geographical locations. Furthermore, the significantly higher expression of *IgM, IL-1β, TNF-α and MHCII* transcripts 72 hpv in the spleen of the vaccinated tilapia provides further evidence of the ability of the vaccine to trigger an effective immune response that contributes to the significant protection of tilapia against *Fno* infection. However, more studies are required to investigate the role of cell-mediated immunity in depth and dissect the relationship between the different immune cell populations (i.e. B cells and T cells) and protection. Considering the short production cycle of tilapia in most tropical countries (~ 4-6 months), a desirable vaccine must induce a significant long-term protection against *Fno*. Thus, more studies are needed to investigate the duration of protection induced by the developed vaccine and test the efficacy under field conditions by performing a field vaccination trial. Moreover, future work should investigate the broad-spectrum protection of the vaccine against more *Fno* isolates. To this end, the whole-cell inactivated vaccine described in the present study may provide the benchmark for developing a broad-spectrum highly protective injectable vaccine against emerging *Fno* outbreaks. Testing the inactivated *Fno* vaccine in immersion trial is required to evalaute the efficacy of this vaccine against *Fno* in tilapia fry.

Chapter Five

Development and efficacy of a whole cell inactivated immersion vaccine against *Francisella noatunensis* subsp. *orientalis* in Nile tilapia

5.1. Introduction

Piscine francisellosis is a significant bacterial disease of fish and molluscs grown in both the wild and aquaculture (Colquhoun and Duodu, 2011; Soto *et al.,* 2014b). In tilapia and other warm water species, the disease is caused by the Gram-negative fastidious intracellular aquatic pathogen, *Francisella noatunensis* subsp. *orientalis* (*Fno*), that has been detected in different geographical locations (Kamishi *et al.,* 2005; Kamishi *et al.,* 2010; Hseih *et al.,* 2006; Mauel *et al.,* 2007; Soto *et al.,* 2009a; Lewish *et al.,* 2016; Nguyen *et al.,* 2016; Ortega *et al.,* 2016). *Fno* induces systemic granulomatosis with high morbidity and variable mortalities up to 95 % have been recorded in all stages of fish (Colquhoun and Duodu, 2011); however, fingerlings (3-30 g) and juveniles (150-500 g) were previously found to be the most affected groups (Sebastião *et al.,* 2017).

Transmission of the disease is associated with the aquatic environment (Colquhoun and Duodu, 2011), however, the portal of entry is still to be defined. Both horizontal and vertical transmission routes have been recorded (Mauel *et al.,* 2003; Soto *et al.,* 2009a; Jeffery *et al.,* 2010; Pradeep *et al.,* 2016) and cases of asymptomatic carriers of tilapia and other susceptible ornamental species were previously reported (Pradeep *et al.* 2016; Soto *et al.,* 2014b). Thus, more research on development of control strategies against *Fno* infection in fish is warranted. The development of vaccines and other strategies to prevent or control piscine francisellosis must take into account the epizootiology of the disease and pathogenesis of *Fno*. Curently there is no commercially available vaccine against francisellosis in tilapia in spite of the serious losses caused by this disease. Various experimental vaccines against *Fno* have been developed to date with variable degree of success. Live attenuated immersion vaccine produced by *Fno* strain with an insertional mutantion in the *iglC* gene (Δ*iglC*) induced high protection (RPS 87.5%) in tilapia against immersion challenge with high dose (LD_{80}) of wild type *Fno* (Soto *et al.,* 2011b). Injection vaccines were also developed against *Fno* infection in fish including an autogenous inactivated oil-adjuvanted injectable vaccine (RPS 100%, Ramirez-Paredes, 2015), the injection vaccine tested against heterologous *Fno* isolates in tilapia in Chapter 4 (RPS 65.9, 69.8 and 82.3%) and *Fno*-derived outer membrane vesicle (OMV) vaccine in zebra fish (RPS 65.5%, Lagos *et al.,* 2017). In comparison to the whole cell inactivated or the OMVs-based vaccines, the live attenuated *Fno* vaccine may offer longer-lasting protection. However, safety issue represents a significant concern when live vaccines are

used in farms and stringent policies should be used in order to provide the safest product for the fish, humans and the environment (Soto and Hawke, 2017). Moreover, the developed *Fno* injection vacciones are not suitable for vaccination of small sized tilapia (< 10 g). Thus, development of an effective vaccination strategy for protection of small fish against *Fno* infection on farms is urgently needed.

Immersion vaccination is one of the first strategies used for immunisation of fish in farms and continues to be used globally (Sudheesh and Cain, 2017). It represents a natural route of antigen entry that has advantages over other means of vaccination including, not only stimulation of the mucosal immunity (Munang'andu *et al.,* 2015a), but also low labour costs, reduced stress on the fish and convenience of vaccinating a large number of different sizes of fish at one time (Nakanishi and Ototake, 1997; Moore *et al.,* 1998). Immersion has been successfully implemented in farms as a primary vaccination procedure to protect fish that are too small to vaccinate during hatchery rearing, and this has contributed to reduction and/or prevention of many threatening infectious diseases in farmed fish (Barnes, 2017). Tilapia are mainly reared in intensive breeding systems to increase production, however these high stocking densities provide a suitable environment for the occurrence of various infectious diseases including francisellosis, where fry are at their most vulnerable stage (Sebastião *et al.,* 2017). The aim of this research was to develop and test the efficacy of an immersion bacterin against *Fno* infection in tilapia fry.

5.2. Materials and methods

5.2.1. Fish

5.2.1.1. Source and acclimation of fish

Healthy tilapia fry, *O. niloticus* (L.) with a mean weight of 5 ± 0.8 g were obtained from a commercial tilapia farm in Chachoengsao province, Thailand, a source believed to be free of francisellosis, and were transported to the wet laboratory of Fish Vet Group Asia Ltd. (FVGAL), Chonburi, Thailand. Fish were acclimated for 10 days in 100 L recirculation glass aquaria (Fleuren & Nooijen, Netherlands) with continuous aeration supplied with dechlorinated municipal water. The water parameters were maintained as described in Section 4.2.1.1, Chapter 4. Fish were fed at a rate of 3% body weight per day with a commercial tilapia feed containing 40% crude protein (CPF, Thailand). All experimental procedures with live fish were carried out in accordance with the UK animals (Scientific Procedures) Act 1986 and associated guidelines (EU Directive 2010/63/EU for animal

experiments) and were approved by the Animal Welfare and Ethical Review Body (AWERB) of the Institute of Aquaculture, University of Stirling, UK.

5.2.1.2. Screening tilapia fry for previous exposure to Fno infection

Six fish were randomly collected and euthanized with 5 mL/L of benzocaine 10% (w/v) in ethanol (Sigma-Aldrich, UK) and head kidney and spleen were sampled, preserved in 95% ethanol (Sinopharm, China) and checked by *Francisella* genus specific PCR to confirm they were free from *Fno* before the experiment following the protocol published by Forsman *et al.* (1994) as described in Section 4.2.1.2, Chapter 4.

5.2.2. Vaccine preparation

The *Fno* UK isolate, that was used to produce the injection vaccine in Chapter 4, was also used for preparation of the current immersion vaccine. The bacterial cells were prepared, formaline inactivated, checked for sterility and the concentration of the inactivated *Fno* was adjusted to a final OD_{600} of 0.4 (~1.2×10^9 CFU\mL) using sterile 1X PBS as described in Section 4.2.2, Chapter 4. No adjuvants were used in the current immersion vaccine and after OD adjustment, the prepared vaccine was kept at 4°C till use.

5.2.3. Pre-challenge

The aim of the pre-challenge trial was to determine the bacterial dose that caused 60-70 % mortality in the control group. A total of 50 fish of mean weight 7 ± 0.6 g were used for the pre-challenge. The fish were distributed between 5 tanks containing 100 L of recirculating chlorine-free water and each tank had 10 fish. The vaccine isolate (*Fno* UK or STIR-GUS-F2f7) was used for the pre-challenge trial. The bacteria were prepared and the viable number of *Fno* cells (CFU/mL) was confirmed as described in Section 4.2.2, Chapter 4. Fish were then placed in a bathing solution of 2 L of live *Fno*-UK isolate at 1.2×10^3, 1.2×10^4, 1.2×10^5, 1.2×10^6 or 1.2×10^7 CFU/mL in a static condition at a temperature of 23 ± 2°C with aeration for 3 h. Fish were then moved to 100 L recirculation tanks and kept for 15 days at 23±2°C. Daily mortalities in the different treatments were recorded. Moribund fish or mortalities were removed and sampled by streaking head kidney or spleen on CHAH to confirm specific mortality. A sub-sample of colonies recovered were analysed by PCR for detection of *Fno* as described in section 4.2.1.2, Chapter 4.

5.2.4. Immersion vaccination

After acclimatization of fish, 160 fish (6± 0.2 g) were divided into three groups: vaccinated group (*n*= 70), non-vaccinated group (MMHB-vaccinated/control group) (*n*= 70) and naïve group (*n*= 20). The fish were stocked in 100 L recirculation tanks filled with chlorine-free water. Vaccination and control groups were divided into duplicate tanks with 35 fish per tank, while the naïve group included 1 tank of 20 fish. The formalin-inactivated vaccine was diluted 1:10 in tank water to a final concentration of 1×10^8 CFU/mL. The fish in the vaccination group were dipped in the vaccine for 30 s, while the non-vaccinated fry were mock vaccinated by dipping in sterile MMHB mixed with tank water for 30 s (1:10 dilution) (Figure 5.1). The naïve fish remained untreated throughout the experiment. Fish were fed 3% of their body weight 3 times/day and water temperature was maintained at 28°C. Water quality was monitored through a Seneye monitoring system (Seneye®, UK) as described in Chapter 4. At day zero (D_0) and 30-days post vaccination (dpv), blood samples were collected from five fish per tank and processed as described in Section 4.2.5. in Chapter 4.

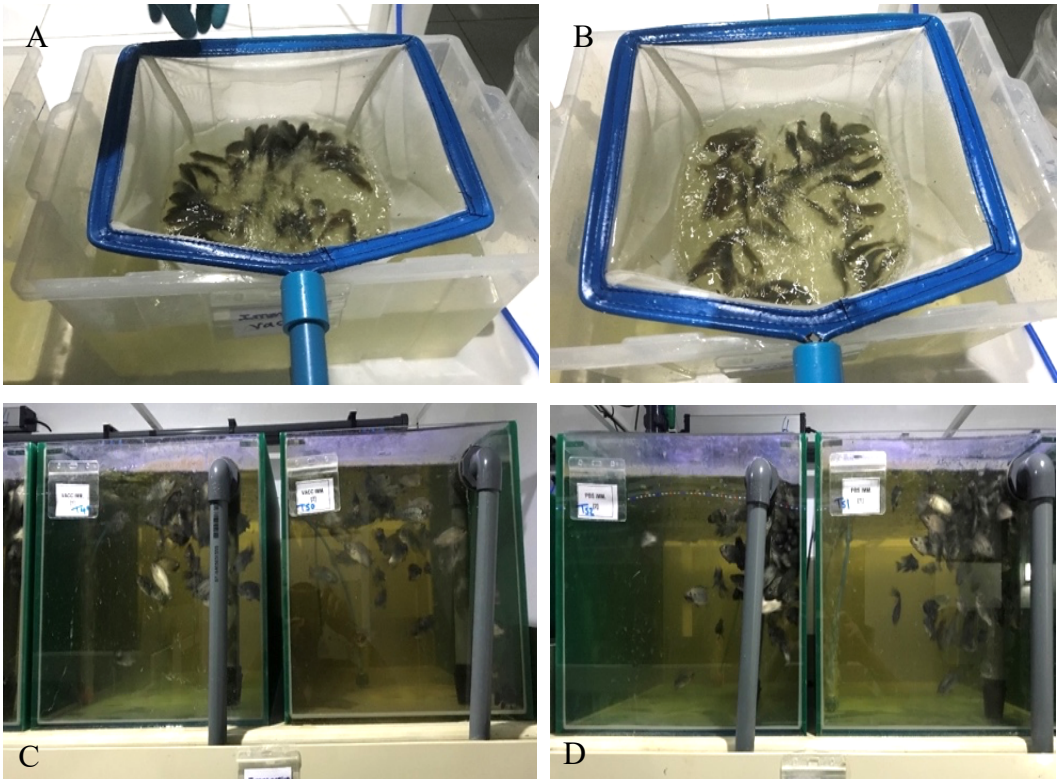


Figure 5.1. Immersion vaccination of tilapia fry. (A) immersion vaccination of tilapia fry in vaccine diluted in tanks water (1:10; final concentration 1×10^8 CFU/mL) for 30 s, (B) mock vaccination of fish by dipping into solution of sterile MMHB diluted in tank water (1:10), (C) and (D) are holding tanks for vaccinated and control groups after bath immersion challenge, respectively.

5.2.5. Immersion challenge

The bacterial suspension was prepared following the same procedures as described in Section 4.2.2, Chapter 4. Thirty dpv, 50 fish (~ 9 ± 0.3 g) (duplicate tanks of 25 each) from vaccinated and control groups were used for the challenge. Fish were starved for 48 h before the challenge. Vaccinated (n = 50) and non-vaccinated fry (n = 50) were immersed into a static bath of 6 L of live *Fno* UK isolate at 1×10^8 CFU/ mL with aeration for 3 h. After the challenge, the fish were then placed back into the holding tanks from which they had originated and were maintained at 23± 2°C for 20 days. Daily mortalities in the different treatments were recorded. Moribund fish or recent mortalities (<6 h) were sampled and subjected to bacteriological culture and screening using the PCR for *Fno* as described in Section 4.2.1.2, Chapter 4. At 20 dpc, the survivors of the different groups were euthanized, and blood was sampled, serum was collected as described in Section 4.2.5, Chapter 4, and preserved at -20°C for measuring IgM by ELISA following the protocol described in Section 4.2.7., Chapter 4.

5.2.6. Statistical analyses

Cumulative percent mortality (CPM) of each treatment (vaccinated challenged; non-vaccinated challenged) at 20 dpc was calculated. The relative percentage survival (RPS) of fish in the vaccinated group was calculated using the following formula described in Section 4.2.6, Chapter 4. All data were arranged using Excel 2016. GraphPad prism® v7 (GraphPad, San Diego, CA, USA) was used for statistical analysis and generating graphs. Kaplan-Meier survival analysis with subsequent Mantel-Cox log-rank test was applied to the mortality data to calculate the survival probabilities and to compare the survival distributions of the two groups (Kaplan and Meier, 1958). Normality of the data from serum antibody responses was tested using Komogorov-Smirnov test. Significant differences between vaccinated and control groups was analysed by a Kruskal-Wallis test (Non-parametric One-way ANOVA). If the difference was significant, a Dunn's multiple comparison post-hoc test was performed to determine which groups were significantly different.

5.3. Results

5.3.1. Screening of tilapia fry for previous *Fno* infection

Initial screening of a sub-sample of tilapia by conventional PCR prior to vaccination gave negative results for all tested spleen and kidney tissues, confirming the *Fno* free status of the fish (Figure. 5.2).

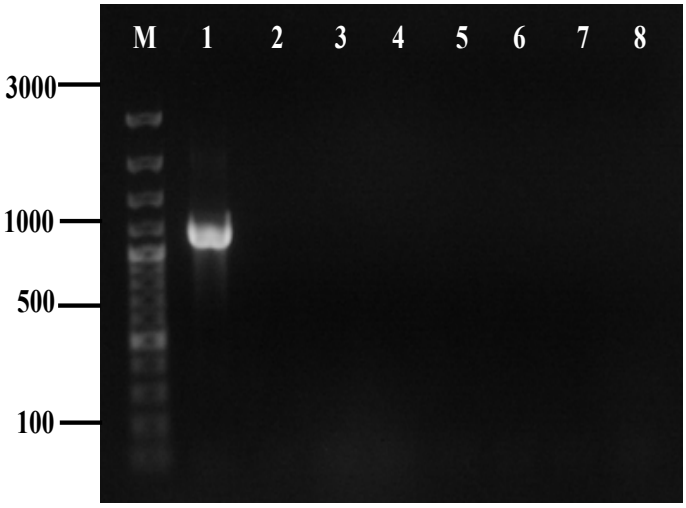


Figure 5.2. Screening of tilapia fry for presence of *Fno* before vaccination by *Francisella* genus specific PCR. 1% agarose gel showing negative results for *Fno* in tested fish. M: 100 bp Molecular marker, Lane 1: Positive control, Lane 2: Negative control, Lane 3-8: head kidney and spleen pool of six naïve tilapia.

5.3.2 Pre-challenge

The cumulative percentage mortalities from the dose response trial were 0 %, 0 %, 10 %, 30 % and 40 % with doses of 10^3, 10^4, 10^5, 10^6, 10^7 CFU/mL, respectively (Figure 5.3). As none of the tested doses achieved the $LD_{60\text{-}70}$, a higher dose of 1×10^8 CFU/mL was used in the main challenge.

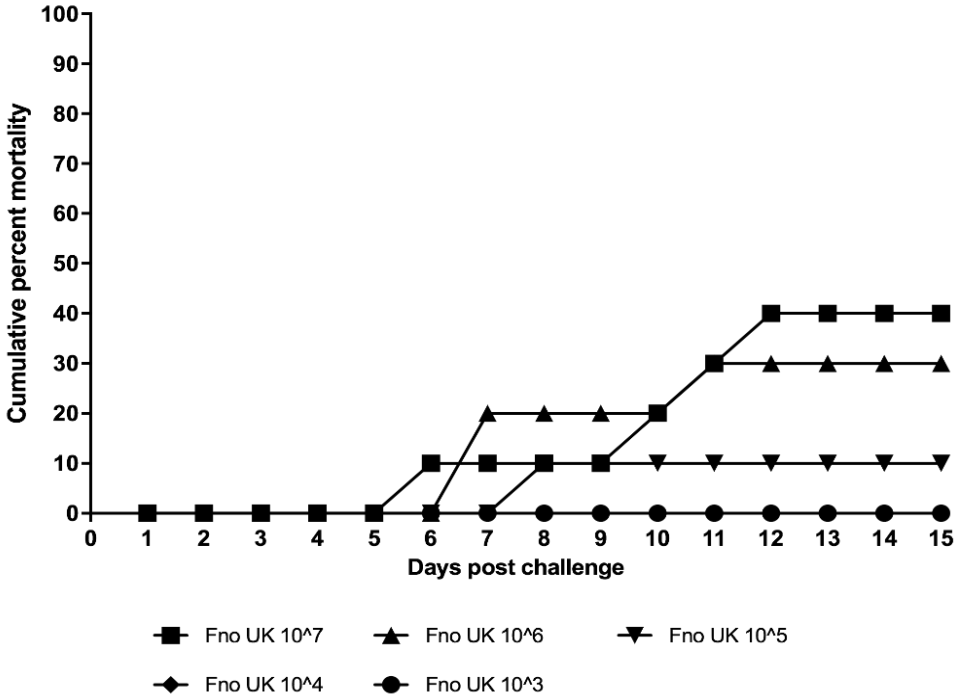


Figure 5.3. Cumulative percentage mortalities of Nile tilapia fry after immersion challenge with five doses of *Fno* UK isolate. Each curve represents the total mortality among 10 fish/tank at 15 days post-challenge.

5.3.3. Vaccine efficacy

Following immersion immunisation of the tilapia fry, fish were behaving and eating normally, and no mortalities were recorded, indicating safety of the vaccine. Following challenge, the non-vaccinated fish appeared lethargic, gathered towards the sides of the tanks, and showed reduced appetite and abnormal swimming. The moribund fish showed abnormal swimming behaviour with distended abdomens. White nodules in the spleen and head kidney with remarkable enlargement of most of the visceral organs and ascetic fluid in the abdominal cavity were also observed in moribund and dead fish post-challenge (Figure 5.4). The bacteriological investigation using spleen and head kidney from moribund fish showed growth of grey, semi translucent and mucoid colonies after streaking on CHAH and incubating for 72 h at 28°C (Figure 5.5A). These fish were also positive by *Francisella* genus-specific PCR (Figure 5.5B).

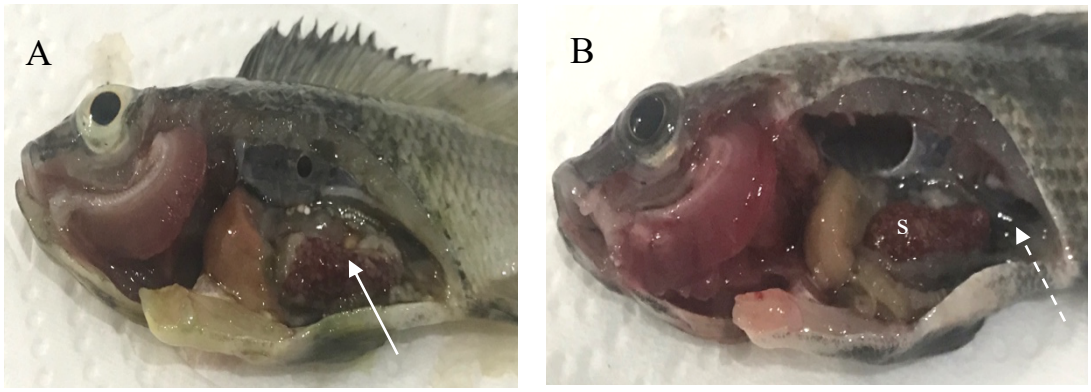


Figure 5.4. Clinical signs of francisellosis in tilapia fry after immersion challenge with *Fno* UK isolate. **(A)** Enlargement of spleen and visceral adhesions (white arrow), (B) whitish nodules on spleen (s) with clear ascetic fluid in abdominal cavity (dotted arrow).

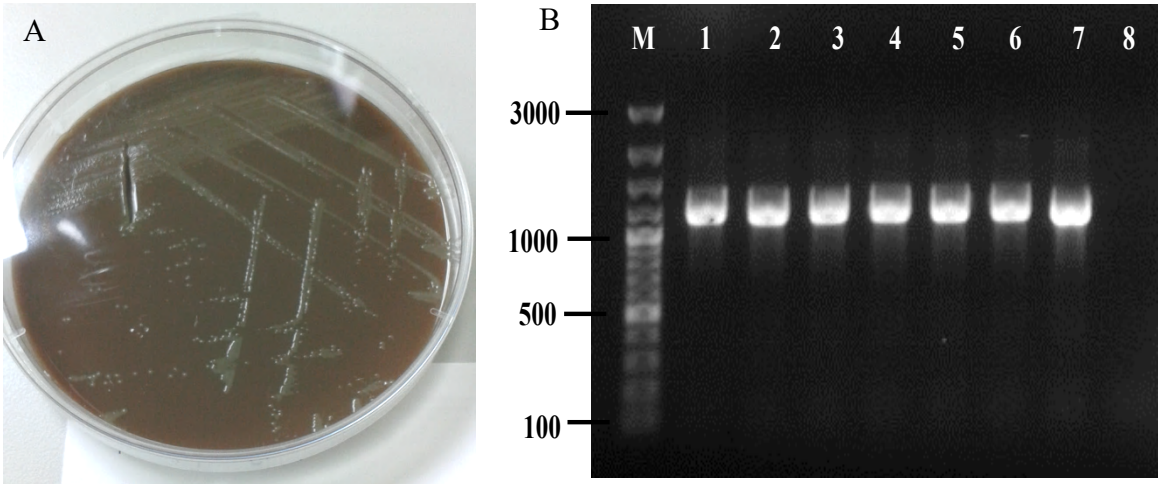


Figure 5.5. Confirmation of *Fno*-specific mortality in tilapia fry post immersion challenge with *Fno* UK by bacteriology and PCR. (A) Grey, semi translucent and mucoid colonies on CHAH retrieved from spleen homogenate of moribund tilapia. (B) 16S rRNA PCR for detection of *Francisella* spp. in moribund and dead tilapia. 1% agarose gel showed amplicon of 1140 bp. M: DNA ladder, Lanes 1 – 6: spleen of representative moribund fish (1-3) and recent mortalities (4-6) post-immersion challenge with 1.2×10^8 CFU/mL of *Fno* UK, Lane 7: positive control (*Fno* DNA), Lane 8: Negative control (Milli-Q water only).

Mortality started at day 5 post-challenge in the non-vaccinated fry, while it started at day 7 post-challenge in vaccinated fry. The mean cumulative percentage mortalities (CPM) were 64 % (± 26.19) and 36 % (±15.3) in non-vaccinated and vaccinated groups, respectively (Table 5.1). The survival analysis showed a significant difference between the survival rates in the vaccinated and the non-vaccinated control groups post-challenge (p<0.001). The survival rates from both treatments are presented as a Kaplan-Meier plot in Figure 5.6. The RPS in vaccinated tilapia fry was 43.7 % at 20 dpc.

Table 5.1. Mortalities of vaccinated and non-vaccinated tilapia fry after immersion challenge with 1x10^8 CFU/mL of *Fno* (n: number of fish; SD: standard deviation)

Group	Replicate	n	% mortality (n)	Cummulative Percent mortality (CPM) ± SD
Non-vaccinated	1	25	76 % (19)	64 % (±26.19)
	2	25	52 % (13)	
Vaccinated	1	25	44 % (11)	36 % (±15.3)
	2	25	28 % (7)	

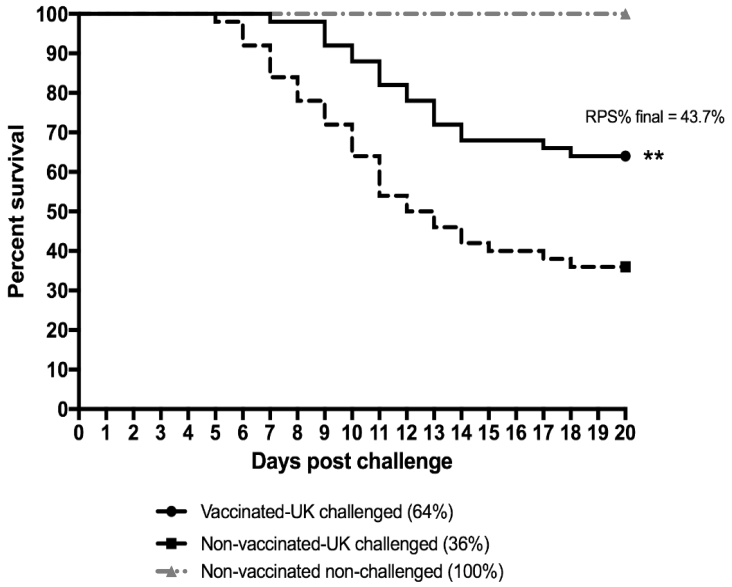


Figure 5.6. Kaplan-meier representation of cumulative survival of vaccinated (n=50) and non-vaccinated tilapia fry (n=50) after immersion challenge with 1.2 × 10^8 CFU/mL of *Fno* UK isolate. Each curve displays the average results for two replicate tanks (n=25 /tank) per treatment group. Asterisks denotes significant differences between treatment pairs (p<0.001)

5.3.4. Specific immune response by ELISA

Levels of specific IgM (OD_{450}, 1:500) in sera of immersion vaccinated fry were above the positive/negative cut-off level in seven out of ten fry at 30 dpv, although the mean OD_{450} value was only just above the cut-off. Similarly following challenge, although the IgM levels in vaccinated fish were higher than in control fish, this difference was not significant ($P > 0.05$) (Figure 5.7).

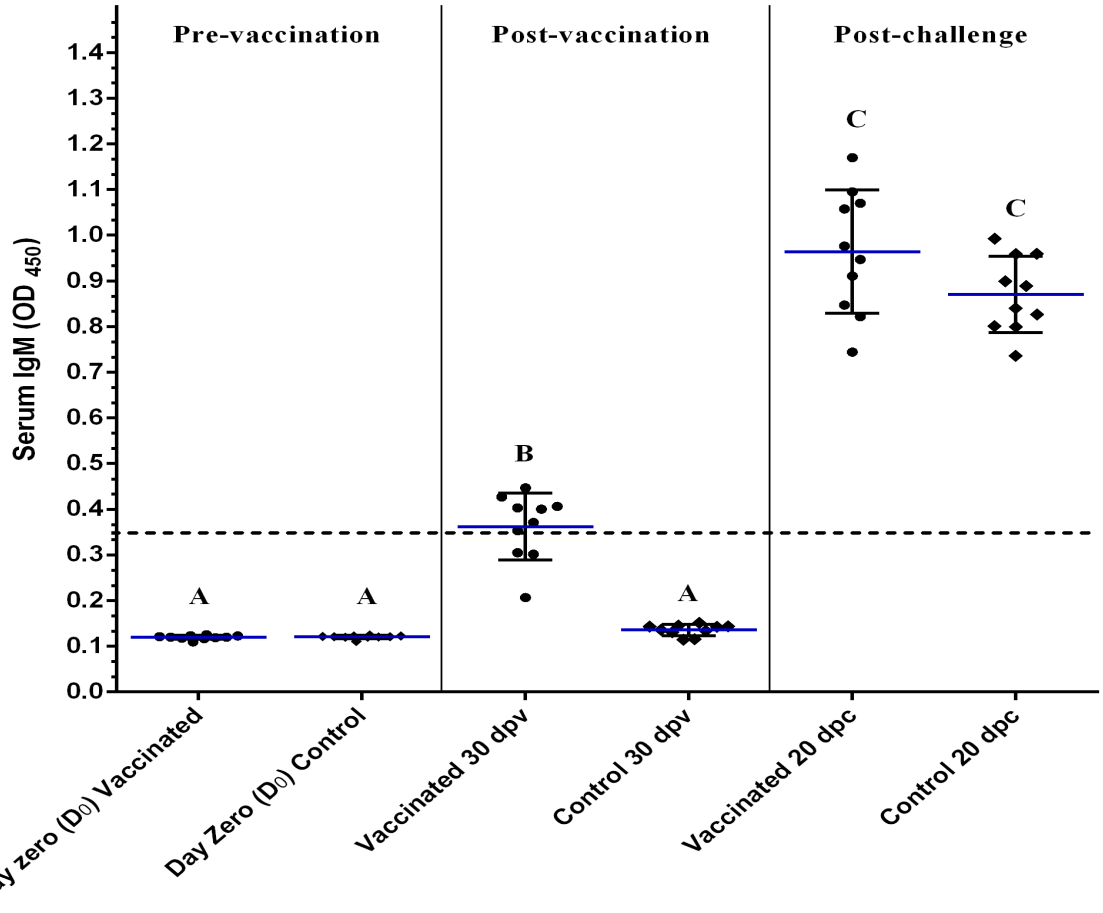


Figure 5.7. Serum IgM responses (Mean ± SD) of vaccinated (n =10) and non-vaccinated (control) (n= 10) tilapia fry at day 0, 30 dpv and 20 dpc. Groups that don't share letters are significantly different (p<0.05) (Kruskal-Wallis test with Dunn's multiple comparison test). The dashed line represents the cut-off as determined by three times the absorbance of the negative control (PBS). The blue line represents mean OD_{450} value of each group. dpv: days post-vaccination, dpc: days post-challenge. Serum dilution was at 1:500.

5.4. Discussion

Francisellosis is an important bacterial disease affecting tilapia farming causing high mortalities in different developmental stages, including fry and fingerlings. In the current study, a whole cell-inactivated immersion vaccine was developed using a virulent isolate of *Fno* (*Fno* UK isolate). The efficacy of this immersion vaccine was investigated by measuring the RPS following immersion immunisation and immersion challenge in tilapia fry. The results showed that the experimental vaccine gave an RPS of 43.7 % in 6 g tilapia after immersion challenge with a homologous *Fno* isolate.

It is of note that the RPS in this study was lower than that previously reported by other vaccines developed against *Fno*. A live attenuated vaccine that was produced by a mutation in the *IglC* locus in *Fno* was reported to induce a protective immune response in tilapia (average weight ~ 6.4 g) and the highest RPS value obtained was 87.5% following immersion vaccination for 180 min and challenging with 1×10^8 CFU/ mL (Soto *et al.,* 2011b). It is not surprising to get a high level of protection with live attenuated vaccines as they are likely to elicit a stronger immune response than a bacterin vaccine. Many studies have investigated the use of live attenuated vaccines against tilapia pathogens such as *Streptococcus agalactaie* (Pridgeon and Klesius, 2013; Li *et al.,* 2015). However, the use of live vaccines is not approved in most countries due to safety concerns. In addition, preparation of live-attenuated vaccines is often costly, thus it may not be cost-effective for, a relatively low-priced fish such as tilapia. An alternative/additional explanation for the low RPS in the current efficacy trial compared to the trial by Soto *et al.* (2011) is the duration of vaccination. Tilapia fry in the previous study were immersed for 180 min in the vaccine comparing to 30 s in this study. However, this long administration time would be impractical under a field vaccination process and most of the commercial immersion vaccines recommend a short time for dipping the fish ranging from 30 to 60 s (Adams *et al.,* 1988; Deshmukh *et al.,* 2012). In addition, Sudheesh *et al.* (2016) reported that administration of an immersion vaccine for more than 3 min in rainbow trout fry did not give any advantage and these authors mentioned that this strategy can be considered only if the vaccine dose was low. It is of note that most recommendations regarding vaccine administration in fish are based on trials with salmonids which are anatomically and physiologically different from tilapia (e.g. tilapia have thicker skin and more tolerance to

oxygen depletion as well as changes in water temperature). Thus, trials need to be conducted to determine the optimal delivery time for the developed vaccine in tilapia.

Route of administration of vaccines has a great influence on the nature, length and magnitude of the host adaptive immune response (Salinas, 2015). A previous trial using an injectable vaccine prepared with the same *Fno* isolate as in this study was shown to give 100 % RPS in vaccinated tilapia of a mean weight of 15 g after a homologous challenge at 690 dd (Ramirez-Paredes, 2015). Also, in a very recent study in a zebra fish model (*Danio rerio*), a *Fno*-OMVs derived injectable vaccine gave RPS of 65% (Lagos *et al.,* 2017). A similar survival variability was observed in vaccines produced against *Streptococcus agalactiae* in Nile tilapia. A microwave killed *S. agalactiae* vaccine produced RPS of 74% and 24-40% in Nile tilapia with a mean weight of 5.6 ± 1.55 g by i.p injection vaccination and bath immersion (BI), respectively (Pasnik *et al.,* 2014). In addition, a live attenuated *S. agalactiae* resulted in RPS of 93.61%, 60.58% and 53.16% in Nile tilapia with a mean weight of 30.15± 1.55 g when administrated by i.p. injection, bath immersion and oral routes, respectively (Li *et al.,* 2015).

In general, injection vaccines confer more protection than mucosal vaccines in fish, including immersion vaccines (Munang'andu *et al.,* 2015a). This is due to the rapid uptake of the injection vaccine after administration inside the fish that causes a quicker and stronger systemic immune response compared to the immersion vaccines that require more time to cross-mucosal barriers before gaining entry to the fish's systemic milieu (Munang'andu *et al.,* 2016). In addition, the injectable vaccine produced by Ramirez-Paredes, (2015) was formulated with an adjuvant, which is well-known to effectively stimulate the adaptive arm of the immune system and contributes to improving the immune-availability of the vaccine antigen (Tafalla *et al.,* 2013). Taken together, this may explain the superior protection of the previously mentioned injection vaccines (Ramirez-Paredes, 2015; Lagos *et al.,* 2017) compared with the bacterin vaccine described here.

Triggering of an immune response in fish following mucosal immunisation depends mainly on either local response in the mucosal surfaces or uptake of antigens from the external surfaces and/or the gut lumen for systemic distribution to head kidney and/or spleen (Evensen, 2017). The high exposure of the fish mucosal surfaces to various pathogens present in their environment can adversely affects the immune response following immersion vaccination (Salinas, 2015). Thus, mucosal adjuvants are required to

enhance the immunogenicity of immersion vaccines and improve the fish mucosal immune status to achieve a high level of protection (Kim *et al.,* 2012; Munang'andu *et al.,* 2015b). Use of mucosal adjuvants was previously reported in the formulation of immersion vaccines for fish. An immersion adjuvanted vaccine against *Yersinia ruckeri* was formulated by addition of Polyethylenimine (PEI), a polycation with a potent mucosal and systemic adjuvant activity, to a commercial *Y. ruckeri* immersion vaccine. Immersion vaccination of rainbow trout by this vaccine showed an increased serum antibody response 6, 8 and 10 weeks post-vaccination, however, no evidence of enhancement of the pro-inflammatory genes expression in gills or skin was detected (Heidari, *et al.,* 2016). In addition, olive flounder, *Paralichthys olivaceus,* was immersion vaccinated against viral haemorrhagic septicaemia (VHS) with a heat killed vaccine containing Montanide IMS 1312 VG. This induced higher RPS levels than that observed in fish vaccinated with the vaccine without the adjuvant (Hwang *et al.,* 2017). There is no available information for using mucosal adjuvants so far in tilapia. Therefore, more studies need to be performed to test the potential use of adjuvants in formulating immersion vaccines against *Fno* and test their effect on the immune response of tilapia post-vaccination and post-challenge.

One of the key factors in fish vaccination is the size of the fish to be vaccinated. In tilapia, the development of the immune system has been reported to start at two days post-fertilisation and differentiation of thymus cortex and medulla has been observed in Nile tilapia (*O. niloticus*) larvae at the age of 20 days (Cao *et al.,* 2017). In addition, detection of changes in an IgM-like protein were detected in *Oreochromis mosambicus* larvae at 14 days post-hatch (Takemura*,* 1993) and the age of immune-competency in tilapia is believed to be at approximately 21 days (Evans *et al.,* 2004). Thus, fish of ~ 6 g will apparently be suitable for vaccination. However, in a previous experiment, 10 g tilapia fingerlings were shown to produce a significantly higher protective immune response against *Fno* infection than 5 g fry (Soto *et al.,* 2014a). This in part, may explain the increased RPS and protection in the vaccinated fingerlings (~10 g or more) after challenge. More studies are required for optimisation of the size and/or age at first vaccination in tilapia to identify optimal vaccination regimen for this species.

Onset of mortality post-challenge was delayed in vaccinated fry (7th dpc) when compared with control fry (5th dpc). Similar finding has been reported in a previous study, where mock vaccinated Nile tilapia died at 7 dpc after immersion challenge with dose of

1×10^8 CFU/mL of *Fno* (Soto *et al.,* 2011b). In contrast, the first mortality in zebra fish in both vaccinated and control fish was recorded on the 2nd day after i.p. challenge with *Fno*, however mortality continued in the controls where only 20 % of the fish were left after 7 dpc. This variation may be attributed to difference in susceptibility of the different fish species to *Fno,* the bacterial strain virulence and route of challenge (i.p. versus immersion).

The mean specific serum IgM levels in the immersion vaccinated fry 30 dpv were considered to be negative when compared with the positive/negative threshold level. This can be attributed to the limited ability of immersion vaccination to stimulate a systemic antibody response in contrast to injection vaccination, although protection against *Y. ruckeri* in immersion vaccinated trout was shown to be strongly associated with plasma IgM levels (Raida *et al.,* 2011). This stimulation of IgM responses by the immersion route in trout could be pathogen specific (Hoare *et al.,* 2017). In the current work, there was high variability in the immune response of the vaccinated fry with IgM levels in serum of 3 of 10 fish considered negative (i.e. below the threshold), while 7 fish had IgM levels above the threshold level. The reason for the variability between fish is not clear but may be attributed to the genetic make-up of the fish. Similar results were reported by Hoare *et al.* (2017), where immersion vaccination against *Flavobacterium psychrophilum* in rainbow trout fry induced high protection, however, levels of specific serum IgM of 2/5 fish were below the threshold 6 weeks post-vaccination. At 20 dpc with *Fno*, a higher serum IgM response was detected in both vaccinated and control fry, although there were no significant differences between these levels in both groups. This result is in contrast to that previously obtained by Soto *et al.* (2011b) who reported a significantly higher secondary antibody response 8- 10 weeks post-immunisation using an immersion mutant *Fno* vaccine in serum and mucus of vaccinated tilapia compared to mock-vaccinated tilapia post-challenge with 1×10^8 CFU/mL. This again could be attributed to the superior effect of the live attenuated *Fno* vaccine compared to the formalin killed vaccine in triggering a stronger immune response.

Measurement of antibody levels post-vaccination and post-challenge is a widely used correlate of protection in many vaccines including fish vaccines (Munang'andu and Evensen, 2018). In the current study, higher survival rates and higher, but non-significant, serum IgM levels were detected in the immersion vaccinated fry compared to the PBS

control fish post-challenge. However, protection induced by the immersion vaccine was more likely to be due to triggering mucosal immune response (e.g. IgT) along with contribution of non-specific immune mechanisms. In general, immersion vaccination has a strong effect on mucosal immunity, with specific antibody secreting cells (B-cells) detected mostly in skin epithelium and gills following immersion vaccination (Barnes, 2017). Both IgT and IgM were previously detected in all mucosal associated lymphoid tissues (MALT) (Salinas, 2015). However, IgT is the major isotype in mucosal organs (Zhang *et al.,* 2010; Salinas *et al.,* 2011) and it was reported to play a role in preventing attachment and localisation of pathogens, using a mechanism equivalent to the IgA in mammals (Munang'andu *et al.,* 2015; Salinas, 2015). In a previous study, immersion vaccination against *F. psychrophilum* in rainbow trout induced high RPS of 84 % that was associated with high serum IgT and proliferation of IgT positive cells in spleen and head kidney 6 weeks post-vaccination (Hoare *et al.,* 2017). There is little information about the role of IgT in mucosal immunity in tilapia. In addition, there is no so far available anti-tilapia IgT which presents a challenge to use IgT to evaluate efficacy of mucosal vaccines in tilapia. Thus, further studies are required to develop assays for quantification of IgT in mucus of tilapia. Moreover, there is also need for studies to investigate the level of specific immunoglobulin in skin mucus or gills. This will give us more insight to the interaction of the developed immersion vaccine with the mucosal immune system and will enable establishing a more reliable correlate to the protection induced by the *Fno* immersion vaccine.

The interaction of antibody-mediated immunity with other innate immune factors for protecting fish against *Fno* was previously reported by Soto *et al.* (2010b), where serum from tilapia vaccinated by immersion with live attenuated vaccine enhanced phagocytosis of *Fno* in head kidney derived-macrophages. Furthermore, the cellular immune response has been reported to be fundamental in controlling various fish pathogenic bacteria such as *Aeromonas salmonicida* and *Renibacterium salmoninarum* (Ellis, 2001). To this end, investigating the expression of immune-cell markers (e.g. CD4, CD8) post-immersion vaccination against *Fno* in tilapia in conjugation with both humoral and mucosal antibody levels may elucidate the the role of cellular mediated immunity in protection against *Fno* in tilapia. Further research is required to identify cellular immune

response induced by immersion vaccination and elucidate the mechanism of interaction between antibody mediated and cellular mediated responses for controlling *Fno* in tilapia.

5.5. Conclusions

The whole-cell bacterin immersion vaccine developed in this study induced a moderate level of protection against *Fno* infection that was evident by significantly lower mortality and an RPS of 43.7 % in vaccinated tilapia fry compared to the non-vaccinated fry following challenge with a high dose of *Fno*. The protection against immersion challenge with *Fno* is likely to be due to induction of mucosal immune response, however, the levels of specific antibodies, in particular IgT, in mucus of the immersion vaccinated fish need to be evaluated. In addition, further research is required to increase the efficacy of the *Fno* immersion vaccine by optimising delivery time, delivery dose, vaccine formulation by including mucosal adjuvants, booster applications, and investigate duration of immunity and cross-protection against other heterologous *Fno* isolates. In addition, further investigation is needed to understand the mechanism of protection and the expression of immune-related genes following immersion vaccination. What is clear is that vaccines developed in Chapter 4 and 5 have demonstrated potentials to protect tilapia against *Fno* infection. Application of such vaccines in association with convenient, rapid, sensitive, specific and cost-effective diagnostic tool will facilitate controlling of francisellosis in tilapia farms.

Chapter Six

Development of a recombinase polymerase amplification assay for rapid molecular detection of *Francisella noatunensis* subsp. *orientalis*

6.1. Introduction

Francisella noatunensis subsp. *orientalis* (*Fno*) is the causative agent of piscine francisellosis in warm water fish including tilapia. The disease induces chronic granulomatous inflammation with high morbidity and can result in high mortality. Early and specific detection of *Fno* is crucial for initiation of appropriate outbreak control measures in tilapia farms. Diagnosis of fish *Francisella* is a challenging issue due to its nature as a fastidious intracellular microorganism and the insufficient availability of sensitive and specific detection methods for this aquatic pathogen (Soto *et al.,* 2010a; Soto and Hawke, 2017). Conventional diagnosis of fish francisellosis via bacterial isolation in culture media has many constrains as it takes several days to grow and is often overgrown by concomitant bacteria (Duodu *et al.,* 2012; Assis *et al.,* 2016). Furthermore, affected tissue samples need homogenisation for maximum bacterial recovery. Antibody-based immunological assays such as enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC), have also been used for diagnosis of francisellosis in fish (Soto *et al.* 2011a), but they were reported to have low sensitivity and limited throughput (Ottem *et al.,* 2008).

Nucleic acid-based methods have been applied for detection of fish pathogenic *Francisella*, including a conventional *Francisella* genus-specific polymerase chain reaction (PCR) (Nguyen *et al.,* 2016; Hsieh *et al.,* 2006; Ramirez-Paredez *et al.,* 2017b), quantitative real-time PCR (qPCR) (Soto *et al.,* 2010a; Duodu *et al.,* 2012; Assis *et al.*, 2016; Ortega *et al.,* 2016; Sebastião *et al.,* 2017), duplex PCR, *in situ* hybridisation (Dong *et al.,* 2016a) and loop mediated isothermal amplification (LAMP) (Pradeep *et al.,* 2016). Despite the fact that these techniques have their own points of interest, downsides make them more challenging to use for pond-site diagnosis, for instance, they can be time consuming (Dudou *et al.,* 2012; Assis *et al.,* 2016), labour intensive, there is a prerequisite need for skilled staff, some tests are prone to show false negatives or false positives because of low sensitivity or low specificity liability (Soto *et al.,* 2010a), highly influenced by reaction inhibitors (Rodrigues *et al.,* 2012; Hall *et al.,* 2013) and requirement of complex design (Pradeep *et al.,* 2016).

The isothermal amplification technology recombinase polymerase amplification (RPA) has been successfully used for field diagnostics of various pathogens. The technique has been widely used recently due to its affordable price (~ 4.5 USD per test),

sensitivity (limits of detection as low as 1 copy), short reaction time (results can be obtained in less than 10 min), robustness and simplicity as only minimum equipment and hands-on manipulation are required (Daher *et al.,* 2016).

The RPA performs DNA amplification at a constant temperature between 37 and 42 °C by employing phage-derived recombinase (UvsX), single-strand binding protein (SSB) and strand-displacing *Staphylococcus aureus*-derived-DNA polymerase (Sau). The recombinase protein promotes binding of the primers to their homologous target sequence in the double stranded DNA (dsDNA) after invasion and displacement of the non-template strand by Sau enzyme, which is assisted by reaction stabilisation by SSB proteins (Pipenburg *et al.,* 2006). By the end of the reaction, complementary dsDNA containing the target sequence will be synthesised following the same strategy as PCR, however, all RPA steps are run at lower temperatures, giving a minimal chance for generating undesired products that could oppose target amplification (Sharma *et al.,* 2014).

Since its first introduction in 2006, RPA has been widely used in diagnosis of various pathogens of clinical importance in human medicine including Methicilline-resistant *Staphylococcus aureus* (Pipenburg *et al.,* 2006), *Francisella tularensis* (Euler *et al.,* 2012a and 2013), Middle East respiratory syndrome coronavirus (Abd El Wahed *et al.,* 2013a), Ebola virus (Faye *et al.,* 2015), Sudan virus (Euler *et al.,* 2013), Dengue virus (Toeh *et al.,* 2015), *Bacillus anthracis* (Euler *et al.,* 2013), Zika virus (Abd El Wahed *et al.,* 2017), Noro viruses (Moore and Jaykus, 2017) and *Mycobacterium tuberculosis* complex (Ma *et al.,* 2017). RPA was also used in diagnosis of animal diseases such as Rift Valley Fever virus (Euler *et al.,* 2012b), Foot-and-mouth disease virus (Abd El Wahed *et al.,* 2013b), Bovine viral diarrhoea virus (Aebischer *et al.,* 2014), lumpy skin disease virus (Shalaby *et al.,* 2016) and Feline herpes virus (Wang *et al.,* 2017)), avian diseases such as Influenza A H7N9 (Abd El Wahed *et al.,* 2015) and plant diseases like little cherry virus 2 (Mekuria *et al.,* 2014) and plum pox virus (Zhang *et al.,* 2014). In the aquatic veterinary field, RPA has recently been developed for *Penaeus stylirostris* densovirus (Jaroenram and Owens, 2014), shrimp white spot syndrome virus (Xia *et al.,* 2014), and infectious hypodermal and hematopoietic necrosis virus (Xia *et al.,* 2015), Cyprinid Herpes virus 3 (Prescott *et al.,* 2016; Soliman and El-Matbouli, 2018), Abalone Herpes-like virus and red-spotted grouper nervous necrosis virus (Gao *et al.,* 2017) and Carp edema virus (Soliman and El-Matbouli, 2018).

While vaccines are important for developing preventative measures to control *Fno* induced francisellosis, development of tools to detect outbreaks of this emerging pathogen is necessary to prevent its spread. The aim of the current study was to develop and validate a real-time RPA for a rapid and specific detection of *Fno* to be applied as a point-of-care diagnostic tool for monitoring and preventing the spread of francisellosis.

6.2. Material and methods

6.2.1. Bacterial isolates and DNA extraction

In this study isolates of *Fno* and other bacteria, including closely related *Francisella* spp. and other non-related bacteria, were used to test the specificity of the RPA. The bacterial isolates used are listed in Table 6.1. All *Francisella* strains including *Francisella noatunensis* subsp. *orientalis* (*Fno*)*, F. noatunensis* subsp. *noatunensis* (*Fnn*) and *F. philomiragia* (*Fp*) were cultured from stock cultures on cysteine heart agar with 2% bovine haemoglobin (CHAH; BD, Oxford, UK). The agar plates were incubated at 28°C for 3 days for *Fno* isolates, 22°C for 5 days for *Fnn* isolates and 28°C for 24 h for *Fp* isolates. After incubation, growth and purity of the colonies were checked then a loop-full of bacteria from each plate was inoculated into modified Mueller Hinton broth (MMHB) with 2% IsoVitaleX and 0.1% glucose (Difco, BD, USA) and incubated in a shaking incubator (Kühner, Switzerland) at 28°C, 150 rpm for 20 h. Colonies of *Aeromonas hydrophila, Streptococcus agalactiae*, *Streptococcus iniae*, *Escherichia coli, Yersinia ruckeri* and *Pseudomonas* sp. were grown on Tryptic Soy agar (Sigma-Aldrich, UK) at 28°C for 48 h, then inoculated into tryptic soy broth (Sigma-Aldrich, UK) and incubated for 24 h. Strains of *Vibrio anguillarum* and *Photobacterium damselae* were cultured on marine agar at 28°C for 48 h (Difco, USA) then inoculated into tryptic soy broth (Sigma-Aldrich, UK) with 2% salt (Sigma-Aldrich, UK) and incubated for 24 h at 28°C.

The genomic DNA from the different bacterial cultures was extracted using a real pure extraction kit (RBMEG01, Real Laboratory, Valencia, Spain) following the protocol for extraction of genomic DNA from bacterial cells with slight modifications. Briefly, 1.5 mL of overnight bacterial culture was centrifuged at 14,000 × *g* for 30 s then the supernatant was removed, and the bacterial pellets were re-suspended in 300 µL of lysis solution and 5 µL of proteinase-K (10 mg/µL) then incubated at 55 °C overnight. For *S. agalactiae* and *S. iniae*, the pellets were re-suspended in 500 µL of 50 mM EDTA

contained 60 µL of lysozyme (10 mg/mL) first then samples were incubated at 37 ºC for 60 min followed by centrifugation at 14,000 × *g* for 2 min. Supernatants were removed and cells were lysed as described above. Samples were left to cool at room temperature (RT) (~ 22 ºC) for 5 min then 3 µL of RNAse (10 mg/µL) was added followed by a quick mix using vortex for 30 s and incubation at 37°C for 1 h. Samples were left to cool at RT for 5 min, then 200 µL of protein precipitation solution was added followed by mix on vortex (1010, VNWR, China) at high speed for 30 s and incubation at 4°C for 15 min. Samples were centrifuged at 14,000 × *g* for 5 mins and the clear supernatant was transferred to fresh Eppendorf tubes (Eppendorf, UK). 600 µL of isopropanol (Sigma-Aldrich, UK) was added to the tubes containing the DNA and mixed by inversion 10 times followed by centrifugation for 5 min at 14,000 × *g*. All traces of isopropanol were removed by pipetting then bacterial DNA pellet were re-suspended in 1 mL of 70 % ethanol (Sigma-Aldrich, UK) and mixed for 1 h on a paddle mixer (VNW, China). After ethanol washing, samples were centrifuged for 5 min at 14000 × *g* and pellets were left to be air dried for 3 min. The DNA pellets were resuspended in 50 µL rehydration solution and properly mixed by vortexing then left for overnight rehydration. The concentration of DNA samples was measured using a nanodrop (Nanodrop 1000, ThermoFisher Scientific, UK). Each DNA sample was standardised to 100 ng/µL and stored at -20°C until used.

Table 6.1. Bacterial strains used in the assay.

Bacterial species	Strain ID	Source
F. noatunensis subsp. *orientalis*[*]	STIR-GUS F2f7	Tilapia (UK)
F. noatunensis subsp. *orientalis*[#]	NVI-PQ1104	Tilapia (Costa Rica)
F. noatunensis subsp. *orientalis* [#]	DSMZ21254[T]	Three-line grunt (Japan)
F. noatunensis subsp. *orientalis*[#]	NVI-9449	Malawi cichlids (Austria)
F. noatunensis subsp. *orientalis*[*]	Fran-Cos1	Tilapia (Mexico)
F. noatunensis subsp. *orientalis*[*]	STIR-HON1	Tilapia (Central America)
F. noatunensis subsp. *noatunensis*[#]	NCIMB 14265 [T]	Atlantic Cod (Norway)
F. noatunensis subsp. *noatunensis* [#]	NVI-7601	Atlantic Cod (Ireland)
F. noatunensis subsp. *noatunensis*[#]	PQ1106	Atlantic Salmon (Chile)
F. philomiragia[#]	ATCC® 25015[T]	Muskrat (USA)
F. philomiragia[#]	ATCC® 25017	Water (USA)
F. philomiragia[#]	CCUG 12603	Human abscess (Sweden)
Aeromonas hydrophila[*]	ATCC® 7966[T]	Milk with fish odour (USA)
Streptococcus agalactiae[§]	ATCC® 51487[T]	Tilapia (Israel)
Streptococcus iniae[§]	ATCC® 29178[T]	Amazon fresh water dolphin
Vibrio anguillarum[*]	ATCC® 19264[T]	Atlantic Cod (UK)
Photobacterium damselae subsp. *piscicida* [§]	ATCC® 51736 [T]	Yellow tail fish (Japan)
Escherichia coli[*]	ATCC® 11775[T]	Urine (Sweden)
Yersinia ruckeri[*]	ATCC® 29473[T]	Rainbow trout (USA)
Pseudomonas spp.[*]	STIR-Ps17	Lump sucker (UK)

[(T)] Type strains, DSMZ: The German Collection of Microorganisms and Cell Cultures, NVI: The Norwegian Veterinary Institute, NCIMB: The National Collection of Industrial Food and Marine Bacteria, ATCC: American Type Culture Collection. [*]Bacterial strains provided by aquatic vaccine unit, Stirling University; [§] bacterial strains kindly provided by Dr. Kim Thompson, Aquatic Research Group, Moredun Research institute, UK; [#] bacterial strain kindly donated by Dr. Duncan Colquhoun, Norwegian Veterinary Institute.

6.2.2. Field samples

Samples of spleen (n=78), head kidney (n= 78) and water (n= 5) were used in the current study. The tissue samples were obtained from 78 moribund and clinically healthy Nile tilapia that were randomly collected from two different geographical locations including 38 fish from three tilapia farms in the UK (Farm one (Lincolinshire): 10 fish / 40 ± 0.6 g; Farm two (Lincolinshire): 10 fish /45 ± 0.4 g; Farm three (London): 18 fish / 12 ± 0.3 g) and 40 fish from a commercial tilapia farm in central Thailand (10 ± 0.6 g). A history of natural outbreak of francisellosis was reported in the first and second UK farms during 2011-2012 (Ramirez-Paredes *et al.,* 2017b) and in 2008, 2013-2016 in the Thai farm (Pradeep *et al.,* 2016). The third UK farm had no history of francisellosis, but it supplied red tilapia fry to an aquaponics farm in London, UK, where a natural outbreak of francisellosis occurred during spring 2017 (Ramirez-Paredes personal communication). The fish showed non-specific clinical signs including loss of scales and eroded fins. On necropsy, some of the examined fish displayed creamy/white nodules on spleen and head kidney and pale liver. Five water samples of 500 mL were collected from different sections at the infected aquaponics farm that received fish from the third UK farm including one sample from UV filter unit, two samples from bio-filter tanks and two samples from individual fish holding tanks.

Isolation of *Fno* was attempted from spleen samples from the first and second UK farms and the Thai farm using CHAH supplemented with Polymixin B (100 units/mL, Sigma, UK) and plates were incubated as described above. DNA from all the collected spleen and head kidney samples was extracted using the kit used for extraction of bacterial gDNA, as described in Section 6.2.1. DNA from 350 mL of each water sample was extracted using DNeasy Blood and Tissue Kit (QIAGEN, Germany) as described by Renshaw *et al.* (2015) with slight modifications. In brief, 350 mL of the water samples were filtrated using 0.45-µm filter (Millipore-Merck, Germany). After filtration, the filter paper was removed from the filtration unit and inserted into 2 mL micro-centrifuge tube then 576 µL of buffer ALT from the kit and 60 µL proteinase-K were added. The tubes containing the filter paper were briefly mixed for 30 s then incubated in a heat block (QBD, Grant, Thomas Scientific, USA) at 65°C for 3 h. After incubation, 630 µL of buffer AL from the kit and 630 µL of 100% ethanol (Sigma, UK) were added then the whole tube content was transferred into DNeasy Mini spin columns placed in 2 mL collection tubes

followed by centrifugation at 10,000 × *g* using a refrigerated centrifuge (SIGMA 2-16K Centrifuge, Sci-quib Ltd., UK) for 1 min. The DNeasy spin columns were transferred to new 2 mL collection tubes and 500 µL of buffer AW1 added and centrifugation repeated as described before. The spin columns were transferred again to new collection tubes and 500 µL of buffer AW2 were added followed by centrifugation at 20,000 × g for 3 min and the flow-through with the collection tube were discarded. Following transfer of the spin columns to 1.5 mL Eppendorf tubes, 150 µL of buffer AE were used to elute the DNA then incubation was done for 2 min at RT. Centrifugation was performed for 2 min at 8000 × *g* and spin columns were discarded. The concentration of the eluted DNA was then measured by nanodrop as described previously. All the extracted DNA samples were standardised to 100 ng/µL and stored at -20°C until used.

6.2.3. Preparation of plasmid DNA standard containing the *Francisella noatunensis* subsp. *orientalis* hypothetical protein gene

6.2.3.1. Plasmid DNA description

A specific DNA sequence unique to *Fno,* previously identified by Duodu *et al.,* (2012), representing the hypothetical protein gene (Genbank accession no. JQ780323.1) was synthesised and ligated into vector backbone pENTR221 (Geneart, Life Technologies Ltd, Paisely, United Kingdom). The resulting *Fno*-standard plasmid was transformed into an *E. coli* vector (OmniMAX™ 2 T1R) and purified from transformed bacteria using QIAprep8 Miniprep Kit (QIAGEN, UK) (Figure 6.1.). The final construct was verified by sequencing and the sequence congruence within the insertion sites was 100%.

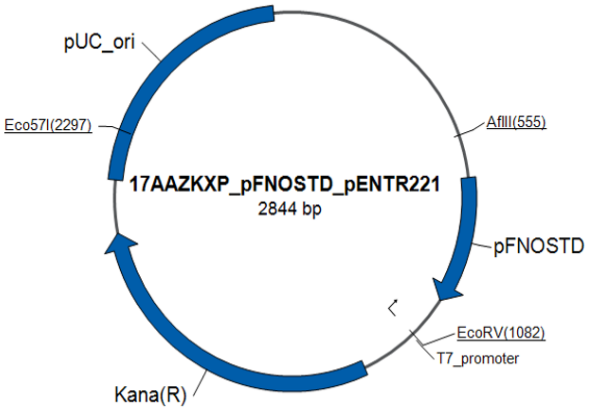


Figure 6.1. *Fno* plasmid standard containing the hypothetical protein gene (JQ780323.1)

6.2.3.2. Restriction, concentration and quantification of the Fno-plasmid standard

A total of 6 µL of the *Fno*-plasmid standard DNA was used for extraction in a 60 µL reaction containing 3 µL of Eco-RV restriction enzyme (R01955 NEB, New England laboratories, UK), 6 µL of 10X buffer 3.1 (B72035 NBE, New England laboratories, UK) and 45 µL of RNAse free water (ThermoFisher Scientific, UK). The mix was incubated in a heat block at 37°C for 1 h. After digestion, the plasmid DNA was run on a 1% agarose gel with non-restricted plasmid DNA to confirm restriction for 40 min at 85 volts. The bands were visualised using gel documentation system (Bio-RAD, USA) (Figure 6.2.).

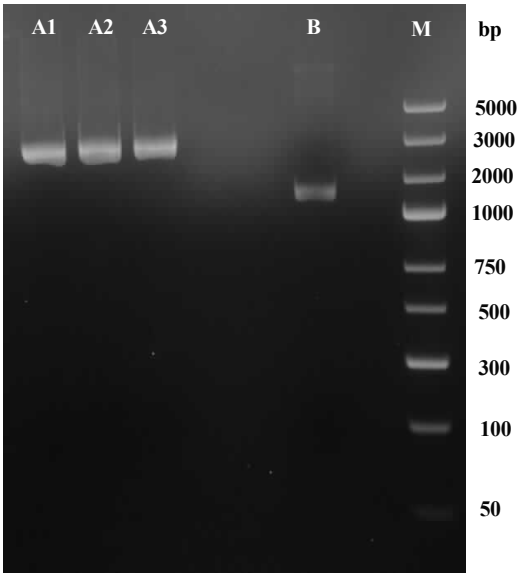


Figure 6.2. Visulaisation of the restricted *Fno* plasmid DNA containing the hypothetical protein gene on 1% agarose gel. A1-A3: restricted *Fno* plasmid DNA; B: non-restricted *Fno* plasmid DNA; M: DNA marker; pb: Base pair.

The restricted plasmid bands were cut using a sterile scalpel and transferred to 1.5 mL Eppendorf tubes. The bands were cleaned and concentrated using Qia Ex II Gel extraction kit (Qiagen) following the manufacturers protocol. Briefly, the weight of the bands was calculated then a volume of QX1 buffer equal to 3X of the band weight was added followed by quick vortex for 30s to re-suspend the bands. 10 µL of Qia EX II buffer were added to the mix to solubilise any extra agarose and bind to the DNA followed by incubation at 50°C for 10 min with vortexing every 2 min. The mix was centrifuged

briefly for 30 s and supernatant was discarded. 500 µL of QX1 buffer was added to wash the DNA pellet followed by quick vortex then centrifugation for 30 s and supernatant was discarded. 500 µL of buffer BE were added followed by centrifugation for 30 s and the supernatant discarded. The last step was repeated twice for proper washing of the product. The pellet was left to air dry for 10 min then 20 µL of 10 mM Tris-HCl (ThermoFisher, Scientific, UK) was added followed by 5 min incubation at RT (~22°C). The pellet was centrifuged for 30 s and the clear supernatant was transferred to new tube that was kept at -20°C till being used.

Quantification of the *Fno*-plasmid DNA was done using Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes, ThermoFisher Scientific, UK) following the manufacturer's protocol. In brief, a five-point high-range standard curve was prepared from a stock solution of 2 µg/mL Lambda dsDNA standard in 1X Tris-EDTA buffer (TE buffer, Sigma-Aldrich, UK) including 1µg/mL; 100 ng/mL; 10 ng/mL; 1ng/mL and a blank. The *Fno*-plasmid DNA was diluted in 1X TE buffer to a final volume of 1 mL. 1 mL of the aqueous working solution of Quant-iT™ PicoGreen® reagent was added to the standard curve dilutions and and the *Fno* plamisd DNA followed by incubation at RT (~ 22°C) for 5 min in the dark. 100 µL of the standard curve dilutions and the plasmid was added to a black flat bottom 96 well plate (Sigma-Aldrich, UK) and the fluorescence (excitation = 480 nm and emission = 520 nm) was measured using a plate reader (BioTek, Synergy HT). The fluorescence value of the reagent blank was substracted from that of each of the samples and the corrected data was used to generate a standard curve of fluorescence versus DNA concentration and the concentration of *Fno*-plasmid DNA in nanogram/mL (ng/mL) was calculated from the generated standard curve. The results were used to calculate the number of copies of the *Fno*-plasmid DNA based on the following equation:

$$\text{Number of copies/µL} = \frac{\text{M x } 6.02 \text{ x } 10^{23} \text{x } 10^{-6}}{\text{n x } 660}$$

where (M) is the amount of DNA in nanogram (ng/mL), (n) is the number of nucleotides in the plasmid in base pair and 660 Da is the average weight of one base pair. Following calculation of the copy number of the *Fno*-plasmid DNA, a quantitative plasmid standard ranging from 10^7: 10^1 copies / µL was prepared and stored at -20 °C till used.

6.2.4. Real-time qPCR for the *Fno*-plasmid standard

Real-time qPCR was performed using the primers listed in Table 6.2. The assay was performed on a LightCycler® 2.0 (ROCHE, Germany) in a 20 µL reaction volume consisting of 0.3 µM from each primer (Eurofins Genomics, UK), 1X Luminaris color HiGreen™ qPCR master mix (ThermoFisher Scientific, UK), 1 µL *Fno* Plasmid DNA template and nuclease free water (ThermoFisher Scientific, UK) up to 20 µL total volume. The PCR cycling conditions were adopted from Duodu *et al.* (2012) as following, 50°C for 2 min for activation of uracil-DNA glycosylase enzyme, 95°C for 10 min to start denaturing the UNG enzyme and activate the DNA polymerase enzyme then 45 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve analysis was formed of 1 cycle at 95°C for 30 s, 55°C for 30 s and 95°C for 30 s. All samples were run in duplicates and each run included non-template control (Milli-Q water only). A standard curve was created from the data of three independent runs (n=3).

Table 6.2. List of primers and probe used in the experiment

Name	Sequence (5' – 3')	Amplicon size	Reference
RPA (F1) RPA (R2) RPA (P)	ATGAGATATGTGTTAATTTGGCTGTTCCTGTACGA TAGTTGTATCAGTAATAGGCGTAACTCCTTTTAGC GTATAATCTTTTCGTTCTAACTGAGATTGAXTXFTT CTAGGAAGCTAA-**PH**	153 bp	This study
qPCR (F) qPCR (R)	CATGGGAAACAAATTCAAAAGGA GGAGAGATTTCTTTTTTAGAGGAGCT	85 bp	Duodu *et al.* (2012)
PCR (F5) PCR (F11)	CCTTTTTGAGTTTCGCTCC TACCAGTTGGAAACGACTG	1140 bp	Forsman *et al.* (1994)

(F) Forward primers, (R) Reverse primer, (P) probe, (PH) Phosphate group to block elongation

6.2.5. Real-time RPA for the *Fno* plasmid standard

6.2.5.1. Fno-RPA primers and probe design

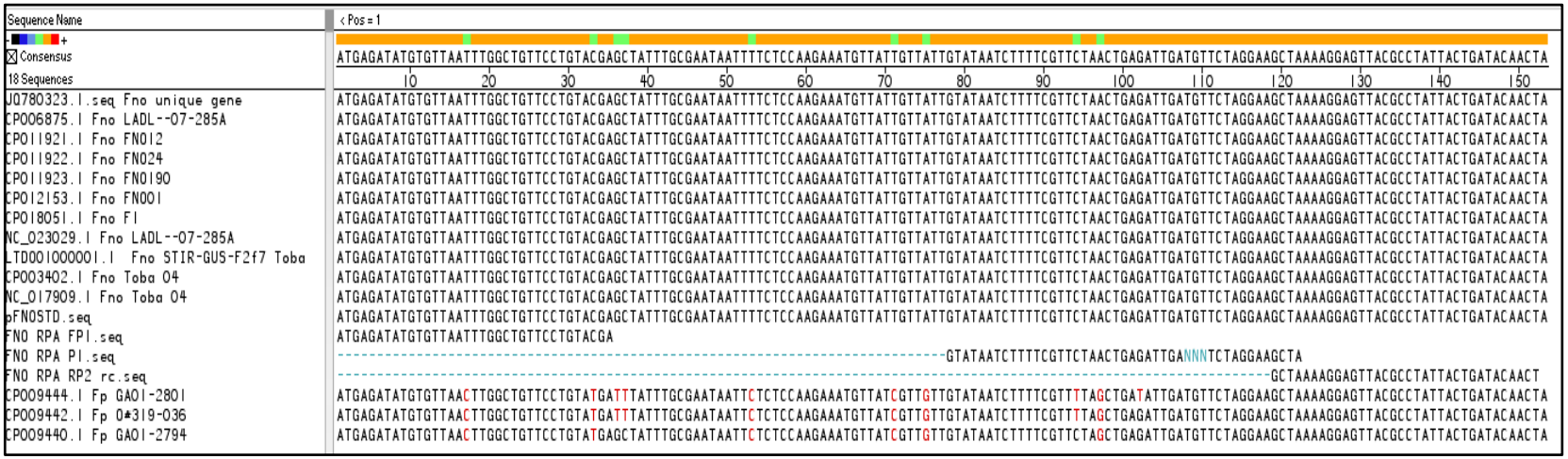
Two primers and a probe for RPA were designed using Primer Express™, V.3.0.1 (ThermoFisher Scientific, UK) following the manufacturer's instructions **(**http://www.twistdx.co.uk/images/uploads/docs/Appendix.pdf**)** using the target sequence (GenBank Accession no. JQ780323) representing the hypothetical protein gene (456 pb) and all available genome sequences for fish pathogenic *Fno, Fnn*, as well as the human pathogenic and environmental *Fp* strains (Sridhar *et al.,* 2012; Sjodin *et al.,* 2012; Johnson *et al.,* 2015; Goncalves *et al.,* 2016; Ramirez-Paredez *et al.,* 2017a) (Table 6.3 and Figure 6.3) to exclude cross detection by RPA.

Table 6.3. *Francisella* genomes used in this study.

Bacteria	Strain ID	Genbank accession no.	Reference
Fno	STIR-GUS-F2f7	LTD00000000.1	Ramirez-Paredes *et al.* (2017a).
Fno	FNO01	CP012153	Figueiredo *et al.* (2016)
Fno	FNO12	CP011921	Gonçalves *et al.* (2016)
Fno	FNO24	CP011922	Gonçalves *et al.* (2016)
Fno	FNO90	CP011923	Gonçalves *et al.* (2016)
Fno	LADL--07-285A	CP006875	Un-published
Fno	F1	CP018051	Un-published
Fno	LADL--07-285A	NC_023029	Un-published
Fno	Toba 04	CP003402	Sridhar *et al.* (2012)
Fno	Toba 04	NC_017909	Sridhar *et al.* (2012)
Fnn	FSC772	CP022207	Sjodin *et al.,* (2012)
Fp	GA01-2801	CP009444	Johnson *et al.* (2015)
Fp	O#319-036	CP009442	Johnson *et al.* (2015)
Fp	GA01-2794	CP009440	Johnson *et al.* (2015)

Fno: *Francisella noatunensis* subsp. *orientalis, Fnn*: *Francisella noatunensis* subsp. *noatunensis, Fp*: *Francisella philomiragia.*

[A]



[B]

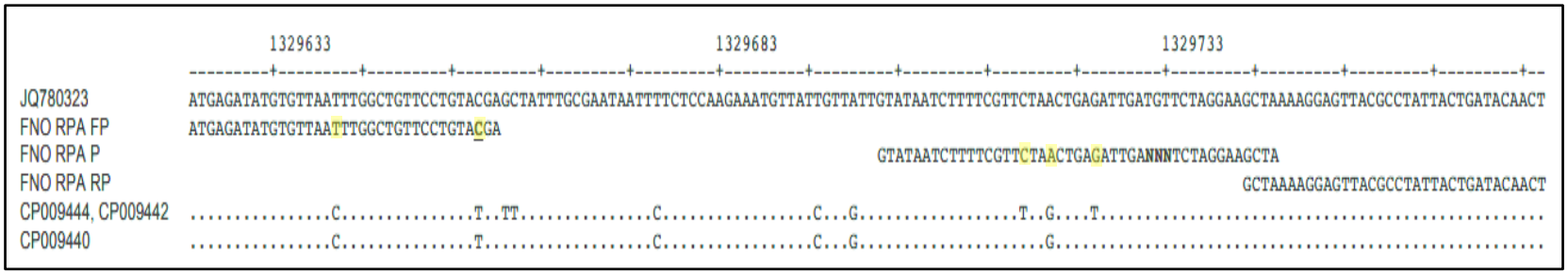


Figure 6.3. Alignment of RPA target region in the hypothetical protein gene. (A) *Fno* unique sequence (JQ780232) at the top, followed by *Fno* sequences, RPA-primers (F: FNO RPA-P1, R: FNO RPA-P2) and probe (FNO RPA-P1) sequences and *F. philomiragia* sequences at the bottom. The *Fno* target gene JQ780323 (shown) is 100% conserved and present in *Fno* genomes and *Fp* genomes, but not in *Fnn*. (B) Nucleotides mismatching with *Fp* sequences at the *Fno*-RPA probe (n = 3) and the RPA forward primer (n= 2) are marked by yellow. Underlined neucleotide refers to mismatch at the 3' end of RPA-FP. NNN represents the tetrahydrofuran bridge of the probe. Positions are given in relation to *Fno* genome (CP006875) and *Fp* genomes (CP009444, CP009442, CP009440).

The primers and the probe for RPA were synthesised by TIB Molbiol (Berlin, Germany). The probe contained a tetrahydrofuran spacer (THF) with a 3' quencher (BHQ1-dT; thymidine nucleotide carrying Blackhole quencher) and 5' fluorescence reporter (FAM-dT; thymidine nucleotide carrying 6 carboxy-flourescein). The sequences of the final primers and probe used in this study are listed in Table 6.2.

6.2.5.2. Fno-RPA reaction

The RPA reaction was performed in a 50 µL volume using a TwistAmp™ exo lyophilized kit (TwistDX, Cambridge, United Kingdom). The reaction mixture included 420 nM of each primer, 120 nM FAM-tagged RPA probe, 14 mM magnesium acetate, 1X rehydration buffer and 1 µL of template. All the reagents except the template and Mg acetate were prepared in a master mix which was distributed into 0.2 mL tubes (Eppendorf, Germany). Four µL of Mg acetate were pipetted into the lid of the reaction tubes containing the dried reaction-pellet. One µL of the template was added to the mixture aliquots and quickly centrifuged using a mini-spin centrifuge (MCF 2360, LMS Co-Ltd., Korea) then transferred to the reaction tubes and lids were closed carefully. The tubes were vigorously mixed by inversion 10 times and centrifuged for 20 s. The tubes were immediately placed in an ESE Quant Tube Scanner device (Qiagen Lake Constance, Stockach, Germany). The tubes were incubated at 42°C for 20 min where the fluorescence measurement including excitation at 470 nm and detection at 520 nm for FAM channel was performed. After four min, the tubes were taken out of the ESE Quant Tube Scanner device for a quick spin then returned to complete the scanning. The ESE Quant scanner software enabled threshold validation including evaluation of fluorescence by increasing the fluorescence above three standard deviations over the background detected in the first minute of the reaction. In addition, the curve slope represented in mV/time can be utilised (slope adaptable) and a second derivative window for calculation of the turning point of the upward fluorescence development can be used for verifying curves with a very low slope.

6.2.6. Analytical sensitivity and specificity of *Fno*-RPA reaction

The quantitative *Fno*-plasmid DNA standard was used to evaluate the sensitivity of the RPA and qPCR reactions using 1 µL of a dilution range of 10^7 to 10^1 copies/µL. Both RPA and qPCR were repeated 10 times using 10 individual master mixes, each run

included duplicate reactions and non-template control (Milli-Q water only). To evaluate the specificity of the RPA reaction, the assay was tested using 1 µL of gDNA (100 ng/µL) from the different bacterial strains listed in Table 6.1.

6.2.7. Clinical testing of the *Fno*-RPA

DNA extracted from fish tissues (78 spleen and 78 head kidney samples) and water samples (*n*= 5) were used to test the developed RPA. The tissue and water samples were firstly tested by both conventional PCR using previously published *Francisella* genus-specific primers (Table 6.2; Forsman *et al.,* 1994) and qPCR using *Fno*-specific primers (Table 6.2; Duodu *et al.,* 2012) targeting a region slightly downstream from the region used for RPA amplicon design as previously described in sections 4.2.1.2 and 4.2.10 in Chapter 4. One µL from the total DNA (100 ng/µL) was amplified using RPA and results were compared with data obtained from qPCR. All positive RPA results were additionally verified by secondary derivative analysis as implemented in the analysis software. Samples tested negative by qPCR and positive by RPA were further diluted to 1:10 and 1:100 dilutions and re-tested to investigate potential inhibition of qPCR. The diagnostic performance of the developed RPA was evaluated by calculation of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) using free statistical calculators "Diagnostic Test Evaluation Calculator" (https://www.medcalc.org/calc/diagnostic_test.php) and results were presented as a percentage.

6.2.8. Evaluation of the effect of reaction temperature on the *Fno*-RPA amplification

To evaluate the temperature ranges at which the developed RPA can be performed, 10 copies of the plasmid standard DNA and a non-template negative control (Milli-Q water) were used to run the RPA assay at 42°C, 39°C, 37°C and 35°C for 20 min and amplification results and time were recorded.

6.2.9. Statistical analysis

Microsoft Excel 2016 was used to arrange the data for analysis. GraphPad® prism v.6 (GraphPad, San Diego, CA, USA) was used to calculate a semi-log regression of the data set of 10 runs of *Fno*-RPA and qPCR by blotting the threshold time in minutes (Tt) for RPA or the cycle threshold (Ct) for qPCR against the molecules detected of the *Fno*-

plasmid standard DNA dilutions (10^7: 10^1 copies/µL). A probit regression analysis was performed in Minitab® v.17 (Minitab Ltd., UK) to calculate limits of detection (LOD) in 95 % of the cases following both assays.

6.3. Results

6.3.1. Analytical sensitivity

To determine the analytical sensitivity, the *Fno*-DNA plasmid standard was used for both qPCR and RPA. RPA needed approximately 6 min (6.2± 0.22 min) to reach the detection limit (Figure 6.4A) while the qPCR required about 90 min (Ct 35.2 ± 0.6) (Figure 6.4B). The highest detection sensitivity of both qPCR and RPA was 10 copies (Figure 6.5). Probit analysis showed that, the limits of detection with 95% probability were 15 molecules and 11 molecules of the *Fno*-plasmid standard DNA/µl in RPA and qPCR, respectively (Figure 6.6)

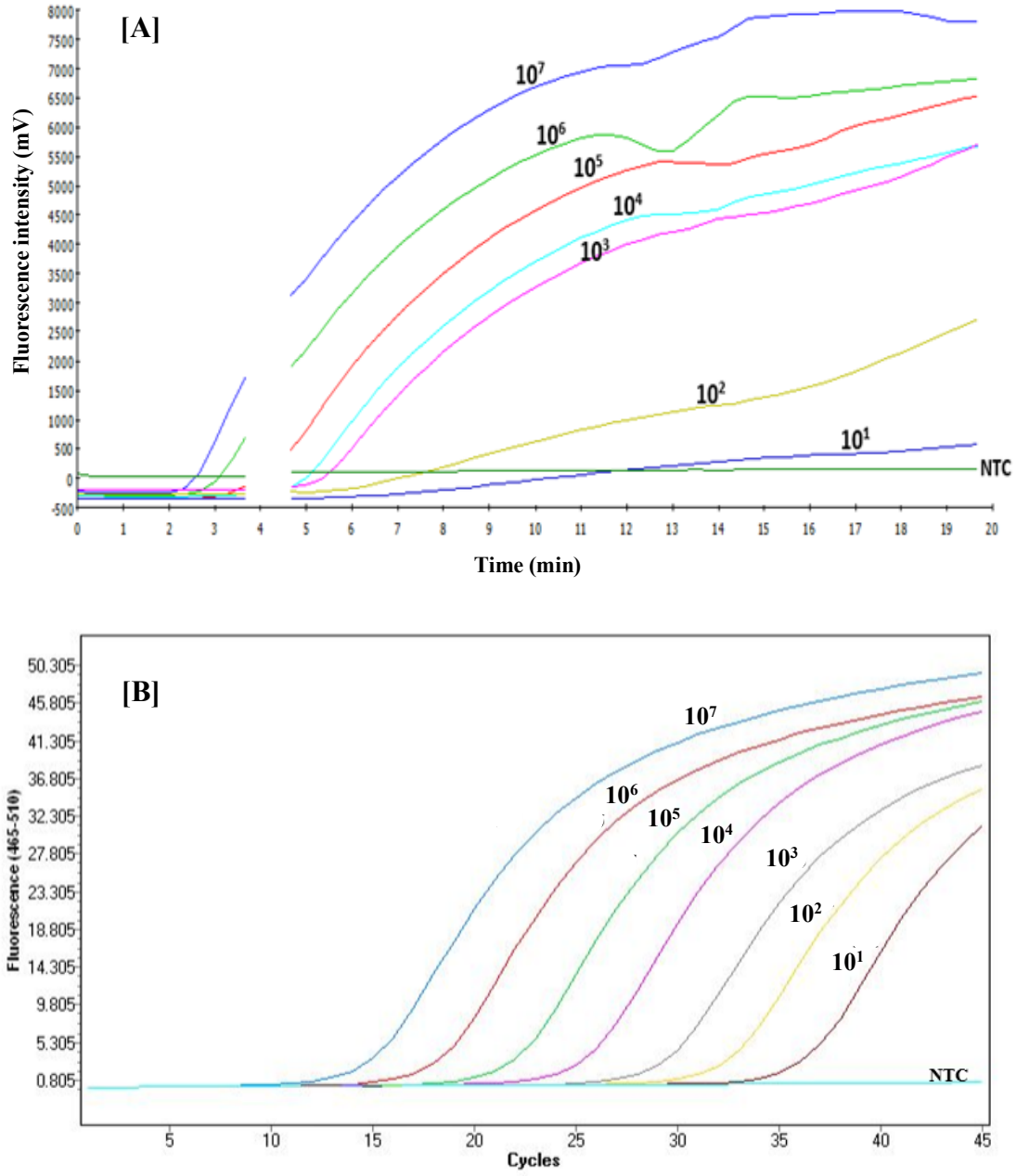


Figure 6.4. Performance of RPA and qPCR using dilutions of *Fno*-plasmid DNA standard. Representatives amplification curves from three runs of both RPA (A) and qPCR (B) (n=3) showing the fluorescence development over time in both assays using a dilution range of 10^7 to 10^1 copies /µL of the *Fno*-plasmid standard DNA.

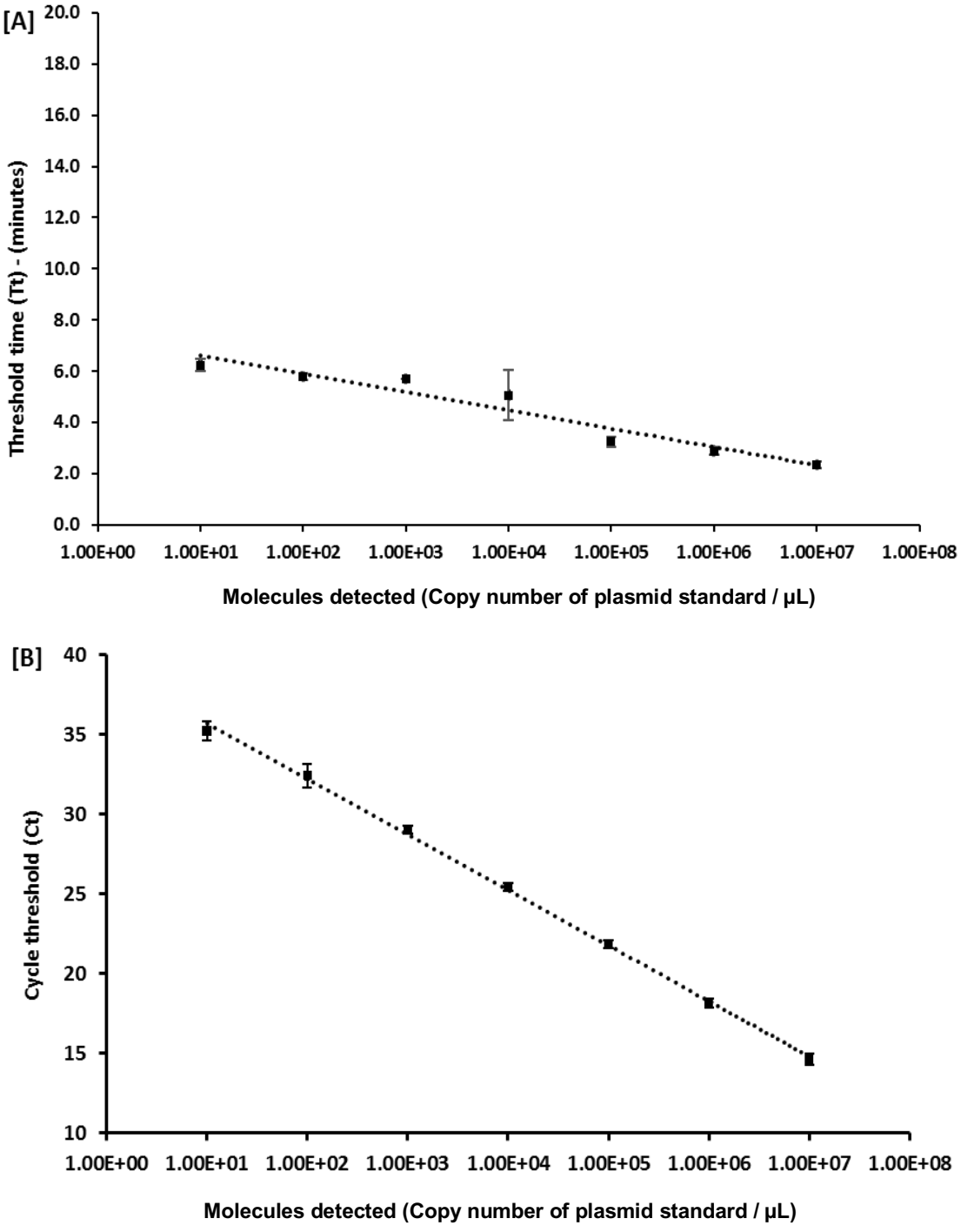


Figure 6.5. Reproducibility of RPA and qPCR assays in detection of dilutions of *Fno*-plasmid standard. The semi-log regression generated by 10 data sets of RPA (A) and qPCR (B). Threshold time (Tt in RPA) and cycle threshold (Ct in qPCR) were represented as a mean ± standard deviation (±SD).

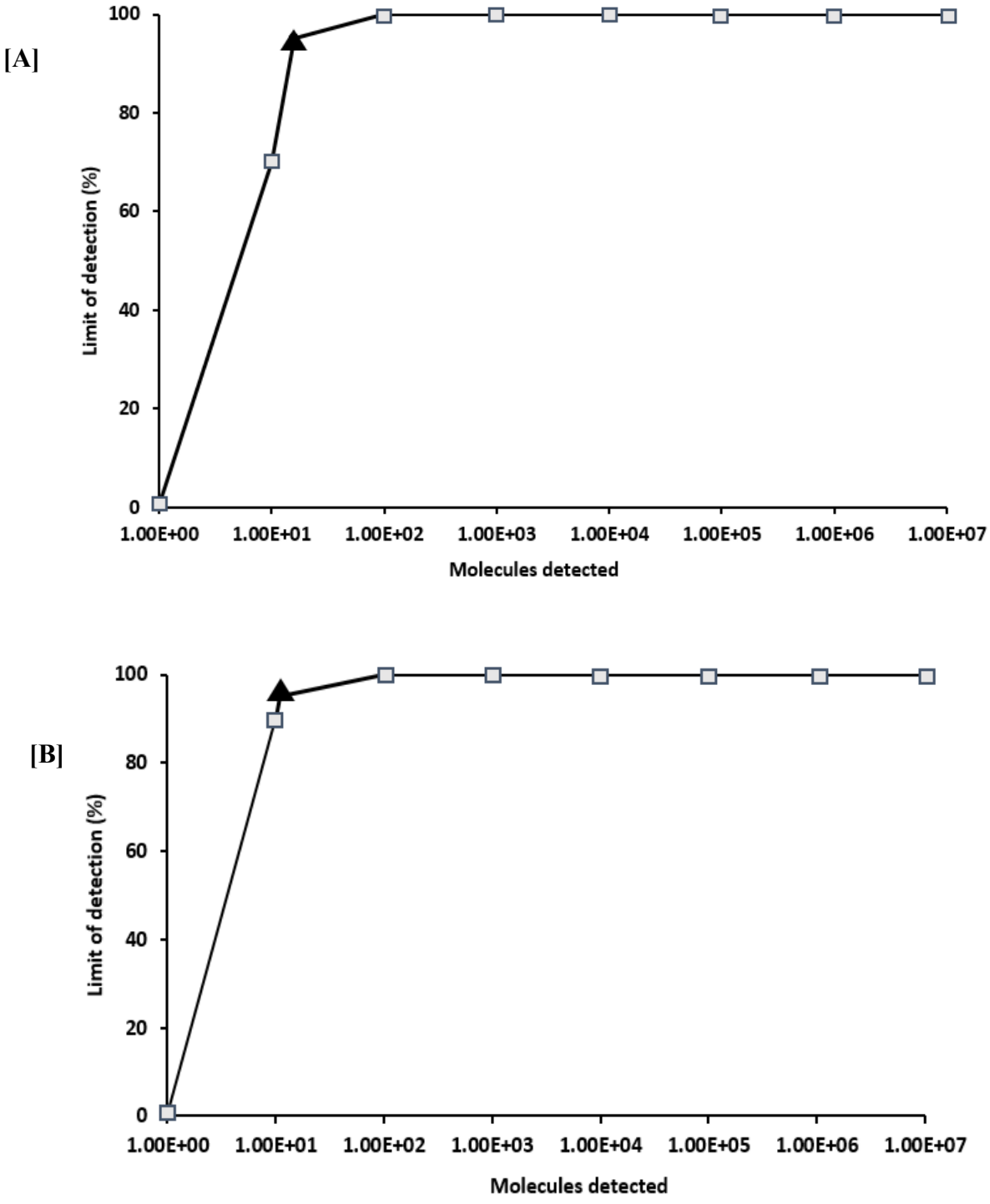


Figure 6.6. Probit analysis of data set of 10 runs of RPA and qPCR. The black triangle indicates limits of detection at 95% probability which were 15 and 11 copies/reaction of *Fno*-plasmid standard DNA in RPA (A) and qPCR (B), respectively.

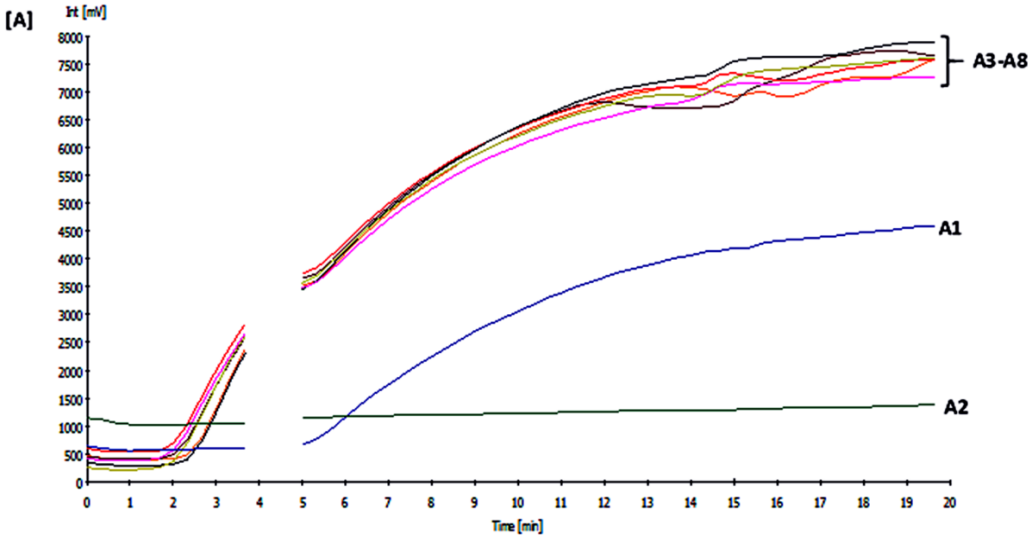
6.3.2. Analytical specificity

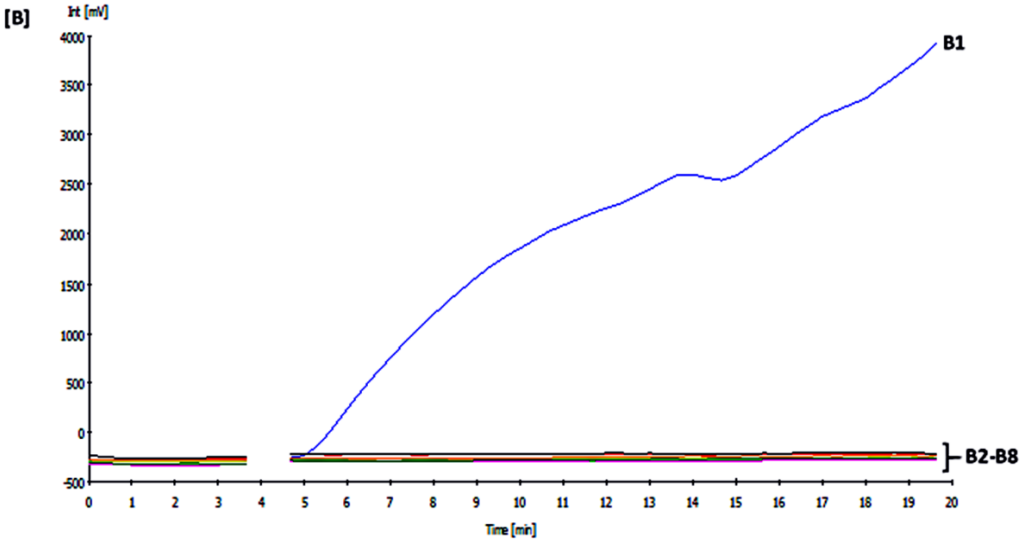
The RPA developed was highly specific as results showed that it only detected the gDNA of the *Fno* isolates and did not detect the gDNA of either the closely related *Francisella* spp. (*Fnn* and *Fp*) or the other bacterial species used in the study (Table 6.4 and Figure 6.7).

Table 6.4. Analytical specificity of *Fno*-RPA

Bacterial species	Strain	Detection by RPA
F. noatunensis subsp. *orientalis*	STIR-GUS F2f7	+
F. noatunensis subsp. *orientalis*	NVI-PQ1104	+
F. noatunensis subsp. *orientalis*	DSMZ21254^{T}	+
F. noatunensis subsp. *orientalis*	NVI-9449	+
F. noatunensis subsp. *orientalis*	Fran-Cos1	+
F. noatunensis subsp. *orientalis*	STIR-HON1	+
F. noatunensis subsp. *noatunensis*	NCIMB 14265^{T}	-
F. noatunensis subsp. *noatunensis*	NVI-7601	-
F. noatunensis subsp. *noatunensis*	PQ1106	-
F. philomiragia	ATCC® 25015^{T}	-
F. philomiragia	ATCC® 25017	-
F. philomiragia	CCUG 12603	-
A. hydrophila	ATCC® 7966^{T}	-
S. agalactiae	ATCC® 51487^{T}	-
S. iniae	ATCC® 29178^{T}	-
V. anguillarum	ATCC® 19264^{T}	-
Photobacterium damselae subsp. *piscicida*	ATCC® 51736^{T}	-
E. coli	ATCC® 11775^{T}	-
Y. ruckeri	ATCC® 29473^{T}	-
Pseudomonas Spp.	STIR-Ps17	-

$^{(T)}$ Type strains, (+) Positive, (-) Negative


[A]
Irt [mV]
A3-A8
A1
A2
Time [min]


[B]
Irt [mV]
B1
B2-B8
Time [min]

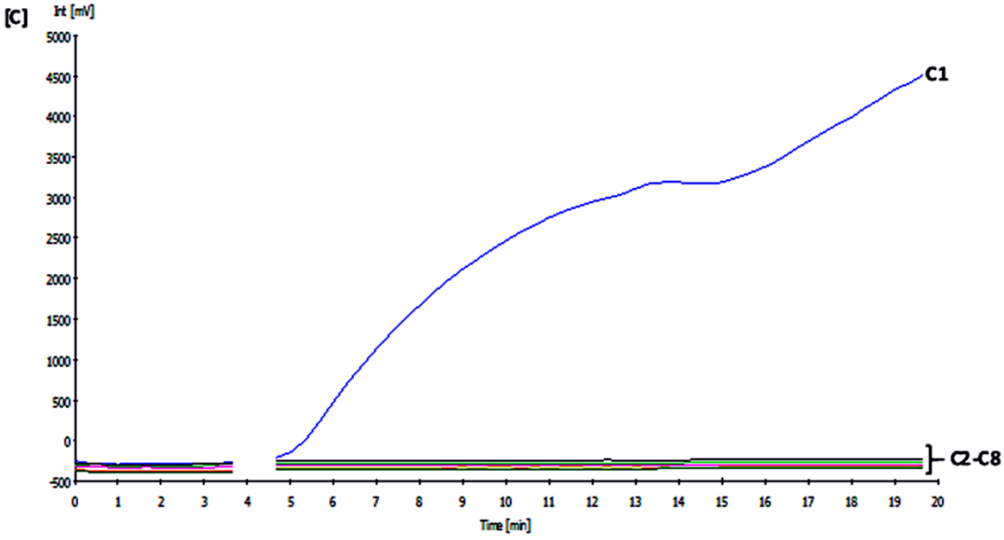

[C]
Irt [mV]
C1
C2-C8
Time [min]

Figure 6.7. Analytical specificity of *Fno*-RPA. Positive amplification only with *Fno* isolates [A], while negative results were obtained with *Fnn*, *Fp* and non-*Francisella* isolates [B and C]. A1 (violet line), B1 (Blue line) and C1 (blue line): Positive control , A2: Negative control (dark green line) , A3: A8 : *Fno* isolates (A3: *Fno* UK isolate (black line), A4: *Fno* Costa Rican isolate (red line), A5: *Fno* Japanese isolate (green line), A6: *Fno* Austrian isolate (orange line), A7: *Fno* Mexican isolate (pink line), A8: *Fno* Central American isolate (brown line), B2: B7 : *Fnn* isolates (B2: *Fnn* Norwegian isolate (black line), B3: *Fnn* Irish isolate (red line), B4: *Fnn* Swedish isolate (green line), *Fp* isolates (B5: *Fp* from muskrat (orange line) , B6: *Fp* from water (pink line), B7: *Fp* human (brown line) and B8: *A. hydrophila* (dark green line). C2: *S. agalactiae* (black line), C3: *S. iniae* (red line), C4: *V. anguilarum* (green line), C5: *P. damselae* subsp. *piscicida* (orange line), C6: *E. coli* (pink line), C7: *Y. ruckeri* (brown line), C8: *Pseudomonas* spp. (dark green line).

6.3.3. Evaluation of clinical sensitivity of *Fno*-RPA with field samples

The collected fish were tested by bacteriological tests, PCR, qPCR and RPA. *Fno* was successfully recovered on CHAH from spleen samples of 7/10, 3/10 and 14/40 from the first and second UK and the Thai tilapia, respectively. *Fno* was successfully detected by conventional PCR (38/78 spleens; 33/78 head kidneys), by qPCR (46/78 spleens; 42/78 head kidneys) and by RPA (47/78 spleens, 47/78 head kidneys). Screening of water samples showed that all samples were negative by PCR and qPCR, while they were positive by RPA (Table 6.5 and Figure 6.8). The clinical sensitivity and specificity of the developed RPA assay were 100 % (95 % CI: 95.89 % to 100 %) and 84.93 % (95 % CI: 74.64 % to 962.23 %), respectively (Table 6.6). The crude DNA extracts of 1 spleen, 5 head kidneys and the 5 water samples that had tested negative by qPCR (Ct>40) and positive by RPA, were diluted to 1:10 and 1:100 dilutions and retested by qPCR. One spleen sample and three head kidney samples (HK 2, 7, and 10) tested positive at 1:10 and 1:100 dilutions, one head kidney sample (HK16) was positive only at 1:100 dilution, 1 sample (HK11) was negative at both dilutions. In addition, 3 samples of water (UV filter; Bio-filter tank1; Fish tank1) tested positive at 1:10 and 1:100 dilutions, while the other two samples (Bio-filter 2; Fish tank 2) tested negative at both dilutions (Table 6.7).

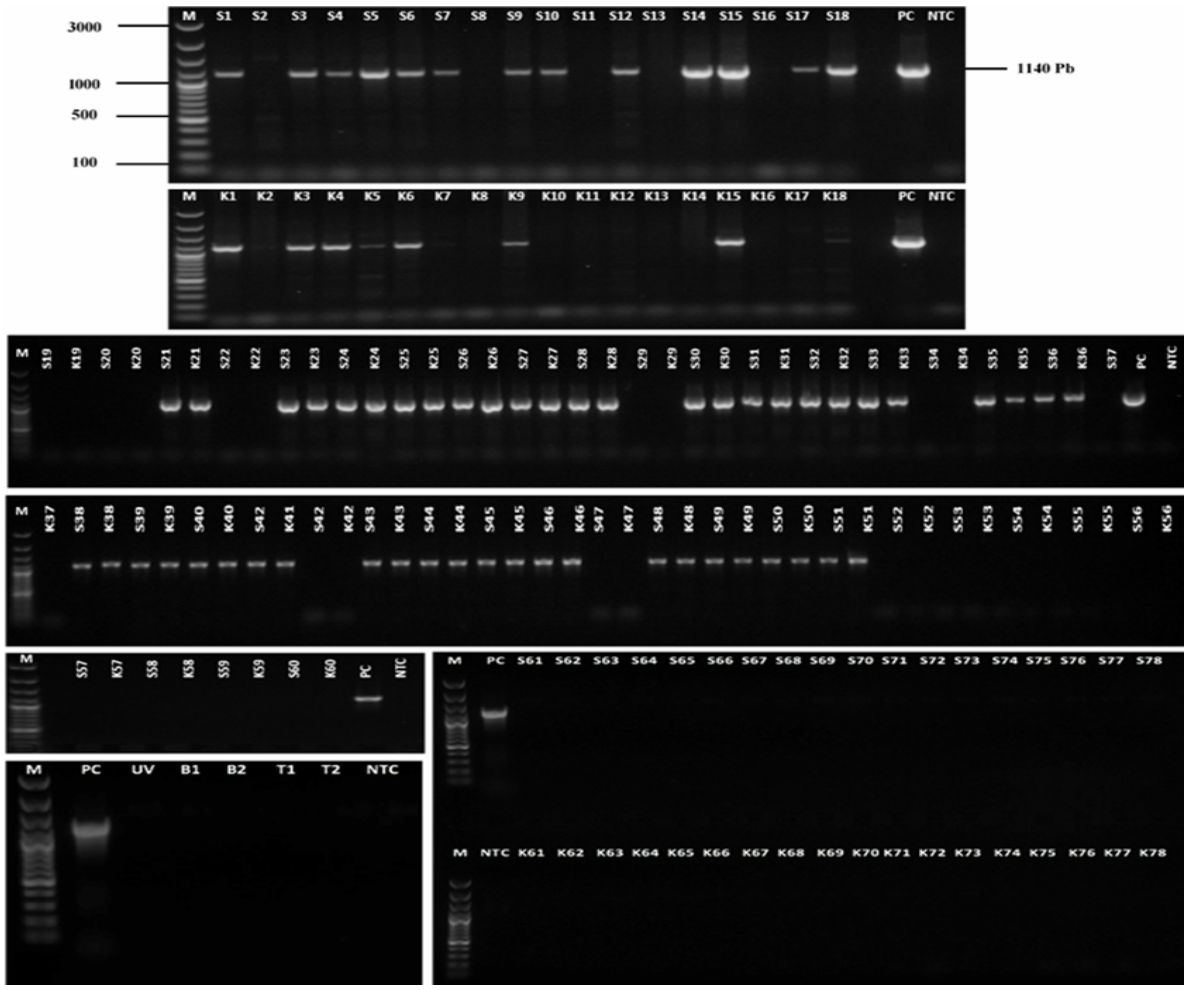


Figure 6.8. PCR results of 156 tissue samples from tilapia and 5 water samples collected from UK and Thai farms after electrophoresis on a 1% agarose gel. M: 100Pb DNA marker, S: spleen, K: Head kidney, UV: ultraviolet filter, B1: Bio-filter tank 1, B2: Bio-filter tank 2, T1: Fish tank 1, T2: Fish tank2, PC: Positive control (*Fno* gDNA), NTC: Negative control (Milli-Q water).

Table 6.5. Screening of field samples (tilapia tissues and water samples) by bacteriology, conventional PCR, qPCR and RPA.

Sample ID.	Sample type	Bacterial isolation	Conventional PCR	qPCR Cycles threshold (Ct)		RPA threshold time (Tt) in minutes	
Fish 1	Spleen	-	+	30.06	+	3.7	+
	Head Kidney		+	36.97	+	5.7	+
Fish 2	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	5.7	+
Fish 3	Spleen	+	+	30.8	+	5.7	+
	Head Kidney		+	25.1	+	3.3	+
Fish 4	Spleen	+	+	29.44	+	5.7	+
	Head Kidney		+	32.3	+	3.7	+
Fish 5	Spleen	+	+	27.45	+	3.3	+
	Head Kidney		+ (w)	31.07	+	5.7	+
Fish 6	Spleen	+	+	-	-	5.7	+
	Head Kidney		+	27.68	+	3.7	+
Fish 7	Spleen	+	+	32.38	+	5.7	+
	Head Kidney		-	-	-	5.7	+
Fish 8	Spleen	-	-	-	-	-	-
	Head Kidney		-	34.86	+	5.7	+
Fish 9	Spleen	+	+	38.82	+	5.7	+
	Head Kidney		+	30.16	+	5.7	+
Fish 10	Spleen	+	+	28.1	+	3.3	+
	Head Kidney		-	-	-	5.7	+
Fish 11	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	6.0	+

Table 6.5. continued

Fish 12	Spleen	-	+	30.83	+	3.7	+
	Head Kidney		-	-	-	-	-
Fish 13	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 14	Spleen		+	23.42	+	3.0	+
	Head Kidney		-	-	-	-	-
Fish 15	Spleen	+	+	27.98	+	2.7	+
	Head Kidney		+	25.14	+	3.3	+
Fish 16	Spleen	-	-	32.53	+	5.7	+
	Head Kidney		-	-	-	5.7	+
Fish 17	Spleen	+	+	28.19	+	3.0	+
	Head Kidney		-	-	-	-	-
Fish 18	Spleen	+	+	23.66	+	3.0	+
	Head Kidney		+ (w)	35.21	+	5.7	+
Fish 19	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 20	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 21	Spleen	+	+	23.94	+	5.7	+
	Head Kidney		+	21.24	+	4	+
Fish 22	Spleen	-	-	23.49	+	5.7	+
	Head Kidney		-	23.30	+	3.7	+
Fish 23	Spleen	+	+	23.57	+	5.7	+
	Head Kidney		+	20.51	+	3.3	+

Table 6.5. continued

Fish 24	Spleen	-	+	21.88	+	5.7	+
	Head Kidney		+ (w)	23.67	+	5.7	+
Fish 25	Spleen	+	+	22.19	+	5.7	+
	Head Kidney		+	22.34	+	3.7	+
Fish 26	Spleen	+	+	21.67	+	5.7	+
	Head Kidney		+	21.28	+	3.3	+
Fish 27	Spleen	-	+	22.91	+	5.7	+
	Head Kidney		+	19.49	+	5.7	+
Fish 28	Spleen	-	+	18.04	+	3.3	+
	Head Kidney		+	21.01	+	3.7	+
Fish 29	Spleen	-	-	19.99	+	5.7	+
	Head Kidney		-	22.33	+	3.7	+
Fish 30	Spleen	+	+	23.71	+	5.7	+
	Head Kidney		+	25.76	+	5.7	+
Fish 31	Spleen	+	+	21.81	+	3.7	+
	Head Kidney		+ (w)	30.94	+	5.7	+
Fish 32	Spleen	-	+	22.04	+	5.7	+
	Head Kidney		+ (w)	23.70	+	3.7	+
Fish 33	Spleen	+	+	24.41	+	3.7	+
	Head Kidney		+	21.85	+	3	+
Fish 34	Spleen	-	-	22.09	+	3.7	+
	Head Kidney		-	25.93	+	3.7	+
Fish 35	Spleen	+	+	24.66	+	3.7	+
	Head Kidney		+	20.32	+	3	+

Table 6.5. continued

Fish 36	Spleen	-	+	20.84	+	4	+
	Head Kidney		+	20.56	+	3	+
Fish 37	Spleen	-	-	21.78	+	3	+
	Head Kidney		-	26.36	+	5.7	+
Fish 38	Spleen	-	-	18.15	+	3	+
	Head Kidney		-	21.60	+	3.3	+
Fish 39	Spleen	+	+	24.65	+	5.7	+
	Head Kidney		+	22.90	+	4.7	+
Fish 40	Spleen	+	+	24	+	5.3	+
	Head Kidney		+	20.72	+	3.3	+
Fish 41	Spleen	+	+	20.81	+	3.3	+
	Head Kidney		+	21.14	+	3.7	+
Fish 42	Spleen	-	+	24.74	+	5.7	+
	Head Kidney		+	20.58	+	3	+
Fish 43	Spleen	-	-	29.67	+	5.7	+
	Head Kidney		-	22.37	+	3.3	+
Fish 44	Spleen	+	+	23.05	+	3.7	+
	Head Kidney		+	35.20	+	6	+
Fish 45	Spleen	-	+	29.06	+	5.7	+
	Head Kidney		+	21.52	+	3.3	+
Fish 46	Spleen	-	+	23.97	+	3.7	+
	Head Kidney		+	23.32	+	3.3	+
Fish 47	Spleen	+	+	20.32	+	3	+
	Head Kidney		+	22.73	+	3.3	+

Table 6.5. continued

Fish 48	Spleen	-	-	26.20	+	5.7	+
	Head Kidney		-	24.67	+	3.7	+
Fish 49	Spleen	-	+	22.54	+	3.7	+
	Head Kidney		+	21.96	+	3.3	+
Fish 50	Spleen	-	+	20.1	+	3	+
	Head Kidney		+	21.29	+	3	+
Fish 51	Spleen	-	+	21.59	+	3.3	+
	Head Kidney		+	18.75	+	3	+
Fish 52	Spleen	+	+	28.09	+	5.7	+
	Head Kidney		+	23.85	+	3.7	+
Fish 53	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 54	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 55	Spleen	-	-	34.53	+	5.7	+
	Head Kidney		-	33.9	+	5.7	+
Fish 56	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 57	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 58	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 59	Spleen	-	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 60	Spleen	-	-	-	-	-	-

Table 6.5. continued.

	Head Kidney		-	-	-	-	-
Fish 61	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 62	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 63	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 64	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 65	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 66	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 67	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 68	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 69	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 70	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 71	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 72	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 73	Spleen	N/A	-	-	-	-	-

Table 6.5. continued

	Head Kidney		-	-	-	-	-
Fish 74	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 75	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 76	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 77	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Fish 78	Spleen	N/A	-	-	-	-	-
	Head Kidney		-	-	-	-	-
Water	UV filter	N/A	-	-	-	5.7	+
Samples	Bio-filter 1	N/A	-	-	-	5.7	+
	Bio-filter 2	N/A	-	-	-	3.7	+
	Tank 1	N/A	-	-	-	6	+
	Tank 2	N/A	-	-	-	5.7	+

(+) Positive, (-) Negative, (w) weak positive/negative, (N) negative Ct (Ct > 40), (N/A) not done.

Table 6.6. Diagnostic performance of the *Fno*-RPA using field samples

		Real-tim qPCR		Performance characteristics (%)			
		Positive	Negative	Sensitivity	Specificity	PPV	NPV
RPA	Positive	88	11	100%	84.93%	88.89%	100%
	Negative	0	62				

(PPV: Positive predictive value, NPV: Negative predictive value)

Table 6.7. Results of testing diluted crude DNA preparations from fish tissues and water samples by qPCR.

Sample	Crude DNA		1:10 dilution		1:100 dilution	
	Ct value	Results*	Ct value	Results*	Ct Value	Results*
Spleen 6	>40.0	-	26.95	+	30.04	+
Head Kidney 2	>40.0	-	29.50	+	32.88	+
Head Kidney 7	>40.0	-	30.20	+	34.30	+
Head Kidney 10	>40.0	-	31.14	+	33.54	+
Head Kidney 11	>40.0	-	>40.0	-	>40.0	-
Head Kidney 16	>40.0	-	>40.0	-	>34.04	+
UV filter water	>40.0	-	34.88	+	37.02	+
Bio-filter 1 water	>40.0	-	>40	-	>40	-
Bio-filter 2 water	>40.0	-	34.99	+	36.72	+
Tank 1 water	>40.0	-	36.80	+	39.9	+
Tank 2 water	>40.0	-	>40	-	>40.0	-

(+) Positive, (-) Negative, (Ct) cycle threshold. * All results are mean of duplicate reactions.

6.3.4. Evaluation of the effect of reaction temperature on the *Fno*-RPA

Running *Fno*-RPA at temperature range of 35-42°C showed positive amplification of *Fno* at all temperatures used but at different reaction time (Table 6.8).

Table 6.8. Results of RPA amplification of *Fno* DNA at different reaction temperatures

Temperature (°C)	Threshold time (Min)
42°C	6 min
39°C	7.3 min
37°C	8.7 min

6.4. Discussion

Francisellosis is one of the most serious bacterial pathogens affecting tilapia industry worldwide. Mortality rates of up to 95% were documented in cultured tilapia in Taiwan (Chen *et al.,* 1994; Chern and Chao, 1994) and recently mortalities up to 40% in broodstock in Mexico (Ortega *et al.,* 2016) and 5-50% in fingerlings and juveniles in Brazil (Sebastião *et al.,* 2017). Application of rapid, sensitive and robust monitoring represents the most reliable strategy for early identification of outbreaks and initiation of control measures to prevent spreading of the disease outbreak. In the current study, a rapid isothermal RPA assay targeting the unique hypothetical protein gene which is present only in *Fno* and not in the very closely related fish pathogen *Fnn* was developed (Duodu *et al.,* 2012). *Fno* specific qPCR targeting this gene could detect up to 20 fg (~10 genome copies) of the genomic DNA of *Fno* in a previous experiment (Duodu *et al.,* 2012). Three recently published sequences of *Fp* isolates (Accession numbers CP009444; CP009442; CP009440; Johnson *et al.,* 2015) containing the hypothetical protein gene like sequence, which were not available at the time of the qPCR assay development, were included in the alignment in the current study. These genome sequences were derived from *Fp* isolates recovered from water and two human clinical samples. In a previous cross detection experiment, the published qPCR did not pick up any *Fp* strains tested (Dudou *et al.,* 2012). Sequence alignment in the current study now confirms that this is due to 3 mismatches in the *Fp* target region for the *Fno*-RPA probe and one mismatch on the 3`-end of the designed RPA forward primer (Figure 6.3).

Additionally, the cold-water fish pathogen *Francisella noatunensis* subsp. *noatunensis* used in the current cross-detection study, was found to be a genetically, biochemically and morphologically distinct sub species from *Fno* (Ottem *et al.,* 2007). Alignments of the existing genome sequences showed that *Fnn* was lacking the hypothetical protein gene sequence. This finding is now confirmed as neither the developed RPA assay nor the previously published qPCR (Duodu *et al.,* 2012) could detect it. By using the principles of the amplification refractory mutation system (ARMS) concept, that stated that oligonucleotides with a mismatched 3'-residue will not function as primers in the PCR under appropriate conditions (Newton *et al.,* 1989), and including a mismatch at position 3 from the 3'-end of the RPA forward primer (Figure 6.3), detection of *Fp* was avoided as confirmed by the cross-detection assays (Table 6.4). Thus, even in

the case of environmental contamination, false positive results due to of *Fp* are excluded when RPA is used.

In this study, a rapid RPA was developed which showed high performance in detection of *Fno*. The analytical sensitivity of the developed RPA was highly comparable to the published qPCR (Dudou *et al.,* 2012), as the limits of detection were determined at 15 molecules of *Fno* target gene for RPA and sensitivity of the published qPCR at 11 molecules was confirmed. However, there was a contrast in the time required to reach the limits of detection in both assays, where qPCR required 90 min, while the RPA could achieve that in 6 min, needing only 2.7- 3 min to determine the *Fno* in clinical samples. The short time of detection in RPA makes it an attractive tool for an on-site detection and monitoring strategy for francisellosis especially on large farms. Also, the quick turnaround of RPA would likewise be of advantage in a standard research facility set-up empowering high-throughput testing. The RPA was performed at a relatively low temperature with isothermal conditions (42°C) and real-time monitoring was performed using an ESE-Quant tube scanner which is less expensive than a mobile cycler. This tube scanner is very convenient with a footprint of 17.5 × 19 cm and an approximate weight of 2 Kg including the attached laptop. Other readers are commercially available such as the Axxin TSO-ISO reader (Axxin. http://www.axxin.com/Molecular-T8.php) or the Genie III (Optigene. http://www.optigene.co.uk/instruments/instrument-genie-iii/). These devices or others currently being developed, such as hand-held detection devices, can contribute to the development of mobile pond-side or point-of-care diagnosis of *Fno* in tilapia farms.

The developed RPA assay was highly specific and only detected gDNA of *Fno* isolates and did not cross detect the closely related *Fnn* nor *Fp* or any other bacteria tested. Moreover, results showed that the crude DNA extracts of 11 clinical samples (1 spleen and 5 head kidneys and 5 water samples) were positive by RPA while they were negative by conventional PCR or qPCR. Calculating the sensitivity and specificity in comparison to qPCR, RPA showed a sensitivity and specificity of 100% and 84.93%, respectively. The lower specificity was due to the RPA scoring 11 samples which the qPCR failed to detect. The qPCR assay used here also gave false positive results. This was demonstrated by achieving positive qPCR results upon diluting the crude DNA extracts which indicates the presence of reaction inhibitors in the DNA extracts that affected qPCR performance but not the RPA.

The robustness of the RPA when crude clinical specimens are used is often featured as benefit of this technique. The developed RPA was found to be more robust than the published qPCR in detection of *Fno* when clinical samples were used. In previous studies, RPA was performed with nucleic acid extracted from various samples including blood (Aebischer *et al.,* 2014), serum (Teoh *et al.,* 2015), plasma (Euler *et al.,* 2013), stool (Crannell *et al.,* 2014), nasal swabs (Boyle *et al.,* 2014), vaginal swabs (Daher *et al.,* 2014), milk (Santiago-Felip *et al.,* 2015), urine (Krolove *et al.,* 2014), sputum and respiratory wash (Ma *et al.,* 2017), foodstuff (Santiago-Felip *et al.,* 2014a), animal tissues (Xia *et al.,* 2014) and plant tissues (Zhang *et al.,* 2014). This is due to the tolerance of the RPA to common PCR inhibitors (Daher *et al.,* 2016). Additionally, it was shown that RPA can work in presence of 15-25% of milk, 50 g/L haemoglobin, 4% (v/v) ethanol and 0.5 U of heparin, all of which have an inhibitory effect on PCR. In an experimental trial, 10^3 copies of gDNA of *S. agalactiae* could be detected in the presence of up to 5 µL of stool sample (Daher *et al.,* 2014). This finding highlights the robustness of the developed *Fno*-RPA for detection of nucleic acids in different crude biological samples if the appropriate extraction protocol is carried out. Other isothermal assays have been adopted for diagnosis of francisellosis in tilapia including LAMP that was successfully used for detection of *Fno* with LOD at 1 fg (Pradeep *et al.,* 2016). However, LAMP has many drawbacks in comparison with RPA, as it depends on turbidity index measurement with a Loopamp® Realtime Turbidimeter that weighs ~ 5 Kg, uses a complex-design of 6 oligonucleotides targeting four target sequences, requires a high temperature of 60 °C, and has a longer reaction time (~ 45 min). Thus, the recent findings ultimately favour the usage of RPA instead of LAMP for mobile isothermal detection of *Fno*.

The RPA assay developed in this study could detect *Fno*-DNA across wide temperature range (35- 42°C) with minimum effect of temperature fluctuation on the amplification results. These findings provide more flexibility and feasibility for the RPA assay over the conventional PCR and qPCR which require a thermocycler and other sophisticated equipment for reliable amplification. Therefore, the developed RPA assay could potentially be used by people with minimal training for on-site diagnosis of francisellosis which will contribute to the decrease and/or prevention of spread of the disease by minimising the movement of potentially infected tilapia. One of the main benefits of the RPA is the convenience of the assay, as the kit used (TwistAmp Exo kit,

Twist DX, UK) is commercially available in the form of dried pellets accompanied with the rehydration buffer and reagents required for the reaction mixture. The only step required is the addition of primers, probe and template DNA. In addition, the detection is performed with an ESE-Quant tube scanner that is very compact. Nevertheless, a major constraint of using the RPA in field is ability to extract good quality nucleic acids to perform the test. However, there are many commercially available DNA extraction methods at present which are simple, cheap and suitable for field application, including magnetic bead-based technology, heated NaOH method (Xing *et al.,* 2017) and mobile extraction devices like QuickGene-Mini80 (Autogen®, USA) (Pereira *et al.,* 2011; Shipley *et al.,* 2012). Using any of these methods will considerably reduce the cost of the assay and provide more flexibility to use RPA in the field conditions.

Recently, RPA assays were used in combination with other tools including lateral flow dip sticks (LFD) (Xing *et al.,* 2017; Guimin *et al.,* 2017; Hou *et al.,* 2017; Tu *et al.,* 2017), ELISA (Santiago-Felipe *et al.,* 2014b), aptamer-based bio-barcodes (ABC) (Loo *et al*., 2013), and hybridisation in microarray format (Kersting *et al*., 2014). These tools have enhanced the performance of the RPA assay and elucidated its significance as a versatile next-generation molecular diagnostic test. Overall, the RPA developed in the current study can be considered a potential user-friendly method for the simple, accurate and rapid detection of *Fno* that can be applied for field screening of tilapia for francisellosis.

6.5. Conclusions

A novel real time *Fno*-RPA was developed for the rapid and accurate detection of *Fno* that showed high analytical sensitivity and specificity. The sensitivity, specificity and reproducibility were highly comparable to the published qPCR with better tolerance to amplification inhibitors in clinical samples. The RPA assay with the mobile tube scanner and proper fast DNA extraction protocol could be used as a promising “pen test” to be applied on fish farms for the rapid detection of *Fno*. Such specific and sensitive diagnostic tools are imperative to development of successful control and management strategies for devastating diseases like aquatic francisellosis.

Chapter Seven
General discussion and Final conclusions

7.1. General discussion

Tilapia is one of the oldest fish species to be farmed around the world. The high consumer demand, the fast growth rate, short production cycle and adaptability to various culture conditions, makes tilapia one of the leading farmed fish species. Tilapia is ranked as the third most important finfish aquaculture species after carp (*Cyprinus carpio*) and salmon (*Salmo salar*) (Munang'andu *et al.,* 2016). At present, tilapia farming is carried out in more than 135 countries worldwide, with 72% of the production taking place in Asia, 19% in Africa and 9% in North and South America (FAO, 2017). Tilapia is also produced in Europe, but on a very minor scale that is estimated to be about 0.05% of the total production (Falch, 2013). Despite the rapid development of global tilapia farming, the intensification of production using high stocking densities, aimed at increasing production outputs, has been associated with great challenges for the industry due to the increased incidence of various diseases. Francisellosis, caused by *Francisella noatunensis* subsp. *orientalis* (*Fno*), is a significant bacterial disease affecting tilapia production globally. The disease has been reported in Asia, Europe, and North, Central and South America over the past 13 years, resulting in massive losses in farms ranging between 5-95% (Soto *et al.,* 2009a; 2012b; 2014b). Up till now, there is no commercially available prophylactic treatment against piscine francisellosis for use on tilapia farms. Therefore, the development of an effective vaccine, that protects against current *Fno* isolates globally is crucial to the tilapia aquaculture industry.

While *Fno* outbreaks in tilapia are increasing, there have been few studies examining the diversity of *Fno* isolates recovered from tilapia published in the literature (Figueiredo *et al*., 2016; Gonçalves *et al*., 2016; Ramirez-Paredes *et al.,* 2017b). In Chapter 2, the proteomic and antigenic profiles of the whole cell proteome of five *Fno* isolates from diverse geographical locations were described using a combination of immunoproteomic and mass-spectrometric methods. Although some of these isolates showed a high degree of homogeneity in a previous study based on phenotypic and genomic comparisons, in addition to antimicrobial susceptibility scheme (Ramirez-Paredes, *et al.,* 2017), the study in Chapter 2 showed minor, but significant differences between the isolates, represented by difference in the abundance of 15 proteins between the different isolates. This finding broadly supports the presence of a "clonal-like behaviour" between *Fno* isolates from wide spread origins as reported in previous studies

(Figueiredo *et al*., 2016; Gonçalves *et al*., 2016; Ramirez-Paredes *et al.,* 2017b). Limited studies have been performed linking the virulence of *Fno* isolates with francisellosis severity in fish farms. Djainal, (2018) showed that the *Fno* UK isolate was more virulent than isolates from Costa Rica, Mexico, Austria or Japan in both tilapia and wax moth larvae models. Differences in abundance of virulence-associated proteins between the *Fno* isolates were examined in Chapter 2, and the UK isolate displayed a significantly higher abundance of some of these proteins (e.g. iglC, GroEL, DnaK, ClpB). This may help to explain the findings of Djainal, (2018), and may provide insight into our understanding of the basis of difference in the virulence between the circulating *Fno* isolates. A combination of proteomics (1-DE and 2-DE) and mass spectrophotometry methods (LC-EDI-MS/MS and MALDI-TOF/MS) was employed in Chapter 2 to characterise the *Fno* proteome and create a data base of the proteins identified. This database is potentially useful for determining which proteins can be used for typing and/or diagnosis of *Fno* as previously shown for *F. tularensis* (Hubalek *et al.,* 2003). More importantly, identification of proteins that are related to virulence or pathogenicity in the *Fno* proteome is a key step to understanding the molecular basis of pathogenicity of this significant pathogen, which in turn will help the development of therapeutics, diagnostics, and prophylactic tools against francisellosis in tilapia.

Bacterial culture is a valuable experimental tool for studying growth characteristics of *Fno in vitro*, however, it is not a true reflection of the conditions present in the host when the bacteria is growing *in vivo*. This may have limited the information on *Fno* virulence obtained with the proteomic analysis performed. Thus, *Fno* isolates grown in the host environment may have different growth and protein expression kinetics *in vivo* compared to the lab adapted growth in culture media, as was reported for *Aeromonas hydrophila* (Poobalane *et al.,* 2008). Therefore, future proteomic studies comparing the protein profiles of *Fno* isolates grown under different conditions (e.g *in vivo* vs *in vitro* or in media lacking essential elements like iron) are required. This will help provide information on the metabolic networks involving the different *Fno* proteins when grown under different culture conditions, and will enhance genome annotations of *Fno*, which are frequently based on bioinformatics predictions and discovery of uncharacterised proteins classified as hypothetical proteins.

While analysis of the whole cell lysate identifies proteins in the soluble portion of the *Fno* bacterial proteome, the outer membrane (OMPs) reflects the proteins in the non-soluble fraction of the bacterium. In Chapter 3, the sub-cellular fraction of *Fno* OMPs displayed a mixture of proteins involved in important biological functions, especially those putatively associated with pathogenicity and host-pathogen interactions. Notably, the majority of the pathogenicity-related proteins were more abundant in the OMPs of *Fno* than in the whole cell preparation, supporting the importance of OMPs in *Fno* pathogenesis. Identification and characterisation of proteins present in the OMP fraction is useful for the development of new generation prophylactic tools such as recombinant OMPs based nano-particles that have been recently reported to provide high levels of protection in Rohu (*Labeo rohita*) and Fringed-lipped Peninsula Carp (*Labeo fimbriatus*) against *A. hydrophila* and *Edwardisella tarda*, respectively (Dubey *et al.,* 2016 a,b). More importantly, simplifying the protein sample preparation using emriched OMPs fraction has contributed to successful identification of the proteins of *Fno* proteome.

One of the key findings in Chapters 2 and 3 was that the antigenic profile of the different *Fno* isolates appeared very homogenous, with the presence of a dominant immuno-reactive protein band (~ 17-28 kDa) present in all isolates, as shown by 1D-western blotting of both whole cell and OMPs preparations. Interestingly, this antigenic protein band has also been reported in other *Francisella* studies, including *Fno* (Kay *et al.,* 2006) and *Fnn* (Schrøder *et al.,* 2009). The ability of the tilapia serum antibodies raised against the *Fno* UK isolate to cross-react with the other *Fno* isolates, supported the hypothesis of the potential cross-protection of the *Fno* UK isolate (vaccine isolate) against other heterologous isolates from different geographical locations. Equally important, identified and validated proteins that showed immunoreactivity against anti-*Fno* antibodies could be used as potential candidates for an improved *Fno* vaccine. Silver staining of the whole cell proteinase-K digest of the five *Fno* isolates used in Chapter 2, revealed an abundant band that was similar to the immunodominant lipo-oligo-polysaccharide band identified in *F. victoria* isolated from tilapia in a previous research study (Kay *et al.*, 2006). Further work is required to investigate the immuno-reactivity of the tilapia anti-*Fno* hyper immune serum against this abundant band in the proteinase-K digested proteins by western blot to show if these antibodies will have a similar affinity to the carbohydrate rich component as opposed to the proteins in the present study. In addition, performing 2D-

SDS-PAGE and 2D western blot analyses for the OMPs of different *Fno* isolates is necessary to improve the protein resolution and to confidently identify the immunogenic proteins within this fraction. Determining similarity in the antigenic profile of *Fno* isolates investigated in Chapters 2 and 3, with only minor difference in their proteomic profile, and the significant abundance of an important set of proteins in the *Fno* UK isolate, that are involved in pathogenicty and/or virulence, energy production and other high biological activities, were results that formed a benchmark to design the vaccination experiments performed in Chapters 4 and 5.

In the absence of a commercial vaccine against *Fno*, the traditional method of raising water temperature to 30°C and/or using prolonged antibiotic treatment for up to 10 days are the current practices for controlling *Fno* outbreaks (Soto *et al.,* 2012d; 2014 a,b). Although antimicrobials showed an effect on *Fno* in tilapia and other susceptible ornamental species, antibiotics do not control the disease and there are increasing concerns regarding the development of antimicrobial resistance by *Fno* (Soto and Hawke, 2017). Vaccination is one of the most effective measures for preventing diseases in farmed fish, contributing to a sustainable aquaculture industry with low use of antimicrobials (Gudding, 2014). Unlike salmonids, vaccination in tilapia is still in its infancy and there are only few available commercial vaccines for use in tilapia farms, such as AQUAVAC® Sa for *S. agalactiae* serotype lb, AQUAVAC® Sa1 for *S. agalactiae* serotypes la and III, AQUAVAC® Si for *S. iniae* and AQUAVAC® IridoV for *Irido* virus. Thus, more research is required to develop effective vaccines against other infectious diseases in tilapia, including francisellosis. Based on the immuno-proteomic study using a diverse selection of *Fno* isolates in Chapter 2 and 3, an injectable whole-cell, formalin killed adjuvanted vaccine against *Fno* was developed using the *Fno* UK isolate. The cross-reactivity of serum raised against heterologous *Fno* isolates would be expected to confer a broad-spectrum protection against *Fno* infection. The protective efficacy of this vaccine in Nile tilapia (mean weight 15 ± 0.2 g) after i.p. challenge with either homologous or heterologous *Fno* isolates, was investigated in Chapter 4. Immunisation of tilapia with this vaccine by i.p. administration, resulted in significant protection against the homologous isolate (RPS 82.3%) and the heterologous isolates (RPS 69.8% and 65.9%). The results of this study were comparable to a previous study using an autogenous immersion live attenuated vaccine (RPS of 68.75 % to 87.5%, Soto *et al.,* 2011b). The use of live

attenuated vaccines on farms, despite promising results, have safety constrains due to concerns of the bacteria regaining virulence under certain biological and environmental conditions (Munang'andu *et al.,* 2016) and generally unacceptable in most countries. As tilapia is one of the most widely farmed fish globally, any live attenuated vaccine used in farmed tilapia must be safe, not only for tilapia, but also for other aquatic organisms. Thus, a killed vaccine would be more acceptable if it provides desirable level of protection against francisellosis in tilapia. The RPS obtained with the vaccine developed in Chapter 4 was higher than that obtained with the injectable OMVs-based vaccine administrated in zebrafish (RPS 65.5%, Lagos *et al.,* 2017). This could be due to the use of adjuvant in the current vaccine study or the weak stimulation of the immune system by the OMVs-vaccine. It is of note that cross-protection against heterologous *Fno* isolates has not been studied previously (Soto *et al.,* 2011b; Lagos *et al.,* 2017), which highlights the importance of the study in Chapter 4 by providing valuable information towards the establishing of control strategies against the circulating *Fno* isolates.

Fish vaccination is generally targeted toward stimulation of antibody responses to provide protection against subsequent infection with the pathogens (LaFrentz *et al.,* 2012). In the current study, the serum antibody level 30 days post initial vaccination was significantly higher in the vaccinated fish than the adjuvant-alone or the PBS control fish. This finding was similar to that reported by Ramirez-Paredes (2015), but contrasted with other reports, where no significant difference of the serum antibody response between vaccinated and control groups before challenge were observed (Soto *et al.,* 2011b; Lagos *et al.,* 2017). The reason for these differences in antibody induction is unclear, but could involve the vaccine formulation (e.g. addition of adjuvant), route of administration of the vaccine, size and species of the fish used. A significant increase in serum antibody response in vaccinated fish was observed after challenge, in agreement with other studies (Soto *et al.*, 2011b; Ramirez-Paredes, 2015; Lagos *et al.,* 2017), but the most important observation was that, higher specific IgM levels were also found against the heterologous isolates as well as the vaccine isolate. In addition, serum sampled from vaccinated fish 30 dpv reacted with the different *Fno* antigens, highlighting an immunogenic band similar to that observed in the proteomic studies in Chapters 2 and 3, which was also seen when anti-sera raised against the different *Fno* isolates 15 dpc reacted with the vaccine isolate. The significantly higher survival rates and serum IgM levels observed in the vaccinated fish

compared to adjuvant-alone and PBS control fish, in addition to observation of cross-reactivity with the different *Fno* antigens, may provide strong evidence to support the efficacy of this vaccine. It also suggests the potential of the vaccine to confer broad ranging protection against heterologous *Fno* isolates. Association between the increased IgM levels and the high survival rate in the vaccinated fish after challenge, may indicate that humoral immunity significantly contributes to protection against *Fno* infection in tilapia. It is not surprising that significantly higher survival and serum IgM levels were observed in the homologous challenge group compared to the heterologous challenge groups, however, these isolates exhibited homologous antigenic profiles with the antiserum raised against the vaccine isolate. This may be attributed to the abundance of important virulence-related proteins in the *Fno* UK isolate (the vaccine isolate) in contrast to the other isolates as observed in the 2-DE analysis in Chapter 2. Thus, larger challenge experiments involving other *Fno* isolates from a broader geographical distribution may explain these findings by comparing the IgM and survival levels post-challenge with the diverse *Fno* isolates.

The expression levels of selected immune-related genes (*MHCII*; *IL-1β*; *TNF-α*; *IgM*) in the spleen samples of tilapia fingerlings in response to the i.p. vaccination was examined to investigate the effect of the vaccine on the fish immune system at the molecular level. The significant up-regulation of *MHCII* in the spleen samples of vaccinated fish 6-72 hpv indicates a successful stimulation of the immune response in vaccinated fish (i.e. contact of the inactivated *Fno* cells in the vaccine with TCR and uptake of *Fno* cells/antigens by APCs which in turn triggered a cascade of adaptive immune responses) (Castro and Carolina, 2015). Future study to quantify the number of macrophages in the spleen of tilapia following vaccination may validate this result. An early pro-inflammatory response (6-72 hpv) was observed in vaccinated fish, indicated by significantly up-regulated levels of *IL-1β* and *TNF-α* genes, providing an evidence of the immune priming and migration of the inflammatory cells to the systemic environment of the fish and stimulation of the fish's innate immune system in response to vaccination. Previous study reported down-regulations or late up-regulation of the *IL-1β* and *TNF-α* transcripts after vaccinations against *Fno* in zebra fish (Lagos *et al.,* 2017). The reason for this finding is not clear, but may be attributed to difference in the composition of the vaccine, dose of vaccine, route of vaccination and fish species used in the trial.

Furthermore, the significant up-regulation of *IgM* transcripts in spleen samples that correlated with the significantly higher levels of serum IgM in vaccinated fish, points to a successful triggering of the humoral immune system post-vaccination against *Fno*. More importantly, it supports the role of humoral immunity in protection of tilapia against subsequent *Fno* infection. In the current study, only spleen samples were analysed post-vaccination. Further studies should be conducted to investigate the immune response in other tissues (e.g. liver, head kidney, gills, gut) at different time points (i.e. pre-vaccination, post-vaccination and post-challenge) to establish the effect of the vaccine on immune gene expression, not only in the systemic organs, but also the mucosal organs would be useful. This will help to improve our understanding of molecular mechanism of induction of immunity by i.p. vaccination against *Fno* infection. In addition, cellular immunity was reported to play an important role against *F. tularensis* in mammals (Celli and Zahrt, 2013). Therefore, further studies are required to examine immune cell markers such as CD8 and CD4 expression in order to investigate the importance of cell-mediated immunity in protection of tilapia against *Fno* infection.

Quantification of the bacterial load by qPCR proved to be a very useful approach in Chapter 4, where it indicated the ability of the vaccine to reduce bacterial replication in comparison to the relatively higher load observed in the adjuvant-alone and non-vaccinated fish post-challenge. This finding demonstrated that vaccination against *Fno* appears to be more advantageous than antimicrobials, which demonstrated a limited ability to reduce the bacterial replication and spread in the infected fish tissues, as previously reported by Soto *et al.* (2012d; 2014b). It can also be used to determine the optimal dose of the vaccine that is able to induce protection, while preventing colonisation of the invading bacteria on mucosal surfaces and penetration to the systemic milieu within the vaccinated fish. Additional histopathological studies are required to investigate the extent of tissue damage post-challenge in vaccinated fish, which can then be used as a correlate of vaccine efficacy in association with the other parameters (e.g. IgM levels, RPS, quantification of bacterial burden).

The use of adjuvant was shown to be beneficial in the vaccine study performed in Chapter 4. The role of adjuvants in assisting the efficacy of injectable vaccines and inducing long-lasting protection in fish has been reported, and previous studies showed lower survival rates with non-adjuvanted vaccines compared to adjuvanted vaccines

(Firdaus-Nawi *et al.* 2013; Jaafar *et al.,* 2015). It is important to consider the short life cycle of tilapia (~ 4-6 months) when developing vaccines for this fish species. Thus, a suitable vaccine for *Fno* in tilapia should confer long-life immunity against the diseases without the need for a booster vaccination that would increase the cost of vaccination for this relatively inexpensive fish. In the present study, vaccination was performed 30 days prior to challenging the fish for 15 days. It would be informative to perform a potency test for the vaccine, by evaluating antibody response over a longer period of vaccination followed by challenge, so that the duration of immunity elicited by the developed *Fno* vaccine can be predicted.

While the injectable vaccine developed in Chapter 4 showed potential efficacy and cross-protection against heterologous *Fno* in tilapia fingerlings, from a practical point of view it cannot be used in fish less than 10 g. Thus, another *Fno* vaccine efficacy trial was performed in Chapter 5 with an immersion vaccine for smaller sized tilapia (~ 6 g). The immersion vaccine was developed using the same isolate as used in the injectable vaccine and was administered by dip immersion without adjuvant. Although a relatively moderate level of protection was obtained with this immersion vaccine (RPS of 43.7%), this level was lower than that obtained with both the current i.p. vaccination described in chapter 4 (RPS 65.9 – 82.3 %) and other recent autogenous i.p. *Fno* vaccine study (RPS 100%, Ramirez-Paredes, 2015). In addition, the RPS induced by this immersion vaccine was lower than that reported for a live attenuated immersion vaccine (RPS 68.75% - 87.5%, Soto *et al.,* 2011b). It is still unclear as to the best parameters to use for vaccination of tilapia fry against *Fno* (e.g. optimum vaccine dose, time of vaccination and fish size at the time of vaccination). Therefore, more studies are required to optimise these factors, taking into account the nature of tilapia's physiology and anatomy. This may enable establishment of an efficient vaccination regimen for tilapia fry against subsequent infection with *Fno.*

Evaluation of serum IgM level was the only parameter studied after immersion vaccination of tilapia in the present study. Notably, the IgM levels of 30% of the immersion vaccinated fish were below the threshold at 30 dpv and serum IgM levels were not-significantly different between immersion vaccinated and non-vaccinated fry 20 dpc, although the average IgM level in the immersion vaccinated and challenged fry was higher compared to the non-vaccinated and challenged fry. It was not surprising to detect a low

serum IgM response in the immersion vaccinated fish. This may be attributed to the limited ability of the immersion vaccination to trigger systemic immune response (i.e. serum IgM response) and the greater effect is more likely to be observed on the level of mucosal surfaces. Thus, performing other investigations including analysing of mucus IgM, and perhaps more importantly IgT levels, will provide more information regarding the effect of the immersion vaccine on the mucosal immunity of tilapia. Triggering of mucosal immunity was reported to be important against *Fno* infection (Soto *et al.,* 2011b). In addition, further studies with different *Fno* vaccine formulations (e.g. bivalent, polyvalent vaccines, OMVs or OMPs derived vaccines) are required. Use of mucosal adjuvants to enhance the efficacy of the *Fno* immersion vaccine should be also investigated. Analysis of immune gene expression in mucosal and systemic organs at different time points pre-challenge and post-challenge should also be performed to elucidate the mechanism of interaction of the *Fno* immersion vaccine with the tilapia immune system.

One of the challenges for controlling piscine francisellosis is the nature of the causative agent, which is a fastidious, intracellular organism that can also form biofilms (Soto *et al.,* 2015) and exists in a viable but non-culturable state (Duodu and Colquhoun, 2010). Development of control measures for *Fno* on tilapia farms is urgently needed, but *Fno* is more difficult to isolate and detect than other common bacterial pathogens found during clinical disease outbreaks in tilapia. Thus, having a diagnostic tool that would enable early and accurate detection of the pathogen in the diseased/carrier fish or its surrounding environment would benefit the tilapia industry. In Chapter 6, a novel isothermal recombinase polymerase amplification assay (RPA) was developed and validated for rapid and accurate detection of *Fno*. Comparing the *Fno*-RPA with bacterial isolation, conventional PCR and the previously published *Fno*-specific qPCR (Duodu *et al.,* 2012), which considered to be the gold standard molecular test in diagnostic labs, showed that RPA can provide more benefits to detection of *Fno*. The *Fno*-RPA displayed a faster reaction time of 6 min to reach a detection limit of 15 molecules of a DNA plasmid standard, prepared using the *Fno*-unique hypothetical protein gene (JQ780323), in contrast to 90 min required by qPCR to achieve a detection limit of 11 molecules. Furthermore, the RPA showed high specificity, where it only detected the DNA of *Fno* isolates without cross-detection of DNA from the other fish, human and environmental pathogenic

Francisella spp. (*Fnn*; *Fp*) or other common fish bacterial pathogens used in this study. More importantly, the *Fno*-RPA demonstrated a greater performance than qPCR using field samples including tissue samples (spleen, head kidney) and water samples. The tolerance of *Fno*-RPA to reaction inhibitors was evident by 100 % clinical sensitivity and 84.89 % clinical specificity. This enabled detection of *Fno* in 47/78 spleen, 47/78 kidney and 5/5 water samples, in contrast to qPCR that failed to initially detect *Fno* in the crude DNA of 6 tissues samples and 5 water samples, but after dilution of the DNA to 1:10 and 1:100, it detected the bacteria in 2/6 and 3/5 of these samples. The robustness of *Fno*-RPA with field samples was in agreement with previous reports of RPA (Daher *et al.,* 2014; 2016; Bonney *et al.,* 2017). The cost effectiveness of RPA, convenience of performing the assay using very simple equipment without the requirement for skilled personnel, the ability to perform the reaction at a relatively wide range of temperatures (37- 42°C) along with the high diagnostic performance, make the RPA a highly recommended diagnostic assay for *Fno*, especially in a poorly equipped diagnostic laboratory. This will provide high throughput, sensitive, specific, affordable, fast, user friendly molecular detection of *Fno*, avoiding the disadvantages of other previously reported diagnostic tools (e.g. bacteriology; immunological assays (ELISA, IHC); nucleic acid-based assays (ISH; PCR; duplex-PCR; qPCR) (Soto *et al.,* 2009b, 2010a, 2012c; Duodu *et al.,* 2012; Assis *et al.,* 2016; Rodrigues *et al.,* 2016; Sebastião *et al.,* 2017; Dong *et al.,* 2016a; Ramirez-Paredes *et al.,*2017b). Future studies are required to test and optimise different protocols for DNA extraction and coupling the RPA with other quick detection assays (e.g. Lateral flow dip stick). This will contribute to decreasing costs and enhancing the performance of the RPA to be applied as a pen-side test for detection of *Fno* on fish farms.

7.2. Final conclusions

Piscine francisellosis is recognised as one of the most significant emergent diseases in farmed tilapia. The fastidious nature of *Fno*, the high infectivity, ability to survive in a wide range of environments and its world-wide distribution, highlight the importance of this aquatic pathogen, yet we know very little about it. *Fno* diversity, pathogenesis and mechanisms of interaction with its host, require further investigation. Prophylactic measures and accurate diagnostic tools are needed for controlling this important disease.

Observation of minor differences in the protein profile of geographically diverse *Fno* isolates, exhibiting a largely homogenous antigenic profile, provides a valuable

information about the diversity of *Fno* and supports the hypothesis of the genomic-based studies of existence of "clonal-like characteristics" between the different *Fno* isolates.

Identification of various virulence-related proteins in the OMP fraction of *Fno* reveals the importance of this simple protein fraction in the pathogenic life-style of *Fno* and its role in host-pathogenic interactions. Furthermore, identification of the immuno-reactive proteins within the *Fno* proteome will help in future development of a new generation of diagnostic and prophylactic tools (e.g. recombinant vaccines).

The whole cell *Fno* vaccine stimulated the expression of genes related to innate and adaptive immunity post-vaccination and provided promising protection not only against the homologous *Fno* isolate, but also against heterologous isolates in tilapia fingerlings. Significantly higher IgM levels and significantly lower bacterial burden following i.p. vaccination and challenge correlated with protection, suggesting a protective role of antibody-mediated immunity against *Fno* in tilapia. Taken together, the results from this study provide a benchmark to develop a broad-spectrum vaccine for protection against diverse *Fno* isolates in tilapia.

Immersion vaccination provided tilapia fry a relatively moderate level of protection (RPS of 43.7%). Therefore, further optimisation is required to enhance the efficacy of this vaccine (e.g. optimisation of delivery time, vaccine composition and using immersion adjuvants). Understanding molecular mechanism of protection induced by immersion vaccination against *Fno* in tilapia fry also requires further research.

Finally, a novel isothermal RPA was developed for cost-effective, fast, accurate, sensitive, specific and user-friendly diagnosis of *Fno*. The short reaction time, with high robustness in detection of *Fno* from clinical samples, make the RPA a suitable diagnostic assay for *Fno* with special consideration to be applied in poor-setting diagnostic labs and in field-diagnostics if accompanied with a proper extraction protocol.

In general, the current study provides an insight into our understanding of the diversity and virulence mechanisms of the pathogenic *Fno* isolates affecting fish. It also lays the groundwork for improving *Fno* vaccine design and aids in enabling surveillance strategies for future *Fno* outbreaks.

7.3. Future prospects

The results obtained in this thesis pave the way to additional research for future studies. This includes, but is not restricted to:

1- Comparing the expression of proteins of *Fno* grown under certain conditions that mimic the host environment such as stress conditions (i.e. iron restricted media) to normal culture conditions or *in vivo* versus *in vitro*.

2- Performing 2D-SDS-PAGE for the OMPs of *Fno* to have a better separation of the proteins that co-migrated in a single band in the 1D-SDS-PAGE.

3- Performing 1D and 2D-SDS-PAGE with more *Fno* isolates from other geographical locations.

4- Cloning of proteins with promising immunoreactivity and testing their efficacy as recombinant vaccines or coupling them with nanoparticles to formulate nano-based vaccines against *Fno* in tilapia.

5- Testing the performance of the developed injection vaccine under field conditions and investigation of the duration of immunity provided by this vaccine.

6- Optimising of dose, duration and suitable fish size for immersion vaccination of tilapia fry and more importantly, investigation of the age of immunocompetency of tilapia that is currently unknown.

7- Investigation of the role of cell mediated immunity in protection of tilapia against *Fno* following i.p and immersion vaccinations.

8- Testing different DNA extraction protocols and optimising rapid, cheap and effective methods to enable the use of *Fno*-RPA in the field and enhance *Fno* detection using recent tools such as the lateral flow dip stick. Other possibilities include set-up of two separate mobile suitcases, one for sample preparation and another for detection of *Fno*. This will enable a more robust field screening of tilapia for francisellosis.

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Appendix 1: Media, buffers and chemicals

Bacteriological media

Cystein heart agar media enriched with hemoglobine (1% v/v) (1 L)

Cystein heart agar	51 g
Bovine haenoglobine	10 g
Distilled water	up to 1L

Modified Muller Hinton broth with 0.1 % (w/v) glucose and 2% (v/v) IsoVitaleX (1L)

Modified Muller Hinton powder	22 g
Glucose	1 gb
IsoVitaleX	20 µL
Distilled water	up to 1L

Buffers used for proteomics experiments

Lysis buffer for extraction of whole cell lysate (stored at -20 ºC)
(7M Urea, 2M thiourea, 4% CHAPS, 40 mM DDT, 25 mL)

Urea (7 M)	10.5 g
Thiourea (2 M)	3.8 g
CHAPS (4% (w/v))	1 g
DDT (40 mM)	154 mg
Double-distilled water	Up to 25 mL
Nuclease mix (1% (v/v))	250 µL
Protease inhibitor (0.1 % (v/v))	25 µL

Thiourea rehydration solution (stored at -20 ºC)
(7M Urea, 2M thiourea, 2% CHAPS, 0.002% bromophenole blue, 2% IPG buffer, 25 mL)

Urea (7 M)	10.5 g
Thiourea (2 M)	3.8 g
CHAPS (2% (w/v))	0.5 g
IPG buffer pH 4-7 (0.5% (v/v))	125 µL
1% Bromophenole blue stock solution (0.002%)	50 µL
DDT	70 g
Double-distilled water	Up to 25 mL

Bromophenol blue stock solution (stored at room temeprature)

Bromophenol blue (1%)	100 mg
Tris-base (50 mM)	60 mg
Double-distilled water	Up to 10 mL

10X Lamelli SDS electrophoresis buffer (250mM Tris base, 1.92M glycine, 1% SDS, 10L)

Tris base (250 M)	303 g
Glycine (1.92 M)	3.8 g
SDS (1% (w/v))	100 g
Double-distilled water	Up to 10 mL

Equilibration buffer I (6M urea, 75mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, DDT 1% (w/v), 200 mL)

Urea (6 M)	72.1 g
Tris-HCl, pH 8.8 (75 mM)	10 mL
Glycerol (29.3%)	69 mL
SDS (2% (w/v))	4 g
1% Bromophenole blue stock solution (0.002%)	400 µL
DDT (1% (w/v)) *	2 g
Double-distilled water	Up to 200 mL

* add before equilibration

Equilibration buffer II (6M urea, 75mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, iodacetamide 2.5% (w/v), 200 mL)

Urea (6 M)	72.1 g
Tris-HCl, pH 8.8 (75 mM)	10 mL
Glycerol (29.3%)	69 mL
SDS (2% (w/v))	4 g
1% Bromophenole blue stock solution (0.002%)	400 µL
Iodacetamide (2.5% (w/v)) *	5 g
Double-distilled water	Up to 200 mL

* add before equilibration

Agarose sealing solution (25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue, 100 mL)

1X Lamelli SDS electrophoresis buffer	100 mL
Agarose	0.5 g
1% Bromophenole blue stock solution (0.002%)	200 µL

Coomassie blue stain fixing solution (1 L)

Methanol (40% (v/v))	400 mL
Glacial acetic acid (10% (v/v))	100 mL
Double-distilled water (50% (v/v))	500 mL

Silver stain fixing solution (1 L)

Absolute ethanol (50% (v/v))	500 mL
Glacial acetic acid (10% (v/v))	100 mL
Double-distilled water (40% (v/v))	400 mL

Ethanol wash solution (1 L)

Absolute ethanol (30% (v/v))	300 mL
Double-distilled water (70% (v/v))	700 mL

Sensitizer solution (100 mL)

Proteosilver sensitizer	1 mL
Double-distilled water	99 mL

Silver solution (100 mL)

Proteosilver solution	1 mL
Double-distilled water	99 mL

Silver stain developer solution (100 mL)

Proteosilver developer solution 1	5 mL
Proteosilver developer solution 2	100 µL
Double-distilled water	95 mL

Protein destaining solution (1:1 (v/v) Acetonitrile (ACN): 50 mM NH_4HCO_3)

Ammonium bicarbonate (NH_4HCO_3)	79.06 mg
Double-distilled water	20 mL
Acetonitrile (ACN)	20 mL

Protein dehydration solution (2:1 (v/v) Acetonitrile (ACN): 50 mM NH_4HCO_3)

Ammonium bicarbonate (NH_4HCO_3)	79.06 mg
Double-distilled water	20 mL
Acetonitrile (ACN)	40 mL

Protein reduction solution (10 mM DDT in 100 mM NH_4HCO_3)

DDT	7.7 mg
NH_4HCO_3 (50 mM (79.06 mg in 20 mL DW))	5 mL

Protein alkylation solution (55 mM iodacetamide in 50 mM NH_4HCO_3)

Iodacetamide	10.16 mg
NH_4HCO_3 (50 mM (79.06 mg in 20 mL DW))	1 mL

Protein digestion solution (Trypsin solution) (20 ng/ µL)

Trypsin lypholized powder	20 µg
HCl (1 mM)	100 µL
NH_4HCO_3 (50 mM (79.06 mg in 20 mL DW))	900 µL

Buffers used for ELISA

Coating buffer (1% (w/v) poly-lysine in carbonate bi-carbonate buffer)

Carbonate-bicarbonate buffer	
(1 capsule of carbonate-bicarbonate in 100 mL DW)	99 mL
Poly-lysine buffer	1 mL

Low salt wash buffer (LSWB), 10X, pH 7.3

Tris-Base (0.2 M)	24.2 g
NaCl (3.8 M)	222.2 g
Tween-20	5 mL
Distilled water	up to 1L

High salt wash buffer (HSWB), 10X, pH 7.7

Tris-Base (0.2 M)	24.2 g
NaCl (5M)	292.2 g
Tween-20	10 mL
Distilled water	up to 1L

Blocking buffer (5% w/v marvel in distilled water)

Marvel	5 g
Milli-Q water	100 mL

Phosphate Buffer Saline (PBS) 10x (1L), pH 7.4.

NaCl	80 g
Na_2HPO_4	14.4 g
NaH_2PO_4	2.4 g
Milli-Q water	up to 1L

Antibody buffer

LSWB	100 mL
BSA	1 g

Substrate buffer (pH 5.4) (Stored at -4 ºC)

Citric acid (0.1 M)	21 g
Na acitate (0.1 M)	2 g
Distilled water	Up to 1 L

Chromogen solution (Stored at -4 ºC)

3’3’5’5’-Tetramethylbenidine dihydrochloride (42 mM)	0.07896 g
Acetic acid: distelled water	6 mL

Chromogen in substrate buffer (Stored at -4 ºC)

Substrate buffer	15 mL
Chromogen solution	150 µL
H_2O_2	5 µL

Buffers used for Western blot

Tris-buffered saline (TBS) (pH 7.5)

Tris-base (0.02 M)	2.42 g
NaCl	29.24 g
Distilled water	Up to 1 L

Tris-buffered saline with Tween-20 (TBST)

Tris-base (0.02 M)	2.42 g
NaCl	29.24 g
Distilled water	Up to 1 L
Tween-20	1 mL

***Francisella* genus specific conventional PCR mixture (25 µL reaction)**

1X ReddyMix PCR master mix	12.5 µL
Forward Primer (0.2 µM)	1 µL
Reverse Primer (0.2 µM)	1 µL
DNA template (100 ng/ µL)	1 µL
Milli-Q water	9.5 µL

***Francisella noatunensis* subsp. *orientalis* qPCR mixture (20 µL reaction)**

1X Luminaris color HiGreen qPCR master mix	10 µL
Forward Primer (0.3 µM)	0.6 µL
Reverse Primer (0.3 µM)	0.6 µL
DNA template (100 ng/ µL)	1 µL
Milli-Q water	7.8 µL

***Francisella noatunensis* subsp. *orientalis* RPA mixture (50 µL reaction)**

1X Rehydration buffer	29.5 µL
Forward Primer (10 µM)	2.1 µL
Reverse Primer (10 µM)	2.1 µL
Probe (10 µM)	0.6 µL
DNA template (100 ng/ µL)	1 µL
Milli-Q water	10.7 µL
Mg acetate (14 mM)	4 µL

Appendix 2:

Table S1. Proteins identified in the OMPs of *Fno* UK isolate.

No.	Accession no.	Protein name	Mw[kDa]	pI	Scores	Peptides	SC [%]
1	gi|300193845|	PdpD	139.9	6.2	4477.2	72	61.4
2	gi|386872131|	Chaperone ClpB	96.0	5.4	2588.9	49	57.7
3	gi|169589436|	PdpD	139.6	6.1	2478.8	39	52.1
4	gi|300193842|	IglC	22.1	5.3	2388.4	14	84.2
5	gi|386871181|	Chaperonin GroEL	57.1	4.9	1857.1	33	63.7
6	gi|103012949|	Ribosomal L29e protein family	126.8	8.9	1839.5	42	46.6
7	gi|386870877|	OmpA family peptidoglycan-associated lipoprotein	23.4	4.8	1585.1	29	75.6
8	gi|386872079|	Bifunctional proline dehydrogenase/pyrroline-5-carboxylate	149.5	7.8	1539.6	33	34.6
9	gi|300193843|	IglB	57.5	4.7	1526.5	30	59.9
10	gi|386870689|	30S ribosomal protein S1	61.5	5.2	1389.2	26	58.1
11	gi|386870694|	Cell division protein FtsZ	39.3	4.6	1354.0	24	85.4
12	gi|386871696|	Outer membrane associated protein	41.3	5.2	1307.4	22	42.8
13	gi|300193831|	PdpA	94.9	8.9	1244.6	15	47.8
14	gi|386870866|	OmpA family protein (FopA)	47.2	6.0	1090.3	30	62.1
15	gi|169589422|	PdpB	126.5	9.2	1046.8	24	44.6
16	gi|386871950|	Ribonuclease E	101.4	8.3	1036.3	19	27.4
17	gi|300193844|	IglA	20.4	8.6	1010.3	32	57.2
18	gi|386871082|	Alpha-ketoglutarate decarboxylase	105.5	6.1	1009.2	21	27.9
19	gi|386870797|	Heat shock protein 90	72.2	5.3	992.4	21	36.9
20	gi|386871083|	2-oxoglutarate dehydrogenase complex, E2 component	52.5	5.0	991.1	18	45.6
21	gi|359325479|	Succinate dehydrogenase	65.7	5.9	978.2	18	40.4
22	gi|386871045|	NADH dehydrogenase subunit G	87.2	5.3	969.6	16	34.8
23	gi|564749340|	Hypothetical protein M973_05085	154.4	5.6	961.0	53	23.2
24	gi|386871702|	Putative cyanophycin synthetase	103.8	5.7	909.0	20	26.6
25	gi|386872051|	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	43.4	7.6	907.0	16	55.6

26	gi|386871615|	Enolase	49.4	4.8	890.7	18	56.5
27	gi|386871214|	Pyruvate dehydrogenase subunit E1	100.3	5.7	869.3	19	30.0
28	gi|386871625|	Peroxidase/catalase	82.1	5.7	859.1	17	36.8
29	gi|386870767|	Elongation factor G	77.7	4.8	851.8	16	35.7
30	gi|386871057|	Translation initiation factor IF-2	91.8	8.9	818.4	17	27.4
31	gi|386871419|	Serine-type D-Ala-D-Ala carboxypeptidase	48.2	9.0	802.8	15	46.8
32	gi|386871186|	Glutamate dehydrogenase	49.0	6.4	782.8	15	43.7
33	gi|386870911|	LemA-like protein	21.9	6.0	754.5	17	66
34	gi|386871126|	Elongation factor	43.3	5.0	735.9	36	46.9
35	gi|386870580|	Carbamoyl phosphate synthase large subunit	120.4	5.0	740.9	16	22.1
36	gi|860224409|	Non-ribosomal peptide synthetase	249.4	5.2	734.6	27	18.2
37	gi|386871071|	ATP synthase subunit beta	49.8	4.9	689.2	15	49.3
38	gi|386870999|	DnaK, molecular chaperone	69.0	4.8	669.8	11	28.7
39	gi|386871203|	tRNA adenyle transferase	35.2	6.8	665.2	11	41.9
40	gi|752588110|	Murein transglycosylase	77.2	9.0	661.9	14	28.5
41	gi|386870616|	Universal stress protein	30.1	5.4	645.3	10	48.2
42	gi|386871260|	DTDP-4-dehydrorhamnose 3,5-epimerase	83.4	9.0	637.2	11	20.1
43	gi|386871056|	Transcription elongation factor NusA	55.3	4.5	629.9	12	29.7
44	gi|386871375|	Beta-ketoacyl-ACP reductase	26.3	9.6	617.3	10	55.9
45	gi|386871595|	Uncharacterized protein OOM_1173	64.6	10.2	614.3	9	20.2
46	gi|855345043|	Pyruvate kinase	51.7	8.4	614.1	11	32.8
47	gi|386871457|	DsbA_Com1_like, DsbA family, Com1-like subfamily	40.4	4.7	608.2	17	46.6
48	gi|564748617|	Peptidyl-prolyl cis-trans isomerase DNA-directed	29.6	9.1	594.0	9	44.5
49	gi|386871134|	RNA polymerase, b’ta' subunit/160 kD subunit	157.2	6.2	590.9	12	13.1
50	gi|564749241|	Hypothetical protein M973_02230	15.1	7.7	576.0	8	47.1
51	gi|386870783|	30S ribosomal protein S8	14.4	9.2	563.2	9	61.4
52	gi|386871081|	Succinate dehydrogenase iron-sulfur subunit	26.5	8.8	555.8	12	54.9
53	gi|386870794|	DNA-directed RNA polymerase subunit alpha	35.3	4.9	550.2	11	49.2
54	gi|386871259|	Putative glucose-1-phosphate thymidyl transferase	100.5	5.3	537.2	13	22.1
55	gi|386870813|	Sel1-like protein	45.4	5.7	533.6	11	36.2
56	gi|386870781|	50S ribosomal protein L5	20.0	9.7	526.4	11	61.5

57	gi|386870724|	Cytochrome ubiquinol oxidase subunit I	63.7	6.0	519.4	8	13.7
58	gi|386870758|	Elongation factor Ts	30.9	5.2	495.8	9	41.5
59	gi|386871568|	Acetyl-CoA carboxylase, biotin carboxylase subunit	49.8	6.9	477.2	10	37.0
60	gi|386870942|	Hypothetical protein OOM_0413	15.7	9.2	471.3	8	48.3
61	gi|386871669|	Microtubule-severing ATPase	70.8	5.4	470.0	10	25.0
62	gi|386871478|	AhpC/TSA family peroxiredoxin	21.8	5.0	510.0	9	59.8
63	gi|386871972|	Enoyl-ACP reductase I	27.7	5.5	449.0	11	55.4
64	gi|386870822|	Putative lipoprotein	18.0	8.8	419.0	6	51.9
65	gi|386871594|	Succinyl-CoA synthetase subunit beta	41.5	5.2	398.0	7	28.9
66	gi|386870957|	Hypothetical protein OOM_0430	58.1	4.4	395.5	7	12.3
67	gi|386871251|	Hypothetical protein OOM-0776	22.5	9.8	388.6	9	41.3
68	gi|386871449|	Alanyl-tRNA synthetase	96.1	5.7	377.0	5	8.7
69	gi|300193838|	Hypothetical protein	24.3	5.6	376.8	5	33.5
70	gi|386870607|	Membrane protein of unknown function	62.1	7.7	374.3	6	13.6
71	gi|386871130|	50S ribosomal protein L1	24.5	9.5	374.1	9	39.4
72	gi|386871311|	Phosphomannomutase	54.5	5.2	372.7	7	15.6
73	gi|386870601|	Pyruvate phosphate dikinase	97.7	5.4	371.1	7	12.7
74	gi|386871607|	Polynucleotide phosphorylase/polyadenylase	75.4	5.8	364.3	9	20.4
75	gi|386871122|	Glycerol-3-phosphate dehydrogenase	58.0	8.6	362.3	7	16.3
76	gi|386871423|	RNA polymerase sigma-70 factor	67.4	5.3	361.4	9	22.2
77	gi|386870769|	50S ribosomal protein L3	22.1	9.5	359.4	6	40.5
78	gi|386871866|	Amino acid adenylation	396.7	5.9	357.6	10	4.6
79	gi|386871131|	50S ribosomal protein L10	18.7	9.1	351.8	10	55.2
80	gi|386871651|	Phosphoglyceromutase	57.6	5.6	349.9	5	12.1
81	gi|386871129|	50S ribosomal protein L11	15.4	9.8	347.8	7	46.2
82	gi|386871662|	Leucyl aminopeptidase	51.7	5.2	347.7	8	22.5
83	gi|752587925|	SAM-dependent methyltransferase	24.7	9.0	346.8	7	39.5
84	gi|386871735|	HflC protein	34.4	9.2	342.5	8	32.1
85	gi|386870644|	Riboflavin synthase	16.3	7.7	341.4	6	54.4
86	gi|386872152|	Putative ABC transporter ATP-binding protein	63.0	5.2	341.2	8	20.6
87	gi|386870642|	Hypothetical protein OOM_0085	35.1	6.7	340.2	5	22.4
88	gi|386870664|	Aspartyl-tRNA synthetase	66.7	6.0	339.4	7	16.3
89	gi|386870854|	Septum formation inhibitor-activating ATPase	30.0	7.6	338.8	8	44.7

90	gi|386870693|	Cell division protein FtsA	44.9	4.9	334.8	7	24.3
91	gi|386871293|	AMP-dependent synthetase/ligase	58.6	5.2	334.5	7	20.2
92	gi|386870757|	30S ribosomal protein S2	26.5	8.8	333.7	5	21.3
93	gi|752587918|	Transcription termination/antitermination factor NusG	20.3	6.8	333.1	7	40.8
94	gi|386872038|	Gamma-glutamyltransferase	62.2	8.1	333.1	6	18.9
95	gi|386871665|	Glucose-6-phosphate isomerase	60.6	5.7	331.3	8	22.2
96	gi|855345177|	Hypothetical protein OOM_1530	27.9	9.4	330.4	6	27.2
97	gi|564748853|	Phosphoglycerate kinase	41.9	5.2	324.3	6	31.1
98	gi|386870979|	Putative cystathionine beta-synthase	34.5	9.0	318.0	11	39.7
99	gi|386871715|	Hypothetical protein OOM_1308	18.6	9.4	316.2	6	40.7
100	gi|386871133|	DNA-directed RNA polymerase subunit beta	151.3	5.6	309.5	9	10.7
101	gi|386871154|	Pyrrolo-quinoline quinone repeat protein	55.7	5.6	309.2	8	24.7
102	gi|386871439|	50S ribosomal protein L9	16.0	5.1	303.6	5	47.7
103	gi|386870820|	Hypothetical protein OOM_0272	33.3	6.7	303.3	5	21.6
104	gi|386871446|	Transaldolase B	36.3	9.2	300.5	6	24.0
105	gi|564748023|	ABC transporter substrate-binding protein	34.1	5.5	300.2	6	37.3
106	gi|386870940|	Oligopeptidase A	76.6	5.4	286.7	3	6.7
107	gi|386870846|	VacJ like lipoprotein	40.0	4.3	286.4	5	20.2
108	gi|386870587|	Alpha/beta fold family hydrolase	32.6	5.3	279.7	7	36.6
109	gi|386871343|	Prolyl-tRNA synthetase	64.0	5.4	275.9	6	13.6
110	gi|564749152|	NADH dehydrogenase subunit D	47.4	6.1	274.1	6	19.4
111	gi|386871593|	Succinyl-CoA synthetase, alpha subunit	29.9	5.9	273.2	7	38.3
112	gi|386870594|	Chorismate mutase	20.3	9.2	267.1	5	40.6
113	gi|386870793|	30S ribosomal protein S4	23.2	10.4	265.0	4	24.8
114	gi|386871240|	DNA-binding response regulator	25.5	6.2	261.9	5	32.9
115	gi|195973818|	DNA-directed RNA polymerase subunit beta	145.2	5.5	258.0	6	6.7
116	gi|386872012|	Ribose-5-phosphate isomerase A	24.5	6.2	254.6	4	37.1
117	gi|386871889|	Chorismate binding family protein	120.0	5.6	253.9	26	31.3
118	gi|386871827|	DEAD-box subfamily ATP-dependent helicase	65.8	9.1	247.8	4	10.1
119	gi|386871215|	Dihydrolipoamide acetyltransferase	56.0	5.1	247.6	5	11.1

120	gi|386870623|	Hypothetical protein OOM_0066	25.3	5.6	246.1	7	34.9
121	gi|386870770|	50S ribosomal protein L4	22.5	10.0	242.5	5	31.9
122	gi|386872041|	GTP binding translational elongation factor Tu and G family protein	67.5	5.2	240.7	6	14.5
123	gi|386870647|	Pyrimidine reductase/pyrimidine deaminase	39.4	9.7	240.6	5	21.9
124	gi|386872101|	Malate dehydrogenase	34.0	6.1	238.6	6	27.3
125	gi|300193839|	Hypothetical protein	46.0	4.5	236.1	5	22.9
126	gi|386871026|	Adenylosuccinate lyase	49.3	6.0	235.9	5	11.1
127	gi|300193833|	Lipoprotein	14.3	6.2	232.5	4	40.0
128	gi|386871069|	ATP synthase subunit alpha	55.5	4.9	230.1	4	9.7
129	gi|386870956|	Malate dehydrogenase	67.0	5.5	227.8	6	18.7
130	gi|386871411|	GMP synthase	57.6	5.7	227.1	7	19.4
131	gi|386870640|	GTP-binding protein LepA	65.6	5.5	227.1	5	12.6
132	gi|504527492|	NADH-quinone oxidoreductase subunit C	25.3	6.5	226.1	5	34.9
133	gi|386871562|	Organic solvent tolerance protein, OstA	98.9	4.9	223.0	6	10.3
134	gi|386870766|	30S ribosomal protein S7	17.8	10.1	220.9	4	31.8
135	gi|386871067|	ATP synthase subunit B	17.4	7.7	215.2	4	23.1
136	gi|386871365|	Hypothetical protein OOM_0903	27.7	8.4	212.1	5	22.7
137	gi|386871865|	ATP binding protein	48.4	6.9	211.5	4	13.0
138	gi|386870786|	30S ribosomal protein S5	17.5	10.0	208.6	4	24.1
139	gi|752595689|	Trigger factor	49.6	5.0	207.3	6	19.9
140	gi|386870792|	30S ribosomal protein S11	13.7	11.5	202.7	6	62.8
141	gi|386871489|	UDP-N-acetylglucosamine pyrophosphorylase/glucosamine-1-phosphate	49.1	6.7	202.2	5	14.3
142	gi|386871068|	ATP synthase subunit delta	19.3	4.6	200.3	5	38.5
143	gi|386871786|	Acyl dehydratase	19.7	6.6	197.2	5	35.1
144	gi|386870784|	50S ribosomal protein L6	19.2	9.7	197.0	4	51.1
145	gi|386871937|	Phenylalanyl-tRNA synthetase subunit beta	87.8	5.0	194.9	4	7.1
146	gi|386871155|	Hypothetical protein OOM_0658	23.7	6.1	193.9	4	22.2
147	gi|386871627|	Glycerophosphoryl diester phosphodiesterase	38.7	5.3	191.0	5	21.1
148	gi|386870788|	50S ribosomal protein L15	15.2	10.4	189.6	3	27.3
149	gi|386871348|	Hypothetical protein OOM_0882	40.1	5.1	186.6	5	24.1

150	gi|564749116|	Succinate dehydrogenase	13.4	7.7	185.9	3	17.9
151	gi|386871908|	Phospho-2-dehydro-3-deoxyheptonate aldolase	41.0	6.2	185.1	4	14.1
152	gi|386870831|	Fructose 1,6-bisphosphatase II	34.6	4.9	182.2	4	12.5
153	gi|386871600|	Glutamine amidotransferase subunit PdxT	20.0	5.3	179.6	3	26.8
154	gi|386871020|	Protein-L-isoaspartate O-methyltransferase	23.6	5.6	179.2	3	23.3
155	gi|386871414|	ABC transporter, ATP-binding protein	27.1	8.5	175.7	4	28.4
156	gi|386871725|	ATP-dependent protease ATP-binding subunit ClpX	45.9	8.9	174.4	4	13.0
157	gi|386870775|	30S ribosomal protein S3	24.5	10.0	174.4	3	14.9
158	gi|386870861|	Fumarate hydratase, class I	54.8	5.1	172.1	4	14.1
159	gi|386872053|	M24 family peptidase	69.1	6.8	170.8	5	8.6
160	gi|386871801|	UTP-glucose-1-phosphate uridylyltransferase	31.9	6.2	169.4	4	18.1
161	gi|386871216|	Pyruvate dehydrogenase complex, E3 component, dihydrolipoamide	50.3	5.9	164.6	5	21.5
162	gi|386870687|	Membrane protein of unknown function	21.1	8.9	164.1	3	16.3
163	gi|386870772|	50S ribosomal protein L2	30.4	11.4	161.7	3	13.5
164	gi|386871673|	Preprotein translocase subunit SecA	103.3	5.3	159.8	5	8.2
165	gi|386871652|	D-lactate dehydrogenase (cytochrome)	114.3	9.6	157.6	4	6.3
166	gi|386871878|	Glutamate-1-semialdehyde-2,1-aminomutase	47.7	5.3	156.7	3	11.3
167	gi|386872072|	D-lactate dehydrogenase (cytochrome)	102.2	9.2	156.5	6	9.6
168	gi|386871268|	Hypothetical protein OOM_0777	43.1	5.5	156.2	4	16.6
169	gi|386870654|	Rhodanese-related sulfurtransferase	15.8	9.4	153.1	3	30.7
170	gi|386871000|	Heat shock protein DnaJ	41.5	7.9	151.0	3	10.5
171	gi|386871230|	Hypothetical protein OOM_0748	22.3	6.7	150.4	3	23.2
172	gi|386870703|	Hypothetical protein OOM_0151	20.2	6.6	149.7	3	18.9
173	gi|386870771|	50S ribosomal protein L23	11.1	9.8	146.0	3	40.4
174	gi|386872075|	Shikimate kinase I	19.7	5.4	145.7	3	18.8
175	gi|386871947|	D-3-phosphoglycerate dehydrogenase	45.0	6.0	145.2	5	16.1
176	gi|386871549|	Phosphoenolpyruvate carboxykinase	60.6	8.7	144.3	3	7.0
177	gi|386872099|	Bifunctional gluaredoxin/ribonucleoside-diphosphate reductase subunit	47.2	5.1	143.4	3	8.1
178	gi|386871490|	Glucosam—e--fructose-6-phosphate aminotransferase	67.5	5.7	142.6	5	12.1

179	gi|386870951|	Hypothetical protein OOM_0424	18.0	7.6	140.4	3	19.4
180	gi|386871408|	CBS domain pair protein	22.9	6.8	138.7	3	28.0
181	gi|386871933|	Lipoprotein	37.2	5.9	137.3	4	10.4
182	gi|386871680|	Polyamine transporter, ABC transporter	42.2	7.8	133.9	3	17.7
183	gi|386870652|	S49 family peptidase	34.2	8.6	131.8	3	17.9
184	gi|386872052|	Hypothetical protein OOM_1699	27.1	8.4	129.5	5	22.1
185	gi|386870779|	50S ribosomal protein L14	13.3	10.4	127.3	3	29.5
186	gi|386870645|	GTP cyclohydrolase II	44.5	6.5	126.9	3	8.9
187	gi|386870968|	Ubiquinone/menaquinone biosynthesis methyltransferase	28.1	9.5	126.8	2	11.6
188	gi|855345336|	aconitate hydratase	99.1	5.1	126.4	3	4.2
189	gi|386871238|	HAM1 protein	21.5	8.5	124.3	3	20.3
190	gi|386871225|	Outer membrane protein OmpH	18.5	9.0	122.6	3	28.9
191	gi|386871909|	Signal recognition particle protein	50.5	9.5	119.9	4	14.8
192	gi|386871569|	Hypothetical protein OOM_1143	24.8	5.1	119.8	3	20.5
193	gi|386871016|	Competence lipoprotein ComL	30.2	7.6	118.6	4	23.6
194	gi|504527146|	Membrane protein	55.7	5.4	118.6	3	11.9
195	gi|386871961|	Acid phosphatase	26.3	8.6	118.4	3	16.7
196	gi|386871612|	Hypothetical protein OOM_1191	20.6	8.6	117.3	3	18.7
197	gi|386871734|	HflK-HflC membrane protein complex, HflK	40.3	9.4	116.3	2	8.7
198	gi|386872049|	2-alkenal reductase	49.4	8.8	115.3	3	7.4
199	gi|752587895|	30S ribosomal protein S9	14.7	10.5	114.7	3	36.4
200	gi|564748965|	Hypothetical protein M973_08435	36.2	8.5	114.5	4	19.0
201	gi|386870994|	50S ribosomal protein L13	16.0	9.9	112.4	3	22.5
202	gi|386871084|	Adenine phosphoribosyltransferase	19.1	5.9	111.5	3	23.4
203	gi|639195041|	30S ribosomal protein S10	11.7	9.8	110.5	2	23.3
204	gi|386871775|	ATP-dependent protease peptidase subunit	19.8	5.6	110.0	2	19.7
205	gi|386871712|	Preprotein translocase family protein	12.8	9.6	107.3	2	22.0
206	gi|386871004|	Membrane fusion protein	38.4	9.3	107.3	2	9.8
207	gi|386871727|	DNA-binding protein HU-beta	9.4	9.8	107.2	2	17.8
208	gi|386870776|	50S ribosomal protein L16	15.7	11.5	107.1	2	16.8
209	gi|386870925|	Superoxide dismutase (Cu-Zn) precursor	20.2	5.2	106.8	3	38.4
210	gi|386870591|	Hypothetical protein OOM_0024	15.2	7.8	102.2	2	24.4

211	gi|386871516|	Phosphoglucomutase	59.4	5.3	102.0	3	10.1
212	gi|386870765|	30S ribosomal protein S12	13.7	12.2	101.2	3	18.5
213	gi|386872008|	50S ribosomal protein L20	13.3	11.0	100.2	3	8.5
208	gi|386870776|	50S ribosomal protein L16	15.7	11.5	107.1	2	16.8
214	gi|386871066|	ATP synthase subunit gamma	10.1	4.9	99.5	3	10.9
215	gi|386871976|	Arabinose-5-phosphate isomerase	34.4	5.8	98.3	2	3.8
216	gi|386871463|	Glutaredoxin like protein	9.7	8.9	97.8	2	31.3
217	gi|386871114|	Type IV pili, pilus assembly protein	48.6	4.6	97.8	2	8.3
218	gi|386872007|	50S ribosomal protein L35	7.3	11.8	96.4	3	46.2
219	gi|386870656|	Recombinase A	38.6	5.6	96.0	3	12.7
220	gi|564749085|	Peptide chain release factor 3	59.3	5.7	95.0	2	6.5
221	gi|386871466|	Chorismate mutase type II	10.6	7.1	94.2	3	48.9
222	gi|564748402|	50S ribosomal protein L21	11.7	10.0	94.0	2	26.9
223	gi|386871713|	Preprotein translocase subunit SecD	69.8	8.7	93.9	2	5.7
224	gi|386870870|	CDP-alcohol phosphatidyltransferase	30.2	7.6	93.7	2	3.7
225	gi|386871051|	NADH dehydrogenase (quinone)	57.8	8.4	93.6	3	4.3
226	gi|386870933|	Phosphoribosylaminoimidazole synthetase	37.9	5.0	90.5	3	15.3
227	gi|386871726|	Endopeptidase La	86.1	8.8	89.0	3	4.4
228	gi|386871258|	GDP-D-mannose dehydratase	41.7	6.1	85.7	2	10.6
229	gi|386871928|	Rare lipoprotein B family protein	20.3	9.6	85.2	2	16.4
230	gi|386870969|	Glucokinase	37.8	6.0	80.6	2	9.7
231	gi|386870782|	30S ribosomal protein S9	14.7	10.4	80.4	2	30.7
232	gi|386871763|	Ribose-phosphate diphosphokinase	35.0	5.8	73.3	2	8.1
233	gi|752588102|	Beta-ketoacyl-[acyl-carrier-protein] synthase II	44.0	5.9	72.8	2	7.4
234	gi|386870604|	Membrane protein	18.5	9	71.7	2	24.1
235	gi|386871142|	50S ribosomal protein L19	13.3	10.7	68.2	2	30.4
236	gi|386870855|	Septum formation inhibitor	24.7	6.3	65.3	2	18.0
237	gi|386870819|	Hypothetical protein OOM_0271	17.1	8.9	65.3	2	8.1
238	gi|198417057|	DnaA, partial	49.0	7.7	62.5	2	5.5
239	gi|386871047|	NADH dehydrogenase subunit I	18.8	6.8	60.4	2	16.0

Table S2. Proteins identified in the immunoreactive band (17-28 kDa) of the OMPs of the *Fno* UK isolate

No.	Accession	Protein name	MW [kDa]	pI	Scores	Peptides	SC [%]
1	gi|300193842|	IglC	22.1	5.3	2388.4	14	84.2
2	gi|504527828|	IglA	20.4	8.6	754.5	12	74.2
3	gi|504527329|	OmpA family peptidoglycan-associated lipoprotein	23.4	4.8	753.9	9	64.9
4	gi|504527815|	beta-ketoacyl-ACP reductase	26.3	9.6	601.2	10	55.9
5	gi|504527529|	succinate dehydrogenase iron-sulfur subunit	26.5	8.8	540.4	11	54.9
6	gi|504527238|	50S ribosomal protein L5	20.0	9.7	526.4	11	61.5
7	gi|504527915|	AhpC/TSA family peroxiredoxin	21.8	5.0	510.0	9	59.8
8	gi|504528404|	enoyl-ACP reductase	27.7	5.5	449	11	55.4
9	gi|386871251|	Hypothetical protein OOM-0776	22.5	9.8	388.6	9	41.3
10	gi|504527834|	hypothetical protein	24.3	5.6	376.8	5	33.5
11	gi|504527577|	50S ribosomal protein L1	24.5	9.5	374.1	9	39.4
12	gi|504527226|	50S ribosomal protein L3	22.1	9.5	359.4	6	40.5
13	gi|504527216|	30S ribosomal protein S2	26.5	8.8	333.7	5	21.3
14	gi|855345305|	Transcription termination/antitermination protein nusG	20.0	6.8	333.1	7	41.2
15	gi|855345177|	Hypothetical protein	27.9	9.4	330.4	6	27.2
16	gi|504527578|	50S ribosomal protein L10	18.7	9.1	274.5	8	45.3
17	gi|504527053|	Chorismiteate mutase	20.3	9.2	267.1	5	40.6
18	gi|504527683|	DNA-binding response regulator	25.5	6.2	261.9	5	32.9
19	gi|504527248|	30S ribosomal protein S4	23.2	10.4	245.0	4	24.8
20	gi|504527363|	LemA-like protein	21.9	6.0	235.4	6	37.2
21	gi|504527082|	hypothetical protein OOM_0066	25.3	5.6	226.3	6	31.2
22	gi|504527492|	NADH-quinone oxidoreductase subunit C	25.3	6.5	226.1	5	34.9
23	gi|504527227|	50S ribosomal protein L4	22.5	10.0	225.7	5	31.9
24	gi|504527224|	30S ribosomal protein S7	17.8	10.1	220.9	4	31.8
25	gi|504528150|	hypothetical protein	18.6	9.4	218.9	5	40.7
26	gi|504527806|	hypothetical protein OOM_0903	27.7	8.4	212.1	5	22.7

27	gi|504527242|	30S ribosomal protein S5	17.5	10.0	208.6	4	24.1
28	gi|504528221|	Acyl dehydratase	19.7	6.6	197.2	5	35.1
29	gi|504527240|	50S ribosomal protein L6	19.2	9.7	197.0	4	51.1
30	gi|504527599|	hypothetical protein OOM_0658	23.7	6.1	193.9	4	22.2
31	gi|504528036|	Glutamine amidotransferase subunit PdxT	20.0	5.3	179.6	3	26.8
32	gi|386871600|	protein-L-isoaspartate O-methyltransferase	23.6	5.6	179.2	3	23.3
33	gi|504527852|	Polyamine transporter, ABC transporter ATP-binding	27.1	8.5	175.7	4	28.4
34	gi|504527232|	30S ribosomal protein S3	24.5	10.0	174.4	3	14.9
35	gi|504527673|	Hypothetical protein OOM_0748	22.3	6.7	150.4	3	23.2
36	gi|504528505|	shikimate kinase I	19.7	5.4	145.7	3	18.8
37	gi|504527846|	CBC domain pair protein	22.9	6.8	138.7	3	28.0
38	gi|386872052|	hypothetical protein OOM_1699	27.1	8.4	129.5	5	22.1
39	gi|386870968|	Ubiquinone/menaquinone biosynthesis methyltransferase	28.1	9.5	126.8	2	11.6
40	gi|504527146|	hypothetical protein	21.1	8.9	118.6	2	12.4
41	gi|504528393|	acid phosphatase	26.3	8.6	118.4	3	16.7
42	gi|504528047|	hypothetical protein	20.6	8.6	117.3	3	18.7
43	gi|504527668|	membrane protein	18.5	9.0	71.7	2	20.5
44	gi|386870855|	septum formation inhibitor protein	24.7	6.3	65.3	2	18.0

Table S3. Bioinformatics analyses of the protein identified in the OMPs of the *Fno* UK isolate

No.	Protein	PSORTb[a]	COGs[b]	LipoP[c]	SignalP[d]
1	PdpD	Outer membrane	S	N	N
2	Chaperone ClpB	Cytoplasmic	O	N	N
3	PdpD	Outer membrane	S	N	N
4	IglC *	Unknown	S	N	N
5	Chaperonin GroEL	Cytoplasmic	O	N	N
6	Ribosomal L29e protein family	Outer membrane	S	N	N
7	OmpA family peptidoglycan-associated lipoprotein *	Outer membrane	M	Y	Y (SpII)
8	Bifunctional proline dehydrogenase	Cytoplasmic	C	N	N
9	IglB	Cytoplasmic	S	N	N
10	30S ribosomal protein S1	Cytoplasmic	J	N	N
11	Cell division protein FtsZ	Cytoplasmic	D	N	N
12	Outer membrane associated protein	Outer membrane	M	Y	Y (SpI)
13	PdpA	Unknown/multiple localization	S	N	N
14	OmpA family protein	Outer membrane	M	Y	Y (SpII)
15	PdpB	Outer membrane	M	N	N
16	Ribonuclease E	Cytoplasmic	E	N	N
17	IglA *	Cytoplasmic	S	N	N
18	Alpha-ketoglutarate decarboxylase	Cytoplasmic	G	N	N
19	Heat shock protein 90	Cytoplasmic	O	N	N
20	2-oxoglutarate dehydrogenase complex, E2 component	Cytoplasmic	C	N	N
21	Succinate dehydrogenase	Cytoplasmic membrane	C	N	N
22	NADH dehydrogenase subunit G	Cytoplasmic	C	N	N
23	Hypothetical protein M973_05085	Unknown	Q	N	N
24	Putative cyanophycin synthetase	Cytoplasmic	M	N	N
25	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	Cytoplasmic	I	N	N
26	Enolase	Cytoplasmic	G	N	N
27	Pyruvate dehydrogenase subunit E1	Cytoplasmic	C	N	N
28	Peroxidase/catalase	Unknown/multiple localization	P	Y	Y (SpI)
29	Elongation factor G	Cytoplasmic	J	N	N

30	Translation initiation factor IF-2	Cytoplasmic	J	N	N
31	Serine-type D-Ala-D-Ala carboxypeptidase	Unknown/multiple localization	M	Y	Y (SpI)
32	Glutamate dehydrogenase	Unknown/multiple localization	E	N	N
33	LemA-like protein *	Cytoplasmic	S	N	N
34	Elongation factor	Cytoplasmic	J	N	N
35	Carbamoyl phosphate synthase large subunit	Unknown/multiple localization	F	N	N
36	Non-ribosomal peptide synthetase	Cytoplasmic	I	N	N
37	ATP synthase subunit beta	Cytoplasmic/ multiple localization	C	N	N
38	DnaK, molecular chaperone	Cytoplasmic	O	N	N
39	tRNA adenyle transferase	Cytoplasmic	I	N	N
40	Murein transglycosylase	Periplasmic	M	Y	Y (SpI)
41	Universal stress protein	Cytoplasmic	T	N	N
42	DTDP-4-dehydrorhamNse 3,5-epimerase	Unknown	I	N	N
43	Transcription elongation factor NusA	Cytoplasmic	K	N	N
44	Beta-ketoacyl-ACP reductase *	Cytoplasmic	I	N	N
45	Uncharacterized protein OOM_1173	Cytoplasmic membrane	S	N	N
46	Pyruvate kinase	Cytoplasmic	G	N	N
47	DsbA_Com1_like, DsbA family, Com1-like subfamily	Unknown	O	Y	Y (SpII)
48	Peptidyl-prolyl cis-trans isomerase	Outer membrane	O	Y	Y (SpII)
49	DNA-directed RNA polymerase, b'ta' subunit/160 kD subunit	Cytoplasmic	K	N	N
50	Hypothetical protein M973_02230	Unknown	S	Y	Y (SpI)
51	30S ribosomal protein S8	Cytoplasmic	J	N	N
52	Succinate dehydrogenase iron-sulfur subunit *	Cytoplasmic Membrane	C	N	N
53	DNA-directed RNA polymerase subunit alpha	Cytoplasmic	K	N	N
54	Putative glucose-1-phosphate thymidyl transferase	Cytoplasmic	I	N	N
55	Sel1-like protein	Unknown	S	Y	Y (SpI)
56	50S ribosomal protein L5 *	Cytoplasmic	J	N	N
57	Cytochrome-D ubiquinol oxidase subunit I	Cytoplasmic Membrane	C	N	N
58	Elongation factor Ts	Cytoplasmic	J	N	N
59	Acetyl-CoA carboxylase, biotin carboxylase subunit	Cytoplasmic	I	N	N

60	Hypothetical protein OOM_0413 *	Outer membrane	S	Y	Y (SpII)
61	Microtubule-severing ATPase	Cytoplasmic	O	N	N
62	AhpC/TSA family peroxiredoxin *	Cytoplasmic	O	N	N
63	Enoyl-ACP reductase I *	Cytoplasmic membrane	I	N	N
64	Putative lipoprotein	Unknown	C	Y	Y (SpII)
65	Succinyl-CoA synthetase subunit beta	Cytoplasmic	C	N	N
66	Hypothetical protein OOM_0430	Unknown/multiple localization	S	Y	Y (SpI)
67	Hypothetical protein OOM_0776 *	Unknown/multiple localization	S	Y	Y (SpI)
68	Alanyl-tRNA synthetase	Cytoplasmic	J	N	N
69	Hypothetical protein *	Cytoplasmic	S	N	N
70	Membrane protein of Unknown function *	Cytoplasmic membrane	U	Y	Y (SpI)
71	50S ribosomal protein L1 *	Cytoplasmic	J	N	N
72	Phosphor-mannomutase	Cytoplasmic	G	N	N
73	Pyruvate phosphate dikinase	Cytoplasmic	G	N	N
74	Polynucleotide phosphorylase/polyadenylase	Cytoplasmic	J	N	N
75	Glycerol-3-phosphate dehydrogenase	Unknown/multiple localization	C	N	N
76	RNA polymerase sigma-70 factor	Cytoplasmic	K	N	N
77	50S ribosomal protein L3 *	Cytoplasmic	J	N	N
78	Amino acid adenylation	Unknown/multiple localization	Q	N	N
79	50S ribosomal protein L10 *	Cytoplasmic	J	N	N
80	Phosphoglyceromutase	Cytoplasmic	G	N	N
81	50S ribosomal protein L11	Cytoplasmic	J	N	N
82	Leucyl aminopeptidase	Cytoplasmic	E	N	N
83	SAM-dependent methyltransferase	Unknown	Q	N	N
84	HflC protein	Cytoplasmic	O	N	N
85	Riboflavin synthase	Cytoplasmic	H	N	N
86	Putative ABC transporter ATP-binding protein	Cytoplasmic	S	N	N
87	Hypothetical protein OOM_0085	Unknown/multiple localization	S	Y	Y (SpII)
88	Aspartyl-tRNA synthetase	Cytoplasmic	J	N	N
89	Septum formation inhibitor-activating ATPase	Cytoplasmic/multiple localization	D	N	N
90	Cell division protein FtsA	Cytoplasmic	D	N	N

91	AMP-dependent synthetase/ligase	Cytoplasmic	Q	N	N
92	30S ribosomal protein S2 *	Cytoplasmic	J	N	N
93	Transcription termination/anti termination factor NusG *	Cytoplasmic	K	N	N
94	Gamma-glutamyl transferase	Periplasmic	E	Y	Y (SpI)
95	Glucose-6-phosphate isomerase	Cytoplasmic	G	N	N
96	Hypothetical protein_1530 *	Outer membrane	S	Y	Y (SpI)
97	Phosphoglycerate kinase	Cytoplasmic	G	N	N
98	Putative cystathionine beta-synthase	Cytoplasmic	E	N	N
99	Hypothetical protein OOM_1308 *	Unknown	S	Y	Y (SpI)
100	DNA-directed RNA polymerase subunit beta	Cytoplasmic	K	N	
101	Pyrrolo-quinoline quinone repeat protein	Unknown/multiple localization	M	Y	Y (SpII)
102	50S ribosomal protein L9	Cytoplasmic	J	N	N
103	Hypothetical protein OOM_0272	Cytoplasmic	O	N	N
104	Transaldolase B	Cytoplasmic	G	N	N
105	ABC transporter substrate-binding protein	Unknown	P	Y	Y (SpI)
106	Oligopeptidase A	Cytoplasmic	E	N	N
107	VacJ like lipoprotein	Outer membrane	M	Y	Y (SpII)
108	Alpha/beta fold family hydrolase	Cytoplasmic	C	N	N
109	Prolyl-tRNA synthetase	Cytoplasmic	J	N	N
110	NADH dehydrogenase subunit D	Cytoplasmic/multiple localization	C	N	N
111	Succinyl-CoA synthetase, alpha subunit	Cytoplasmic	C	N	N
112	Chorismate mutase*	Unknown/multiple localization	E	Y	Y (SpI)
113	30S ribosomal protein S4 *	Cytoplasmic	J	N	N
114	DNA-binding response regulator *	Cytoplasmic	T	N	N
115	DNA-directed RNA polymerase subunit beta	Cytoplasmic	K	N	N
116	Ribose-5-phosphate isomerase A	Cytoplasmic	G	N	N

117	Chorismate binding family protein	Cytoplasmic	J	N	N
118	DEAD-box subfamily ATP-dependent helicase	Cytoplasmic	L	N	N
119	Dihydrolipoamide acetyltransferase	Cytoplasmic	C	N	N
120	Hypothetical protein OOM_0066	Cytoplasmic	P	N	N
121	50S ribosomal protein L4 *	Cytoplasmic	J	Y	Y (SpI)
122	GTP binding translational elongation factor Tu	Cytoplasmic membrane	J	N	N
123	Pyrimidine reductase/pyrimidine deaminase	Cytoplasmic	H	N	N
124	Malate dehydrogenase	Cytoplasmic	C	N	N
125	Hypothetical protein *	Cytoplasmic	S	N	N
126	Adenyl succinate lyase	Cytoplasmic	F	N	N
127	Lipoprotein	Unknown	S	Y	Y (SpII)
128	ATP synthase subunit alpha	Cytoplasmic	C	N	N
129	Malate dehydrogenase	Cytoplasmic	C	N	N
130	GMP synthase	Cytoplasmic	E	N	N
131	GTP-binding protein LepA	Cytoplasmic membrane	M	N	N
132	NADH-quinone oxidoreductase subunit C *	Cytoplasmic/multiple localization	C	N	N
133	Organic solvent tolerance protein, OstA	Outer membrane	M	Y	Y (SpI)
128	ATP synthase subunit alpha	Cytoplasmic	C	N	N
129	Malate dehydrogenase	Cytoplasmic	C	N	N
130	GMP synthase	Cytoplasmic	E	N	N
131	GTP-binding protein LepA	Cytoplasmic membrane	M	N	N
132	NADH-quinone oxidoreductase subunit C *	Cytoplasmic/multiple localization	C	N	N
133	Organic solvent tolerance protein, OstA	Outer membrane	M	Y	Y (SpI)
134	30S ribosomal protein S7 *	Cytoplasmic	J	N	N
135	ATP synthase subunit B	Cytoplasmic membrane	C	N	N
136	Hypothetical protein OOM_0903 *	Unknown	S	N	N
137	ATP binding protein	Cytoplasmic	S	N	N
138	30S ribosomal protein S5 *	Cytoplasmic	J	N	N

139	Trigger factor	Cytoplasmic	O	N	N
140	30S ribosomal protein S11	Cytoplasmic	J	N	N
141	UDP-N-acetylglucosamine pyrophosphorylase /glucosamine-1-phosphate	Cytoplasmic	M	N	N
142	ATP synthase subunit delta	Cytoplasmic	C	N	N
143	Acyl dehydratase *	Unknown	I	N	N
144	50S ribosomal protein L6 *	Cytoplasmic	J	Y	Y (SpI)
145	Phenylalanyl-tRNA synthetase subunit beta	Cytoplasmic	J	N	N
146	Hypothetical protein OOM_0658 *	Extracellular	S	N	N
147	Glycerophosphoryl diester phosphodiesterase	Periplasmic	C	Y	Y (SpI)
148	50S ribosomal protein L15	Cytoplasmic	J	N	N
149	Hypothetical protein OOM_0882	Unknown	S	N	N
150	Succinate dehydrogenase	Cytoplasmic membrane	C	N	N
151	Phospho-2-dehydro-3-deoxyheptonate aldolase	Cytoplasmic	E	N	N
152	Fructose 1,6-bisphosphatase II	Cytoplasmic	G	N	N
153	Glutamine amidotransferase subunit PdxT *	Unknown	E	N	N
154	Protein-L-isoaspartate O-methyltransferase *	Cytoplasmic	O	N	N
155	ABC transporter, ATP-binding protein *	Cytoplasmic/multiple localization	S	N	N
156	ATP-dependent protease ATP-binding subunit ClpX	Cytoplasmic	O	N	N
157	30S ribosomal protein S3 *	Cytoplasmic	O	N	N
158	Fumarate hydratase, class I	Cytoplasmic	C	N	N
159	M24 family peptidase	Cytoplasmic	E	N	N
160	UTP-glucose-1-phosphate uridylyltransferase	Cytoplasmic	M	N	N
161	Pyruvate dehydrogenase complex, E3 component, dihydrolipoamide	Cytoplasmic	C	N	N
162	Membrane protein of Unknown function *	Cytoplasmic membrane	S	N	N
163	50S ribosomal protein L2	Cytoplasmic	J	N	N

164	Preprotein translocase subunit SecA	Cytoplasmic/multiple localization	U	N	N
165	D-lactate dehydrogenase (cytochrome)	Cytoplasmic	C	N	N
166	Glutamate-1-semialdehyde-2,1-amiNmutase	Cytoplasmic	H	N	N
167	D-lactate dehydrogenase (cytochrome)	Cytoplasmic	C	N	N
168	Hypothetical protein OOM_0777	Cytoplasmic	M	N	N
169	Rhodanese-related sulfur transferase	Unknown	P	N	N
170	Heat shock protein DnaJ	Cytoplasmic	O	N	N
171	Hypothetical protein OOM_0748	Unknown	S	N	N
172	Hypothetical protein OOM_0151	Cytoplasmic membrane	L	N	N
173	50S ribosomal protein L23	Cytoplasmic	J	N	N
174	Shikimate kinase I *	Cytoplasmic	E	N	N
175	D-3-phosphoglycerate dehydrogenase	Cytoplasmic	E	N	N
176	Phosphoenolpyruvate carboxykinase	Cytoplasmic	C	N	N
177	Bifunctional gluaredoxin/ribonucleoside-diphosphate reductase subunit	Cytoplasmic	F	N	N
178	Glucosamine--fructose-6-phosphate aminotransferase	Cytoplasmic	M	N	N
179	Hypothetical protein OOM_0424 *	Unknown	S	Y	Y (SpII)
180	CBS domain pair protein *	Cytoplasmic	S	N	N
181	Lipoprotein	Unknown	S	Y	Y (SpII)
182	ABC transporter, ATP-binding protein	Cytoplasmic membrane	E	N	N
183	S49 family peptidase	Cytoplasmic membrane	O	N	N
184	hypothetical protein OOM_1699 *	Cytoplasmic	S	N	N
185	50S ribosomal protein L14	Cytoplasmic	J	N	N
186	GTP cyclohydrolase II	Cytoplasmic	H	N	N
187	Ubiquinone/menaquinone biosynthesis methyltransferase *	Cytoplasmic	H	N	N
188	Aconitate hydratase	Cytoplasmic	C	N	N
189	HAM1 protein	Cytoplasmic	F	N	N
190	Outer membrane protein OmpH	Periplasmic	M	Y	Y (SpI)

191	Signal recognition particle protein	Cytoplasmic membrane	U	N	N
192	Hypothetical protein OOM_1143 *	Unknown	S	N	N
193	Competence lipoprotein ComL	Outer membrane	M	N	N
194	Membrane protein	Outer membrane	M	N	N
195	Acid phosphatase *	Periplasmic	I	Y	Y (SpI)
196	Hypothetical protein	Unknown	S	Y	Y (SpI)
197	HflK-HflC membrane protein complex, HflK	Cytoplasmic	O	N	N
198	2-alkenal reductase	Cytoplasmic membrane	G	N	N
199	30S ribosomal protein S9	Cytoplasmic	J	N	N
200	Hypothetical protein M973_08435	Unknown	S	Y	Y (SpI)
201	50S ribosomal protein L13	Cytoplasmic	J	N	N
202	Adenine phosphoribosyltransferase	Cytoplasmic	F	N	N
203	30S ribosomal protein S10	Cytoplasmic	J	N	N
204	ATP-dependent protease peptidase subunit	Cytoplasmic	O	N	N
205	Preprotein translocase family protein	Cytoplasmic membrane	U	Y	Y (SpI)
206	Membrane fusion protein	Unknown/multiple localization	V	N	N
207	DNA-binding protein HU-beta	Cytoplasmic	L	N	N
208	50S ribosomal protein L16	Cytoplasmic	J	N	N
209	Superoxide dismutase (Cu-Zn) precursor	Periplasmic	P	Y	Y (SpII)
210	Hypothetical protein OOM_0024	Unknown	S	Y	Y (SpII)
211	Phosphoglucomutase	Unknown	G	N	N
212	30S ribosomal protein S12	Cytoplasmic	J	N	N
213	50S ribosomal protein L20	Cytoplasmic	J	N	N
214	ATP synthase subunit gamma	Cytoplasmic membrane	C	N	N
215	ArabiNse-5-phosphate isomerase	Cytoplasmic	M	N	N
216	Glutaredoxin like protein	Unknown	O	N	N
217	Type IV pili, pilus assembly protein	Unknown	S	Y	Y (SpI)

218	50S ribosomal protein L35	Cytoplasmic	J	N	N
219	Recombinase A	Cytoplasmic	L	N	N
220	Peptide chain release factor 3	Cytoplasmic	J	N	N
221	Chorismate mutase type II	Unknown	S	N	N
222	50S ribosomal protein L21	Cytoplasmic	J	N	N
223	Preprotein translocase subunit SecD	Cytoplasmic membrane	U	N	N
224	CDP-alcohol phosphatidyltransferase	Cytoplasmic membrane	I	N	N
225	NADH dehydrogenase (quinone)	Cytoplasmic membrane	C	N	N
226	PhosphoribosylamiNimidazole synthetase	Cytoplasmic	F	N	N
227	Endopeptidase La	Cytoplasmic	O	N	N
228	GDP-D-mannose dehydratase	Cytoplasmic	I	N	N
229	Rare lipoprotein B family protein	Unknown	M	Y	Y (SpII)
230	Glucokinase	Cytoplasmic	G	N	N
231	30S ribosomal protein S9 *	Cytoplasmic	J	N	N
232	Ribose-phosphate diphosphokinase	Cytoplasmic	F	N	N
233	Beta-ketoacyl-[acyl-carrier-protein] synthase II	Cytoplasmic	I	N	N
234	Membrane protein *	Extracellular	U	N	N
235	50S ribosomal protein L19 *	Cytoplasmic	J	N	N
236	septum formation inhibitor protein *	Cytoplasmic	D	N	N
237	Hypothetical protein OOM_0271	Unknown	S	Y	Y (SpII)
238	DnaA, partial	Cytoplasmic	L	N	N
239	NADH dehydrogenase subunit I	Cytoplasmic/multiple localization	C	N	N