Whole cell inactivated autogenous vaccine effectively protects red Nile tilapia (*Oreochromis niloticus*) against francisellosis via intraperitoneal injection

**Short running title:** *Francisella* vaccine for Nile tilapia

José Gustavo Ramírez-Paredes*∞*, Miguel Ángel Mendoza Roldán*∞*, Benjamin López-Jimena*∞*, Khalid Shahin*, Matthijs Metselaar*, Kim Thompson*, David James Penman*, Randolph Richards* and Alexandra Adams*

j.g.ramirezparedes@stir.ac.uk  miguelangeldoze@hotmail.com  benjamin.lopez-jimena@lifearc.org  k.e.shahin@stir.ac.uk  matthijs.metselaar@bmkanimalhealth.com  kim.thompson@moredun.ac.uk  d.j.penman@stir.ac.uk  r.h.richards@stir.ac.uk  alexandra.adams@stir.ac.uk

*∞*Equal contribution to the study.

*Corresponding author: José Gustavo Ramírez-Paredes, Aquatic Vaccine Unit, Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, FK9 4LA, Scotland, UK, jgr1@stir.ac.uk
Acknowledgements

JGRP was a Ph.D. candidate in Aquatic Veterinary Studies at the Institute of Aquaculture University of Stirling partially sponsored by the National Council for Science and Technology in Mexico (CONACyT) under the call “Convocatoria de Becas al Extranjero 2010 segundo periodo” Register number: 214096.

MAMR was an MSc student in Aquatic Veterinary Studies at the Institute of Aquaculture University of Stirling class 2014 partially sponsored by Fundación para el futuro de Colombia COLFUTURO.

Rearing, breeding, and maintenance of naïve, immunised and challenged Nile tilapia was carried out by Mr. Keith Ranson at the Tropical Aquarium and Mr. Niall Auchinachie at the Aquatic Research Facility, Institute of Aquaculture, University of Stirling.

The hypodermic needles to vaccinate the fish were kindly provided by Robert Wittmann and Phil Brown at Aqualife Services Ltd. www.aqualifeservices.com

The adjuvant used in this study was kindly provided by SEPPIC, Puteaux Cedex, France https://www.seppic.com

Abstract

Francisella noatunesis subsp. orientalis is a pathogen of tilapia and other warm water fish for which no vaccines are commercially available. In this study a whole cell formalin inactivated
vaccine was developed using the highly virulent isolate STIR-GUS-F2f7 and the oil-based adjuvant Montanide™ ISA 763A VG. The efficacy of the vaccine was assessed in red Nile tilapia via intraperitoneal (i.p.) injection using homologous experimental infection and correlates of protection such as seral antibody production and bacterial loads in the spleen. For immunisation, fish were i.p. injected with 0.1mL of the vaccine, the adjuvant alone or PBS. At 840 degree days post vaccination all fish were i.p. injected with 4.0x10³ CFU/fish of pathogenic bacteria. The RPS at the end of the trial was 100% in the vaccinated group with significantly higher survival than in the adjuvant and control groups. The RPS in the adjuvant group was 42%, and no significant difference was seen in survival between this and the PBS group. Moreover, significantly higher antibody titres in the serum and significantly lower bacterial loads in the spleen were detected in the vaccinated fish by ELISA and qPCR, respectively. These findings highlight the potential of autogenous vaccines for controlling francisellosis in tilapia.

**Keywords:** tilapia vaccines; *Francisella* vaccines; *Francisella noatunensis* subsp. *orientalis*; francisellosis in tilapia; autogenous vaccines; tilapia diseases.
1 Introduction

*Francisella noatunensis* subsp. *orientalis* is a facultative intracellular pathogen responsible of francisellosis in several warm water fish species (Colquhoun & Duodu, 2011). In farmed Nile tilapia (*Oreochromis niloticus* L.), outbreaks caused by *Francisella noatunensis* subsp. *orientalis* usually occur in intensive culture systems with high stocking densities when water temperature drops below 25°C (Ramirez-Paredes et al., 2017b; Soto et al., 2009; Soto et al., 2012; Soto et al., 2018). Acute episodes of francisellosis in tilapia are commonly triggered by husbandry handling procedures and result in high levels of morbidity (80-90%) and mortality (50-90%) that can affect fish at all production stages (Birkbeck et al., 2011; Colquhoun and Duodu, 2011).

*Francisella noatunensis* subsp. *orientalis* is one of the most pathogenic bacteria for tilapia (Klinger-Bowen et al., 2016; Pulpipat et al., 2019; Ramirez-Paredes 2017b; Soto et al., 2009) and one of the main challenges that this industry currently faces globally. Nevertheless, despite the relevance of this pathogen, there are still no commercial vaccines available for its prevention and control.

Soto et al. (2011) developed the first experimental vaccine for tilapia against *Francisella noatunensis* subsp. *orientalis*. This was a live attenuated strain, generated with an insertional mutation in the intracellular growth locus C gene (*iglC*) of strain recovered in Costa Rica which conferred a relative percentage survival (RPS) of 87.5% after experimental infection by immersion. However, due to the inherent risks of using live genetically modified microorganism (virulence reversion, dissemination into the environment and potential transmission to other species), most of the countries where tilapia is farmed do not grant licences to live genetically modified vaccines. Therefore although such vaccine was shown to
be reasonably efficacious in that and further trails (Soto et al., 2014), it has not being licensed and is not commercially available in any country.

In the present study, a whole cell inactivated autogenous vaccine against *Francisella noatunensis* subsp. *orientalis* was developed in 2014 for the first time. The vaccine was formulated using the commercial oil adjuvant Montanide™ ISA 763A VG and a highly virulent isolate (STIR-GUS-F2f7) recovered in 2012 from diseased red tilapia fingerlings in the United Kingdom (Ramirez-Paredes et al., 2017b). The efficacy of the vaccine was assessed by measuring survival rates after experimentally infecting vaccinated fish by intraperitoneal injection and its potency expressed in terms of RPS. Additionally, relevant correlates of protection including specific antibody (IgM) kinetics and quantification of bacterial loads in the spleen by qPCR were assessed.

**2 Materials and Methods**

**2.1 Bacterial strain and experimental fish**

*Francisella noatunensis* subsp. *orientalis* (STIR-GUS-F2f7) was isolated in November 2012 from a moribund red Nile tilapia, farmed in UK (Ramirez-Paredes et al., 2017b). The strain had been stored at -80 °C in Modified Mueller-Hinton II cation-adjusted broth supplemented with 2% IsoVitaleX (MMHB) and 20% sterile glycerol. The strain was cultured in cystine heart agar with 2% bovine haemoglobin (CHAH) and MMHB following culture conditions indicated by Ramirez-Paredes et al. (2017b).

Healthy naïve red Nile tilapia, 6-7 months/ ~11g (7-13 g), were obtained from the Tropical Aquarium (TA) at the Institute of Aquaculture, University of Stirling, Scotland UK. Fish in these facilities are maintained in recirculation systems with water at 28 °C +/- 2 °C, and
confirmed to be free of francisellosis by bacteriological and molecular methods prior to the study as outlined by Ramirez-Paredes et al. (2017b).

### 2.2 Vaccine preparation and stability

For vaccine preparation, an overnight culture (~18 h) was prepared in 15 mL of broth using 5x 50 mL centrifuge tubes. The next day, the culture was centrifuged at 3500 x g for 20 min, the bacterial pellet washed 3 times with sterile 1x phosphate buffered saline (PBS) and adjusted, with PBS, to an OD$_{600}$ of 1.0 ($2.6 \times 10^9$ CFU/mL).

For bacterial inactivation, the suspension was left mixing overnight at 4 °C in a sterile glass vessel, slow stirring at 100 rpm with 0.5% formalin (Formaldehyde 40% w/v) (Sigma-Aldrich, Dorset, UK). The formaldehyde was neutralised using 1/100 dilution of 15% sodium metabisulphite. This solution was then centrifuged, washed 3 times with PBS and readjusted to an OD$_{600}$ of 1.0. Inactivation of the bacteria and sterility was confirmed by inoculating a subsample of the suspension onto CHA and incubating at 28 °C for 7 days.

For emulsification, the formalin killed bacterial suspension was mixed with a commercial oil adjuvant Montanide™ ISA 763A VG (SEPPIC, Puteaux Cedex, France), following the manufacturer’s guidelines for water-in-oil (W/O) emulsion in a 30/70 distribution of the continuous and dispersed phases (30% antigen to 70% adjuvant). Briefly, the emulsion was homogenised for 5 min with a hand blender as follows: 4000 rpm for the first 3 min, 4500 rpm for 30 s, 4000 rpm for 1 min and 4500 rpm for 30 s. The initial concentration of the inactivated bacteria ($2.6 \times 10^9$ CFU/mL) was reduced with the emulsification to obtain a final concentration in the vaccine of $1.3 \times 10^9$ CFU/mL, to provide a dose of $1.3 \times 10^8$ CFU/fish when 0.1 mL of the vaccine was administered a normal dose.
The final volumes to produce the vaccine were 100 mL of bacteria in PBS, 500 µL of 40% formalin and 1 mL of 15% sodium metabisulphite. The total volume of formalin-killed bacteria obtained was 60 mL of which 33 mL were added to 77 mL of adjuvant to obtain a final vaccine volume of 110 mL (Supplementary File 1).

As indicated by the adjuvant manufacturer and Aucouturier et al. (2001), the vaccine emulsion was stored at 4 °C and its stability checked after 1, 15 and 60 days post preparation by performing a visual inspection of the emulsion. A droplet test was also performed to confirm that a water-in-oil emulsification had been successfully prepared by dropping 20 µL of the emulsion into a beaker containing bi-distilled water and confirming the droplet retained its shape. The conductivity of the emulsion was tested using an electrical conductivity meter (Cole-Parmer, England, UK) in a 50 mL tube at 4 °C. The droplet size was observed under light microscope at 400X by placing a drop of vaccine on a slide glass with a cover slip without crushing the preparation. Finally, the syringeability in and out of fish was measured using a 1 mL disposable insulin syringe with an integrated 30G x 8 mm needle and with stainless steel removable needles of different gauges i.e. 22G x 7 mm and 21G x 8 mm (Aqualife Services Ltd., Stirling, UK).

2.3 Vaccine safety test

To rule out possible side effects during the trial, thirty fish were intraperitoneally (i.p.) injected with twice the normal dose, i.e. 0.2 mL of the vaccine and kept at 28 °C in the TA for 30 days. Fish were anaesthetised with a dose of 100 mg/L of Tricaine Pharmaq 1000 mg/g (TPQ) (Pharmaq, Hampshire, UK) prior to the injection. After injection, the fish were checked twice a day during the first week examining them for acute side effects, such as changes in behaviour, lesions around the injection zone, sudden mortalities due to toxicity or other signs that could be related to the formulation of the vaccine. Fish were euthanized after ~840 degree days (DD)
(30 days at 28 °C) and necropsied to examine them for signs of chronic side effects such as internal lesions or adhesions.

2.4 Vaccination

A total of 468 red Nile tilapia were used in this experiment. The fish were equally distributed into 9 experimental tanks i.e. 3 treatment groups with 3 replicate tanks per group and 52 fish per tank. The treatments groups included (1) vaccinated fish, (2) fish injected with 1x sterile PBS and (3) fish injected with adjuvant only i.e. emulsified with 1x PBS (Table 1). The vaccinated and control groups were used to investigate the efficacy of the vaccine, while the adjuvant-only group was included to investigate possible immunostimulatory effects of the Montanide™ ISA 763A VG in the tilapia and if such stimuli could result in protection.

All the fish were i.p. injected with 0.1 mL of their respective treatment i.e. vaccine, adjuvant or PBS using stainless hypodermic needles 22G x 7 mm and 21G x 8 mm (Aqualife Services Ltd., Stirling, UK). Prior to injection, fish were anaesthetised with a dose of 100 mg/L of TPQ. All fish were maintained in the TA at 28 °C for 30 days (~840 DD) for development of immunity. During this period, fish were fed twice at a daily rate of 2% biomass and sampled at four time points during the trial for serology. Twenty fish per replicate tank (60 per treatment) were used for the experimental challenge to evaluate vaccine efficacy.

2.5 Experimental infections

A pre-challenge was performed to confirm the dose that resulted in 60% mortality (LD₆₀) in a group of naïve fish, housed in similar conditions to those in the vaccine study. This was carried out by injecting 20 fish with 2.4 x 10⁴ CFU/fish in a 21 days trial. The dose was selected based on previous studies (Ramirez-Paredes 2015; Soto et al., 2009). For the main challenge experiment 20 fish in replicate tanks (n=60 fish per treatment) were infected with a single dose
of $4.0 \times 10^3$ CFU/fish live bacteria at 31 days post vaccination (d.p.v.) (Table 1 and Supplementary File 2).

The pre-challenge and main challenge were performed following the methodology previously described (Ramirez-Paredes et al., 2017b). Briefly, fish were moved from the TA into a flow-through system at the Aquatic Research Facility (ARF), where the water was maintained at 23 ± 2 °C to replicate the natural environmental conditions at which the disease occurs.

Prior to performing the challenges, fish were acclimated for 10 days and fed twice daily at a rate of 2% biomass. For the infection process, fish were anaesthetised with a dose of 100 mg/L of TPQ and i.p. injected with the dose of bacteria as stated above. During the course of the infection the numbers of mortalities, as opposed to moribund and near-moribund fish, were kept to a minimum with continuous observations. The following criteria were considered as humane endpoints of infection, based on whether the fish were moribund or near moribund and the clinical signs they presented with one of the following severe signs: poor or no response to stimuli (slow or unable to swim off when touched with net), bilateral exophthalmia, total loss of equilibrium or total loss of buoyancy, or three of the following signs: unilateral exophthalmia, emaciation, hypo/hyperventilation, oedema, irregular swimming or tank placement, partial loss of balance, marked darkening of skin, lesions, haemorrhaging or natural concurrent infection.

Diseased fish classed as moribund or near moribund (humane endpoint) were euthanised by Schedule 1 method (S1-M) i.e. with an overdose of TPQ followed by confirmation of death by brain destruction immediately after detection. All the euthanised fish were recorded throughout the experiments and accounted for posterior mortality and survival statistical analyses.

To confirm specific mortalities, all moribund fish were necropsied, checked for gross pathology and sampled for bacteriology and histopathology as previously described (Ramirez-
Paredes et al., 2017b). The experiment was concluded when there was a period of at least five days with no mortalities. At the end of the trial all surviving fish were euthanized by S1-M and blood sampled to measure specific antibody levels in the serum by ELISA. Additionally the spleen of all survivors was collected and preserved in 95% ethanol for later bacterial quantification in the spleen by qPCR.

2.6 ELISA development for specific IgM detection in tilapia serum

An indirect enzyme linked immunosorbent assay (ELISA) was developed to measure the level of specific anti *Francisella noatunensis* subsp. *orientalis* IgM in the serum of fish. This was used to monitor the antibody kinetics throughout the immunisation period and at the end of the challenge in the survivors. For this, eight fish per replicate tank were euthanised by S1-M and bled from the caudal vein at 4, 9, 15 and 30 d.p.v. Additionally, all surviving fish were euthanised and sampled at 40 days post challenge (d.p.c.) (Table 1). Blood samples were stored overnight at 4 °C and centrifuged at 3000×g for 5 min for serum collection which was subsequently stored at -20 °C until analysis.

The ELISA was performed according to Adams *et al.*, (1995) with modifications. The 96-well ELISA plates (Immune ⁴ HBX-USA) were coated with 100 µL of 1% w/v poly-L-lysine in carbonate–bicarbonate buffer and incubated for 60 min at room temperature (~21 °C). Plates were then washed three times with a low salt wash buffer (LSWB) (0.02 mol/L Trizma base, 0.38 mol/L NaCl, 0.05% (v/v) Tween 20, pH 7.2). Plates were coated with a bacterial suspension of isolate STIR-GUS-F2f7 re-suspended to an OD₆₀₀ of 0.4 (~1.0 x 10⁹ CFU/mL) with PBS. This was then added to each well (100 µL/well) and the plates were incubated overnight at 4 °C. The following day 50 µL of a 0.05% v/v solution of glutaraldehyde in LSWB was added to the plates and these were incubated at 21 °C for 20 min to fix the bacteria to the plate before washing the ELISA plates three times with LSWB.
Non-specific binding was prevented by first adding 100 µL/well of 1/10 dilution of a 30% stock solution of hydrogen peroxide (Sigma-Aldrich, Dorset, UK) and incubating for 1h at room temperature. The plates were washed three times as before and further blocking was performed by incubating them after adding 250 µL/well of 5% w/v marvel (Premier Foods Group Ltd, England UK) in distilled water for 3 h at room temperature. Thereafter the plates were washed three times with 1x LSWB, and 100 µL/well of serum from 5 fish from the 3 different vaccinated groups (4, 9 and 30 dpv) and challenge survivors were added to the plates at a 1/500 dilution in LSWB containing 1 % bovine serum albumin (BSA, Fisher scientific). Both positive (vaccinated and challenged fish serum) and negative (naïve fish serum) were used in each plate using the same dilution as tested sera. The plates were then incubated overnight at 4 °C and after the incubation, plates were washed five times with high salt wash buffer (HSWB) (0.02 mol/L Trizma base, 0.5 mol/L NaCl, 0.01% (v/v) Tween 20, pH 7.4) and left to soak for 5 min on the last wash. An anti-tilapia IgM monoclonal antibody (Aquatic Diagnostics Ltd, Stirling, UK) was added (100 µL/well) and the plates were incubated at room temperature for 60 min. The plates were again washed using 1x HSWB before adding 100 µL/well of goat anti-mouse immunoglobulin-G, conjugated with horseradish peroxidase (Sigma-Aldrich, Dorset, UK) diluted 1/3000 in LSWB with 1% BSA. Plates were incubated for 60 min at room temperature and then washed again with HSWB as previously described. Substrate/chromogen (15 mL of substrate buffer [5.25 g citric acid, 2.05 g of sodium acetate, distilled water up to 15 mL, pH 5.4] containing 5 µL of hydrogen peroxide (Fisher) and 150 µL of trimethyl-benzidine (TMB) di-hydrochloride (Sigma-Aldrich, Dorset, UK) was added to the plates, which were incubated for 5 min at room temperature. The reaction was terminated with the addition of 5 µL/well of 2M H₂SO₄ and the absorbance measured at OD₄₅₀ using a 96-well plate spectrophotometer (Biotek Instruments, Friedrichshall, Germany).

2.7 Determination of bacterial load by quantitative PCR (qPCR)
The bacterial load in the spleen of surviving fish was determined at the end of the challenge experiment using a qPCR protocol previously described by Duodu et al. (2012). For this, 10 of the spleens previously preserved in 95% ethanol were randomly selected from each treatment for genomic DNA extraction. The DNA was extracted from 20 mg of the fixed spleen samples using the DNeasy blood and tissue kit (Qiagen, UK). The concentration of the extracted DNA was measured using a nanodrop ND-1000 Spectrophotometer (Thermo Scientific, UK) and standardised to 100 ng/µL with Milli-Q water (Thermo Scientific, UK). One µL of each DNA sample was visualised in a UV illuminator (Bio Imaging, Syngene) after electrophoresis on a 1% (w/v) agarose gel (Sigma-Aldrich, Dorset, UK) containing 0.1 µg/mL ethidium bromide (Sigma-Aldrich, Dorset, UK) in Tris-EDTA (TAE) buffer. The assay was performed in a LightCycler® 2.0 (ROCHE) using a 20 µL reaction volume consisting of 0.3 µM from each primer (Eurofins Genomics, UK), 1x Luminaris color HiGreen™ qPCR master mix (ThermoScientific), 1 µL DNA template and nuclease free water (ThermoScientific) up to 20 µL. The PCR cycling conditions were 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 was performed with 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 quantification cycles (Cq values).

2.8 Statistical analysis

The vaccine efficacy was estimated by calculating the relative percent survival (RPS). This value indicates the proportional relationship between mortality in the vaccinated group and the unvaccinated group according to Amend (1981) using the following equation:
\[ RPS = \left[ 1 - \left( \frac{\text{\% mortality in vaccinated fish}}{\text{\% mortality in non-vaccinated fish}} \right) \right] \times 100\% \]

The RPS of the adjuvant-only group was also calculated as a comparison. Differences in survival were determined using the product limit method of Kaplan and Meier and the Log-rank (Mantel–Cox) test was used to compare survival curves. The specific antibody levels in the 3 different treatments, at the different time points were analysed by one-way ANOVA followed by Welch's test. The bacterial loads quantified by qPCR were also analysed with a one way ANOVA, paired comparisons between vaccinated and unvaccinated fish were analysed using a Tukey's test. In all cases, a p-value of < 0.05 was considered significant. All statistical analyses were carried out using the GraphPad Prism 8.02 software package (GraphPad Software Inc., San Diego, CA, USA).

2.9 Ethics

The vaccination, infection and associated procedures were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and the University of Stirling Animal Welfare and Ethical Review Body (AWERB) regulations. All the protocols were approved by the University of Stirling AWERB.

3 Results

3.1 Vaccine stability

An appropriate water-in-oil emulsion was observed in the drop test, with a conductivity of 30 \( \mu \text{s/cm} \). The microscopic particles observed were 1 \( \mu \text{m} \) in size and were homogenously distributed in the continuous and dispersed phase of the mixture, showing a dense liquid compatible with the 70/30 emulsion. No separation of the liquid phases was observed when examined 24 h after emulsification, indicating that the vaccine was suitably stable for injection into the fish. The results of these tests when performed at 15, 30 and 60 days post-preparation
confirmed that the emulsion remained stable during this time. During the syringeability test, the needles in the insulin syringes were not successful administering the vaccine as due to the viscosity of the final product the process was slow, complicated and some of the syringes broke while injecting the fish. For this reason hypodermic needles 22G x 7 mm and 21G x 8mm were used to vaccinate the fish (Supplementary File 3).

3.2 Vaccine safety

The 30 fish injected with the double dose of vaccine remained healthy, with no acute side effects evident, such as changes in behaviour i.e. lethargy, lack of feeding, aggression, gaping, flashing etc. No adhesions between mesentery and the internal wall of the peritoneal cavity or between sections of the intestines or the intestines and other organs were seen and therefore the fish were classified as “0” according to the Speilberg scoring system (Midtlyng et al., 1996). All fish had a droplet of the vaccine in the peritoneal cavity, always located dorsal, caudal and lateral to the right (below the swim-bladder, posterior section of the peritoneal cavity). Smaller vaccines droplets could also be observed distributed randomly within the peritoneal cavity in about 30% of the fish. The droplets were contained in a smooth soft transparent sac with vascularisation. Although 12 fish (40%) had dark pigments within the peritoneal cavity and mesenteric fat, these were not linked to the vaccine as peritoneal pigmentation is a common feature of tilapia. As expected, during the immunisation period (~840 DD) all fish vaccinated with the normal dose remained healthy and no mortalities or signs of acute toxicity or chronic side effects were seen.

3.3 Bacterial infection

Administration of $2.4 \times 10^4$ CFU/fish produced a cumulative mortality of 90% by 21 d.p.c. in the pre-challenge test. On the basis of this result, it was decided to challenge the vaccinated
fish with a log10 lower dose to test the efficacy of the vaccine, with the actual dose used
determined to be 4.0 x 10^3 CFU/fish based on colony counts.

When moribund fish were dissected, granulomas were observed in their spleens and kidneys,
with varying degrees of severity (Figure 1). Some spleens were larger than normal, with dark
red coloration and white nodules in 80-90% of the parenchyma, others were bright red with 90-
100% granulomas and some others showed splenomegaly with large nodules and a white
membranous lining of the capsule of the spleen, which extended over the majority of the
peritoneal cavity. Head kidneys appeared enlarged, haemorrhagic and full of granulomas, and
in some cases the organ protruding ventrally towards the anterior section of the peritoneal
cavity, in contact with the spleen, liver, and gut sections. Other organs such as the gonads, gut
and posterior kidney were also affected. Bacterial recovery was achieved from 100% of the
moribund fish sampled.

Histopathological sections of the affected organs showed typical granuloma lesions. The
bacteria were seen frequently contained within enlarged macrophages or found covered with
fibrin and enclosed by accumulation of phagocytic cells, that outlined by inflammatory cells
and fibroblasts. Concomitant mononuclear infiltration and increased vascularisation were also
noted around the in the affected areas (Figure 2).

### 3.4 Vaccine efficacy

A significant level of protection against the bacterial challenge was obtained for the vaccinated
group compared to the control and adjuvant groups (p<0.0001). (Figure 3). However, no
significant difference was seen between adjuvant and control treatment (Table 2). The
mortalities in the control group started by 8 d.p.c. and reached an average of 63.3 ± 2.9% by
34 d.p.c. The mortalities in the adjuvant group started at 13 d.p.c. and reached an average of
36.6 ± 18.9% by 32 d.p.c. All the fish in the vaccinated group survived until the end of the
experiment by 40 d.p.c. The RPS value at the end of the experiment was 100% for the vaccinated group and 42% for the adjuvant group. The mortality data is presented in Supplementary File 4.

3.5 Kinetics of specific IgM response of vaccinated fish

The results of the ELISA confirmed that there was no significant difference in the levels of specific anti *Francisella noatunensis* subsp. *orientalis* IgM between vaccinated, adjuvant and control groups in the first 15 d.p.v. However, the vaccinated fish started to produce significantly higher levels of specific IgM by 30 d.p.v. (p<0.001) compared to the other two groups (Figure 4). No specific antibody response was seen in serum sampled from the adjuvant or control groups prior to challenge. All the survivors showed increased antibody production after the infection with the vaccinated fish displaying the highest titres (Figure 4).

3.6 Quantitative PCR (qPCR) for estimation of bacteria load in the spleens of infected fish

Molecular quantification of bacterial loads in the spleen of survivors revealed significantly lower *Francisella noatunensis* subsp. *orientalis* loads i.e. pathogen genome copies, in the vaccinated than in the adjuvant-only (p<0.05) and PBS-injected (p<0.001) fish. The fish immunised only with the adjuvant displayed significantly lower loads than the control fish (p<0.05) (Figure 5).

4 Discussion

Francisellosis remains one of the most important infectious diseases in aquaculture for which no licenced vaccines are available. Autogenous vaccines are custom made formulations that have the potential to be rapidly developed and deployed when no off-the-shelf fully licensed vaccines exist. In principle autogenous vaccines must be inactivated (killed) and derived from
pathogens isolated directly from the sites where they will be delivered i.e. have the capacity to confer immunity against homologous challenges (Haskell et al, 2004).

Under these circumstances, the formulation of autogenous vaccines is a logical scenario but as yet, remains as an unexplored option to control francisellosis in tilapia. The present study was therefore carried out using a whole cell inactivated vaccine against *Francisella noatunensis* subsp. *orientalis*, which had an excellent efficacy in red Nile tilapia using a homologous isolate to assess its efficacy.

The vaccine here developed was found to be safe with adequate bacterial inactivation achieved, residual formaldehyde neutralisation and absence of acute or chronic side effects to the fish during the safety test. Although the safety test was run for 30 days, the first 7 days are considered the most critical for this assessment.

The vaccine was also considered stable as the use of a high speed mixer resulted in an efficient water-in-oil 70/30 (70% adjuvant / 30% antigen) emulsion that was easy to administer using stainless steel 21-22 gauge needles. In the emulsion, the antigenic component was the primary inducer of the specific adaptive immune response while the adjuvant enhanced and extended such response.

According to Tafalla et al. (2014) such role of the adjuvants is achieved in part by acting as a delivery vehicles for the antigens and also by stimulating immune system through inherent immunostimulatory properties. Although the exact mechanisms of how adjuvants work are mostly unknown, according to Cox & Coulter (1997) five modes of action have been recognised: (1) Immunomodulation: the ability to modify the cytokine network. (2) Presentation: the ability to preserve the conformational integrity of an antigen and to present the antigen to appropriate immune effector cells. (3) Cytotoxic T-lymphocyte (CTL) induction: induction of CD8+ CTL responses. (4) Targeting: the ability to deliver an immunogen to
immune effector cells, generally via antigen presentation cells. (5) Depot generation: generation of a short-term or long-term depot to give a continuous or pulsed release.

In the present study, fish in the adjuvant-only group had a better survival rate (RPS 42%) than the control fish, making clearly evident that the commercial adjuvant Montanide™ ISA 763 A VG acted as an immunostimulant for Nile tilapia. It is thought that this could have been caused by immunomodulation, CTL induction and/or depot generation mode of actions as no specific antibody production was seen in this group at any point after vaccination (Cox & Coulter, 1997; Tafalla et al., 2014). The immunostimulation properties of the adjuvant allowed the fish to control bacterial replication to some degree and this was reflected in the qPCR results of the only adjuvant group where significantly lower bacterial loads than in the control fish were seen.

If the immunostimulation properties were seen in the only adjuvant group it can be assumed that these properties were also present in the vaccine group. However further research comparing the expression of cell mediated immune genes in adjuvant, vaccinated and control groups is needed to clarify if these mechanisms of immunity are stimulated by the adjuvant ISA 763A VG and to better understand the interactions between this adjuvant and the tilapia immune system.

As discussed by Munang’andu & Evensen (2015) the type of adaptive immune response is highly influenced by the site of antigen uptake i.e. antigens deposited extracellularly primarily evoke humoral immune responses, while antigens deposited intracellularly evoke both humoral and cellular mediated immune responses. It is believed that in this study the external aqueous phase of the water-in-oil emulsion secured a correct preservation and incorporation of the killed antigen (antigen delivery role) into the intracellular space of the immune effector cells (local antigen presenting cells, CD8+, CD4+, etc.) and this resulted in a gradually increasing and
continuous release of specific IgM (Aucouturier et al., 2001; Cox & Coulter, 1997; Tafalla et al. 2014).

It is possible that such a strong humoral immune response controlled the extracellular stage of the bacterial infection especially during the early stages of the infection presumably preventing entry and replication of live pathogenic bacteria into the immune cells of the fish (Soto et al., 2010). Most importantly although some individual variation was seen amongst the vaccinated fish, the average humoral response reached a signature of protective immunity as defined by Pulendran et al. (2010) by day 25 p.v. that fell well within the concept of herd immunity (Anderson and May, 1985; Gudding, 2014). Further research is needed to understand the exact mechanisms that induced the potent and rapid humoral response seen in the vaccinated fish and if the vaccine is also able to stimulate cellular immune response.

Previous reports of Francisella infections in humans have reported development of immunity in naturally infected individuals (Koskela & Salminen, 1986). Interestingly the antibody titres in the survivors of the control group was above the threshold of protection confirming that this group of fish had already developed humoral immunity to Francisella noatunensis subsp. orientalis infection. It is possible that this immunity had potentiated the immunity conferred by the vaccine and adjuvant. These observations correlated with the bacterial loads seen in spleen determined from the qPCR in all the treatments. Whether the survivors from these groups would have eventually eliminated the infection or became carriers or vertical/horizontal transmitters stills remains unknown.

The correlation between the RPS values, differences in the survival rate, antibody titres and bacterial loads observed in this study confirmed that the vaccine developed here was 100% effective in protecting red Nile tilapia from the bacterial challenge administered by i.p. injection. These results provide a strong support that this approach could be further explored
at a commercial level to develop autogenous vaccines as a solution to control francisellosis in farms or areas where the problem is endemic.

Further research is needed to investigate the efficacy of this vaccine in scenarios such as higher challenge doses and other infection routes i.e. immersion and cohabitation. Moreover, this approach could also be used to explore the possibility of cross protection between isolates from different origins, as previous studies have revealed high genetic homogeneity at genome level between isolates obtained from different countries, fish species, and over time (Gonçalves et al., 2016; Ramirez-Paredes 2015; Ramirez-Paredes et al., 2017a; Sjödin et al., 2012).

5 Conclusion

The whole cell inactivated vaccine developed in this study provided excellent results, protecting red Nile tilapia against experimentally induced francisellosis (RPS = 100%) using homologous isolate. The protection correlated with systemic IgM responses and bacterial loads as confirmed by ELISA and qPCR, respectively.

6 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 References


Aucouturier, J., Dupuis, L. & Ganne, V. (2001). Adjuvants designed for veterinary and human vaccines. *Vaccine*, 19, 2666-2672. [https://doi.org/10.1016/S0264-410X(00)00498-9](https://doi.org/10.1016/S0264-410X(00)00498-9)


Gudding R. (2014). Adjuvants in Fish Vaccines. In Gudding, Lillehaug & Evensen (Eds.), *Vaccination as a Preventive Measure* (pp. 12-20)


Table 1. Experimental design of vaccination trial and sampling point for correlates of protection.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Vaccine</th>
<th>Adjuvant</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunisation (No. fish/replicate)</td>
<td>52 x 3</td>
<td>52 x 3</td>
<td>52 x 3</td>
</tr>
<tr>
<td>Inoculum (100 μl via i.p. injection)</td>
<td>$1.3 \times 10^9$ CFU/ml + Adjuvant</td>
<td>70% Adjuvant + 30% PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>Sampling points for serology (d.p.v.)</td>
<td>4, 9, 15 and 30</td>
<td>4, 9, 15 and 30</td>
<td>4, 9, 15 and 30</td>
</tr>
<tr>
<td>No. fish/No. replicates</td>
<td>20 x 3</td>
<td>20 x 3</td>
<td>20 x 3</td>
</tr>
<tr>
<td>Challenge dose</td>
<td>$10^3$ CFU/fish</td>
<td>$10^3$ CFU/fish</td>
<td>$10^3$ CFU/fish</td>
</tr>
<tr>
<td>Sampling for pathology, serology and qPCR</td>
<td>40 d.p.c.</td>
<td>40 d.p.c.</td>
<td>40 d.p.c.</td>
</tr>
</tbody>
</table>

*d.p.c.* = days post challenge.

---

Table 2. Survival analysis of different treatment groups showing results between treatments and the overall comparisons results.

<table>
<thead>
<tr>
<th>Comparisons between treatments</th>
<th>Log-rank Mantel (and Cox) df</th>
<th>significance</th>
<th>Are the survival curves significantly different?</th>
<th>Overall Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated control</td>
<td>38.6</td>
<td>5</td>
<td>&lt;0.0001</td>
<td>41.39</td>
</tr>
<tr>
<td>Vaccinated adjuvant control</td>
<td>30.12</td>
<td>5</td>
<td>&lt;0.0001</td>
<td>yes</td>
</tr>
<tr>
<td>Control adjuvant adjuvant</td>
<td>7.357</td>
<td>5</td>
<td>0.1954</td>
<td>no</td>
</tr>
</tbody>
</table>
Figure 1. Gross pathology of experimentally infected naïve red Nile tilapia showing varying degrees of affection. A. Necropsy of moribund fish displaying erratic swimming and ascites, the black arrows point at enlarged and haemorrhagic organs including gills, wall of the peritoneal cavity and liver. The white arrows point at formation of white nodules in head kidney and mesentery. B. Spleen from fish shown in A, the organ presents severe affection with white nodules covering 100% of the parenchyma. C. Head kidney with moderate white nodule formation. D. Spleen from fish shown in C, the organs displays moderate affection with white nodules in the parenchyma.

Figure 2. Histological sections of the kidney of tilapia experimentally infected with francisellosis stained with H&E. A. The arrows indicate fully formed granulomas. Scale bar = 100 μm. B. Higher magnification 100x of one the granulomas with necrotic centre, encased by macrophages, mononuclear leukocytes and fibroblasts.

Figure 3. Cumulative percentage survival of red Nile tilapia during the main experimental challenge. The three replicate tanks of the three treatment groups (vaccinated, adjuvant alone and control) were experimentally infected with the homologous strain STIR-GUS-F2f7 at 840 degree days post vaccination. Survival for each replicate tank is shown. Relative percent survival (RPS) of the vaccinated group was 100% and RPS of the adjuvant only group was 42%.
Figure 4. Serum antibody levels of vaccinated, adjuvant-injected and PBS control tilapia post vaccination and post challenge. Each bar represents average OD$_{450}$ values of 5 fish per treatment ± SD. Horizontal dashed line represents cut-off calculated from average of the background multiplied by 3. Vertical dashed line divides vaccinated fish at days 4, 9, 15 and 30 post vaccination from challenge survivors at day 40 post challenge. Asterisks indicates significant difference, ns= not significant different (p≥0.05), * (p≤0.05), *** (p≤0.0001) d.p.v. = days post vaccination, d.p.c. = days post challenge.

Figure 5. Francisella noatunensis subsp. orientalis loads on the spleen of vaccinated, adjuvant-injected and control group survivors at day 40 post challenge. Each bar represents average copy number of 10 spleen samples per treatment ± SD. Asterisks indicate significance difference * (p≤0.05), *** (p≤0.0001).