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

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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 $4.0x10^3$ 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 (2.6×10^9 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 CHAH and incubating at 28 ^{o}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 (2.6×10^9 CFU/mL) was reduced with the emulsification to obtain a final concentration in the vaccine of 1.3×10^9 CFU/mL, to provide a dose of 1.3×10^8 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 (LD_{60}) 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 2.4×10^4 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 x 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 ^{o}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 (Immulone®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 OD_{600} of 0.4 (~1.0 x 10^9 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 H_2SO_4 and the absorbance measured at OD_{450} 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:

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

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 μs/cm. The microscopic particles observed were 1 μm 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

292 confirmed that the emulsion remained stable during this time. During the syringeability test,
293 the needles in the insulin syringes were not successful administering the vaccine as due to the
294 viscosity of the final product the process was slow, complicated and some of the syringes broke
295 while injecting the fish. For this reason hypodermic needles 22G x 7 mm and 21G x 8mm were
296 used to vaccinate the fish (Supplementary File 3).

297 **3.2 Vaccine safety**

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

312 **3.3 Bacterial infection**

313 Administration of 2.4×10^4 CFU/fish produced a cumulative mortality of 90% by 21 d.p.c. in
314 the pre-challenge test. On the basis of this result, it was decided to challenge the vaccinated

315 fish with a log10 lower dose to test the efficacy of the vaccine, with the actual dose used
316 determined to be 4.0 x 10^3 CFU/fish based on colony counts.

317 When moribund fish were dissected, granulomas were observed in their spleens and kidneys,
318 with varying degrees of severity (Figure 1). Some spleens were larger than normal, with dark
319 red coloration and white nodules in 80-90% of the parenchyma, others were bright red with 90-
320 100% granulomas and some others showed splenomegaly with large nodules and a white
321 membranous lining of the capsule of the spleen, which extended over the majority of the
322 peritoneal cavity. Head kidneys appeared enlarged, haemorrhagic and full of granulomas, and
323 in some cases the organ protruding ventrally towards the anterior section of the peritoneal
324 cavity, in contact with the spleen, liver, and gut sections. Other organs such as the gonads, gut
325 and posterior kidney were also affected. Bacterial recovery was achieved from 100% of the
326 moribund fish sampled.

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

332 **3.4 Vaccine efficacy**

333 A significant level of protection against the bacterial challenge was obtained for the vaccinated
334 group compared to the control and adjuvant groups ($p<0.0001$). (Figure 3). However, no
335 significant difference was seen between adjuvant and control treatment (Table 2). The
336 mortalities in the control group started by 8 d.p.c. and reached an average of 63.3 ± 2.9% by
337 34 d.p.c. The mortalities in the adjuvant group started at 13 d.p.c. and reached an average of
338 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

362 pathogens isolated directly from the sites where they will be delivered i.e. have the capacity to
363 confer immunity against homologous challenges (Haskell et al, 2004).

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

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

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

378 According to Tafalla et al. (2014) such role of the adjuvants is achieved in part by acting as a
379 delivery vehicles for the antigens and also by stimulating immune system through inherent
380 immunostimulatory properties. Although the exact mechanisms of how adjuvants work are
381 mostly unknown, according to Cox & Coulter (1997) five modes of action have been
382 recognised: (1) Immunomodulation: the ability to modify the cytokine network. (2)
383 Presentation: the ability to preserve the conformational integrity of an antigen and to present
384 the antigen to appropriate immune effector cells. (3) Cytotoxic T-lymphocyte (CTL) induction:
385 induction of CD8+ CTL responses. (4) Targeting: the ability to deliver an immunogen to

386 immune effector cells, generally via antigen presentation cells. (5) Depot generation:
387 generation of a short-term or long-term depot to give a continuous or pulsed release.

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

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

402 As discussed by Munang'andu & Evensen (2015) the type of adaptive immune response is
403 highly influenced by the site of antigen uptake i.e. antigens deposited extracellularly primarily
404 evoke humoral immune responses, while antigens deposited intracellularly evoke both humoral
405 and cellular mediated immune responses. It is believed that in this study the external aqueous
406 phase of the water-in-oil emulsion secured a correct preservation and incorporation of the killed
407 antigen (antigen delivery role) into the intracellular space of the immune effector cells (local
408 antigen presenting cells, CD8+, CD4+, etc.) and this resulted in a gradually increasing and

409 continuous release of specific IgM (Aucouturier et al., 2001; Cox & Coulter, 1997; Tafalla et
410 al. 2014).

411 It is possible that such a strong humoral immune response controlled the extracellular stage of
412 the bacterial infection especially during the early stages of the infection presumably preventing
413 entry and replication of live pathogenic bacteria into the immune cells of the fish (Soto et al.,
414 2010). Most importantly although some individual variation was seen amongst the vaccinated
415 fish, the average humoral response reached a signature of protective immunity as defined by
416 Pulendran et al. (2010) by day 25 p.v. that fell well within the concept of herd immunity
417 (Anderson and May, 1985; Gudding, 2014). Further research is needed to understand the exact
418 mechanisms that induced the potent and rapid humoral response seen in the vaccinated fish and
419 if the vaccine is also able to stimulate cellular immune response.

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

429 The correlation between the RPS values, differences in the survival rate, antibody titres and
430 bacterial loads observed in this study confirmed that the vaccine developed here was 100%
431 effective in protecting red Nile tilapia from the bacterial challenge administered by i.p.
432 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

Adams, A., Thompson, K.D., Morris, D., Farias, C. & Chen, S.C. (1995). Development and use of monoclonal antibody probes for immunohistochemistry, ELISA and IFAT to detect bacterial and parasitic fish pathogens. *Fish and Shellfish Immunology*, 5, 537-547. https://doi.org/10.1016/S1050-4648(95)80040-9

Amend, D. F. (1981). Potency testing of fish vaccines. *Developments in Biological Standardization*, 49, 447–54.

456 Anderson, R.M. & May, R.M. (1985). Vaccination and herd immunity to infectious diseases.
457 *Nature*, 318, 323-329. https://www.nature.com/articles/318323a0

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

460 Birkbeck, T.H., Feist, S.W. & Verner-Jeffreys, D.W. (2011). *Francisella* infections in fish and
461 shellfish. *Journal of Fish Diseases,* 34, 173–187. https://doi.org/10.1111/j.1365-
462 2761.2010.01226.x

463 Colquhoun, D.J. & Duodu, S. (2011). *Francisella* infections in farmed and wild aquatic
464 organisms. *Veterinary Research*, 42, 47–62. https://doi.org/10.1186/1297-9716-42-47

465 Cox, J. C., & Coulter, A. R. (1997). Adjuvants—a classification and review of their modes of
466 action. *Vaccine*, 15, 248-256. https://doi.org/10.1016/S0264-410X(96)00183-1

467 Duodu, S., Larsson, P., Sjödin, A., Soto, E., Forsman, M., & Colquhoun, D.J. (2012). Real-
468 time PCR assays targeting unique DNA sequences of fish-pathogenic *Francisella noatunensis*
469 subspecies *noatunensis* and *orientalis*. *Diseases of Aquatic Organisms*, 101, 225–234.
470 https://doi.org/10.3354/dao02514

471 Gonçalves, L. A., de Castro Soares, S., Pereira, F. L., Dorella, F. A., de Carvalho, A. F., de
472 Freitas Almeida, G. M., Leal, C.A.G., Azevedo, V., & Figueiredo, H.C.P. (2016). Complete
473 genome sequences of *Francisella noatunensis* subsp. *orientalis* strains FNO12, FNO24 and
474 FNO190: a fish pathogen with genomic clonal behavior. *Standards in genomic sciences*, 11,
475 30. https://doi.org/10.1186/s40793-016-0151-0

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

478 Haskell, S.R., Carberry-Goh, K., Payne, M.A. & Smith, S.A. (2004). Current status of aquatic
479 species biologics. *Journal of the American Veterinary Medical Association*, 225, 1541-1544.
480 https://doi.org/10.2460/javma.2004.225.1541

481 Klinger-Bowen, R., Tamaru, C., McGovern-Hopkins, K., Li, Y., Sithole, F., Kearney, M.T.,
482 Francis, S. & Soto, E. (2016). Dynamics of piscine francisellosis differs amongst tilapia species
483 (*Oreochromis* spp.) in a controlled challenge with *Francisella noatunensis* subsp. *orientalis*.
484 *Journal of Fish Diseases*, 39, 1305-1312. https://doi.org/10.1111/jfd.12461

485 Koskela, P. E. N. T. T. I., & Salminen, A. I. M. O. (1985). Humoral immunity against
486 Francisella tularensis after natural infection. Journal of clinical microbiology, 22(6), 973-979.
487 https://jcm.asm.org/content/22/6/973.short

488 Midtlyng, P.J., Reitan, L.J. & Speilberg, L. (1996). Experimental studies on the efficacy and
489 side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against
490 furunculosis. *Fish and Shellfish Immunology*, 6, 335-350.
491 https://doi.org/10.1006/fsim.1996.0034

492 Munang'andu, H. M., & Evensen, Ø. (2015). A review of intra-and extracellular antigen
493 delivery systems for virus vaccines of finfish. *Journal of immunology research*, 2015.
494 http://dx.doi.org/10.1155/2015/960859

495 Pulendran, B., Li, S. & Nakaya, H.I. (2010). Systems vaccinology. *Immunity*, 33, 516-529.
496 https://doi.org/10.1016/j.immuni.2010.10.006

497 Pulpipat, T., Lin, K.H., Chen, Y.H., Wang, P.C. & Chen, S.C. (2019). Molecular
498 characterization and pathogenicity of *Francisella noatunensis* subsp. *orientalis* isolated from
499 cultured tilapia (*Oreochromis* sp.) in Taiwan. *Journal of Fish Diseases*. 04 February 2019
500 https://doi.org/10.1111/jfd.12964

Ramirez-Paredes, J.G. (2015). The fish pathogen *Francisella orientalis*: characterisation and vaccine development. http://hdl.handle.net/1893/21822

Ramírez-Paredes, J.G., Larsson, P., Wehner, S., Bekaert, M., Öhrman, C., Metselaar, M., Thompson, K.D., Richards, R.H., Penman, D.J. & Adams, A., (2017a). Draft genome sequence of *Francisella noatunensis* subsp. *orientalis* STIR-GUS-F2f7, a highly virulent strain recovered from diseased red Nile tilapia farmed in Europe. *Genome Announcements*. 5(11), pp.e01555-16. https://doi.org/10.1128/genomeA.01555-16.

Ramírez-Paredes, J.G., Thompson, K.D., Metselaar, M., Shahin, K., Soto, E., Richards, R.H., Penman, D.J., Colquhoun, D.J. & Adams, A. (2017b). A Polyphasic Approach for Phenotypic and Genetic Characterization of the Fastidious Aquatic Pathogen *Francisella noatunensis* subsp. *orientalis*. *Frontiers in Microbiology*, 8:234. https://doi.org/10.3389/fmicb.2017.02324

Sjödin, A., Svensson, K., Öhrman, C., Ahlinder, J., Lindgren, P., Duodu, S., Johansson, A., Colquhoun, D. J., Larsson, P. & Forsman, M. (2012). Genome characterisation of the genus *Francisella* reveals insight into similar evolutionary paths in pathogens of mammals and fish. *BMC genomics*, 13, 268. https://doi.org/10.1186/1471-2164-13-268

Soto, E., Abrams, S. B., & Revan, F. (2012). Effects of temperature and salt concentration on Francisella noatunensis subsp. orientalis infections in Nile tilapia *Oreochromis niloticus*. Diseases of aquatic organisms, 101, 217-223. https://doi.org/10.3354/dao02533

Soto E., Brown N., Gardenfors Z.A., Yount S., Revan F., Francis S., Kearney M.T. & Camus A. (2014). Effect of size and temperature at vaccination on immunization and protection conferred by a live attenuated *Francisella noatunensis* immersion vaccine in red hybrid tilapia. *Fish and Shellfish Immunology*, 41, 593–599. https://doi.org/10.1016/j.fsi.2014.10.009

523 Soto, E., Fernandez, D. & Hawke, J.P. (2009). Attenuation of the fish pathogen *Francisella* sp.
524 by mutation of the iglC* gene. *Journal of Aquatic Animal Health*, 21, 140–49.
525 https://doi.org/10.1577/H08-056.1

526 Soto, E., Fernandez, D., Thune, R. & Hawke, J.P. (2010). Interaction of *Francisella asiatica*
527 with tilapia (*Oreochromis niloticus*) innate immunity. *Infection and Immunity*, 78, 2070–2078.
528 https://doi.org/10.1128/IAI.01308-09

529 Soto, E., Shahin, K., Talhami, J. J., Griffin, M. J., Adams, A., & Ramírez-Paredes, J. G. (2019).
530 Characterization of *Francisella noatunensis* subsp. *orientalis* isolated from Nile tilapia
531 *Oreochromis niloticus* farmed in Lake Yojoa, Honduras. *Diseases of Aquatic Organisms*, 133,
532 141-145. https://doi.org/10.3354/dao03333

533 Soto, E., Wiles, J., Elzer, P., Macaluso, K. & Hawke, J.P. (2011). Attenuated *Francisella*
534 *asiatica* iglC mutant induces protective immunity to francisellosis in tilapia. *Vaccine* 29, 593–
535 98. https://doi.org/10.1016/j.vaccine.2010.06.040

536 Tafalla C., Bøgwald J., Dalmo R.A., Munang'andu H. M. & Evensen O. (2014). Adjuvants in
537 Fish Vaccines. In Gudding, Lillehaug & Evensen (Eds.), *Fish vaccination* (pp. 68-81).

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

Feature	Vaccine	Adjuvant	Control
Immunisation (No. fish/ replicate)	52 x 3	52 x 3	52 x 3
Inoculum (100 µl via i.p. injection)	$1.3x10^9$ CFU/ml + Adjuvant	70% Adjuvant + 30% PBS	PBS
Sampling points for serology (d.p.v.)	4, 9, 15 and 30	4, 9, 15 and 30	4, 9, 15 and 30
No. fish/ No. replicates	20 x 3	20 x 3	20 x 3
Challenge dose	10^3 CFU/fish	10^3 CFU/fish	10^3 CFU/fish
Sampling for pathology, serology and qPCR	40 d.p.c.	40 d.p.c.	40 d.p.c.

d.p.c. = days post challenge.

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

Comparisons between treatments						Overall Comparisons		
Treatment 1	vs	Treatment 2	Log-rank Mantel (and Cox) test	df	significance	Are the survival curves significantly different?	Log-rank Mantel (and Cox) test	df	significance
vaccinated		control	38.6	5	<0.0001	yes	41.39	8	<0.0001
vaccinated		adjuvant	30.12	5	<0.0001	yes			
control		adjuvant	7.357	5	0.1954	no			

538

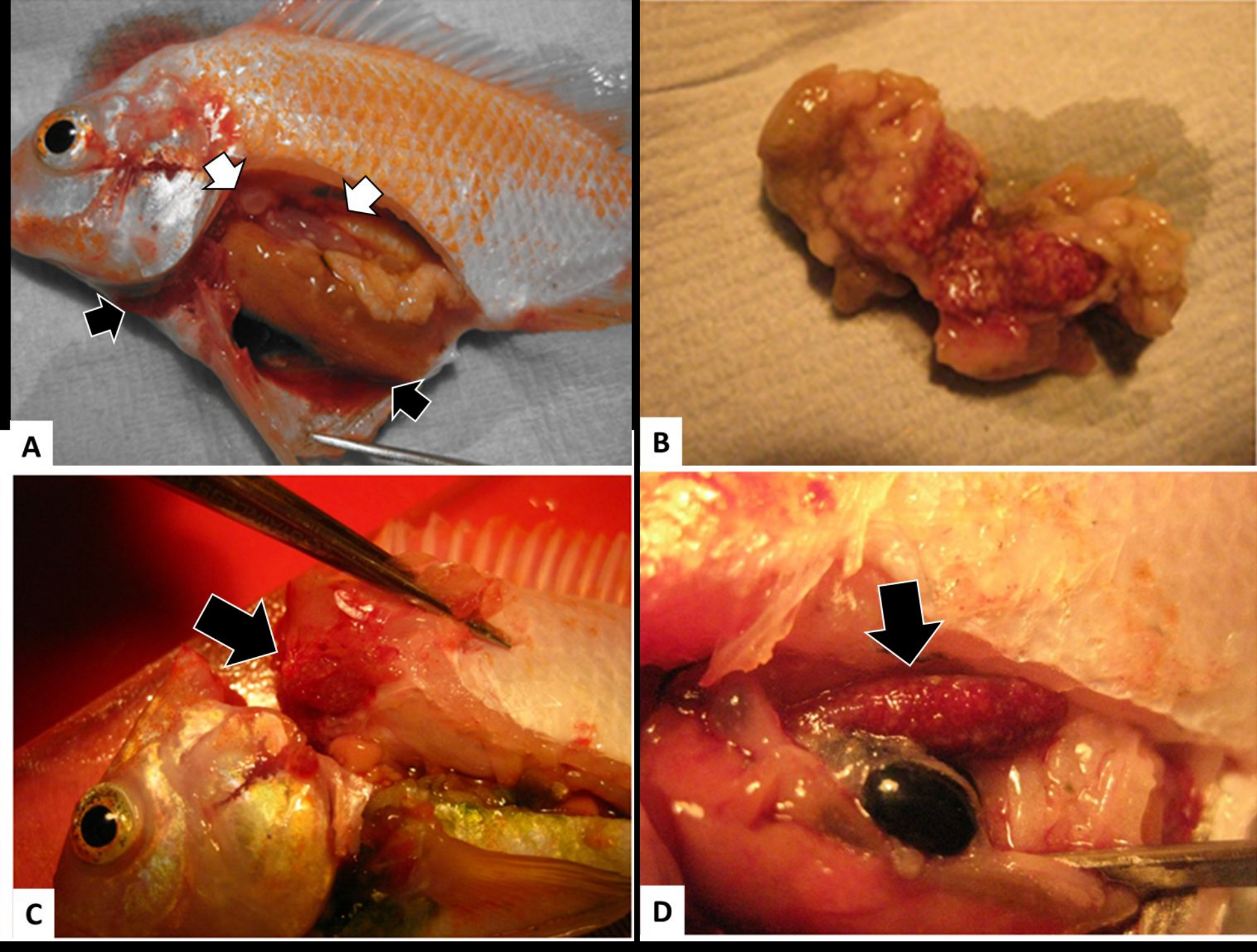
Figure 1. Gross pathology of experimentally infected naïve red Nile tilapia showing varying degrees of affection. A. Necropsy of moribund fish displaying erratic swimming and ascites, the black arrows point at enlarged and haemorrhagic organs including gills, wall of the peritoneal cavity and liver. 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 ($p \geq 0.05$), * ($p \leq 0.05$), *** ($p \leq 0.0001$) 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 * ($p \leq 0.05$), *** ($p \leq 0.0001$).


A
B
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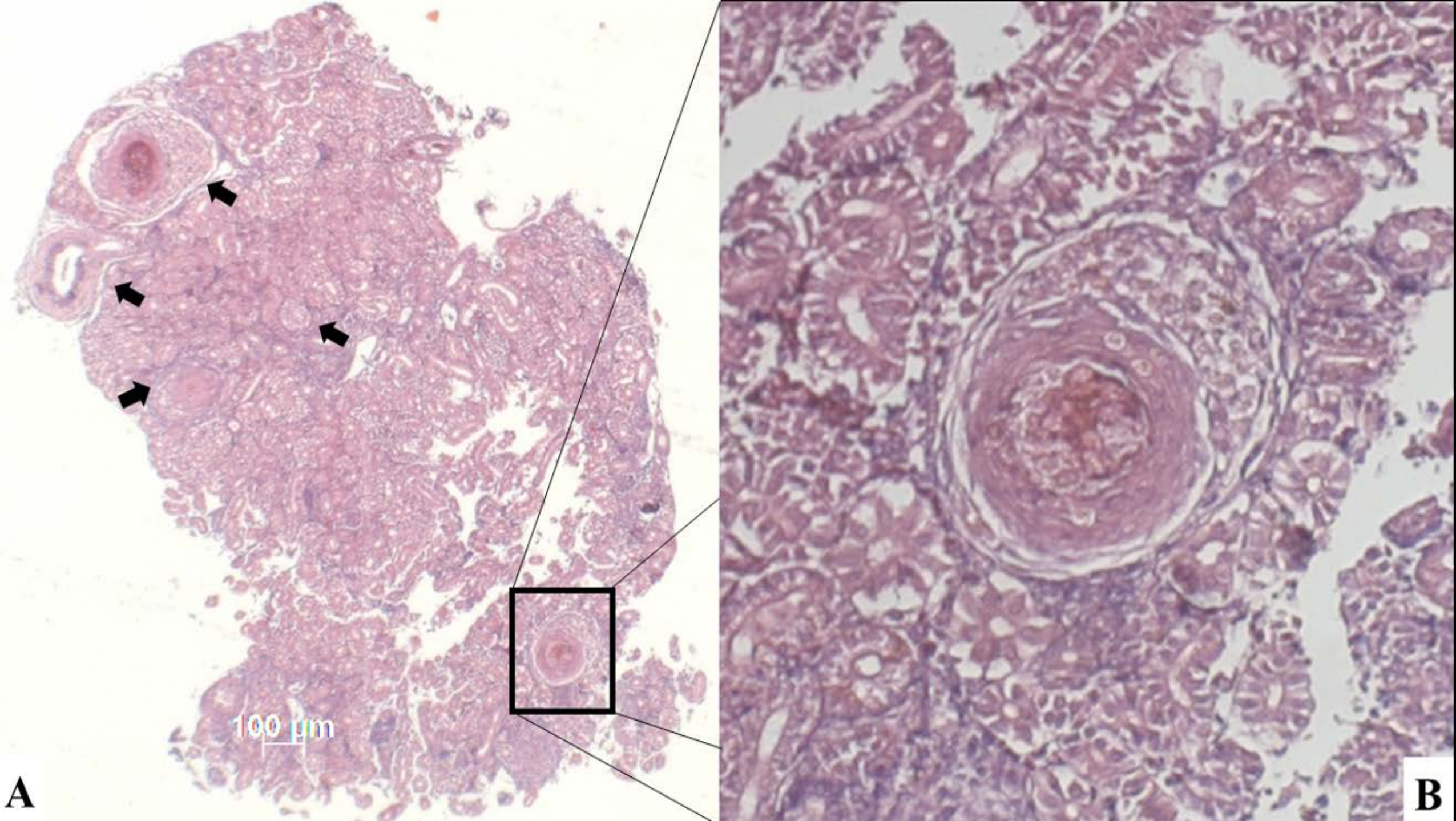
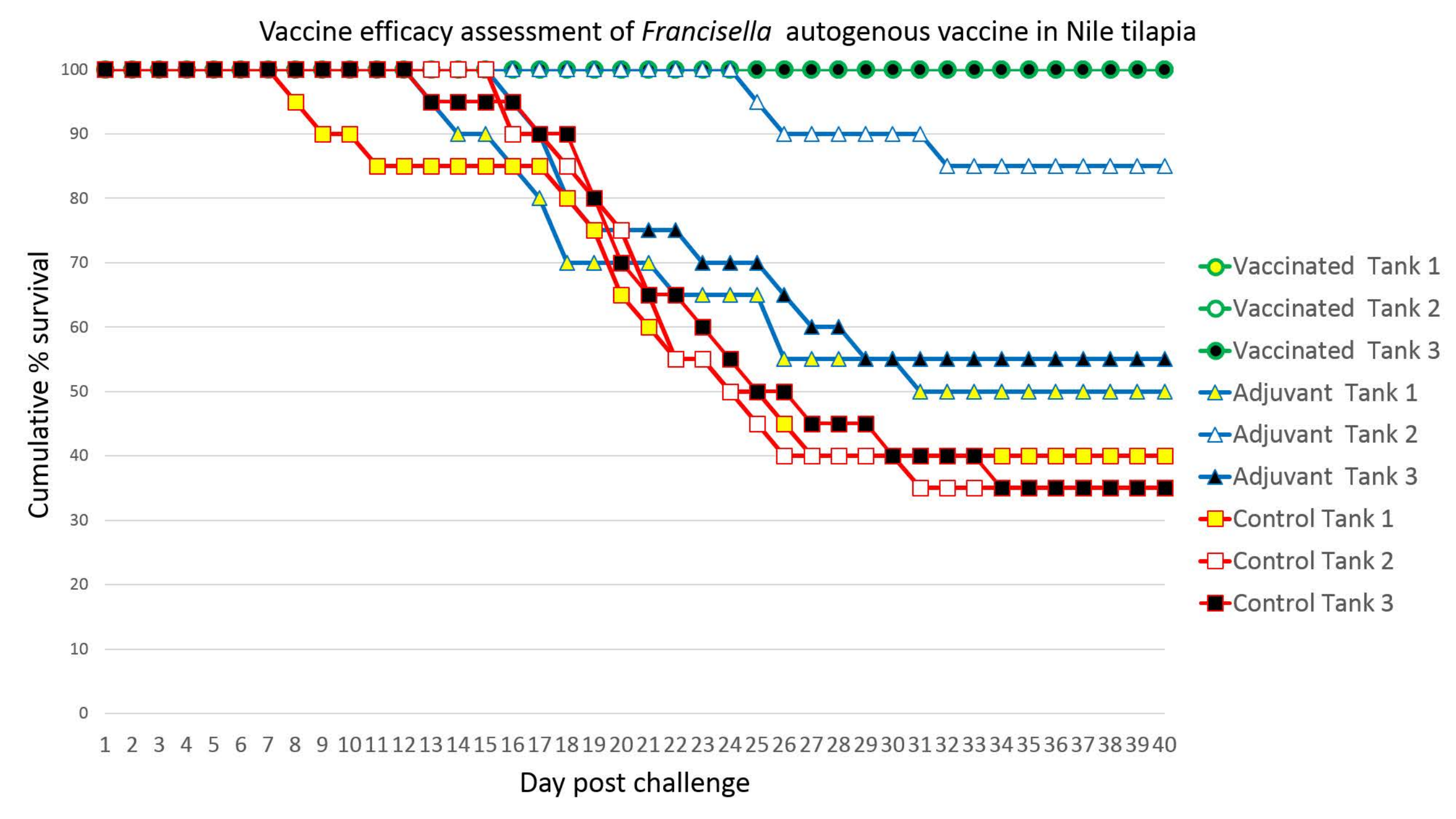

100 µm
A
B


Vaccine efficacy assessment of *Francisella* autogenous vaccine in Nile tilapia
Cumulative % survival
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Day post challenge
Vaccinated Tank 1
Vaccinated Tank 2
Vaccinated Tank 3
Adjuvant Tank 1
Adjuvant Tank 2
Adjuvant Tank 3
Control Tank 1
Control Tank 2
Control Tank 3

IgM at OD $_{450}$

1.200
1.000
0.800
0.600
0.400
0.200
0.000

ns
ns
ns
***
*

4 dpv
9 dpv
15 dpv
30 dpv
40 dpc

Vaccinated
Adjuvant only
PBS control

***

*

*

5

4

3

2

1

0

Log $_{10}$ *Fno* copies/µL

Vaccine

Adjuvant

Control