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NOTE

# Susceptibility of common carp and sunfish to a strain of *Francisella noatunensis* subsp. *orientalis* in a challenge experiment

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ABSTRACT: Francisellosis, an emerging disease in many fish species, can cause high mortality in affected populations. Here we investigated the susceptibility of common carp *Cyprinus carpio* and sunfish *Lepomis gibbosus* to *Francisella noatunensis* subsp. *orientalis* (*Fno*), and possible transmission of the bacteria between the 2 fish species. In a challenge experiment, 3 groups of each species were injected intraperitoneally (IP) with 3 different doses of an *Fno* strain no. 9449 of the Norwegian Veterinary Institute, recovered from naturally infected ornamental Malawi cichlids. Infected carp were cohabitated with sunfish and vice versa. Control groups were injected with 0.9M phosphate-buffered saline and cohabitated accordingly. Fish were sampled at different time points. Mortality of challenged sunfish was observed during the first 96 h and reached 56.1%. In the control sunfish, 4 of 16 fish (25%) died within 48 h. In carp, no mortalities or clinical signs were observed during the experiment. General clinical and patho-anatomical disease signs of affected sunfish were observed. We detected granulomas in 2 cohabitated sunfish and 1 challenged carp, but could not re-isolate *Fno* from these fish. *Fno* was successfully cultured from 6 sunfish and 3 carp specimens until 35 d post injection. PCR of spleen and kidney with 16S rDNA *Francisella*-like bacterium primers 180f and 485r yielded amplicons in 68.3% of challenged sunfish and only 12.2% of challenged carp. We demonstrated that sunfish were susceptible to *Fno* infection while the carp were not. Horizontal transmission of the agent between the 2 fish species could not be demonstrated.

KEY WORDS: Bacteria · Francisellosis · *Cyprinus carpio* · *Lepomis gibbosus* · Transmission



## INTRODUCTION

Aquaculture of various cyprinid fishes, including common carp *Cyprinus carpio*, is the largest provider of freshwater food-fish worldwide (FAO 2014). Because cyprinids are cultured in different climate zones and temperatures, a wide variety of bacterial infections can affect these fish. Moreover, in many countries, carp are held in polyculture systems, with efforts to combine the production of carp and tilapia *Oreochromis* spp. (Pandit et al. 2004, da Silva et al. 2006).

Francisellosis is an emerging disease in many fish species (Hsieh et al. 2006, 2007, Nylund et al. 2006, Birkbeck et al. 2007, Ottem et al. 2007, Kamaishi et al. 2010, Camus et al. 2013). In warm-water fish like tilapia, the disease is caused by *Francisella noatunensis* subsp. *orientalis* (*Fno*), a Gram-negative, aerobic, non-motile, facultative intracellular, pleomorphic bacterium. In general, the disease follows a chronic progression characterized by the formation of multifocal white nodules in the spleen and other organs (Soto et al. 2009a, 2012a). Mortalities can be high (Chen et al. 1994, Leal et al. 2014).

A case of francisellosis was recently reported from ornamental Malawi cichlids in Austria (Lewisch et al. 2014). No data are currently available about the distribution of this disease among ornamental fish. The condition is very likely under-diagnosed, and, be-

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cause of the formation of granulomas, misattributed to *Mycobacterium* spp. The possible threat that *Fno* poses to native fish species such as carp is unknown.

The aim of this study was to assess the susceptibility of common carp and sunfish *Lepomis gibbosus* to a strain of *Fno* recovered from ornamental cichlids. The sunfish were chosen as a species biologically related to the cichlids and often found in Austrian carp ponds. Possible horizontal transmission of the disease between the 2 fish species was also investigated.

## MATERIALS AND METHODS

### Bacteria

The isolate was recovered from naturally infected ornamental Malawi cichlids (Lewisch et al. 2014). The isolate is part of the collection of the Norwegian Veterinary Institute under the number 9449 and its identity was determined by amplification and sequencing of its 16S-23S internal transcribed spacer (ITS) region (Ramírez Paredes 2015). Bacteria were cultured on blood-supplemented cystein-heart agar plates (CHAB) and subsequently suspended in 5 ml aliquots of modified Mueller-Hinton II cation-adjusted broth (Sigma-Aldrich) as described by Soto et al. (2009a) and incubated at 26°C. From the resulting suspension, a $10^{-1}$ and a $10^{-2}$ dilution were prepared to obtain 3 different infection doses. Bacteria were enumerated based on optical density at 600 nm (OD600) and correlated to plate count after serial dilution.

### Challenge

Common carp (mean length 11.5 cm, mean weight 21.5 g) from a local hatchery with no history of koi herpesvirus (KHV) and common sunfish (mean length 9.8 cm, mean weight 13.4 g) from a wholesaler were transferred to 100 l aquaria. The aquaria were individually filtered and offered hiding places for the fish. Water temperature was 24°C and was monitored daily together with ammonia and nitrite levels. Nitrogenous waste products remained in a normal range throughout the experiment. The fish were fed a commercial pellet diet twice a day.

Prior to the challenge, 5 carp and 5 sunfish were submitted to a complete clinical, bacteriological, and molecular investigation and found to be free of *Fno* and parasitic or bacterial diseases. Sunfish and carp were separately acclimatized for 2 wk. Subsequently, fish were divided into 6 groups of 3 different infection doses and acclimatized for 5 more days. In 3 of these groups, carp were infected by intraperitoneal (IP) injection, in the other 3 groups, the sunfish were IP infected. An equal number of the other fish species was cohabitated with the challenged fish. For the highest infection dose, 12 fish were used; for the lower doses we used 13 and 16 fish, respectively. Two control groups comprised 16 fish of each species. In one of the control groups, carp were sham injected with phosphate-buffered saline (PBS), in the other control group, the sunfish were sham injected.

Fish were anesthetized with 100 mg $l^{-1}$ MS-222 (Sigma Aldrich), and the injection site was disinfected with 70% alcohol. In the control groups, the fish were injected with 0.1 ml sterile 0.9M PBS (Thermo Fisher Scientific). Challenge groups received 0.1 ml of $2 \times 10^6$, $2 \times 10^7$ and $2 \times 10^8$ CFU $ml^{-1}$, respectively.

### Sampling

Post-injection (p.i.), fish were monitored several times each day, and dead and moribund fish were removed from the tanks. Moribund fish were euthanized (MS 222, 1 g $l^{-1}$). After 4 d of elevated mortalities, fish from each group were euthanized at different time points (35, 49, 104, 111, 113, 120, 139, 143, 146 d p.i.). Gross patho-anatomical findings were recorded, and in 12 sunfish and 12 carp, Gram stains of spleen and kidney imprints were performed.

Samples of spleen, kidney, and in selected cases, gills, were aseptically transferred to 2 ml Eppendorf tubes and homogenized in 360 µl lysis puffer (Qiagen). An aliquot (100 µl) of the homogenate was cultured on CHAB plates and incubated at 26°C. DNA was extracted from the rest of the homogenate, using a DNeasy® Blood and Tissue Kit (Qiagen). PCR was conducted with *Francisella*-like bacteria (FLB)-specific primers (16S rDNA FLB-primers 180f and 485r) using a published protocol (Hsieh et al. 2006). For histopathology, spleen, kidney, and liver of 16 sunfish and 5 carp were fixed in 4% neutral buffered formalin, processed for routine histology, and stained with hematoxylin and eosin (H&E).

## RESULTS

No mortality of carp occurred during our study. In the challenged sunfish, mortalities occurred in all groups within 24 h of injection. Cumulative mortality reached 56.1% (23/41) in challenged sunfish, 25%

(4/16) in PBS-injected sunfish, and 6.25% (1/16) in sunfish cohabitated with PBS-injected carp, 96 h p.i. One additional mortality was recorded on Day 7 p.i. in the group of sunfish cohabitated with challenged carp ($2 \times 10^6$ CFU ml$^{-1}$). The mortalities did not correlate with infection dose (Fig. 1). Statistical analysis of mortality rates between the groups was conducted by Fisher's exact test. The difference between challenged sunfish and all other groups of sunfish and carp was significant ($p < 0.05$ for all comparisons). Clinical findings of moribund sunfish included lethargy and anorexia, swimming head down, tachypnea, fading of color, and skin hemorrhages (Fig. 2). These signs were observed only during the first 4 d p.i. After that, no other ailments and mortalities were observed, except for the mortality on Day 7.

Necropsy of sunfish that died within 4 d p.i. revealed unspecific signs of disease, including gill pallor, hyperemia of internal organs, and mild splenomegaly. Some of the infected fish that were euthanized at different time points showed enlarged spleens (11 sunfish, 1 carp), hyperemia of internal organs (4 sunfish, 2 carp), enlarged head kidney (2 sunfish, 2 carp), or melanin deposits in the kidney (1 sunfish). One carp

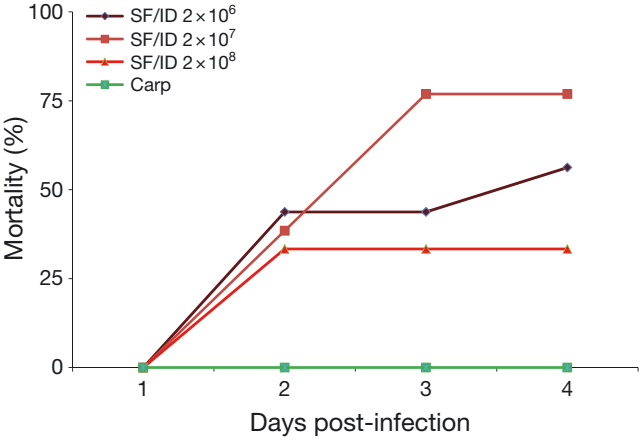


Fig. 1. Cumulative mortality of challenged fish related to infection dose (ID). Sunfish (SF) challenged with $2 \times 10^7$ CFU ml$^{-1}$: 76.9%; SF $2 \times 10^6$ CFU ml$^{-1}$: 56.3%; SF $2 \times 10^8$ CFU ml$^{-1}$: 33%. Carp *Cyprinus carpio*, all groups: 0%

Fig. 2. Sunfish *Lepomis gibbosus* (from challenge group $2 \times 10^7$ CFU ml$^{-1}$) 72 h after being injected with *Francisella noatunensis* subsp. *orientalis*. Note the skin hemorrhages on the ventral body parts

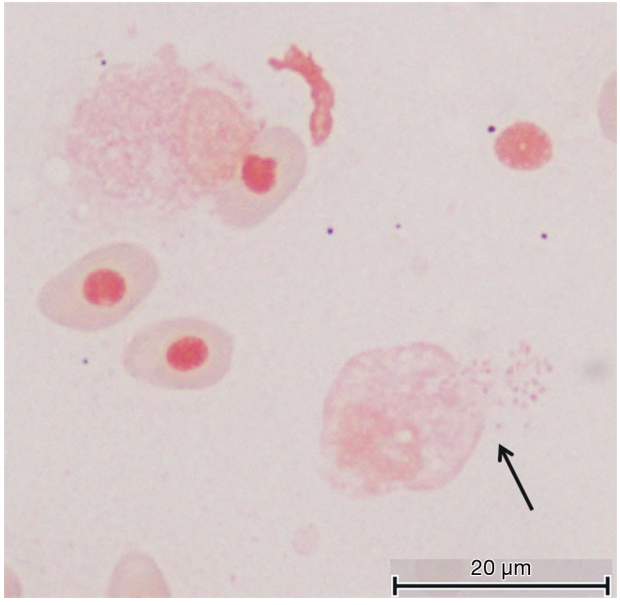


Fig. 3. Spleen imprint of sunfish *Lepomis gibbosus*, Gram stain, 48 h p.i. (from the group $2 \times 10^7$ CFU ml$^{-1}$), showing macrophage releasing *Francisella noatunensis* subsp. *orientalis* from a vacuole (arrow)

($2 \times 10^7$ CFU ml$^{-1}$) developed macroscopic granulomas in the kidney and 1 cohabitated sunfish had microscopic granulomas in the spleen (146 d p.i.). One cohabitated sunfish (carp challenged with $2 \times 10^8$ CFU ml$^{-1}$) showed macroscopic granulomas in the spleen at 113 d p.i. There were no signs of disease in carp during the whole experiment.

Gram stains of spleen and kidney imprints of several sunfish showed intra-and extracellular Gram-negative coccobacilli (Fig. 3). *Fno* was successfully cultured from 6 sunfish and 3 carp specimens until 35 d p.i. In 1 carp ($2 \times 10^8$ CFU, 35 d p.i.), the identity of the bacterium was verified by PCR.

In fish that died within 4 d p.i., histological sections of spleen and liver showed acute tissue necrosis with no inflammatory response. In specimens sampled later, vacuolated macrophages were observed proximal to melanomacrophage centers and blood vessels. Granulomas were evident in the posterior kidney of 1 challenged carp and the spleen of 2 cohabitated sunfish (Fig. 4). Except for 1 fish ($2 \times 10^6$ CFU ml$^{-1}$), spleen and kidney of all challenged sunfish that died within the first 4 d tested positive for *Fno* with FLB-specific PCR (22/23). DNA samples from gills were positive in 2/2 ($2 \times 10^8$ CFU ml$^{-1}$), 1/3 ($2 \times 10^7$ CFU ml$^{-1}$) and 0/4 ($2 \times 10^6$ CFU ml$^{-1}$) tested

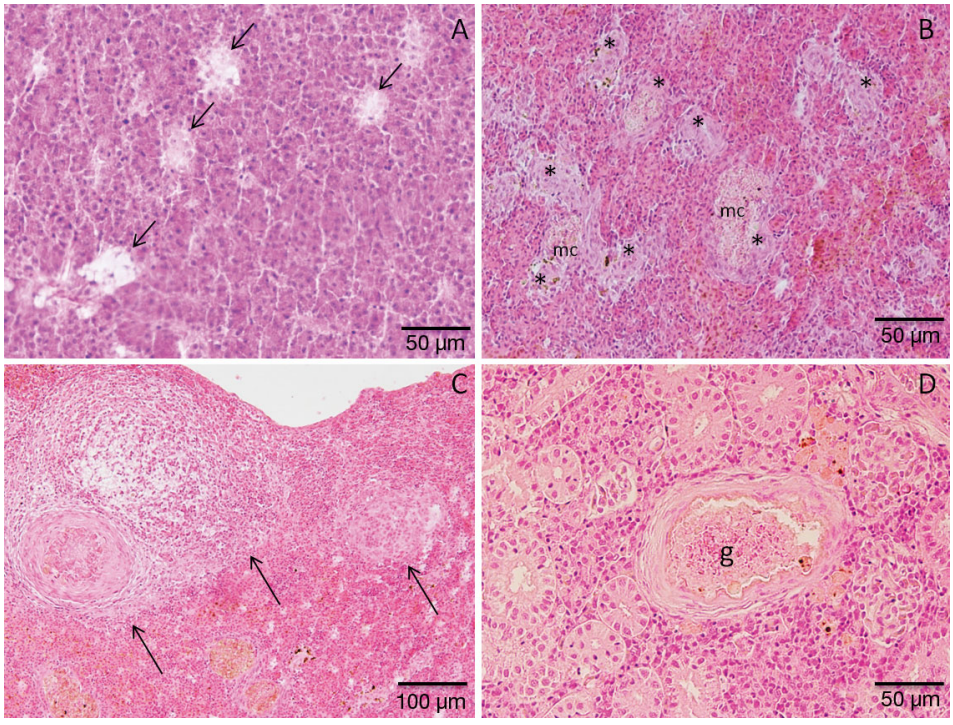


Fig. 4. H&E-stained sections from organs of sampled fish: (A) liver of challenged sunfish *Lepomis gibbosus* that died 48 h following injection with *Francisella noatunensis* subsp. *orientalis*, showing disseminated multifocal necrosis with vacuolation of hepatocytes and karyolysis (arrows). (B) Spleen of challenged sunfish (120 d post injection), showing accumulation of macrophages (*) around blood vessels and proximal to melanomacrophage centers (mc). (C) Spleen of sunfish cohabitated with infected carp *Cyprinus carpio*, showing different development stages of granulomas (arrows). (D) Kidney of challenged carp showing a granuloma (g) with central necrosis surrounded by epitheloid macrophages and fibrous material

sunfish. In fish tested at different time points, DNA extract from spleen of 5 of the remaining 8 sunfish (62.5%) challenged with the highest dose tested positive until 143 d p.i. In the 2 other groups, only DNA extract of spleen from 1/7 sunfish challenged with $2 \times 10^6$ CFU ml$^{-1}$ tested positive 111 d p.i. In carp, DNA extract of spleen from 5/12 (41.67%) challenged with the highest dose tested positive by FLB-specific PCR until day 139 p.i. (Fig. 5). No carp of the other 2 challenge groups tested positive at any time point. Overall, 68.29% of challenged sunfish and only 12.2% of challenged carp tested positive. None of the cohabitated fish and none of the control fish tested positive.

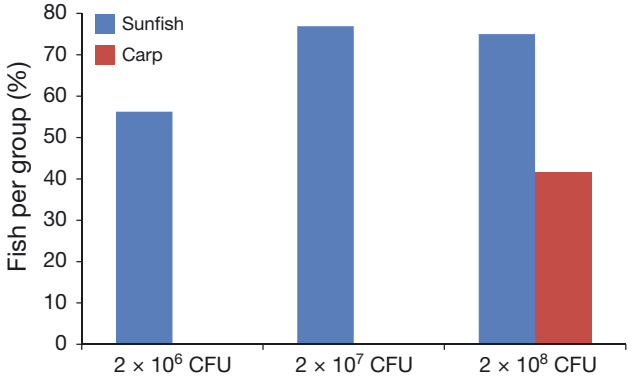


Fig. 5. Evidence of *Francisella noatunensis* subsp. *orientalis* DNA in sunfish *Lepomis gibbosus* (SF) and carp *Cyprinus carpio* dependent on infection dose. Percent of SF challenged with $2 \times 10^6$ CFU ml$^{-1}$: 56.25%; SF $2 \times 10^7$ CFU ml$^{-1}$: 76.92%; SF $2 \times 10^8$ CFU ml$^{-1}$: 75%; carp $2 \times 10^8$ CFU ml$^{-1}$: 41.67%; all other groups 0%

## DISCUSSION

The aim of this study was to investigate the susceptibility of native fish species to *Fno* and possible transmission of the disease between sunfish and carp. The choice of the 2 fish species was based on the importance of carp as food fish in Austria and around the world; sunfish were included as they are sympatric with carp in Austria's standing waters and carp ponds. Sunfish were assumed to be susceptible to *Fno* as they belong to the Percomorphaceae, the same order as cichlids, which are known hosts. Tem-

peratures in Austrian carp ponds often reach 24°C during summer months, a regime that is conducive for outbreaks of *Fno*-induced disease (Mauel et al. 2003, Soto et al. 2012a). Challenged sunfish showed an overall mortality of 56.1% within the first 96 h p.i. Compared to this, the number of mortalities due to handling in the control group was significantly lower (25.0%). In carp, no mortality occurred during the entire study.

Mortality started as early as 24 h p.i. Previous work has shown that for tilapia mortality rates can be 100% in less than 48 to 72 h (Soto et al. 2009a), 86.7 ± 23% in 5 d p.i. (Nguyen et al. 2016), or 90% between 20 h and 8 d (Leal et al. 2014). In our study, mortality did not correlate with the infection dose. This might be attributed to individual immunological condition of the fish as well as to the relatively small number of fish in the groups. Mortality ceased after 96 h, which possibly shows that the remaining fish effectively overcame infection by an innate immune response.

Necropsy of the infected carp and sunfish did not indicate death due specifically to the injection, with general clinical signs of acute disease in accordance with published observations during infection experiments with *Francisella* sp. (Ostland et al. 2006, Soto et al. 2009a,b, Vojtech et al. 2009). Formation of granulomas during the acute stage of the infection was not observed. At later time points, we observed granulomas in spleens of 2 cohabitated sunfish, and in kidney of 1 challenged carp. All 3 of these fish tested negative in PCR. This is in accordance with the study of Klinger-Bowen et al. (2016), who found that several groups of *Fno*-challenged tilapia species developed granulomas without molecular and bacteriological evidence of *Fno*. A possible explanation is the relatively long time since infection and the very low number of remaining bacteria. Other authors found that in advanced cases, FLB-specific PCR was only positive in 39% of cases that tested positive by *in situ* hybridization (ISH) (Hsieh et al. 2007). However, specimens used by those authors were archived, formalin-fixed, paraffin-embedded tissues, with possible DNA damage. In our experiment, ISH and real-time PCR, as proposed by Soto et al. (2012b), would be an excellent tool to clarify whether the granulomas were caused by *Fno*, which would confirm transmission of *Fno* from the carp to the sunfish. This issue will be addressed in further studies.

Until 35 d p.i., we were able to culture *Fno* from kidney and spleen of challenged sunfish and carp. In 1 carp, identity of the cultivated *Fno* was confirmed by PCR. *Fno* DNA could be detected by PCR in spleen, kidney, and gills in early-mortality sunfish, and in spleen and kidneys of carp and sunfish up to 139 and 143 d p.i., respectively. This suggests that bacteremia had taken place. While the sunfish were sensitive to the infection during a very short initial phase, the infection did not compromise the health status of the carp.

The differences between sunfish and carp in mortality due to *Fno* and evidence of *Fno* DNA detected by PCR highlight adaptation of the bacterium to specific hosts. Other authors demonstrated different susceptibility of zebrafish and tilapia, and even of various tilapia species under experimental conditions (Vojtech et al. 2009, Soto et al. 2009b, 2013, Farrell et al. 2014, Klinger-Bowen et al. 2016). In our study, susceptibility of sunfish could be demonstrated whereas carp were not susceptible to this particular strain of *Fno*, and no transmission of the bacterium between the 2 species could be demonstrated.

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## LITERATURE CITED

Birkbeck TH, Bordevik M, Frøystad MK, Baklien A (2007) Identification of *Francisella* sp. from Atlantic salmon, *Salmo salar* L., in Chile. J Fish Dis 30:505–507

Camus AC, Dill JA, McDermott AJ, Clauss TM, Berliner AL, Boylan SM, Soto E (2013) *Francisella noatunensis* subsp. *orientalis* infection in Indo-Pacific reef fish entering the United States through the ornamental fish trade. J Fish Dis 36:681–684

Chen SC, Tung MC, Chen SP, Tsai JF and others (1994) Systematic granulomas caused by a rickettsia-like organism in Nile tilapia, *Oreochromis niloticus* (L.), from southern Taiwan. J Fish Dis 17:591–599

Da Silva LB, Barcellos LJG, Quevedo RM, de Souza SMG and others (2006) Alternative species for traditional carp polyculture in southern South America: initial growing period. Aquaculture 255:417–428

FAO (Food and Agriculture Organization of the United Nations) (2014) World aquaculture production of fish, crustaceans, molluscs, etc., by principal species in 2012. FAO Fishery and Aquaculture Statistics 2012. FAO, Rome

Farrell F, Hansen J, Verma A, Illanes O, Soto E (2014) Disruption of the pathogenicity determinant protein A gene (pdpA) in *F. noatunensis* subsp. *orientalis* results in attenuation and in greater susceptibility to oxidative stress. Proceedings of the Seventh International Symposium on Aquatic Animal Health (ISAAH). American Fisheries Society, Portland, OR, p 71

Hsieh CY, Tung MC, Tu C, Chang CD, Tsai SS (2006) Enzootics of visceral granulomas associated with *Francisella*-like organism infection in tilapia (*Oreochromis* spp.). Aquaculture 254:129–138

Hsieh CY, Wu ZB, Tung MC, Tsai SS (2007) PCR and in situ hybridization for the detection and localization of a new pathogen *Francisella*-like bacterium (FLB) in ornamental cichlids. Dis Aquat Org 75:29–36

Kamaishi T, Miwa S, Goto E, Matsuyama T, Oseko N (2010) Mass mortality of giant abalone *Haliotis gigantea* caused by a *Francisella* sp. bacterium. Dis Aquat Org 89: 145–154

Klinger-Bowen R, Tamaru C, McGovern-Hopkins K, Li Y and others (2016) Dynamics of piscine francisellosis differs amongst tilapia species (*Oreochromis* spp.) in a controlled challenge with *Francisella noatunensis* subsp. *orientalis*. J Fish Dis (in press) doi:10.1111/jfd.12461

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

Lewisch E, Dressler A, Menanteau-Ledouble S, Saleh M, El-Matbouli M (2014) Francisellosis in ornamental African cichlids in Austria. Bull Eur Assoc Fish Pathol 34:63–70

Mauel MJ, Miller DL, Frazier K, Liggett AD, Styer L, Montgomery-Brock D, Brock J (2003) Characterization of a piscirickettsiosis-like disease in Hawaiian tilapia. Dis Aquat Org 53:249–255

Nguyen VV, Dong HT, Senapin S, Pirarat N, Rodkhum C (2016) *Francisella noatunensis* subsp. *orientalis*, an emerging bacterial pathogen affecting cultured red tilapia (*Oreochromis* sp.) in Thailand. Aquacult Res (in press) doi:10.1111/are.12802

Nylund A, Ottem KF, Watanabe K, Karlsbakk E, Krossøy B (2006) *Francisella* sp. (Family *Francisellaceae*) causing mortality in Norwegian cod (*Gadus morhua*) farming. Arch Microbiol 185:383–392

Ostland VE, Stannard JA, Creek JJ, Hedrick RP, Ferguson HW, Carlberg JM, Westerman ME (2006) Aquatic *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* × *M. saxatilis*. Dis Aquat Org 72:135–145

Ottem KF, Nylund A, Karlsbakk E, Friis-Møller A, Krøssoy B (2007) Characterization of *Francisella* sp., GM 2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*). Arch Microbiol 187:343–350

Pandit NP, Shrestha MK, Yi Y, Diana JS, Lin JS (2004) Polyculture of grass carp and Nile tilapia with napier grass as the sole nutrient input in the subtropical climate of Nepal. In: Bolivar R, Mair G, Fitzsimmons K (eds) Proceedings of the 6th International Symposium on Tilapia in Aquaculture, p 558–573

Ramírez Paredes JG (2015) The fish pathogen *Francisella orientalis*: characterisation and vaccine development. PhD thesis, University of Stirling

Soto E, Hawke JP, Fernandez D, Morales JA (2009a) *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica. J Fish Dis 32:713–722

Soto E, Fernandez D, Hawke JP (2009b) Attenuation of the fish pathogen *Francisella* sp. by mutation of the iglC* gene. J Aquat Anim Health 21:140–149

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

Soto E, Illanes O, Hilchie D, Morales JA and others (2012b) Molecular and immunohistochemical diagnosis of *Francisella noatunensis* subsp. *orientalis* from formalin-fixed, paraffin-embedded tissues. J Vet Diagn Invest 24: 840–845

Soto E, McGovern-Hopkins K, Klinger-Bowen R, Fox BK and others (2013) Prevalence of *Francisella noatunensis* subsp. *orientalis* in cultured tilapia on the island of Oahu, Hawaii. J Aquat Anim Health 25:104–109

Vojtech LN, Sanders GE, Conway C, Ostland V, Hansen JD (2009) Host immune response and acute disease in a zebrafish model of *Francisella* pathogenesis. Infect Immun 77:914–925

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