Vol. 137: 125–130, 2019
https://doi.org/10.3354/dao03429

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published online December 19

Outbreak of francisellosis (*Francisella noatunensis* subsp. *orientalis*) in cultured neon jewel cichlids *Hemichromis bimaculatus* from Morelos, Mexico

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ABSTRACT: Francisellosis is a disease caused by different species of the bacterial genus *Francisella* and has been diagnosed in a wide variety of animals, including fish. Francisellosis in fish is characterized by the development of non-specific clinical signs as well as the presence of numerous granulomas in several organs (mainly spleen and kidney). Ten neon jewel cichlids *Hemichromis bimaculatus* were submitted for diagnosis from a farm located in Morelos, Mexico. Gross examination, wet preparations, cytology, histopathology and PCR were performed. Affected fish showed lethargy, erratic swimming, imbalance and gasping. At the post mortem examination, multiple granulomas were observed in the kidney and spleen. Microscopically, granulomatous inflammation was observed in several organs. Species-specific PCR assay using DNA from the affected tissues of *H. bimaculatus* as a template demonstrated the presence of *F. noatunensis* subsp. *orientalis* (*Fno*) by amplifying a hypothetical protein gene of the *Fno* species. The end diagnosis of francisellosis is important for Mexican ornamental aquaculture, since it is necessary to implement measures for treatment, prevention, control and diagnosis. This is the first report of francisellosis in the neon jewel cichlid.

KEY WORDS: *Francisella noatunensis* subsp. *orientalis* · Francisellosis · *Hemichromis bimaculatus* · Histopathology · PCR

1. INTRODUCTION

Francisellosis is a disease caused by different species of the genus *Francisella* and has been diagnosed in a wide variety of animals, including fish (Colquhoun et al. 2014). Currently, the genus *Francisella* contains several recognized species, including *F. noatunensis*, one of the most important and emerging pathogens in cultured fish. *F. noatunensis* is further divided into 2 subspecies, *F. noatunensis* subsp. *noatunensis* (*Fnn*) and *F. noatunensis* subsp. *orientalis* (*Fno*) (Birkbeck et al. 2011, Colquhoun & Duodu 2011). *Fnn* is associated with cold-water fish, whereas *Fno* is observed in warm-water fish (Colquhoun & Duodu 2011). *F. noatunensis* has been implicated as a cause of disease in several important production species and has been reported in the USA, UK, Ireland, Taiwan, Japan, Norway, Costa Rica, Brazil and Chile (Kamaishi et al. 2005, Hsieh et al. 2006, Ostland et al. 2006, Birkbeck et al. 2007, Mauel et al. 2007,

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Mikalsen et al. 2007, Soto et al. 2009b, Ellingsen et al. 2011, Ruane et al. 2015, Pulpipat et al. 2019). It has also been identified in different genera of ornamental cichlids (Hsieh et al. 2007, Lewisch et al. 2014). In Mexico, the first report of piscine francisellosis was in Nile tilapias *Oreochromis* spp. (Ortega et al. 2016), but there are no reports of this disease affecting ornamental species in this country. In farmed and wild fish, the most characteristic gross lesions are multifocal granulomas in the anterior kidney, posterior kidney, spleen and occasionally in gills, gastrointestinal walls, choroidal gland and mesenteric fat, as well as nephromegaly and splenomegaly (Hsieh et al. 2006, Ostland et al. 2006, Birkbeck et al. 2007, 2011, Mauel et al. 2007, Mikalsen et al. 2007, Müller et al. 2007, Soto et al. 2009a,b, Colquhoun & Duodu 2011). The aim of this report is to describe an outbreak of francisellosis in cultured neon jewel cichlids *Hemichromis bimaculatus* from Morelos, Mexico.

2. MATERIALS AND METHODS

A total of 120 neon jewel cichlids *Hemichromis bimaculatus* were purchased from a farm located in the community of Cuautitla in Tetecala, Morelos in November 2017 and moved to the farm where the outbreak later occurred, which is located nearby. Upon arrival at the new farm, fish were acclimated for 30 min and introduced into a clean tank measuring 2.7 × 4.6 × 0.65 m (8.0 m^3) filled with water (23.6°C and 5.7 mg l^{-1} dissolved oxygen) obtained from a deep well. A week later they began to die (1 fish every 3 d). Clinically, they showed lethargy, exophthalmia, distension of the coelomic cavity and hyporexia. Since water quality issues were suspected, it was decided to make a 100% water exchange per day and to apply potassium permanganate at a dose of 100.0 ml per 3000 l of water, diluted from a stock solution composed of 1 g of potassium permanganate and 4.0 l of water. After ca. 2 wk, accumulated mortality reached 116 fish (96.6%), and during this period, the water temperature of the tanks ranged between 22 and 24°C. Ten live fish showing clinical signs were sent to the Department of Pathology of the Faculty of Veterinary Medicine and Zootechnics of the National Autonomous University of Mexico for diagnosis. Upon arrival at the laboratory, each fish underwent a clinical examination and then euthanasia. Euthanasia was accomplished in a 3-step procedure. Fish were left in a solution of clove oil at a dose of 100 mg l^{-1} of water for a minimum of 10 min until cessation of opercular movement and loss of vestibulo-ocular reflex; thereafter cervical transection using a knife was performed; this was followed by pithing. Each individual was weighed, measured and sexed externally. A post mortem study was carried out on each fish and during this procedure, wet preparations of spleen, anterior kidney and posterior kidney were evaluated. Kidney and spleen imprints were also made and were stained with Diff-Quik and Gram stains. Samples of spleen, anterior kidney, posterior kidney, liver, stomach, intestines, gills, eye, brain, skeletal muscle and skin were fixed in 10% buffered formalin and processed according to the standard protocol for histological studies of fixed tissues. Sections of 3.0 µm thickness were cut, mounted on slides and stained with hematoxylin and eosin, Gram, and Ziehl-Neelsen stains. DNA extraction from paraffin blocks was performed using the commercial kit DNeasy Blood & Tissue (Qiagen) according to the manufacturer's instructions. The extracted DNA was used as a template for carrying out the PCR. For this, the FnoF1 (5'-GGC GTA ACT CCT TTT AGC TTC C-3') and FnoR1 (5'-TTA GAG GAG CTT GGA AAA GCA-3') oligonucleotides were used (Dong et al. 2016). They amplify a 203 bp fragment of a hypothetical protein of *Fno*. PCR reactions were performed in a final volume of 25 µl containing: 12.5 µl of TopTaq Master Mix (1.25 units TopTaq DNA polymerase, 1× PCR buffer with 1.5 mM $MgCl_2$, 200 µM of each dNTP), 2 µl of each oligonucleotide at a concentration of 25 pmol and 10 µl of the extracted DNA. The reaction conditions were the same as reported by Dong et al. (2016). DNA amplification was visualized and documented on a UVP UVsolo transilluminator (Analytik Jena). PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions and sequenced in both directions.

3. RESULTS

On gross examination, the affected fish showed exophthalmia, gill paleness, distension of the coelomic cavity, nephromegaly, splenomegaly as well as the presence of multiple nodular, white, firm, pinpoint lesions (nodules) in the anterior and posterior kidney and spleen which measured <1.0 mm in diameter (Fig. 1a–c). On wet preparations of the organs, these nodular lesions were consistent with granulomas, which were composed of a central core of necrosis surrounded by several cellular layers (Fig. 1d). Gram staining of spleen imprints revealed

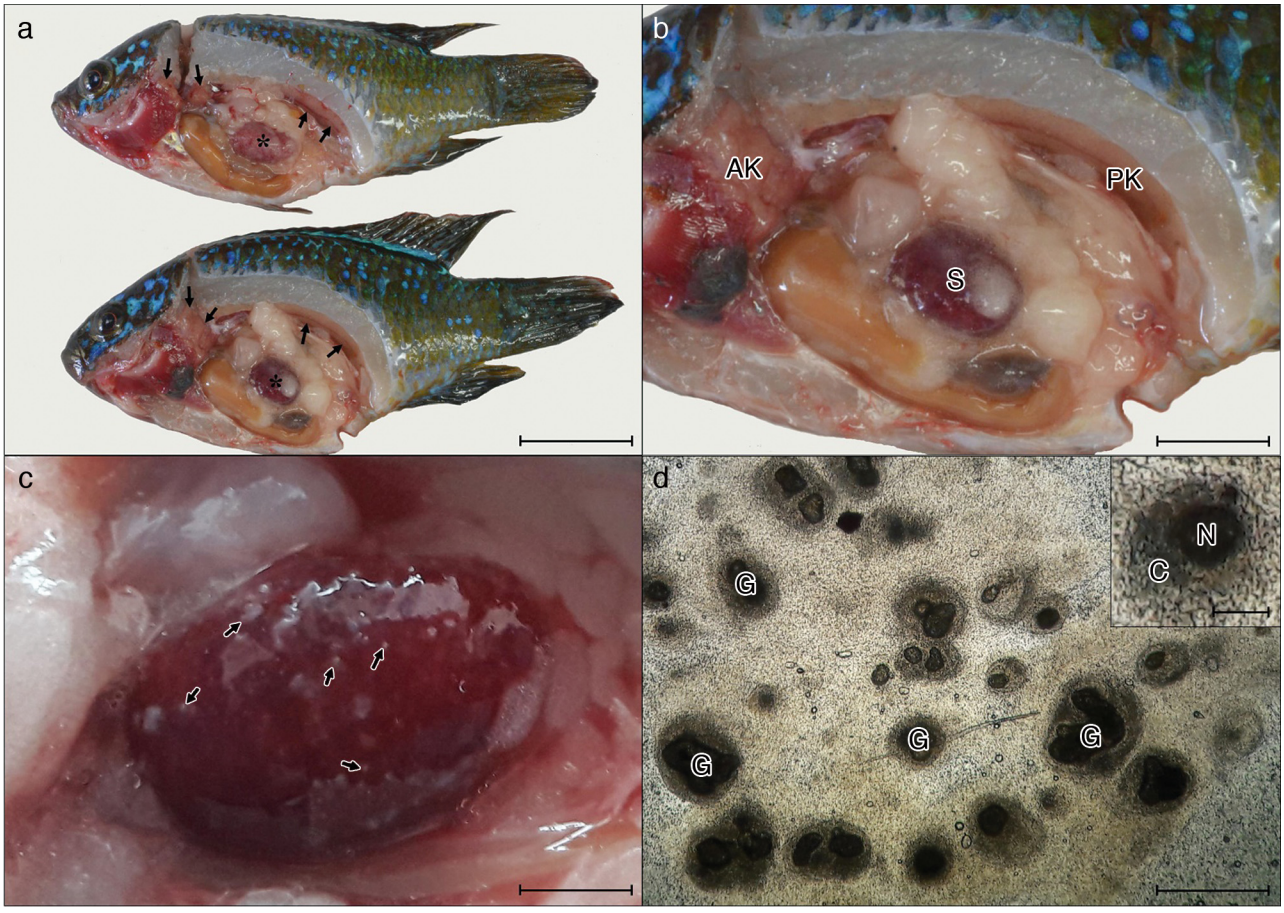

Fig. 1. Gross and sub-gross changes observed during post mortem examination of neon jewel cichlids *Hemichromis bimaculatus* with francisellosis. (a) Two fish showing marked anterior and posterior nephromegaly (arrows) and splenomegaly (asterisk). Scale bar = 2 cm. (b) One of the affected fish showing splenomegaly (S), anterior (AK) and posterior (PK) nephromegaly and marked tissue paleness. Scale bar = 1 cm. (c) Spleen, showing severe splenomegaly and multiple, variably sized, white nodules (granulomas) multifocally distributed (arrows). Scale bar = 5 mm. (d) Fragment of the affected spleen (crushed between 2 glass slides, viewed under a light microscope at low power field), with closed diaphragm showing multiple granulomas (G). Scale bar = 200 µm. Inset: detail of a granuloma, composed of a central core of necrosis (N) surrounded by several cellular layers (C). Scale bar = 50 µm

numerous Gram-negative, pleomorphic, intracellular and free bacteria (Fig. 2a). Histological evaluation of the gills, stomach, skeletal muscle, eye choroidal gland, spleen, anterior kidney, posterior kidney, liver and heart revealed the presence of granulomatous infiltrates and numerous granulomas, except within the brain, which was partially destroyed because of the method of euthanasia (pithing). Granulomas had a central area of necrosis surrounded by several layers of epithelioid macrophages, lymphocytes, plasma cells, as well as fibroblasts and collagen fibers. In some affected organs, small, pleomorphic coccobacilli were visible inside and outside the cells (Fig. 2b–f). No bacterial microorganisms were in the Ziehl-Neelsen stains. The 203 bp fragment corresponding to the hypothetical protein specific for *Fno* was amplified from the organs of all fish (Fig. 3). Although this fragment has been reported as being species-specific for *Fno* (Dong et al. 2016), BLAST analysis shows that this is also present in *Francisella philomiragia*. In order to avoid confusion and confirm the presence of *Fno*, the 203 bp PCR products (Fig. 3) were purified and sequenced in both directions. The sequences were submitted to a BLAST search of all available databases at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and showed 100% homology with *Fno* strains FNO190, FNO24, FNO12, FNO01 and F1, among others. As all the sequences were the same, we only submitted one to GenBank (accession number MN385384).

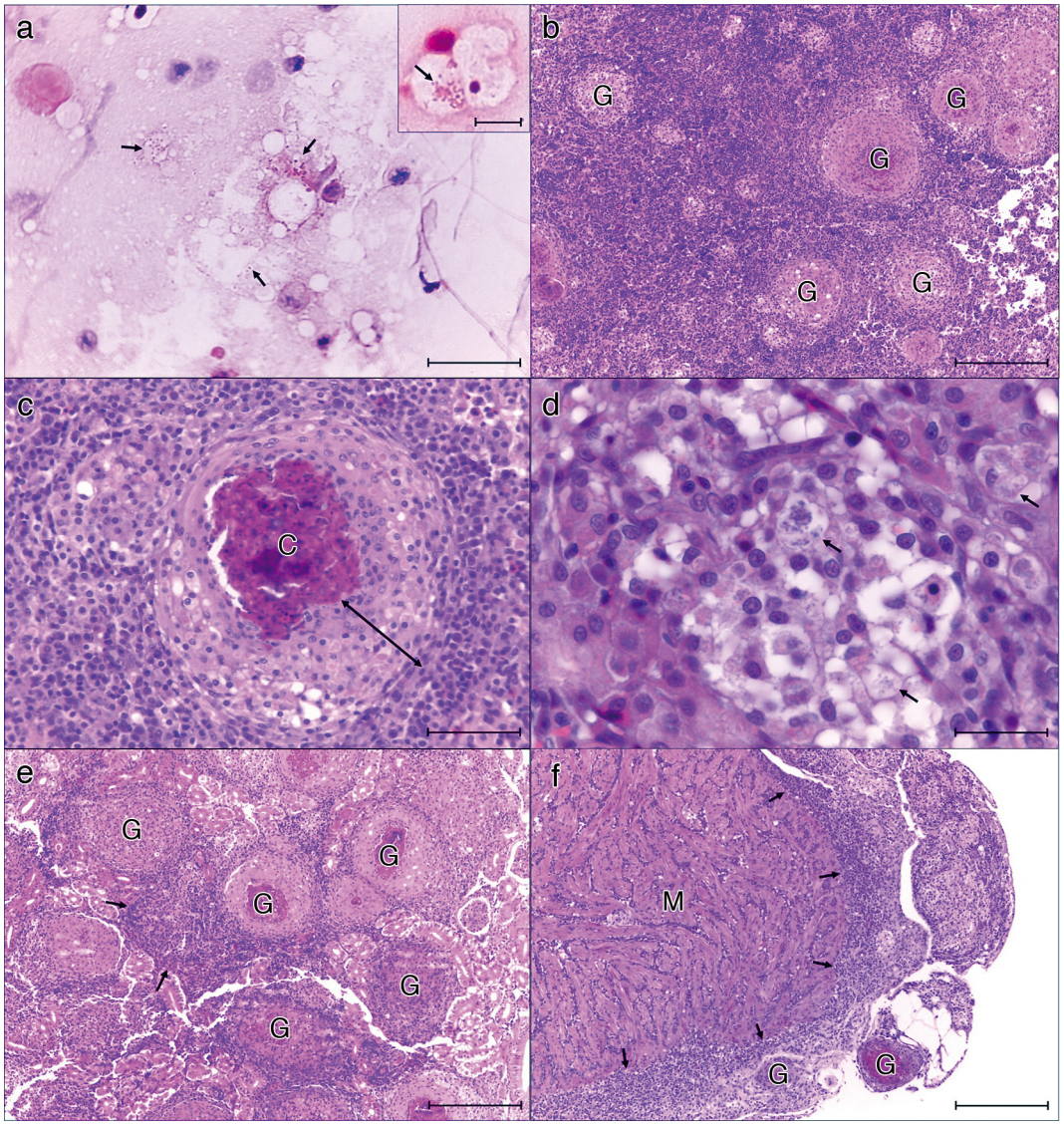

Fig. 2. Cytological and histological analysis (a: Gram stain; b–f: hematoxylin and eosin) of organs obtained from neon jewel cichlids *Hemichromis bimaculatus* with francisellosis. (a) Spleen imprints. Numerous Gram-negative, pleomorphic intracellular and extracellular coccobacilli (arrows). Scale bar = 20 µm. Inset: bacteria within macrophages (arrow). Scale bar = 5 µm. (b) Spleen. Numerous granulomas (G) involving the spleen. Scale bar = 200 µm. (c) Spleen. Detail of a granuloma, composed of a central core of necrosis (C) surrounded by inflammatory cells and fibroblasts (arrow). Scale bar = 50 µm. (d) Spleen. Numerous pleomorphic, intracellular and extracellular bacteria (arrows). Scale bar = 20 µm. (e) Posterior kidney. In the renal interstitium: a diffuse granulomatous reaction (arrows), as well as multiple granulomas (G) effacing the normal architecture. Scale bar = 200 µm. (f) Heart. On the myocardium (M) and epicardial surface: inflammatory infiltrate composed mainly of macrophages and lymphocytes (arrows). Multifocally, some granulomas are also observed (G). Scale bar = 200 µm

4. DISCUSSION

In fish, granulomatous lesions can be caused by a wide variety of bacteria, including *Mycobacterium* spp., *Nocardia* spp. and *Edwardsiella* spp., among others (Mohanty & Sahoo 2007, Colquhoun & Duodu 2011). The presumptive diagnosis of these diseases can be made through cytology (Reavill & Roberts 2007), where it is often easy to detect the type of agent involved through the use of routine and special histochemical stains. In the present study, the presence of pleomorphic, intracellular, Gram-negative bacteria in spleen imprints significantly reduces the range of possibilities, since these are not the morphologic or staining features of *Mycobacterium* spp., *Nocardia* spp. or *Edwardsiella* spp. In addition, there

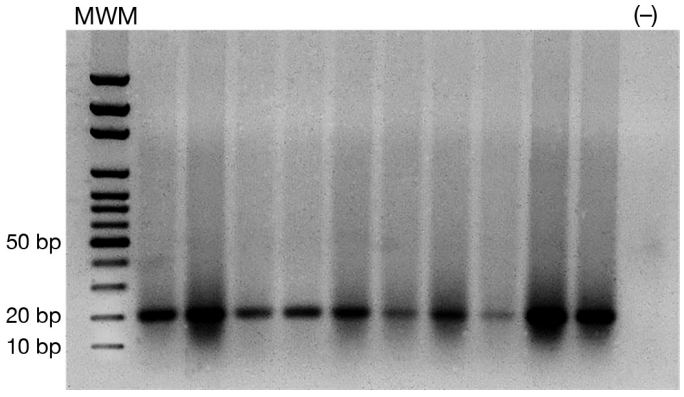

Fig. 3. PCR detection (1.5% agarose gel stained with SYBR Safe) of *Francisella noatunensis* subsp. *orientalis*. DNA obtained from paraffin-embedded tissues of 10 affected neon jewel cichlids *Hemichromis bimaculatus*. Each lane shows DNA amplification from each 1 of the 10 fish using FnoF1 and FnoR1 oligonucleotides (Dong et al. 2016), which amplify a 203 bp fragment of a hypothetical protein of *F. noatunensis* subsp. *orientalis*. MWM: 100 bp molecular weight marker; (–): negative control

was a previous report of francisellosis in tilapias from Central Mexico (Ortega et al. 2016), where the state of Morelos is located, and so our diagnosis was oriented towards the genus *Francisella*. The clinical signs described in the present study, although nonspecific, are similar to those reported previously for francisellosis (Hsieh et al. 2006, 2007, Mauel et al. 2007, Soto et al. 2009b, Ortega et al. 2016), which were mostly characterized by lethargy and distension of the coelomic cavity. In some outbreaks of francisellosis in tilapias, there have been mortality rates of up to 90% (Chern & Chao 1994, Mauel et al. 2005, Soto et al. 2009b, Colquhoun & Duodu 2011), and compared with those references, mortality rate was higher in the present study (96.6%), and even higher than the mortality rate of 40% reported in Mexican tilapias (Ortega et al. 2016). Given the high accumulated mortality in the present study, the rest of the fish (3.4%) were euthanized to avoid transmission of this disease to other fish within the farm. Interestingly, no other fish of the farm were affected during or after the outbreak. Gross findings observed in the affected neon jewel cichlids are in agreement with those reported previously, and one of the most important lesions described is the presence of multiple whitish nodules that can be found in several organs, mainly spleen and kidney (Chern & Chao 1994, Mauel et al. 2005, Hsieh et al. 2006, Mauel et al. 2007, Soto et al. 2009b, Ortega et al. 2016). In the present study, these nodules were only observed grossly in the spleen and the anterior and posterior kidney. Histological changes were consistent with those described previously and are characterized by the presence of granulomatous inflammation within several organs (Chern & Chao 1994, Mauel et al. 2005, Hsieh et al. 2006, Mauel et al. 2007, Soto et al. 2009b, Birkbeck et al. 2011, Ortega et al. 2016). Necrotizing vasculitis and thrombosis of the liver have been previously reported, but they were not observed in the necropsied fish in the present study (Mauel et al. 2007, Colquhoun & Duodu 2011, Ortega et al. 2016). It has been determined that water temperature is a risk factor for the development of this disease, and the highest mortality rates occur between 21.5 and 26.3°C (Mauel et al. 2005, Mauel et al. 2007). During the present outbreak, water temperature ranged between 22 and 24°C, and this, along with the stress caused by the 100% water exchange per day, could have contributed to the development of the disease. PCR has been widely used by other authors to detect francisellosis in aquaculture (Hsieh et al. 2006, Mauel et al. 2007, Soto et al. 2009b); in the present study, the presence of *Fno* was confirmed in all the affected fish using both PCR and sequencing.

In conclusion, *Fno* was identified as the causal agent responsible for high mortality of cultured neon jewel cichlids *Hemichromis bimaculatus* in an ornamental fish farm located in Morelos, Mexico. Clinical history, physical examination, gross lesions, cytology, histopathology and molecular analysis were consistent with that previously described in francisellosis in other fish.

Acknowledgements. We thank Karen Janeth Guitareo Quintana and Maribel Nieto Miranda for tissue processing.

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Editorial responsibility: David Verner-Jeffreys, Weymouth, UK

Submitted: April 23, 2019; Accepted: October 10, 2019
Proofs received from author(s): December 16, 2019