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Note

Molecular evidence of *Francisella noatunensis* sub sp. *orientalis* in *Oreochromis niloticus* in Yunnan Province, south-western China

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ABSTRACT

Francisella sp. is an emerging bacterial pathogen affecting tilapia *Oreochromis niloticus.* This study surveyed presence of *Francisella* sp. in tilapia raised in Yunnan Province, south-western China along with water quality parameters and management practices. A total of 224 tilapia specimens were sampled from 28 farms between June and September 2017. Necropsy of the sampled fish revealed no granulomatous lesions and the standard bacterial isolation protocols proved negative for *Francisella* sp. However, the presence of *Francisella* sp. was confirmed by polymerase chain reaction in 18 samples (8%) from 8 of the 28 farms (28.6%) sampled, though no increasing mortality was reported from these farms. The 16S ribosomal RNA gene sequences revealed 99.7% identity with *F. noatunensis* sub sp. *orientalis*. The presence of *Francisella* sp. infection showed significant association with water temperature.

Keywords: *Francisella* sp., Tilapia, Water quality, Yunnan Province

Francisellosis is an emerging disease caused by Gram negative, intracellular, non-motile, strictly aerobic, coccobacillus bacteria of the genus *Francisella*. *Francisella noatunensis* is responsible for the outbreaks of piscine Francisellosis in cultured and wild aquatic animals. Francisellosis associated with *F. noatunensis* sub sp. *noatunensis* (*Fnn*) and *F. noatunensis* sub sp. *orientalis* (*Fno*) have been diagnosed in both coldwater fish such as Atlantic cod (*Gadus morhua* L.) and warm water fish such as tilapia (*Oreochromis* spp.) (Olsen *et al.*, 2006; Soto *et al.*, 2009). Francisellosis in tilapia is a chronic disease with high morbidity and mortality (>90%) (Chern *et al.*, 1994). It is recognised to cause significant economic losses in aquaculture globally (Nguyen *et al.*, 2016). No clinical signs normally are observed with Francisellosis in tilapia. Necropsy of infected fish mainly exhibited granulomas in internal organs including liver, spleen, kidney and gill (Soto *et al.*, 2009). However, the absence of nodules or granulomas did not exclude the possibility of *Francisella* sp. infection in fish (Rodrigues *et al.*, 2018). The outbreaks of Francisellosis caused by *Fno* in tilapia have been reported throughout the world (Colquhoun *et al.*, 2011). Tilapia is one of the most important farmed fish species globally and China stands on top in production of cultured tilapia (FAO, 2014). The main tilapia culture areas in China are Guangdong, Guangxi, Hainan and Fujian provinces (Chen *et al.*, 2012). Tilapia is also one of the most important freshwater species raised in Yunnan Province in south-western China. Production of tilapia in Yunnan Province in 2016 stood over 180,000 t, which accounted for over 10% of the total tilapia production in China. *Francisella* sp. infection has rarely been reported in China, with no diagnoses reported in Yunnan Province. Therefore, the aim of this research was to survey *Francisella* sp. in tilapia raised in Yunnan Province and to investigate the potential associations between *Francisella* sp. infection, management practices and water quality parameters.

A total of 224 tilapia specimens (*Oreochromis* spp.) were collected from 28 farms located in 4 different cities in Yunnan Province, from June to September 2017. Earthen pond production predominated in Pu'er and Xishuangbanna cities and cage farm production was mostly found in Wenshan City. The sampling locations and number are shown in Fig. 1 and Table 1. Water temperature and dissolved oxygen at 0.5 m under the surface were determined using a hand held DO meter (HACH®, Colorado, USA). Water pH, ammonia and nitrite concentration were measured using digital meter (*Octadem* Technology Co. Ltd., Wuxi, China). Fish were anesthetised and examined for external clinical signs of disease and placed on ice in plastic boxes to transport to the laboratory of Yunnan Agricultural University. Necropsies were performed to look for gross pathology of internal organs. Kidney and spleen were aseptically collected for bacterial isolation and molecular diagnosis.

Table 1. Distribution of *Francisella* sp. infection in Yunnan Province, south-west China

Location	No. of farms	Percentage of positive fish	Percentage of positive farms
Xishuangbanna	7	8.9 (5/56)	28.6 (2/7)
Pu'er	12	12.5 (12/96)	41.7 (5/12)
Wenshan	8	1.6 (1/64)	12.5 (1/8)
Kunming	1	0 (0/8)	0 (0/1)
Total	28	8.0 (18/224)	28.6 (8/28)

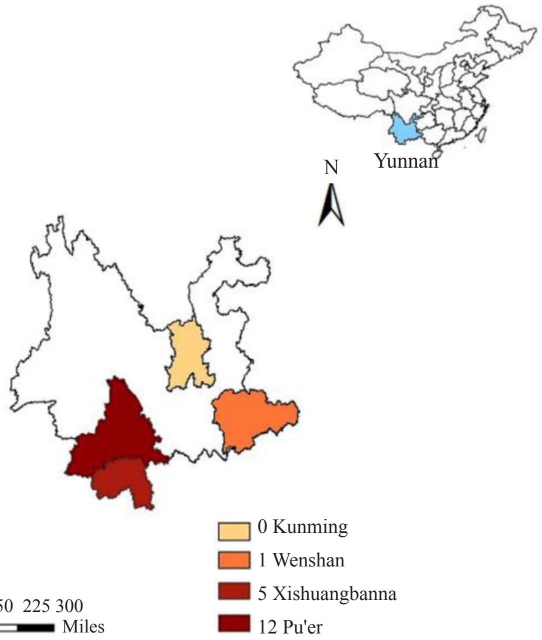

Fig. 1. Geographic distribution of *Francisella* sp. infection in Yunnan Province, China

Kidney and spleen tissues were homogenised in 500 µl phosphate buffered saline (pH 7.4) and homogenates were smeared on Cystine Heart Agar (CHA) (Himedia, India) plates with 2% (w/v) bovine hemoglobin and 100 unit ml^{-1} polymixin B (Sigma-Aldrich, USA). Plates were incubated at 28^{0}C for 2-5 days. No *Francisella* sp. was isolated through standard bacterial culture. Previous studies also reported that bacteriological culture did not identify subclinical infected fish while approximately 11% of fish were positive to *Fno* by qPCR (Assis *et al.*, 2017).

Genomic DNA was extracted from spleen samples using a DNA extraction kit (Takara Biotech Co. Ltd., Dalian, China). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using *Francisella* specific primers (F11: 5' TAC CAG TTG GAA ACG ACT GT 3' and F5 5' CCT TTT TGA GTT TCG CTC C-3') described by Forsman *et al.* (1994). The 25 µl reaction comprised 0.2 µM of each primer, 2x TransHiFi Super Mix (TransGen Biotech Co. Ltd., Beijing, China), approximately 200 ng of template and nuclease-free water to adjust the volume to 25 µl. Reaction conditions comprised of an initial denaturation of 5 min at 94^{0}C, followed by 35 cycles of 30 s at 94^{0}C, 60 s at 55^{0}C and 60 s at 72^{0}C, with a final extension of 10 min at 72^{0}C. The PCR products were subjected to electrophoresis on a 1.5% (w/v) agarose gel, stained with ethidium bromide and visualised under fluorescence with an expected band of 1140 bp (Fig. 2). The bands specific to PCR products were excised from the gels and DNA fragments were recovered using gel extraction kit (Sangon Biotech Co. Ltd., Shanghai, China). Recovered DNA fragments were linked to the pMD18T vector (Takara Biotech Co. Ltd., Dalian, China) and transformed to competent *E. coli* cells following standard protocols. Plasmid purification was done using kit from Takara Biotech Co. Ltd., Dalian, China) and sequenced (Sangon Biotech Co. Ltd., Shanghai, China). Sequences obtained were subjected to similarity search using BLAST in NCBI GenBank 16S rRNA nucleotide sequence database which showed 99.7% identity with *F. noatunensis* sub sp. *orientalis* (*Fno*) sequences obtained from tilapia in Guangdong in China, USA, Brazil, United Kingdom and Costa Rica (Soto *et al.*, 2009; Jeffery *et al.*, 2010; Leal *et al.*, 2014; Lin *et al.*, 2016). The partial 16S rRNA sequence was submitted in GenBank with Accession no. MH470274. A phylogenetic tree was constructed by the neighbor-joining method in Molecular Evolutionary Genetic Analysis (MEGA) (version 7.0) package (Kumar *et al.*, 2016) (Fig. 3). *Francisella* sp. was identified in eighteen samples, isolated from eight tilapia farms which accounted for 28.6% of the farms sampled. *Francisella* sp. infected farms are located in Pu'er, Xishuangbanna and Wenshan cities (Table 1). The results demonstrated that molecular diagnosis could be an appropriate technique for identification, monitoring and surveillance of this pathogen (Rodrigues *et al.*, 2018).

Tilapia cultured in earthen ponds located at Kunming, Pu'er and Xishuangbanna were introduced from Hainan Province, while most tilapia cultured in cages located at Wenshan City were introduced from Guangxi Province. Fingerlings and fry introduced into Yunnan Province

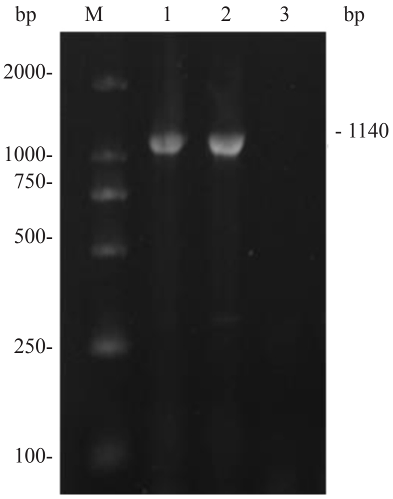

Fig. 2. Agarose gel image of PCR amplification product (1140 bp) of 16S rRNA gene from *Francisella* sp. isolated from cultured tilapia (Lanes 1 and 2). Lane M: 2.0 kbp DNA ladder; Lane 3: Negative control

from other provinces, which may represent the sources of transmission of *Francisella* sp. (*Fno*) in this province. Previous researchers have demonstrated both horizontal and vertical transmission for *Francisella* sp. (Soto *et al.*, 2013; Pradeep *et al.*, 2017).

The water quality parameters recorded are summarised in Table 2. Chi-square test using the R software (version 3.5) was performed to determine the correlation between water quality parameters and presence of *Francisella* sp. in tilapia. The lower the water temperature (<25^0C), the more likely the occurrence of *Francisella* sp. (*Fno*) in Yunnan Province (χ^2=31.1, $p<$ 0.05). Higher *Francisella* sp. infection was observed in Pu'er and Xishuangbanna City where water temperature was below to 25^0C. No significant association was identified with other water quality parameters and *Francisella* sp. (*Fno*) infection in tilapia during the present study. Soto *et al.* (2012) reported that water temperature significantly affected the outbreak of *Fno*, while water salinity did not play a significant role.

The present study investigated *Francisella* sp. infection in tilapia for the first time in south-western China. Identification of the infection combined with the rapid expansion of tilapia culture in the region warrants the need for active surveillance and control strategies to prevent the occurrence of this economically important disease outbreak due to *Fno* infection in tilapia. Further research on virulence mechanisms and virulent factors, immune response and prevention strategies as well as treatment techniques are also needed to plan strategies for effective control of this emerging pathogen.

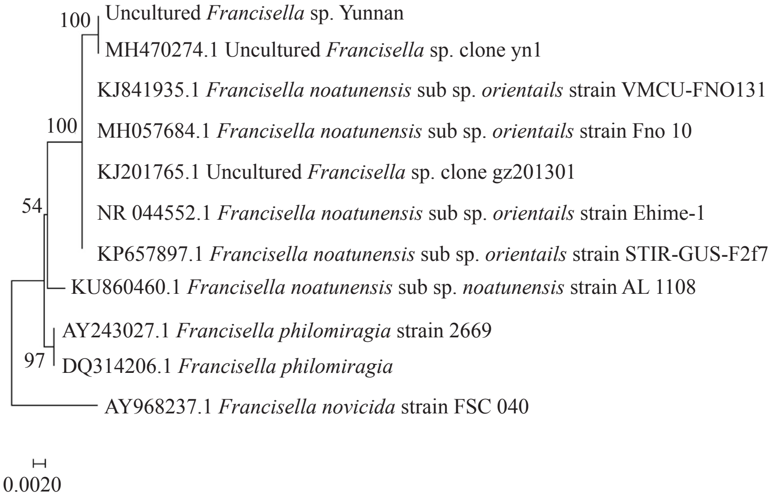

Fig. 3. Phylogenetic tree constructed based on 16S rRNA gene partial sequences from *Francisella* spp. using neighbor-joining method

Table 2. Water quality parameters (Mean±SD) of different study locations in Yunnan Province, South-western China

Location	Temperature (^{0}C)	Dissolved oxygen (mg l^{-1})	pH	Ammonia-N (mg l^{-1})	Nitrite-N (mg l^{-1})
Xishuangbanna	26.00 ± 0.82	8.59 ± 2.45	8.07 ± 0.73	2.68 ± 1.51	0.06 ± 0.07
Pu'er	25.54 ± 0.67	5.02 ± 1.09	7.20 ± 0.21	1.81 ± 0.80	0.09 ± 0.18
Wenshan	30.13 ± 1.13	7.54 ± 0.62	8.21 ± 0.15	0.31 ± 0.12	0.01 ± 0.00
Kunming	26.5	6.8	7.6	0.6	0.05

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References

Assis, G. B. N., de Oliveira, T. F., Gardner, I. A., Figueiredo, H. C. P. and Leal, C. A. G. 2017. Sensitivity and specificity of real-time PCR and bacteriological culture for francisellosis in farm-raised Nile tilapia (*Oreochromis niloticus* L.). *J. Fish Dis.,* 40(6): 785-795. doi:10.1111/jfd.12559.

Chen, M., Li, L. P., Wang, R., Liang, W. W., Huang, Y., Li, J., Lei, A. Y., Huang, W. Y. and Gan, X. 2012. PCR detection and PFGE genotype analyses of streptococcal clinical isolates from tilapia in China. *Vet. Microbiol.,* 159(3-4): 526-530. doi:10.1016/j.vetmic.2012.04.035.

Chern, R. S. and Chao, C. B. 1994. Outbreaks of a disease caused by rickettsia-like organism in cultured tilapias in Taiwan. *Fish Pathol., 29*(2): 61-71.

Colquhoun, D. J. and Duodu, S. 2011. *Francisella* infections in farmed and wild aquatic organisms. *Vet. Res., 42*: 47. doi:10.1186/1297-9716-42-47.

FAO 2014. *The state of world fisheries and aquaculture.* Food and Agriculture Organisation, Rome, Italy, 243 pp.

Forsman, M., Sandstrom, G. and Sjostedt, A. 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilisation for determination of the phylogeny of the genus and for identification of strains by PCR. *Int. J. Syst. Bacteriol.,* 44(1): 38-46. doi:10.1099/00207713-44-1-38.

Jeffery, K. R., Stone, D., Feist, S. W. and Verner-Jeffreys, D. W. 2010. An outbreak of disease caused by *Francisella* sp. in Nile tilapia *Oreochromis niloticus* at a recirculation fish farm in the UK. *Dis. Aquat. Org.,* 91(2): 161-165. doi:10.3354/dao02260.

Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis ver. 7.0 for bigger datasets. *Mol. Biol. Evol., 33*(7): 1870-1874.

Leal, C. A. G., Tavares, G. C. and Figueiredo, H. C. P. 2014. Outbreaks and genetic diversity of *Francisella noatunensis* sub sp. *orientalis* isolated from farm-raised Nile tilapia (*Oreochromis niloticus*) in Brazil. *Genet. Mol. Res.,* 13(3): 5704-5712. doi:10.4238/2014.July.25.26.

Lin, Q., Li, N. Q., Fu, X. Z., Hu, Q. D., Chang, O. Q., Liu, L. H., Zhang, D. F., Wang, G. J., San, G. B. and Wu, S. Q. 2016. An outbreak of granulomatous inflammation associated with *Francisella noatunensis* sub sp. *orientalis* in farmed tilapia (*Oreochromis niloticus* x *O. aureus*) in China. *Chin. J. Oceanol. Limnol.,* 34(3): 460-466. doi:10.1007/s00343-016-4311-2.

Nguyen, V. V., Dong, H. T., Senapin, S., Pirarat, N. and Rodkhum, C. 2016. *Francisella noatunensis* sub sp. *orientalis*, an emerging bacterial pathogen affecting cultured red tilapia (*Oreochromis* sp.) in Thailand. *Aquac. Res.,* 47(11): 3697-3702. doi:10.1111/are.12802.

Olsen, A. B., Mikalsen, J., Rode, M., Alfjorden, A., Hoel, E., Straum-Lie, K., Haldorsen, R. and Colquhoun, D. J. 2006. A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. *J. Fish Dis.,* 29(5): 307-311. doi:10.1111/j.1365-2761.2006. 00714.x.

Pradeep, P. J., Suebsing, R., Sirithammajak, S., Kampeera, J., Turner, W., Jeffs, A., Kiatpathomchai, W. and Withyachumanarnkul, B. 2017. Vertical transmission and concurrent infection of multiple bacterial pathogens in naturally infected red tilapia (*Oreochromis* spp.). *Aquac. Res.,* 48(6): 2706-2717. doi:10.1111/are.13102.

Rodrigues, M. V., Francisco, C. J., David, G. S., da Silva, R. J., Falcone-Dias, M. F. and Araujo, J. P. 2018. Monitoring of *Francisella noatunensis* sub sp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus*) in Brazil. *Aquac. Int.,* 26(1): 127-138. doi:10.1007/s10499-017-0204-4.

Soto, E., Abrams, S. B. and Revan, F. 2012. Effects of temperature and salt concentration on *Francisella noatunensis* sub sp. *orientalis* infections in Nile tilapia *Oreochromis niloticus*. *Dis. Aquat. Org.,* 101(3): 217-223. doi:10.3354/dao02533.

Soto, E., Hawke, J. P., Fernandez, D. and Morales, J. A. 2009. *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica. *J. Fish Dis.,* 32(8): 713-722. doi:10.1111/j.1365-2761.2009.01070.x.

Soto, E., Kidd, S., Mendez, S., Marancik, D., Revan, F., Hiltchie, D. and Camus, A. 2013. *Francisella noatunensis* sub sp. *orientalis* pathogenesis analyzed by experimental immersion challenge in Nile tilapia, *Oreochromis niloticus* (L.). *Vet. Microbiol.,* 164(1-2): 77-84. doi:10.1016/j.vetmic.2013.01.024.

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