

Characterization of *Francisella* species isolated from the cooling water of an air conditioning system

Quan Gu¹, Xunde Li³, Pinghua Qu², Shuiping Hou¹, Juntao Li¹,
Edward R. Atwill³, Shouyi Chen¹

¹Guangzhou Center for Disease Control and Prevention, Guangzhou, China.

²Guangdong Hospital of Traditional Chinese Medicine, University City Branch, Guangzhou, China.

³Department of Population Health and Reproduction, University of California, California, USA.

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Abstract

Strains of *Francisella* spp. were isolated from cooling water from an air conditioning system in Guangzhou, China. These strains are Gram negative, coccobacilli, non-motile, oxidase negative, catalase negative, esterase and lipid esterase positive. In addition, these bacteria grow on cysteine-supplemented media at 20 °C to 40 °C with an optimal growth temperature of 30 °C. Analysis of 16S rRNA gene sequences revealed that these strains belong to the genus *Francisella*. Biochemical tests and phylogenetic and BLAST analyses of 16S rRNA, *rpoB* and *sdhA* genes indicated that one strain was very similar to *Francisella philomiragia* and that the other strains were identical or highly similar to the *Francisella guangzhouensis* sp. nov. strain 08HL01032 we previously described. Biochemical and molecular characteristics of these strains demonstrated that multiple *Francisella* species exist in air conditioning systems.

Key words: *Francisella*, air conditioning, water.

Introduction

Francisella is a genus of Gram-negative, coccobacilli, non-motile and aerobic bacteria (Pechous *et al.*, 2009). Currently, five species have been recognized in the genus: *F. tularensis*, *F. philomiragia*, *F. noatunensis*, *F. hispaniensis* and *F. haliotica* (Brevik *et al.*, 2011; Francis, 1922; Hollis *et al.*, 1989; Huber *et al.*, 2009; Ottem *et al.*, 2009). *F. tularensis* was the first *Francisella* species to be discovered in 1912 in the USA (McCoy *et al.*, 1912) and is the pathogen that causes tularemia in the northern hemisphere. The other species were frequently isolated in the environment or in marine fish with unknown pathogenicity to humans. One of the most common methods for identification of *Francisella* species is 16S rRNA gene sequencing (Barns *et al.*, 2005; Kreizinger *et al.*, 2013; Sjöstedt *et al.*, 1997). In addition to the 16S rRNA gene, some housekeeping genes, such as the RNA polymerase subunit β (*rpoB*) gene and the succinate dehydrogenase A (*sdhA*) gene, have

been utilized in phylogenetic analysis of *Francisella* (Barns *et al.*, 2005).

It is recognized that the aerosol generated by air conditioning systems is one medium of transmission of respiratory pathogens, such as *Legionella pneumophila* (Stout and Yu, 1997). There is a knowledge gap regarding the existence of bacterial species in the cooling water of air conditioning systems. In 2008, we isolated the first strain of *Francisella* sp. (ID 08HL01032) (NCTC 13503) from the cooling water of an air conditioning system in Guangzhou, China (Qu *et al.*, 2009), which was the first report of *Francisella* spp. isolated from air conditioning cooling water. This strain was named *Francisella guangzhouensis* sp. nov. (Qu *et al.*, 2013). Between 2009 and 2011, nine additional *Francisella* spp. strains were isolated from the cooling water of air conditioning systems in the same city. Herein, we describe the biochemical characteristics and multiple gene fingerprinting of these strains, which demonstrate the presence of multiple species of *Francisella* and the consistent presence of *Francisella guangzhouensis* sp. nov. in the

cooling water of air conditioning systems in Guangzhou, China.

Materials and Methods

Isolation of bacteria

A total of 312 water samples were collected from cooling towers of air conditioning systems in metro stations and hotels in Guangzhou, China and were used for the detection of bacterial pathogens. Samples were collected between 2009 and 2011 as a part of the city's continuous surveillance program conducted by the Guangzhou Center for Disease Control and Prevention (GZCDC). Samples were stored at 4 °C and processed in less than 3 days after sampling. Previously described methods were used for isolation of *Francisella* spp. (Qu *et al.*, 2009). Briefly, water samples (250 mL each) were filtered through 0.22 µm polycarbonate filters. Filters with filtrate were suspended in 5.0 mL of sterilized water in a 50 mL centrifuge tube and treated with an equal volume of acid buffer (pH 2.2) for 5 min at room temperature. Then, 0.1 mL of the acidified suspension was placed onto Legionella-BCYE α -agar or BCYE α -GVPC (BCYE α supplemented with 3 g glycine L⁻¹, 80,000 IU polymyxin B sulfate L⁻¹, 1 mg vancomycin L⁻¹ and 80 mg cycloheximide L⁻¹) and incubated for 7 days at 30 °C under an atmosphere of 5% CO₂. Suspected colonies were inoculated onto BCYE α cysteine heart agar supplemented with 9% heated (chocolatized) sheep red blood cells (CHAB) and Columbia media agar, respectively, and incubated for 5 days under the same conditions as above. The BCYE α and CHAB media were cysteine supplemented and suitable for the growth of *Francisella* spp. (Petersen *et al.*, 2009).

Phenotypic characteristics

Bacterial colonies were isolated from BCYE α plates after 3-5 days of cultivation. Colonies of each strain were stained with Gram stain and examined with a bright field microscope (1000x). To determine the most suitable growth temperatures, a colony of each strain was inoculated into brain heart infusion broth (BHI) with L-cysteine and incubated at 20, 25, 30, 35 and 40 °C for 12 h (Qu *et al.*, 2009). The bacterial concentrations after incubation at each temperature were measured by a spectrophotometer at 600 nm.

Biochemical characteristics

Colonies isolated from BCYE α plate after 3 days of cultivation were used for biochemical tests. Oxidase and catalase activity were tested using a BioMerieux API 20STREP kit and Vitek 2 (GN card) kit (BioMerieux). Other biochemical and enzymatic activities were tested by using API ZYM (BioMérieux) kit. Table 1 shows all of the

biochemical tests performed using the three kits and the obtained results.

Molecular analysis

DNA was extracted from bacteria using a Qiagen DNA mini kit according to the manufacturer's manual. DNA concentrations were measured by a spectrophotometer at A260/A280, and those with concentrations between 1.7 and 2.1 were used as templates for PCR to amplify 16S rRNA, *rpoB*, and *sdhA* genes. For PCR of the 16S rRNA gene, approximately 1500 bp were amplified using the primers 27F and 1492R, as described by Lane (1991). Each 50 µL PCR reaction mixture included 10 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 0.1 M of each primer, 2.5 U *Taq* (TAKARA) and 1 µg of genome DNA template. PCR cycles consisted of denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 65 °C for 40 s, and 72 °C for 90 s, and extension at 72 °C for 5 min. For PCR of the *rpoB* gene and *sdhA* gene, the primers used were RL1 and RL2 for the *rpoB* gene (Ko *et al.*, 2002) and *sdhF* and *sdhR* for the *sdhA* gene (Barns *et al.*, 2005). The PCR reaction mixture and conditions for both of these genes were identical to those used for the 16S rRNA gene. PCR cycles consisted of denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 40 s, and 72 °C for 90 s, and extension at 72 °C for 5 min. The *rpoB* primers amplified a 330 bp product, and the *sdhA* primers amplified a 350 bp product. For all PCR experiments, DNA extracted from *F. philomiragia* (ATCC 25015) was used as positive control and sterilized water was used as negative control. PCR products were verified by electrophoresis on 2% agarose gel stained with ethidium bromide.

The PCR products of 16S rRNA, *rpoB* and *sdhA* genes were sequenced by the Invitrogen Laboratory in Guangzhou, China, using the same primers as above. DNA sequences were analyzed using the SeqScanner 1.0 software, and the bases with a QV value less than 20 were deleted. Sequences were analyzed by multiple alignment analysis using Clustal X (Goujon *et al.*, 2010). Sequences of currently available *Francisella* type species in GenBank were used to construct phylogenetic trees that were inferred using neighbor-joining analysis by the MEGA 5.0 software (Tamura *et al.*, 2011). Bootstrap values were determined using 1,000 replicates, and *Legionella pneumophila* was used as out-group. For each gene, sequences were analyzed to match the maximal identical sequence by BLAST analysis using NCBI's online BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Results and Discussion

Nine strains of *Francisella* spp. bacteria (coded GZ-01 to GZ-09) were isolated from 312 air conditioning cooling water locations in the city of Guangzhou. The 9 strains

Table 1 - Comparison of biochemical characteristics of *Francisella* sp. strains, *F. guangzhouensis* sp. nov. (NCTC 13503), and *F. philomiragia* (ATCC25015).

Biochemical test	<i>Francisella</i> spp. ^a	<i>Francisella</i> sp. (GZ-04)	<i>F. philomiragia</i> (ATCC25015)	<i>F. guangzhouensis</i> sp. nov. (NCTC 13503) ^b
Oxidase	-	-	+	-
Catalase	W	-	W	W
Urease	-	-	-	-
Cysteine for growth	+	+	-	+
PYRA	-	+	+	-
αGAL	-	+	+	-
βGUR	-	+	+	-
βGAL	-	+	+	-
PAL	-	+	+	-
LAP	-	+	+	-
RIB	+	+	+	+
Alkaline phosphatase	-	+	+	-
Esterase	+	+	+	+
Esterase lipase	+	+	+	+
Leucine arylamidase	-	+	+	-
Valine arylamidase	-	+	+	-
Acid phosphatase	+	+	+	+
Naphthol phosphohydrolase	+	+	+	+
α-galactosidase	-	+	+	-
β-galactosidase	-	+	+	-
Chitinase	-	+	+	-

^a*Francisella* sp. contains all strains except for GZ-04; +: positive reaction; -: negative reaction; W: weak reaction.

^b*F. guangzhouensis* sp. nov. previously isolated from cooling water of air conditioning system in Guanzhou (Qu *et al.*, 2009; Qu *et al.*, 2013).

were isolated from different cooling towers, except for GZ-02 and GZ-07, which were collected from the same cooling tower but from different samplings. All of these strains were Gram-negative coccobacillus (Gram stain images not shown). Bacteria of all strains grew on BCYE α and CHAB media, as well as on BHI with L-cysteine, but not on other media without L-cysteine, such as Columbia agar. Colonies of all strains grown on BCYE α medium were slightly ivory, round, convex and approximately 0.5-1.0 mm in diameter after 3 days of cultivation at 35 °C. Colonies on CHAB medium were light green, round and larger (1.0-1.5 mm). These strains of bacteria grow in BHI at 20 °C to 40 °C with an optimal temperature of approximately 30 °C but did not grow at 45 °C.

All 9 strains, except for GZ-04, exhibited the same biochemical characteristics, which were almost identical to those of *Francisella guangzhouensis* sp. nov. (NCTC 13503), as previously reported (Qu *et al.*, 2013). The GZ-04 strain exhibited different biochemical characteristics compared to the others but was very similar to *F. philomiragia* (ATCC 25015). Table 1 shows the comparison of biochemical characteristics between these strains, *F. guangzhouensis* sp. nov., and *F. philomiragia*.

PCR and sequencing of 16S rRNA, *rpoB*, and *sdhA* genes were successful for all of the 9 strains, except for sequencing of the *sdhA* gene for GZ-03, 06, 08, and 09. Sequences were deposited in the GenBank with accession numbers in order of the strain numbers: JN620404, KC120990, JN620406, JN620407, JN620408, JN620409, JN620410, JN620411, JN620412 for the 16S rRNA gene; KC253375, KC253382, KC253376, KC405591, KC253377, KC253378, KC253381, KC253379, and KC253380 for the *rpoB* gene; KC253383, KC253386, NA, KC253384, KC253385, NA, KC253387, NA, and NA for the *sdhA* gene (NA=not available for GZ-03, 06, 08, and 09). The identities of the 16S rRNA gene sequences among these strains and the nearest sister species, *F. philomiragia* and *F. guangzhouensis* sp. nov. (NCTC 13503), are shown in Table 2. As shown in the table, GZ-04 was 99% identical to *F. philomiragia* but 95% identical to *F. guangzhouensis* sp. nov. (NCTC 13503). In contrast, the other eight strains were 94-95% identical to *F. philomiragia* but 98-100% identical to *F. guangzhouensis* sp. nov. (NCTC 13503).

Phylogenetic trees (Figures 1, 2, and 3) were constructed with *Francisella* type species and *Francisella*-like strains from environmental samples depending on the avai-

lability of sequences in GenBank. As shown in the figures, all of the *Francisella* sp. strains, with the exception of GZ-04, formed a well-defined branch, including *F.*

guangzhouensis sp. nov. The GZ-04 strain formed a separated branch with *F. philomiragia*. Specifically, the 9 strains formed 3 clusters: cluster 1 (GZ-01, 02, 05), cluster

Table 2 - Comparison of identity (percentage) of 16S rRNA gene of *Francisella* sp. strains, *F. guangzhouensis* sp. nov. (NCTC 13503), and *F. philomiragia* (ATCC25015).

ID of strains	GZ -01	GZ -02	GZ -03	GZ -04	GZ -05	GZ -06	GZ -07	GZ -08	GZ -09
GZ -01	100%								
GZ -02	99%	100%							
GZ -03	98%	98%	100%						
GZ -04	95%	95%	95%	100%					
GZ -05	99%	100%	98%	95%	100%				
GZ -06	98%	98%	100%	95%	98%	100%			
GZ -07	98%	98%	100%	95%	98%	100%	100%		
GZ -08	98%	98%	99%	94%	98%	99%	99%	100%	
GZ -09	98%	98%	99%	95%	98%	99%	99%	99%	100%
<i>F. philomiragia</i>	95%	95%	95%	99%	95%	94%	94%	94%	95%
<i>F. guangzhouensis</i> sp. nov. ^a	99%	100%	98%	95%	100%	98%	98%	98%	98%

^a*F. guangzhouensis* sp. nov. previously isolated from the cooling water of an air conditioning system in Guangzhou (Qu et al., 2009; Qu et al., 2013).

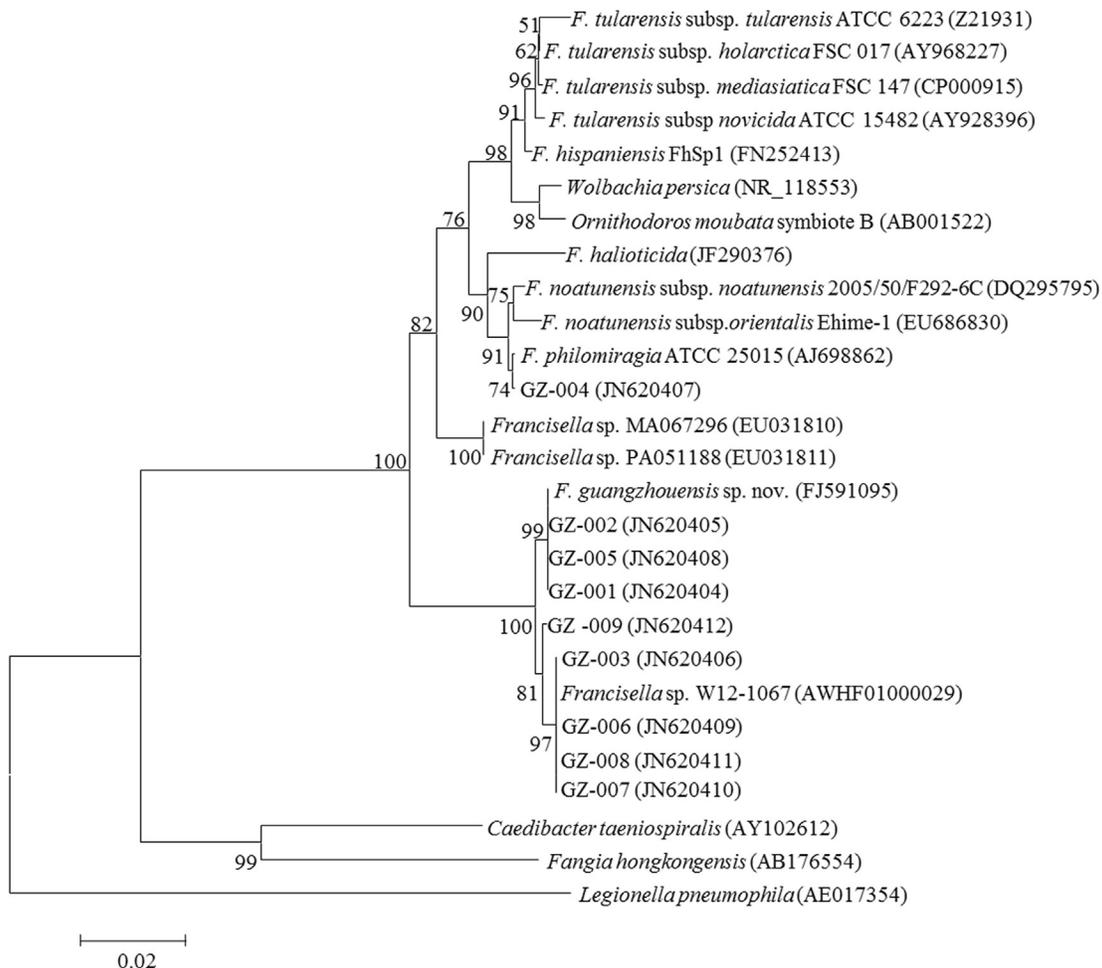


Figure 1 - Phylogenetic analysis of the 16S rRNA gene. The significance of branches is indicated by bootstrap values calculated for 1000 replications. *L. pneumophila* was used as an out-group.

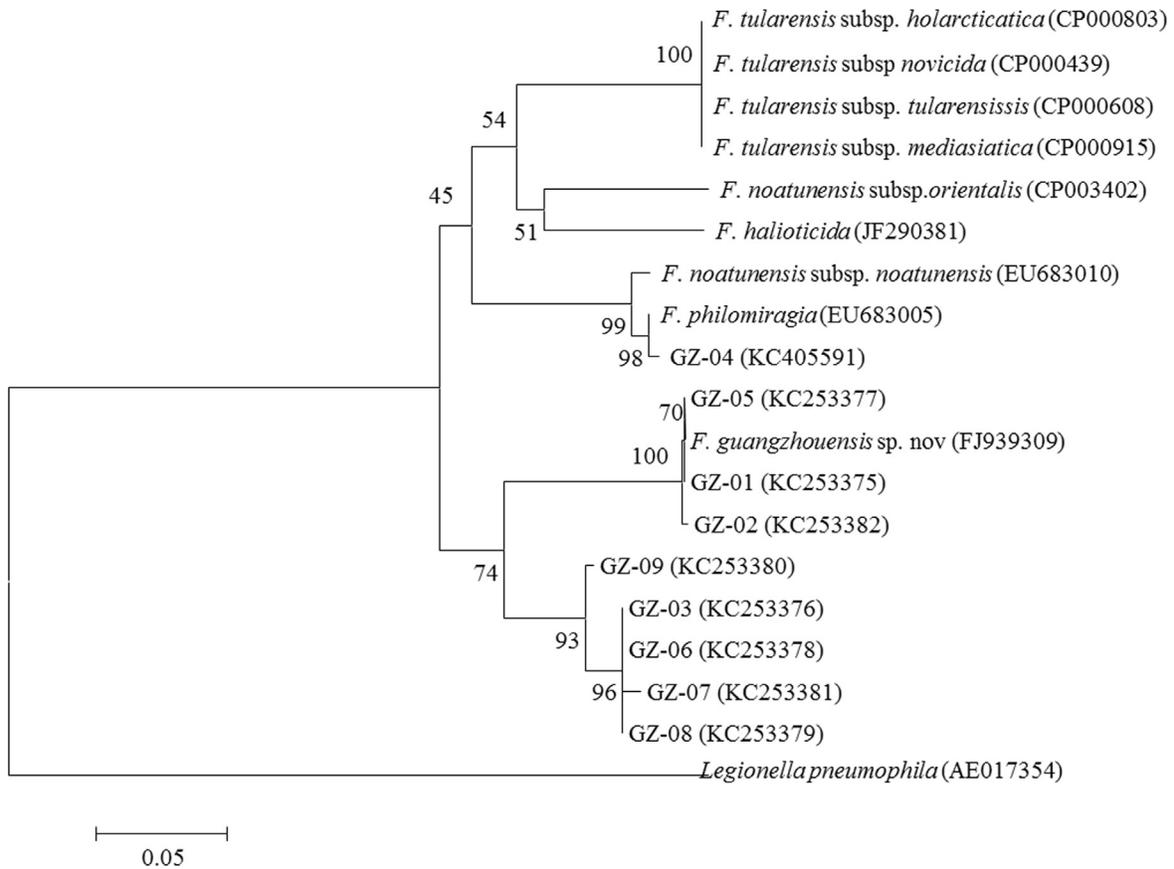


Figure 2 - Phylogenetic analysis of the *rpoB* gene. The significance of branches is indicated by bootstrap values calculated for 1000 replications. *L. pneumophila* was used as an out-group.

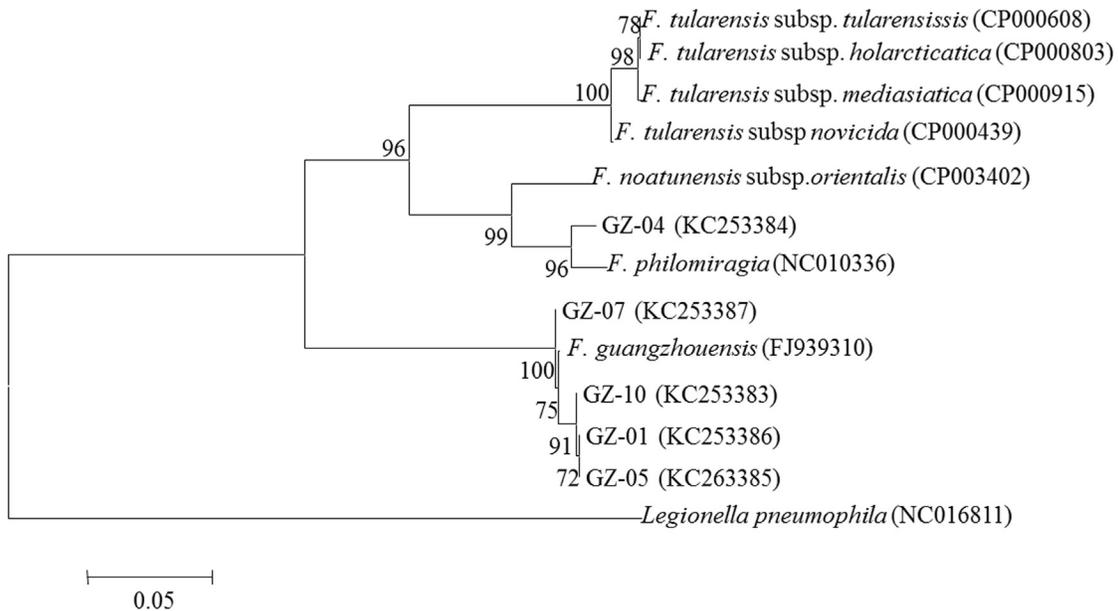


Figure 3 - Phylogenetic analysis of the *sdhA* gene. Sequences of strains GZ-03, GZ-06, GZ-08, and GZ-09 were not available due to the failure of PCR or sequencing of these strains. The significance of branches is indicated by bootstrap values calculated for 1000 replications. *L. pneumophila* was used as an out-group.

2 (GZ-03, 06, 07, 08, 09), and cluster 3 (GZ-04) (sequences of the *sdhA* gene were not available for GZ-03, 06, 08, and 09). For all of the 3 genes, cluster 3 was consistently in the same clade with *F. philomiragia* (Figures 1, 2, and 3). For 16S rRNA and *rpoB* genes, cluster 1 was identical to *F. guangzhouensis* sp. nov. and showed high identity with cluster 2 (Figures 1 and 2). For the *sdhA* gene, cluster 2 showed slightly higher identity to *F. guangzhouensis* sp. nov. than cluster 1 (Figure 3). Interestingly, *Francisella* sp. W12-1067 (AWHF01000029) was grouped in the same clade as cluster 2. This strain was isolated from a cooling tower from a hospital in Germany and was not *F. tularensis* (Rydzewski *et al.*, 2014).

BLAST analysis of the sequences of 16S rRNA and *sdhA* genes consistently demonstrated that all strains of clusters 1 and 2 shared 99-100% identity with each other and between *F. guangzhouensis* sp. nov. but were 95% identical to *F. philomiragia* and *F. tularensis* (16S rRNA gene) and 80-85% to other *Francisella* spp. isolates (*sdhA*). Cluster 3 (GZ-04) was 99% identical to 23 *F. philomiragia*, 5 *F. piscicida*, and 5 *F. noatunensis* isolates with the 16S rRNA gene and 98% identical to 14 *F. philomiragia* isolates with the *sdhA* gene. For the *rpoB* gene, strains of cluster 1 were 100% identical to *F. guangzhouensis* sp. nov. (NCTC 13503) and 87% identical to a strain of *F. noatunensis*; strains of cluster 2 were 91% identical to *F. guangzhouensis* sp. nov. (NCTC 13503) and 77-88% to *Legionella anisa* and *Mycoplasma fermentans* or *F. philomiragia*. Cluster 3 was 99% identical to *F. guangzhouensis* sp. nov. (NCTC 13503) and 87% to 3 isolates of *F. noatunensis* (as of BLAST analysis completed on May 24, 2014).

As described above, the biochemical characteristics, identity of the 16S rRNA gene, and phylogenetic and BLAST analyses with multiple genes were consistent in differentiating the 9 strains of *Francisella*. One of the 9 strains (GZ-04) was very similar to *F. philomiragia* but presented some different molecular and biochemical characteristics indicating that GZ-04 may be a new strain of *F. philomiragia* or perhaps a new species. *F. philomiragia* is a rare and opportunistic species that can cause invasive infection in patients with compromised neutrophil function (Mailman and Schmidt, 2005). The presence of *Francisella* species similar to *F. philomiragia* indicate the potential risk of human infection in the case of exposure to contaminated air conditioning cooling water. Interestingly, regarding the 16S rRNA and *sdhA* genes, 8 of the 9 strains (clusters 1 and 2) were identical or very similar (99-100%) to *F. guangzhouensis* sp. nov. (NCTC 13503) detected in 2008. For the *rpoB* gene, strains of cluster 1 were also 100% identical to *F. guangzhouensis* sp. nov. (NCTC 13503), but strains of cluster 2 were only 91% identical to *F. guangzhouensis* sp. nov. (NCTC 13503). These findings demonstrate the consistent existence of *F. guangzhouensis* sp. nov., including new isolates (isolates in clusters 1 and 2)

and potentially more new species in the cooling water of air conditioning systems in Guangzhou.

Virulence genes and pathogenicity islands have been frequently reported in *Francisella* strains (Faron *et al.*, 2013; Hare and Hueffer, 2014; Law *et al.*, 2014; Long *et al.*, 2013). Although we did not analyze *Francisella* virulence genes and pathogenicity islands due funding limitations and the scope of this work, our findings warrant future studies to understand the pathogenicity of *Francisella* spp. strains from air conditioning systems. *Legionella* was not detected in the samples used in this work (collected between 2009 and 2011) but was detected in routine surveillance samples collected after 2011. Worldwide, *Legionella* was detected in cooling water in Germany (Freudenmann *et al.*, 2011), Greece (Mouchtouri *et al.*, 2010), Poland (Matuszewska and Krogulska, 2008), Spain (Pérez *et al.*, 2011), and the USA (Stout and Yu, 1997). The detection of pathogenic bacteria from the cooling water of air conditioning systems suggests a potential risk to public health and the need for continuous surveillance. In the present work, using biochemical characterizations and multiple gene fingerprinting approaches, we confirmed the consistent presence of *F. guangzhouensis* sp. nov. and potentially novel species of *Francisella* sp. that are very similar to *F. philomiragia* in the cooling water of air conditioning systems in Guangzhou. Isolation, identification and characterization of these bacteria in cooling water filled the gap of knowledge concerning *Francisella* spp. in air conditioning systems. Findings from this study demonstrate a potential public health risk and warrant future work to study the source and routes of contamination, the pathogenicity of *Francisella* spp. and possible interventions to prevent outbreaks associated with contaminated air conditioning cooling water.

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