

## Case Report

# Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia

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Received 3 March 2003  
Accepted 24 May 2003

*Francisella tularensis* is found throughout the Northern Hemisphere, where it is associated with the disease of tularaemia in animals and humans. The isolation and identification is reported of a *novicida*-like subspecies of *F. tularensis* from a foot wound sustained in brackish water in the Northern Territory of Australia.

## Introduction

The disease tularaemia is usually one of small mammals (e.g. rodents, rabbits and hares) and is caused by the bacterium *Francisella tularensis*. The organism is normally transmitted through the bite of arthropod vectors. However, it may also persist in the environment through infected animal excreta or the carcasses of animals that succumb to infection. As tularaemia is a zoonotic disease, human infections are generally self-limiting and are acquired via arthropod bites, handling infected animals, inhalation of infectious aerosols or exposure to contaminated food or water. It presents as an acute febrile illness, with the major clinical forms determined by the route of entry: ulceroglandular tularaemia (from arthropod bite or contact with an infected animal) or respiratory tularaemia (from inhalation of substances such as contaminated dust). The disease may be severe or fatal, but it is not spread from person to person. Infections caused by members of the genus *Francisella* have only been reported from the Northern Hemisphere and, until this report, have never been reported in the Southern Hemisphere. The most virulent strains of *F. tularensis* are highly infectious by the aerosol route and cause severe disease. They are recognized as potential biological warfare agents (Dennis *et al.*, 2001).

The classification of the genus *Francisella* is still in transition and is likely to change as its unique genetic relationships become more apparent. At the time of writing, the genus

contains three species, *Francisella philomiragia*, *F. tularensis* and *Francisella novicida*, although it is widely accepted that the latter is misclassified and is actually a subspecies of *F. tularensis*. In addition to '*F. tularensis* subsp. *novicida*', *F. tularensis* is divided into three subspecies, which differ in their virulence and geographical distribution. *F. tularensis* subsp. *tularensis*, also known as the type A biovar, causes the most severe form of tularaemia and is limited in its distribution to North America. Type A isolates have been recovered from arthropod vectors in Europe but have not been associated with human disease there (Gurycova, 1998). *F. tularensis* subsp. *holarctica* (previously *palaearctica*), the type B biovar, is less virulent and is the most widely distributed subspecies recovered from human and animal cases in North America, Europe and Central and Far-East Asia. *F. tularensis* subsp. *mediasiatica* has only been recovered sporadically from ticks and animals in prescribed regions of Central Asia, without any human disease association. '*F. tularensis* subsp. *novicida*' was first recovered from water in Utah, USA, in 1951 (Utah 112 prototype strain). Subsequently, isolates recovered from four hospitalized patients, first identified as atypical *F. tularensis*, were classified as '*F. tularensis* subsp. *novicida*' or, more conservatively, as *novicida*-like organisms (Clarridge *et al.*, 1996; Hollis *et al.*, 1989). These patients recovered from their infections with comparatively milder disease than type A infections. Human isolates of *F. philomiragia* have been recovered in North America and Europe (Hollis *et al.*, 1989; Wenger *et al.*, 1989). Human disease caused by *F. philomiragia* appears to be associated with two risk groups: chronic granulomatous disease patients and victims of near-drownings.

Growth requirements, biochemical tests, fatty acid analysis

The GenBank/EMBL/DDBJ accession numbers for sequences from isolates 3523 and 2669 are respectively AY243028 and AY243027 (16S rDNA), AY243029 and AY243030 (TUL4 gene partial sequence) and AY243031 (putative RNA helicase gene partial sequence of isolate 2669).

and molecular typing have all been used to distinguish members of the genus *Francisella*. The subspecies of *F. tularensis* (*tularensis*, *holarctica* and *mediasiatica*) are fastidious, requiring thiol compounds for growth on laboratory media. In contrast, '*F. tularensis* subsp. *novicida*' and *F. philomiragia* are non-fastidious in their growth requirements. Glycerol fermentation serves as the standard biochemical test that separates type A biovar (positive) from type B (negative), but subspp. *mediasiatica* and '*novicida*' are also glycerol-positive, so would be grouped as type A if no other distinguishing tests were applied. Since biochemical reactions among the genus *Francisella* are subject to some variation, these tests should be considered as supplementary tests for the identification of *Francisella* species. Fatty acid composition separates the genus *Francisella* from other bacteria, but does not always separate the subspecies accurately (Hollis *et al.*, 1989). *F. tularensis* and *F. philomiragia* can be differentiated by their 16S rDNA sequences (Forsman *et al.*, 1994) and there are a number of molecular techniques that distinguish *F. tularensis* subspp. *tularensis* and *holarctica* (de la Puente-Redondo *et al.*, 2000; Farlow *et al.*, 2001; Garcia del Blanco *et al.*, 2002; Johansson *et al.*, 2000). However, because of the limited number of '*F. tularensis* subsp. *novicida*' and *F. philomiragia* isolates used in these studies, it is presently unclear which molecular tests can identify '*F. tularensis* subsp. *novicida*' and *F. philomiragia* accurately.

### Case report

A 53-year-old man presented with a swollen toe and swollen inguinal lymph nodes as a result of a cut received in brackish water in the Northern Territory of Australia. When initial antibiotic treatment and an unsuccessful needle aspiration did not resolve the infection, the patient was admitted to hospital for further treatment. No fever or other significant clinical symptoms were noted. A swab was taken from the toe for microscopy and culture. The patient was treated with flucloxacillin (1 g, intravenously, four times a day for 2 days then 2 g, intravenously, four times a day for 3 days) and doxycycline (100 mg, orally, twice daily for 5 days) and was discharged on dicloxacillin (500 mg, orally, four times a day for 7 days) and doxycycline (100 mg, orally, twice daily for 7 days). The patient sought medical care within 1 day of receiving the injury and presented to hospital 8 days after the incident. On the ninth day, pus was drained from the toe. Recovery was uneventful.

### Laboratory findings

Although bacteria were not seen in the direct Gram stain, a Gram-negative rod grew in pure culture from the swab. This isolate (3523) was an aerobe, grew well on most laboratory media (weakly on MacConkey agar) and produced translucent colonies that had a slightly 'gluey' texture. The Gram-stained smear showed pleomorphic, Gram-negative coccobacilli. Colonies on horse-blood agar were approx 1–1.5 mm in diameter at 48 h. The isolate was catalase- and ONPG-positive and negative for oxidase, nitrate, urea and indole, with no acid production from carbohydrates glucose, lactose,

maltose or sucrose in BBL cystine trypticase agar (CTA) base with phenol red indicator (Becton-Dickinson Microbiology Systems). An initial identification of an atypical *Acinetobacter* sp. was considered, but this was not consistent with the result for ONPG or with the appearance of the Gram-stained smear. A direct fluorescent antibody (DFA) test, used routinely for the identification of *F. tularensis* type A and type B biovars at the Centers for Disease Control and Prevention (CDC) laboratory in Fort Collins, CO, USA, was negative. Despite the negative test by DFA, the isolate had colonial morphology consistent with *F. tularensis* on cysteine heart agar supplemented with 9% sheep blood (CHAB). In addition, Biolog micro-well GN2 biochemical typing detected acid production from glucose, mannose, sucrose and glycerol and a weak reaction with maltose. Taken together, these results suggested that isolate 3523 belonged to the genus *Francisella*. However, it was clearly not a member of subspp. *tularensis* or *holarctica*, since it was not recognized by the CDC antibody and was of low virulence in Swiss-Webster mice. Thus, additional tests were required to classify the organism as either '*F. tularensis* subsp. *novicida*' or *F. philomiragia*.

To resolve the identity of isolate 3523, molecular techniques were applied. Its 16S rDNA sequence was determined and compared with other sequences in the databases (Altschul *et al.*, 1997). The closest matches were to strains of *F. tularensis*. Subsequent 16S rDNA testing was carried out in parallel with a supraclavicular lymph node biopsy isolate from Victoria, Australia (isolate 2669), which had previously been identified as an atypical *F. philomiragia* on the basis of cellular fatty acid analysis performed by the CDC in Atlanta, GA, USA. The 16S rDNA sequence of isolate 2669 matched that of *F. philomiragia*. To provide the best possible discrimination, the two Australian isolates were compared with two type A strains (Schu 4 and AR011117), two type B strains (LVS, CO976559), '*F. tularensis* subsp. *novicida*' isolate Utah 112 and two *novicida*-like isolates, D9876 and F6168 (Hollis *et al.*, 1989), and three *F. philomiragia* isolates (ATCC 25015<sup>T</sup>, ATCC 25017, ATCC 25018) archived in the collection at the CDC in Fort Collins, CO, USA. The 16S rDNA sequences of the CDC panel correlated with expected 16S rDNA groupings (Forsman *et al.*, 1994). The 16S rDNA sequence derived from the *F. philomiragia* isolate 2669 exhibited 99.8–100% identity to *F. philomiragia* strains. The sequence of isolate 3523 showed 99.2–99.8% similarity to the 16S rDNA sequences of Schu 4 (type A prototype strain), LVS (type B prototype strain) and three '*F. tularensis* subsp. *novicida*' strains (Utah 112, prototype strain; D9876 and F6168, *novicida*-like strains), with the closest match being to strain D9876 (99.8%). In comparison, isolate 3523 matched less well with *F. philomiragia* strains ATCC 25015<sup>T</sup>, ATCC 25017 and ATCC 25018, sharing only 97.3–97.5% identity. Therefore, based on 16S rDNA sequence comparison, isolate 3523 could be confirmed as a member of a subspecies of *F. tularensis*.

Further molecular tests were carried out in an effort to confirm and establish a subspecies identification. The 17 kDa

lipoprotein (TUL4) and its encoding gene were shown previously to be conserved among strains of *F. tularensis* (Sjöstedt *et al.*, 1992). Thus, PCR primers specific for the TUL4 protein precursor gene (Johansson *et al.*, 2000; Sjöstedt *et al.*, 1997) were used to amplify product from isolates 2669 and 3523. Surprisingly, 0.4 kb amplicons were generated from both isolates 2669 and 3523. This was unexpected for the *F. philomiragia* isolate, since it has not been documented previously, although it is known to carry a homologous gene that has less than 85 % but more than 70 % identity to that of *F. tularensis*. Both TUL4 amplicons were sequenced. The amplicon from isolate 3523 (*F. tularensis*) had 91 % identity to the matching region from LVS (EMBL/GenBank accession no. M32059), while that of isolate 2669 (*F. philomiragia*) had only 69 % identity. Ostensibly, isolate 3523 also shares ~91 % identity with *F. tularensis* strain Schu 4, since the ~400 bp region of LVS is 99.7 % identical to the corresponding region of Schu 4 (<http://artedi.ebc.uu.se/Projects/Francisella/>). These results provided additional support for classifying isolate 3523 as belonging to a subspecies of *F. tularensis*.

To discriminate between *F. tularensis* subsp. *holarctica* and other *F. tularensis* subspecies, a PCR targeting a region downstream of the coding region of a putative peptidyl-prolyl *cis-trans* isomerase gene (PPIase) was utilized. This region varies by 30 bp between type A and type B subspecies (Johansson *et al.*, 2000). Using this set of primers, an amplicon of about 180 bp was obtained from the *F. philomiragia* isolate (2669), but no product was obtained from the *F. tularensis* isolate (3523). A product from *F. philomiragia* was not expected (Johansson *et al.*, 2000), and the size did not fit the 300–330 bp described previously (Johansson *et al.*, 2000). This 180 bp amplicon was sequenced and found to match the corresponding regions of *F. tularensis* subsp. *tularensis*, *mediasiatica* and ‘*novicida*’ strains. From comparisons with sequences in GenBank, *F. tularensis* subsp. *holarctica* strains had a sequence of approximately 150 bp for the corresponding region. It is interesting to note that, in another study, a number of *F. tularensis* subsp. *tularensis* strains failed to give a product with this PCR (Farlow *et al.*, 2001). Thus, molecular analysis of this region was not able to identify either isolate 2669 or 3523 accurately.

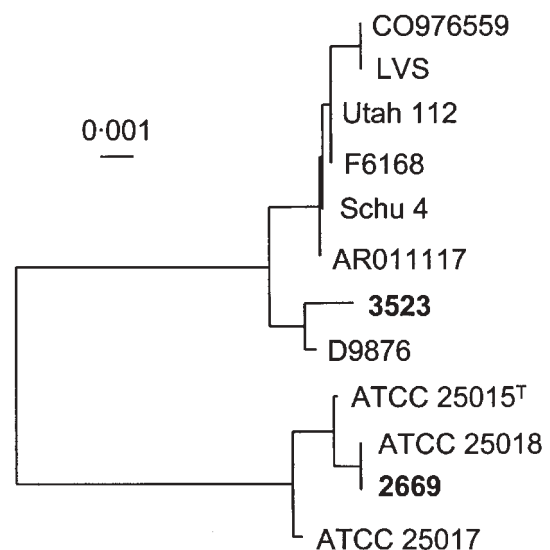
Comparison of GenBank sequences for the putative PPIase from *F. tularensis* subsp. *tularensis*, *holarctica* and *mediasiatica* and *F. philomiragia* revealed that nucleotide differences in this region could be used to differentiate between *F. tularensis* and *F. philomiragia*. Therefore, a new set of PCR primers was developed in order to amplify this region. Using primers 3F (5'-ATGAAAGCTTCAGCTAGACAT-3') and 7R (5'-CTTBACCTAGCACATCTCTAC-3'), a 400 bp fragment was amplified from the Australian isolates and the CDC panel. Alignment of 213 nt of the PPIase coding region showed that isolate 3523 could be grouped with the *F. tularensis* isolates, while the atypical *F. philomiragia* Australian isolate 2669 grouped with the *F. philomiragia* clade

(Fig. 1). With only 13 nucleotide differences, isolate 3523 is most closely associated with the North American ‘*F. tularensis* subsp. *novicida*’ human isolate D9876 (93.9 % identity).

## Discussion

The clinical presentation of the patient and the mode of acquisition are consistent with other human cases of tularaemia caused by *novicida*-like strains (Hollis *et al.*, 1989), non-cysteine-requiring *F. tularensis* strains (Bernard *et al.*, 1994) or ‘*F. tularensis* subsp. *novicida*’ (Clarridge *et al.*, 1996). The Gram stain and growth morphology are consistent with other known *Francisella* species. *F. tularensis* isolates are typically inactive in biochemical tests, and isolate 3523 gave different results for acid production from carbohydrates, depending on the system used. This illustrates the difficulties that may be experienced in the identification of this species by traditional biochemical methods. Isolate 3523 was also not recognized by the CDC antibody specific for *F. tularensis* type A and type B biovars and was of low virulence in Swiss–Webster mice, suggesting that it was either ‘*F. tularensis* subsp. *novicida*’ or *F. philomiragia*. Molecular evidence, matching selected sequences from 16S rDNA and the putative PPIase gene that are unique to the species and subspecies, demonstrated that isolate 3523 most closely resembles ‘*F. tularensis* subsp. *novicida*’ strain D9876.

This is the first report of *F. tularensis* from Australia and from the Southern Hemisphere. This significant discovery suggests that tularaemia-like infections are indeed more widely distributed, as has been often postulated but never proven.



**Fig. 1.** Dendrogram based on nucleotide sequence comparison of 213 bp of the coding region of the PPIase gene. Australian isolates 3523 and 2669 are compared with *F. tularensis* subsp. *tularensis* (Schu 4, AR011117), subsp. *holarctica* (LVS, CO976559), ‘subsp. *novicida*’ (Utah 112), *novicida*-like isolates (D9876, F6168) and *F. philomiragia* (ATCC 25015<sup>T</sup>, ATCC 25017, ATCC 25018). Bar, 0.001 substitutions per site.

In this instance, its discovery and characterization are the result of a combination of factors: microbiologists' skills and persistence, the availability of molecular tools, national and international collaboration and the heightened awareness of tularaemia as a potential biothreat agent.

Infections caused by '*F. tularensis* subsp. *novicida*', though rarely reported, are probably far more frequent and widespread than previously thought. Previous reports (Bernard *et al.*, 1994; Clarridge *et al.*, 1996) described the recovery of several North American human isolates of atypical, non-cysteine-requiring *F. tularensis*, two of which (Clarridge *et al.*, 1996) have been determined molecularly to be '*F. tularensis* subsp. *novicida*' (Johansson *et al.*, 2000). At the CDC laboratory, isolates from Utah and Alabama, previously thought to be *F. tularensis* type A biovar based on positive glycerol fermentation, have recently been molecularly characterized as subspecies '*novicida*'. Taken together, this suggests that '*F. tularensis* subsp. *novicida*' infections in humans may be identified increasingly as more sensitive molecular tools are applied.

The genotypic and phenotypic characteristics of isolate 3523 indicate that it belongs to the species *F. tularensis* and resembles most closely North American isolate D9876 of '*F. tularensis* subsp. *novicida*'. However, differences in the sequences of the genes encoding the TUL4 (data not shown) and PPIase proteins, combined with 16S rDNA dissimilarities (data not shown), suggest divergence between the Australian *F. tularensis* isolate and other '*F. tularensis* subsp. *novicida*' isolates archived at the CDC. Further investigations will determine whether this isolate is classified as an atypical '*F. tularensis* subsp. *novicida*' isolate or as a new subspecies of *F. tularensis*. Its relationship to Northern Hemisphere strains and its epidemiology remain avenues for further study.

## Acknowledgements

We thank staff of the Department of Microbiology and Infectious Disease, Royal Children's Hospital, Parkville, Australia, for referring isolate 2669 and for supplying clinical details.

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