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; Wengert 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
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 subsp. *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* subsp. *tularensis* and *holarctica* (de la Puente-Redondo *et al.*, 2000; Farlow *et al.*, 2001; García 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 ciprofloxacin (1 g, intravenously, four times a day for 2 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 cocobacilli. 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 cyanide tryptose 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 subsp. *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 supravacular 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 25015T, 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 99–100% identity to *F. philomiragia* strains. The sequence of isolate 3523 showed 99–99.8% 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 25015T, 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, <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/ Franciscella/). 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 (PPlase) was utilized. This region varies by 30 bp between type A and type B subspecies (Johansson et al., 2000). The 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. *holarctica*, *F. tularensis* mediaisatica 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 PPlase from *F. tularensis* subsp. *tularensis*, *holarctica* and *mediaisatica* 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′-ATAGAAGTCCTAGCTAGCAT-3′) and 7R (5′-GCTTACCCATGACATCTATAC-3′), a 400 bp fragment was amplified from the Australian isolates and the CDC panel. Alignment of 213 nt of the PPlase coding region showed that isolate 3523 could be grouped with the type *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* (Claridge et al., 1996). The Gram stain and growth morphology are consistent with other known Franciscella 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 PPlase 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.
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 tularemia as a potential bioterror 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; Claridge et al., 1996) described the recovery of several North American human isolates of atypical, non-cysteine-requiring F. tularensis, two of which (Claridge 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 PPase proteins, combined with 16S rRNA 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.

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References


