Legionella cardiaca sp. nov., isolated from a case of native valve endocarditis in a human heart

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A Gram-negative, rod-shaped bacterium, designated H63^T, was isolated from aortic valve tissue of a patient with native valve endocarditis. 16S rRNA gene sequencing revealed that H63^T belongs to the genus *Legionella*, with its closest neighbours being the type strains of *Legionella brunensis* (98.8 % similarity), *L. londiniensis* (97.0 %), *L. jordanis* (96.8 %), *L. erythra* (96.2 %), *L. dresdenensis* (96.0 %) and *L. rubrilucens*, *L. feeleii*, *L. pneumophila* and *L. birminghamensis* (95.7 %). DNA–DNA hybridization studies yielded values of <70 % relatedness between strain H63^T and its nearest neighbours in terms of 16S rRNA gene sequence similarity, indicating that the strain represents a novel species. Phylogenetic analysis of the 16S rRNA, macrophage infectivity potentiator (*mip*) and RNase P (*rnpB*) genes confirmed that H63^T represents a distinct species, with *L. brunensis* being its closest sister taxon. Fatty acid composition and biochemical traits, such as the inability to ferment glucose and reduce nitrate, supported the affiliation of H63^T to the genus *Legionella*. H63^T was distinguishable from its neighbours based on it being positive for hippurate hydrolysis. H63^T was further differentiated by its inability to grow on BCYE agar at 17 °C, its poor growth on low-iron medium and the absence of sliding motility. Also, H63^T did not react with antisera generated from type strains of *Legionella* species. H63^T replicated within macrophages. It also grew in mouse lungs, inducing histopathological evidence of pneumonia and dissemination to the spleen. Together, these results confirm that H63^T represents a novel, pathogenic *Legionella* species, for which the name *Legionella cardiaca* sp. nov. is proposed. The type strain is H63^T (=ATCC BAA-2315^T =DSM 25049^T =JCM 17854^T).

Legionellae are Gram-negative bacteria that are ubiquitous in freshwater environments as well as man-made water systems (Diederen, 2008; Fields *et al.*, 2002). Many legionellae have been associated with human disease, and the most common mode of transmission is through inhalation of aerosolized water droplets containing the bacteria (König *et al.*, 2005; Muder & Yu, 2002; Whiley & Bentham, 2011). Inside the lung, legionellae utilize alveolar macrophages and epithelial cells for intracellular replication, resulting in a severe pneumonia referred to as Legionnaires' disease (Fields *et al.*, 2002). In rare cases, *Legionella* species can be isolated from extrapulmonary sites such as the heart (Lowry & Tompkins, 1993). At the time of writing, the genus *Legionella* comprised 54 species with validly published names and one genomospecies (Benson *et al.*, 1996; Edelstein *et al.*, 2012; Euzéby, 1997; Yang *et al.*, 2012).

We previously described a rare case of native valve endocarditis due to a novel *Legionella* strain (Pearce *et al.*, 2011). The strain, designated H63^T, was isolated from resected aortic valve tissue of a patient requiring aortic valve replacement for treatment of congestive heart failure related to infective native valve endocarditis. Sequencing of the 16S rRNA gene and preliminary BLAST analysis suggested that the strain represented either a novel clinical strain of *Legionella brunensis* or a novel *Legionella* species (Pearce *et al.*, 2011). *L. brunensis* was first isolated from cooling tower water in Czechoslovakia and has been isolated once from a case of Legionnaires' disease in Europe (Ricketts *et al.*, 2007; Wilkinson *et al.*, 1988). Based on DNA–DNA hybridization values of less than 70 % and

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *mip* and *rnpB* gene sequences of strain H63^T are JF831047, JF831048 and JN673956, respectively.

Four supplementary figures and a supplementary table are available with the online version of this paper.

sequence analysis of multiple gene targets, we now report that H63^T represents a novel *Legionella* species. Phenotypic profiling revealed a number of differences between H63^T and its phylogenetically nearest neighbours. Furthermore, H63^T replicated in a human macrophage cell line and in the murine lung, indicating that H63^T represents a virulent strain.

To establish the placement of the novel strain in the genus *Legionella*, the almost-complete 16S rRNA gene (1423 bp) was amplified from purified genomic DNA of H63^T and sequenced using primers 8F (5′-AGAGTTTGATCCTG-GCTCAG-3′), 806R (5′-GGACTACCAGGGTATCTAAT-3′), 515F (5′-TGCCAGCAGCCGCGGTAA-3′) and rP1 (5′-GGTTACCTTGTTACGACTT-3′) (Relman *et al.*, 1992; Weisburg *et al.*, 1991). The EzTaxon service was used to determine the nearest neighbours in the genus *Legionella* on the basis of 16S rRNA gene sequence similarity as recommended for the calculation of pairwise percentage similarity values (Chun *et al.*, 2007; Tindall *et al.*, 2010). H63^T shared highest similarity with the type strains of *L. brunensis* (98.80 %), followed by *Legionella londiniensis* (97.03 %), *L. jordanis* (96.76 %), *L. erythra* (96.20 %), *L. dresdenensis* (95.99 %), *L. rubrilucens* (95.73 %), *L. feeleii* (95.72 %), *L. pneumophila* (95.71 %) and *L. birminghamensis* (95.71 %) (Table 1). To determine whether H63^T represents a novel *Legionella* species, DNA–DNA hybridization was performed at 30 °C using the microplate technique with photobiotin-labelled DNA as described previously (Ezaki *et al.*, 1989, 1990) and modified (Willems *et al.*, 2001), with the exception that the DNA was sheared prior to biotin labelling as opposed to after. Strain H63^T was only 17 and 20 % related to its nearest neighbour, *L. brunensis* ATCC 43878^T, in reciprocal relationships, well below the 70 % cut-off for species delineation (Table 1). Furthermore, H63^T exhibited less than 20 % relatedness to all eight of the remaining type strains tested (Table 1). The difference between reciprocal hybridizations was within 20 % and the standard deviation among replicates was $\leqslant$7 %, both of which are acceptable deviations for the microplate technique (Ezaki *et al.*, 1990; Kuroki *et al.*, 2007; Willems *et al.*, 2001).

To define the relationship between H63^T and other *Legionella* species further, a 584 bp portion of the *mip* gene and a 327 bp portion of the *rnpB* gene of H63^T were sequenced as described previously (Kuroki *et al.*, 2007; Lück *et al.*, 2010; Ratcliff *et al.*, 1998; Rubin *et al.*, 2005; Yang *et al.*, 2012). The European Working Group for *Legionella* Infections (EWGLI) *Legionella mip* gene sequence database was used to determine the similarity based on *mip*, and NCBI BLAST was used to determine similarity based on *rnpB* (Altschul *et al.*, 1990; Fry *et al.*, 2007). Similar to the 16S rRNA gene sequence analysis, the *mip* gene sequence of strain H63^T was most similar to that of *L. brunensis* ATCC 43878^T (85.49 %), followed by *Legionella hackeliae* ATCC 35250^T (85.11 %), *L. jamestowniensis* ATCC 35298^T (85.11 %), *L. feeleii* ATCC 35072^T (83.95 %) and *L. lansingensis* ATCC 49751^T (83.56 %). Based on analysis of *rnpB* sequences, strain H63^T was again most similar to the type strain of *L. brunensis*

Table 1. Genetic analysis of strain H63^T compared with its nearest neighbours based on 16S rRNA gene sequences

Strain	Source	Reference	16S rRNA gene sequence similarity (%) to strain H63^T	DNA–DNA hybridization (%)* with H63^T DNA as:		DNA G+C content (mol%)
				Probe	Covalent DNA	
L. cardiaca sp. nov. H63^T	Clinical	Pearce *et al.* (2011)	(100)	(100)	(100)	41.8
L. brunensis ATCC 43878^T	Environmental	Wilkinson *et al.* (1988)	98.8	17.3±5.8	20.3±7.2	40.5
L. londiniensis ATCC 49505^T	Environmental	Dennis *et al.* (1993)	97.0	5.7±2.4	2.4±1.1	43.0
L. jordanis ATCC 33623^T	Environmental	Cherry *et al.* (1982)	96.8	15.7±1.8	4.5±3.0	45.0
L. erythra ATCC 35303^T	Environmental	Brenner *et al.* (1985)	96.2	4.3±0.6	7.5±6.6	51.0
L. dresdenensis NCTC 13409^T	Environmental	Lück *et al.* (2010)	96.0	5.0±0.4	8.7±2.6	42.5
L. rubrilucens ATCC 35304^T	Environmental	Brenner *et al.* (1985)	95.7	6.2±2.6	9.3±4.7	52.0
L. feeleii ATCC 35072^T	Environmental	Herwaldt *et al.* (1984)	95.7	3.7±2.2	6.4±2.8	46.0
L. pneumophila ATCC 33152^T	Clinical	Brenner *et al.* (1979)	95.7	5.3±2.3	15.1±5.7	39.0
L. birminghamensis ATCC 43702^T	Clinical	Wilkinson *et al.* (1987)	95.7	6.3±2.0	4.5±2.6	42.9

*Means±SD of at least triplicate hybridization experiments are shown.

(91.2 %), followed by the type strains of *L. lansingensis* (89.4 %), *L. jamestowniensis* (89.7 %), *L. hackeliae* (88.3 %) and *L. feeleii* (88.3 %). For phylogenetic analyses, the 16S rRNA, *mip* and *rnpB* sequences of type strains of *Legionella* species and the nearest other relative within the *Legionellaceae*, *Coxiella burnetii*, were obtained from GenBank (Benson *et al.*, 2008). Trimmed sequences were aligned using the CLUSTAL W program (Larkin *et al.*, 2007). Phylogenetic trees were inferred by the neighbour-joining method using TOPALI version 2 and edited using TreeView version 1.6.6 (Milne *et al.*, 2009; Page, 1996). Phylogenetic analysis based on the consensus alignment of 16S rRNA, *mip* and *rnpB* gene sequences indicated that strain H63^T is most closely related to *L. brunensis*, followed by the group of *L. hackeliae* and *L. jamestowniensis* (Fig. 1). The strength of the association was confirmed by bootstrap values $\geqslant$80 based on 100 replicates. For completeness, DNA–DNA hybridizations were performed comparing H63^T with both *L. hackeliae* ATCC 35250^T and *L. jamestowniensis* ATCC 35298^T, because they were closely related according to the consensus tree. According to hybridization analysis, *L. hackeliae* ATCC 35250^T was 8.0 % ($\pm$0.9) related to H63^T when H63^T DNA served as the probe and 31.7 % ($\pm$0.4) similar when H63^T represented the covalent DNA. *L. jamestowniensis* ATCC 35298^T was 10.1 % ($\pm$1.2) similar to H63^T when H63^T DNA was the probe and 7.7 % ($\pm$3.3) similar when H63^T DNA was the covalent DNA. The topologies of the individual gene trees support the consensus assignment of H63^T and *L. brunensis* as sister taxa (Figs S1–S3, available in IJSEM Online).

To complete our genetic analysis, the DNA G+C content of H63^T was determined through HPLC analysis performed by the Identification Service of the DSMZ. The DNA G+C content of H63^T was 41.8 mol%, within the range of values reported for its neighbours (39.0–52.0 mol%; Table 1).

Using the slide agglutination test as described previously (Thacker *et al.*, 1985), antigen from H63^T did not react with antisera generated previously against the type strains of *Legionella* species, including sera raised against all of the nearest neighbours in the 16S rRNA gene tree as well as the type strains of *L. jamestowniensis* and *L. hackeliae* (Table S1).

Initially, we determined the phenotype of strain H63^T by examining a set of 13 physiological traits that are standards for *Legionella* (Table 2) (Hookey *et al.*, 1996). Like most members of the genus *Legionella* (Dennis *et al.*, 1993; Edelstein *et al.*, 2012; Hookey *et al.*, 1996; Yang *et al.*, 2012), including its nearest neighbours, H63^T grew well at 37 °C on buffered charcoal yeast extract (BCYE) agar or in buffered yeast extract (BYE) broth and required supplementary cysteine for growth. Colonies of strain H63^T on BCYE agar did not autofluoresce under UV light, distinguishing the strain from its neighbours *L. erythra*, *L. dresdenensis*, *L. rubrilucens* and *L. birminghamensis* (Table 2). The strain, like many other legionellae (Hookey *et al.*, 1996) but unlike *L. dresdenensis* and *L. birminghamensis*, secreted a brown pigment upon entering stationary phase (Table 2)

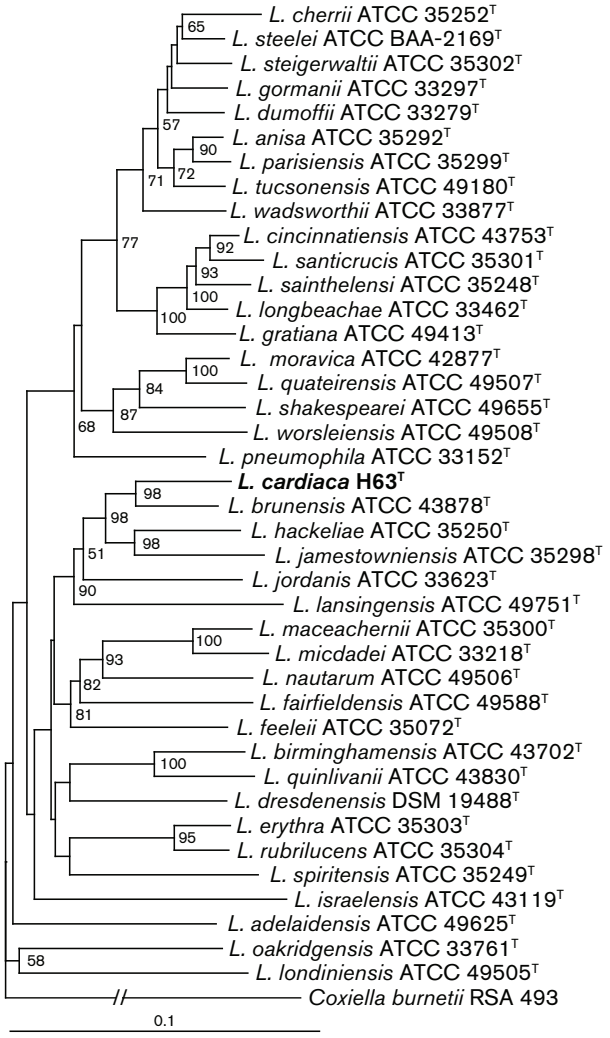

Fig. 1. Neighbour-joining tree showing relationships between strain H63^T and all previously sequenced type strains of *Legionella* species based on the consensus sequence of the 16S rRNA, *mip* and *rnpB* loci. Bootstrap values greater than 50 (from 100 replicates) are shown. *Coxiella burnetii* RSA 493 was used as an outgroup. GenBank accession numbers of the individual sequences used to reconstruct the tree are provided in Figs S1–S3. Bar, 0.1 substitutions per nucleotide site.

(Chatfield & Cianciotto, 2007). Tests for glucose fermentation, nitrate reduction, urease, catalase, gelatinase and oxidase were performed as described previously (Orrison *et al.*, 1983; Weaver & Feeley, 1979) using stationary-phase bacteria obtained from BCYE agar. β-Lactamase and hippurate hydrolysis activities were assessed by disc assays (Becton Dickinson) as described previously (Kuroki *et al.*, 2007). As expected of a member of the genus *Legionella* (Dennis *et al.*, 1993; Weaver & Feeley, 1979; Yang *et al.*, 2012), H63^T was negative for glucose fermentation, nitrate reduction and urease activity (Table 2). However, the strain was positive for catalase, gelatinase, β-lactamase and

Table 2. Differential characteristics of strain H63^T compared with its nearest neighbours based on 16S rRNA gene sequences

Strains: 1, *L. cardiaca* sp. nov. H63^T (data from this study); 2, *L. brunensis* ATCC 43878^T (unless indicated, data from Wilkinson *et al.*, 1988); 3, *L. londiniensis* ATCC 49505^T (Dennis *et al.*, 1993); 4, *L. jordanis* ATCC 33623^T (Cherry *et al.*, 1982); 5, *L. erythra* ATCC 35303^T (Brenner *et al.*, 1985), 6, *L. dresdenensis* DSM 19488^T (Lück *et al.*, 2010); 7, *L. rubrilucens* ATCC 35304^T (Brenner *et al.*, 1985); 8, *L. feeleii* ATCC 35072^T (Brenner *et al.*, 1985); 9, *L. pneumophila* ATCC 33152^T (Brenner *et al.*, 1979); 10, *L. birminghamensis* ATCC 43702^T (Wilkinson *et al.*, 1987). Reactions are scored as follows unless indicated: +, positive; +W, weakly positive; −, negative; ±, variable; ND, no data available. All strains grow on BCYE at 37 °C but do not grow under these conditions without cysteine, and all strains grow in BYE at 37 °C. All strains are positive for catalase and are negative for glucose fermentation, nitrate reduction and urease activity.

Characteristics	1	2	3	4	5	6	7	8	9	10
Pigment production when grown in BYE/BCYE at 37 °C	+	+	+	+	+	−*	+	+W	+	−
Autofluorescence when grown on BCYE at 37 °C	−	−	−	−	+	+W	+	−	−	+
Gelatinase	+	+	+	+	+	+	+	−	+	+
β-Lactamase	+	+	+	+	+	ND	+	−	+	+
Hippurate hydrolysis	+	−	−/+W	−	−	−	−	+W	+	−
Oxidase	+W	−	−	+	+	−	−	−	+	±
Protease*†	+	+	+W	+	++	−	++	−	++	++
Phosphatase*†	+	++	−	+W	+W	+W	+W	−	++	−
Lipase*†	−	+W	+W	−	+	−	+	+W	++	+W
Swimming motility	+	−*	−	+	+	+*	+	+	+	+
Sliding motility*	−	−	−	−	−	−	−	+	+	−
Growth on BCYE at 17 °C*‡	−	+	−	+	+++	+	+	+++	+++	++
Growth on low-iron BCYE at 37 °C*‡	+	++	+	+	+++	+++	+++	+++	+++	+++
Growth in deferrated CDM at 37 °C§	+	++	+/−	+*	+	+*	+++	+++	+++	++
Siderophore secretion§	−	++	+/−	+*	+/−	−*	+++	++	+++	++
Salt sensitivity‖	S	S	R	R	S	S*	S	R	S	R

*Data from this study.

†+++, Strongly positive; +, positive; +W, weakly positive, slightly above background; −, negative.

‡Scored as follows in comparison with growth of spot dilutions on BCYE at 37 °C: +++, equal or 1 log less growth; ++, 2–4 logs less growth; +, 5–6 logs less growth; −, no growth.

§Scored as follows: +++, high CDM growth/chrome azurol S (CAS) reactivity; ++, moderate CDM growth/CAS reactivity; +, slight CDM growth/CAS reactivity; +/−, CDM growth/CAS reactivity varied between experiments for unknown reasons (Starkenburg *et al.*, 2004).

‖R, Resistant (similar level of growth on BCYE with or without NaCl); S, sensitive (reduced efficiency of plating on BCYE in the presence of 100 mM NaCl at 37 °C) (O'Connell *et al.*, 1996).

hippurate hydrolysis (Table 2). The strongly positive hippurate hydrolysis test distinguished H63^T from *L. brunensis*, *L. londiniensis*, *L. jordanis*, *L. erythra*, *L. dresdenensis*, *L. rubrilucens* and *L. birminghamensis*, and the presence of both gelatinase and β-lactamase differentiated H63^T from *L. feeleii*. The fact that H63^T was positive for hippurate hydrolysis and weakly positive for oxidase distinguishes it from *L. hackeliae* and *L. jamestowniensis*, the two other species that showed high similarity to H63^T based on *mip* and *rnpB* sequences (Brenner *et al.*, 1985).

Although we were able to detect phenotypic differences between H63^T and its nearest neighbours using long-established methods, it can be difficult to distinguish *Legionella* species based on the biochemical tests that are typically done, because various species give similar reactions in many of the tests. For example, *L. lansingensis* cannot be distinguished from *Legionella micdadei* and *Legionella maceachernii* based on standard biochemical profiling (Hookey *et al.*, 1996; Thacker *et al.*, 1992). For this reason, we examined 10 additional characteristics that we have recently found to be expressed variably within the genus *Legionella* (Söderberg *et al.*, 2008; Starkenburg *et al.*, 2004; Stewart *et al.*, 2009). To that end, cell-free supernatants from late-exponential BYE broth cultures were analysed for protease, acid phosphatase and lipase activities as measured by azocasein, *p*-nitrophenyl phosphate and *p*-nitrophenyl palmitate hydrolysis, respectively (Aragon *et al.*, 2000, 2001; Thorpe & Miller, 1981). Strain H63^T was positive for both protease and phosphatase activities but lacked lipase activity, a finding that distinguished it from all nine of its nearest neighbours (Table 2). That H63^T had these activities in BYE culture supernatants suggests that the strain has a functional type-II protein secretion system, as has been documented extensively in *L. pneumophila* (Cianciotto, 2009; Pearce & Cianciotto, 2009). Interestingly, in *L. pneumophila*, a functional type-II secretion system has also been linked to sliding on low-agar media (Stewart *et al.*, 2009) and growth at low temperature (Söderberg *et al.*, 2008). H63^T exhibited swimming motility by wet-mount microscopy of 3-day-old

BCYE agar-grown cultures, but did not show sliding motility (surface translocation) and its associated surfactant when grown on 0.5 % agar BCYE plates incubated at 30 °C for 14 days (Stewart *et al.*, 2009). These data indicated further differences between H63^T and *L. brunensis*, *L. londiniensis*, *L. feeleii* and *L. pneumophila* (Table 2). Strain H63^T was unable to grow at 17 °C on BCYE agar, differentiating it from eight of its nine nearest neighbours; *L. londiniensis* was the only other species in the panel that did not grow under this low-temperature condition (Table 2). That H63^T did not exhibit sliding motility nor grow at 17 °C on BCYE agar would suggest that it lacks those type-II-dependent factors associated with sliding and low-temperature growth. Unlike *L. brunensis*, *L. erythra*, *L. dresdenensis*, *L. rubrilucens*, *L. feeleii*, *L. pneumophila* and *L. birminghamensis*, H63^T grew very poorly at 37 °C on BCYE agar depleted for iron by the addition of 14 μM deferoxamine mesylate (Table 2) (Chatfield *et al.*, 2011). This result suggested that the strain has a higher-than-average iron requirement and/or a reduced ability to scavenge iron. In support of this hypothesis, H63^T, unlike most of its nearest neighbours, showed poor growth in deferrated chemically defined medium (CDM) at 37 °C, and cell-free supernatants obtained 15 h post-inoculation showed no evidence of siderophore activity as measured by the chrome azurol S assay (Table 2) (Chatfield *et al.*, 2011; Liles *et al.*, 2000; Starkenburg *et al.*, 2004). Interestingly, strain H63^T secreted a yellow pigment upon culturing in CDM, and its supernatants displayed a green fluorescence under UV light (not shown). We also observed that H63^T was more sensitive to the presence of 100 mM NaCl on BCYE agar at 37 °C than were some of the other species (Table 2), as described previously (O'Connell *et al.*, 1996). In *L. pneumophila*, salt-sensitivity is correlated with a type-IV secretion system known as Dot/Icm (Sadosky *et al.*, 1993; Vogel *et al.*, 1996). In summary, the results from these additional chemotaxonomic assays provide strong evidence for H63^T being phenotypically distinct from its phylogenetically nearest neighbours.

The fatty acid composition of H63^T was determined after 72 h of incubation at 35 °C on BCYE agar using the Microbial Identification System (MIDI Inc.) and MIDI operating software version 6.0 (Diogo *et al.*, 1999) as described previously (Pearce *et al.*, 2011). Typical of the genus *Legionella*, the profile of H63^T consisted primarily of branched-chain fatty acids and a few hydroxyl fatty acids (Lambert & Moss, 1989; Pearce *et al.*, 2011). The three most abundant fatty acids were anteiso-$C_{15:0}$ (29 %), $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$ (22 %) and $C_{16:0}$ (21 %); H63^T contained only small amounts of 14-carbon and cyclic 17-carbon fatty acids (Table 3).

L. pneumophila and some of the other *Legionella* species tested are well known for their capacity to grow in macrophages; indeed, intracellular infection of lung macrophages is critical for the pathogenesis of legionellosis (Brieland *et al.*, 1994; Rossier *et al.*, 2004). Therefore, we assayed the ability of H63^T to replicate within human U937 cell (ATCC CRL-1593.2) macrophages as described previously (Rossier *et al.*, 2004). During the first 48 h of infection, H63^T replicated by approximately two logs, indicating that H63^T is quite adept at intracellular replication (Fig. S4a). In comparison, *L. pneumophila* ATCC BAA-74 displayed approximately 4 logs of growth during 48 h of infection (Fig. S4a). H63^T was next examined for its ability to grow and survive within the lungs of 6- to 8-week-old A/J mice (Jackson Laboratory, Bar Harbor, ME, USA) following intratracheal inoculation with 10^6 c.f.u. stationary-phase BCYE agar-grown bacteria (Rossier *et al.*, 2004). The numbers of H63^T bacteria increased approximately 5-fold by 48 h post-inoculation, indicating that the strain can grow in the mammalian lung (Fig. S4b). This level of growth was comparable to what we and others have observed for *L. pneumophila* inoculated into A/J mice (Brieland *et al.*, 1994; Rossier *et al.*, 2004). After 48 h post-inoculation, the numbers of H63^T bacteria decreased steadily (Fig. S4b), presumably due to bacterial clearance from the lung by the innate immune response. Compatible with the acute infection observed, histopathological examination of haematoxylin/eosin-stained lung sections taken from infected mice at 72 h post-inoculation

Table 3. Fatty acid profiles of strain H63^T and its nearest neighbours based on 16S rRNA gene sequence similarity

Strains: 1, *L. cardiaca* sp. nov. H63^T (data from this study); 2, *L. brunensis* ATCC 43878^T (Wilkinson *et al.*, 1988); 3, *L. londiniensis* ATCC 49505^T(Dennis *et al.*, 1993); 4, *L. jordanis* ATCC 33623^T (Cherry *et al.*, 1982); 5, *L. erythra* ATCC 35303^T (Lambert & Moss, 1989); 6, *L. dresdenensis* DSM 19488^T (Lück *et al.*, 2010); 7, *L. rubrilucens* ATCC 35304^T (Lambert & Moss, 1989); 8, *L. feeleii* ATCC 35072^T (Moss *et al.*, 1983); 9, *L. pneumophila* ATCC 33152^T (Brenner *et al.*, 1979; Cherry *et al.*, 1982); 10, *L. birminghamensis* ATCC 43702^T (Wilkinson *et al.*, 1987). Values are percentages of total fatty acids. tr, Trace amount (<1 %); −, not detected/not reported.

Fatty acid	1	2	3	4	5	6	7	8	9	10
$C_{14:0}$	2	1	−	tr	−	−	−	1	tr	−
iso-$C_{14:0}$	tr	1	tr	tr	tr	−	2	4	8	−
anteiso-$C_{14:0}$	2	−	−	−	−	−	−	−	−	−
$C_{15:0}$	−	1	1	tr	−	−	−	3	tr	3
iso-$C_{15:0}$	3	2	tr	−	1	−	tr	−	−	−
anteiso-$C_{15:0}$	29	39	5	42	8	16	12	18	14	30
$C_{15:0}$ 2-OH	tr	−	−	−	−	−	−	−	−	−
$C_{15:1}$	−	tr	−	2	−	−	−	3	tr	−
$C_{15:1}\omega6c$	tr	−	2	−	−	−	−	−	−	−
$C_{16:0}$	21	12	15	4	26	−	15	21	10	13
iso-$C_{16:0}$	5	5	11	17	4	25	23	17	32	14
$C_{16:1}$	−	9	19	7	−	−	−	18	13	11
iso-$C_{16:1}$	−	−	tr	2	−	−	−	2	2	−
$C_{16:1}\omega7c/C_{16:1}\omega6c$	22	−	−	−	38	24	27	−	−	−
$C_{17:0}$	tr	tr	8	2	−	−	−	1	tr	−
iso-$C_{17:0}$	3	1	0	−	1	−	tr	−	−	−
anteiso-$C_{17:0}$	10	24	23	18	10	9	9	6	11	16
$C_{17:0}$ cyclo	1	1	0	2	−	0	−	−	3	0
$C_{18:0}$	1	1	8	tr	4	−	2	2	2	−
$C_{20:0}$	2	2	−	2	−	−	−	3	2	−

displayed irregular interstitial inflammation with moderate mononuclear infiltrate (not shown), indicative of pneumonia, as has been observed previously in A/J mice infected with *L. pneumophila* for 72 h (Brieland *et al.*, 1994). At 24 h post-inoculation, 4.5 logs of H63^T were present in the spleen, and the bacterial burden remained at that level for an additional 24 h (Fig. S4c). After 48 h, the numbers of bacteria in the spleen decreased and, by the fifth day, H63^T was detectable in the spleen of only one of the five animals. In summary, H63^T replicates significantly within both human macrophages and the murine lung, indicating that the strain is pathogenic, compatible with its isolation from a human patient experiencing severe illness.

Although H63^T was isolated from a patient and no environmental source was identified in the case history, it is undoubtedly true that the novel species exists naturally in freshwaters, as is the case for most other legionellae. However, it is difficult to predict if and when an environmental isolation of the species would occur, based on what has been seen for other *Legionella* species. Indeed, most *Legionella* species were monotypic in their initial description (Brenner *et al.*, 1985; Morris *et al.*, 1980; Thacker *et al.*, 1988, 1989). In the case of *Legionella tucsonensis*, since the publication of its clinical isolation in the 1980s, additional strains have yet to be described, despite years of environmental sampling and clinical surveillance (Thacker *et al.*, 1989).

Of the 55 previously characterized *Legionella* species, 26 have been isolated from clinical cases and, in 25 of those instances, they were believed to be the likely causative agent of disease (Berger *et al.*, 2006; Diederen, 2008; Edelstein *et al.*, 2012; Gobin *et al.*, 2009; König *et al.*, 2005; Marrie *et al.*, 2001; Tang & Krishnan, 1993; Yang *et al.*, 2012). Eleven additional species have been linked to disease through serology (Berger *et al.*, 2006; Fang *et al.*, 1989; Lieberman *et al.*, 2002; Marrie *et al.*, 2001; McNally *et al.*, 2000). We therefore conclude that the novel species represents the fifty-sixth *Legionella* species, and the thirty-seventh to be linked to disease. Although the lung is the typical primary site for *Legionella* infections, extrapulmonary manifestations do occur, and legionellae have been found in various niches within the body including the spleen, lymph node, blood, kidney, liver, skin, bone, sinus and heart (Edelstein *et al.*, 1979; Evans & Winn, 1981; McClelland *et al.*, 2004; Monforte *et al.*, 1989; Schlanger *et al.*, 1984; Waldor *et al.*, 1993; Watts *et al.*, 1980; Weisenburger *et al.*, 1980). In the heart, *Legionella* infection can take the form of myocarditis, pericarditis or endocarditis. There have been 18 documented cases of *Legionella* endocarditis, including the recent isolation of H63^T (Leggieri *et al.*, 2012; Pearce *et al.*, 2011). Four *Legionella* species, *L. pneumophila*, *L. micdadei*, *L. dumoffii* and *L. longbeachae*, have been implicated in prosthetic valve endocarditis; however, only *L. pneumophila* has been isolated from a native heart (Leggieri *et al.*, 2012; Samuel *et al.*, 2011; Tompkins *et al.*, 1988). Therefore, the novel species represents the first non-pneumophila species to be isolated from native valve endocarditis.

Description of *Legionella cardiaca* sp. nov.

Legionella cardiaca (car.di.a′ca. L. fem. adj. *cardiaca* of or pertaining to the heart, in reference to the isolation of the type strain from aortic valve tissue).

Gram-negative rod. Grows on BCYE agar and requires L-cysteine. Negative in tests for glucose fermentation, nitrate reduction, urease and autofluorescence. Positive in tests for swimming motility, catalase, gelatinase, β-lactamase, hippurate hydrolysis and pigmentation in BYE broth. The fatty acid profile consists primarily of branch-chained fatty acids.

The type strain is H63^T (=ATCC BAA-2315^T =DSM 25049^T =JCM 17854^T), isolated from human aortic valve tissue and the causative agent of endocarditis. The DNA G+C content of the type strain is 41.8 mol%.

Note added in proof

A paper describing two novel species of *Legionella*, *Legionella tunisiensis* sp. nov. and *Legionella massiliensis* sp. nov. by Campocasso *et al.* (2012), which was accepted for publication shortly after this paper, is published on pp. 3003–3006 of this issue.

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