**Legionella taurinensis sp. nov., a new species antigenically similar to Legionella spiritensis**

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A group of 42 Legionella-like organisms reacting specifically with Legionella spiritensis serogroup 1 antisera were collected throughout Europe by the Centre National de Référence (French National Reference Centre) for Legionella. This group of isolates differed somewhat from L. spiritensis in terms of biochemical reactions, ubiquinone content and protein profile. The latter two analyses revealed that one of these L. spiritensis-like isolates, Turin I no. 1', was highly related, but not identical to any of the red autofluorescent species of Legionella. In fact, this strain was the first of these particular isolates recognized to emit a red autofluorescence when exposed to UV light. Profile analysis of randomly amplified polymorphic DNA established that the red autofluorescent L. spiritensis-like isolates constituted a homogeneous group distinct from Legionella rubrilucens and Legionella erythra. DNA–DNA hybridization studies involving the use of 51 nuclease confirmed that the indicated group of isolates are a new species of Legionella, for which the name **Legionella taurinensis** is proposed with strain Turin I no. 1' (deposited as ATCC 700508') as the type strain.

**Keywords:** Legionella taurinensis sp. nov., taxonomy, identification

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**INTRODUCTION**

Bacteria of the family *Legionellaceae* are usual inhabitants of both natural and man-made aquatic habitats (Fliermans, 1996; Fliermans *et al.*, 1981; Stout *et al.*, 1985). Their survival in these environments is highly dependent on their ability to invade and multiply within a large diversity of protozoa (Fields, 1996; Rowbotham, 1980). Since the description of this family in 1979 (Brenner *et al.*, 1979), 42 species represented by 64 serogroups have been described including *Legionella lytica*, a species not yet culturable on buffered charcoal-yeast extract (BCYE) media (Benson *et al.*, 1996; Hookey *et al.*, 1996). Other strains of *Legionella*-like amoebal pathogens (LLAP) that are not yet culturable could presumably add five more species to the genus *Legionella* (Adlecke *et al.*, 1996). Twenty-five of the *Legionella* species were initially described on the isolation of a single unidentified strain and some of them are still represented (to our knowledge) by the original strain only (Benson *et al.*, 1991, 1996; Bornstein *et al.*, 1989; Brenner *et al.*, 1985; Dennis *et al.*, 1993; Edelstein *et al.*, 1982; Herwaldt *et al.*, 1984; Thacker *et al.*, 1988, 1989, 1992; Verma *et al.*, 1992; Wilkinson *et al.*, 1987, 1988). *Legionella pneumophila* remains the most frequently isolated species from patients, although eighteen other species, including *L. lytica*, have also been isolated in these instances from pneumonia patients (Hookey *et al.*, 1996; Lo Presti *et al.*, 1997). Some species, such as *Legionella parisiensis*, *Legionella gormanii* and *Legionella jordanis* were first obtained as environmental specimens before being recognized as human pathogens.

During several recent investigations of hospital and community water in Europe, 42 isolates reacting exclusively with antisera raised against *L. spiritensis* serogroup 1 were collected by the Centre National de Référence des *Legionella* (French National Reference Centre for *Legionella*). However, in contrast to *L.
spiritensis, some of these isolates displayed red auto-
fluorescence under longwave UV light. This unusual
feature stimulated the characterization of this group of
organisms. Analysis of phenotypic and genotypic
characteristics presented in this report have led to the
description of this group as a new species, Legionella
taurinensis.

METHODS

Isolation procedure. Water samples (1 l) were collected from
six European countries (Czech Republic, France, Italy,
Portugal, Spain and Switzerland), concentrated by con-
tinuous centrifugation and/or filtration, and either un-
treated or treated by acidification according to previously
described procedures (Bornstein et al., 1989). All samples
were cultured on BCYE agar supplemented with 0-1 %
α-ketoglutarate (BCYEa) and BCYEz supplemented with
glycine, vancomycin and colistin (GVC).

Bacterial strains. Forty-two isolates of Legionella (Table 1)
were obtained from the different sites. In addition, the type
strains of 41 Legionella species (Table 2) were used in this
study; these strains fell into 63 serogroups. Several potential
new species, including "Legionella donaldsonii" (strain 'Glas-
gow' 86/35784; gift from T. G. Harrison, Colindale, UK)
(Hooker et al., 1996), which has not yet been formally
described, were also tested (Table 2). As amoeba cultures
were not available, one validly published species, L. lytica,
could not be used for the DNA hybridization studies.

Cultural and biochemical tests. The type strains and the
isolates were cultured on BCYEa or GVC when needed.
Requirement for cysteine was tested by its omission when
preparing the BCYEa media. The cultures were examined
for autofluorescence using Wood's light (366 nm). Tests for
sugar fermentation, gelatinase, nitrate reduction and the
presence of flagella were done as previously described
previously described (Lo Presti et al., 1997). Sequencing
was done by the dideoxy chain-termination method (Sanger
et al., 1977) under previously described conditions (Lo Presti

<table>
<thead>
<tr>
<th>Origin</th>
<th>No. of isolates</th>
<th>Strain</th>
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</thead>
<tbody>
<tr>
<td>Czech Republic</td>
<td>1</td>
<td>BM-750</td>
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<tr>
<td>France</td>
<td>24</td>
<td>Châlons/Saône eau 1 colonie 25, Châlons/Saône eau 1 colonie 26, Croix-Rousses 2 C9, Croix-Rousses 2 C13, Gap 1 B 41, Gap 1 B42, Hôtel-Dieu 2 B3, Hôtel-Dieu 2 B4, Hôtel-Dieu 2 B12, Henry-Gabrielle 1 eau 1 C7, Henry-Gabrielle 1 eau 3 C7, Henry-Gabrielle 1 eau 3 C10, IB 39 no. 6, Moulins 1 C1, Moulins 1 D3, Moulins 1 D5, Moulins 1 D6, Mercure 96 I eau 8 no. 7, Mercure 96 I eau 8 no. 8, St-Etienne III no. 1, Toulouse 26 no. 14, Toulouse 26 no. 17, Toulouse 28 no. 7, Toulouse 28 no. 8</td>
</tr>
<tr>
<td>Italy</td>
<td>4</td>
<td>Turin I no. 1° (ATCC 700508°), Turin I no. 2, Turin II no. 180, Turin II no. 195</td>
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<td>Portugal</td>
<td>8</td>
<td>Lisbonne III A5, Portugal A12, Portugal A13, Portugal A14, Portugal A16, Portugal A17, Portugal A19, Portugal A20</td>
</tr>
<tr>
<td>Spain</td>
<td>2</td>
<td>Madrid 1 no. 4, Madrid IV no. 1</td>
</tr>
<tr>
<td>Switzerland</td>
<td>3</td>
<td>Genève 10 no. 1, Lausanne 15 no. 6, Lausanne 96 V no. 1</td>
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### Table 2. 16S rRNA similarity and DNA relatedness of strain Turin I no. 1°

<table>
<thead>
<tr>
<th>Source of unlabelled DNA (Legionella strain)</th>
<th>Relatedness (%) to labelled DNA from: Turin I no. 1° (ATCC 700508T)</th>
<th>L. rubrilucens (ATCC 35304T)</th>
<th>Similarity (%) to 16S rDNA gene of Turin I no. 1°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 °C*</td>
<td>∆T_m†</td>
<td>60 °C</td>
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<tr>
<td>Turin I no. 1°†‡</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Turin I no. 2†‡</td>
<td>100</td>
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<td>0</td>
</tr>
<tr>
<td>Lisbonne III A5‡</td>
<td>95</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Madrid IV no. 1 ‡</td>
<td>78</td>
<td>2.5</td>
<td>56</td>
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<tr>
<td>L. rubrilucens (ATCC 35304T)</td>
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<tr>
<td>L. erythra serogroup 2 (LC 217)</td>
<td>64</td>
<td>7.5</td>
<td>100</td>
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<td>L. erythra serogroup 1 (ATCC 35303T)</td>
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<td>L. spiritensis serogroup 2 (NCTC 12082)</td>
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<td>L. spiritensis serogroup 1 (ATCC 35249T)</td>
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<tr>
<td>L. cherrii (ATCC 35252T)</td>
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<td>L. birminghambensis (ATCC 43702T)</td>
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<td>L. jordanis (ATCC 33623T)</td>
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<tr>
<td>L. londiniensis (ATCC 49505T)</td>
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<td>L. nautarum (ATCC 49506T)</td>
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<td>L. hackelliae serogroup 1 (ATCC 35250T)</td>
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<td>L. oakridgensis (ATCC 33761T)</td>
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<td>L. fairfieldensis (ATCC 49588T)</td>
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<td>L. anisa (ATCC 35292T)</td>
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<td>Other Legionella species§</td>
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</tr>
<tr>
<td>LLAP strains</td>
<td>ND</td>
<td></td>
<td></td>
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</tbody>
</table>

* Reactions were done at least in duplicate at 60 °C.
† Divergence from the T_m of Turin I no. 1° (ATCC 700508T), calculated to the nearest 0.5%. Reactions were done in triplicate.
‡ Strains of the cluster 'Turin'.
§ Other Legionella species: Legionella cincinnatiensis, ATCC 43753T; Legionella dumoffii, ATCC 33279T; Legionella genomospecies 1, ATCC 51913T; Legionella gormanii, ATCC 33297T; Legionella gratiana, ATCC 49413T; Legionella jamestowniensis, ATCC 35298T; Legionella moravica, ATCC 43877T; Legionella parisiensis, ATCC 35399T; Legionella quteiensis, ATCC 49507T; Legionella quinlivanii serogroup 1, ATCC 43830T; Legionella sainthelenensis serogroup 1, ATCC 35248T; Legionella santercius, ATCC 35301T; Legionella shakespearei, ATCC 49653T; Legionella steigerwaltii, ATCC 35302T; Legionella tucsonensis, ATCC 49180T; Legionella wadsworthii, ATCC 33877T; L. waltersii, ATCC 51914T; Legionella worsleiensis, ATCC 49508T; strain Greoux 11 D13, ATCC 700509; and strain IB V no. 2, ATCC 700511.
ND, Not determined.

et al., 1997), and the sequence (GenBank no. AF037597) was compared with those previously published (Adeleke et al., 1996; Birtles et al., 1996; Fry et al., 1991; Hookey et al., 1996) and available in the DDBJ, EMBL and GenBank databases with the gapped BLAST program (Altschul et al., 1997) via the Internet (http://www.ncbi.nlm.nih.gov).

**DNA studies.** DNA was extracted and purified as previously described (Brenner et al., 1982) and the native DNA was labelled by nick-translation with 3H-labelled nucleotides (Amersham). DNA–DNA hybridization with all species (Table 2) was performed by the standard S1 nuclease method (Grimont et al., 1980). The thermal stability of heterologous
hybrids was calculated by a standard method (Crosa et al., 1973).

The G+C (mol%) content of DNA from strain Turin I no. 1T was determined by HPLC of nucleotides after hydrolysis of the DNA by P1 nuclease (Kaneko et al., 1986) and also spectrophotometrically by the thermal denaturation method (Marmur & Doty, 1962).

RESULTS

Cultural and biochemical characterization

The 42 strains of the 'Turin' cluster of Legionella collected from hospital and community water in Europe grew on BCYEa agar and GVC medium, but not on blood agar or BCYEa lacking supplemented L-cysteine. The type strain, Turin I no. IT, was a Gram-negative rod with a single polar flagellum. Physiological tests performed on the cluster were positive for gelatin liquefaction and the presence of flagella (100% of isolates), catalase (93%), β-lactamase (98%) and oxidase (76%). Negative reactions were obtained for urease, carbohydrate fermentation and nitrate reduction. The majority of isolates (90%) did not hydrolyse hippurate. No brown pigment was produced on tyrosine-supplemented media by the majority of these strains (96%) when using the 3 d growth criteria. After 10 d growth, 67% of the isolates remained negative while half of the positive strains were detected as only weakly positive. Including the type strain (at the first isolation), 67% of the strains exhibited red autofluorescence under UV light whereas the other isolates were not autofluorescent or produced only a weak red autofluorescence after two subcultures. However, this autofluorescence was often lost after several passages on agar media; this situation is similar to that observed for the bluish-white autofluorescent species of Legionella (unpublished observations). All the isolates also gave rise to yellow fluorescence of the growth medium. Colonies produced a greenish colour on DGVC medium. Strain Madrid IV no. 1 turned out to be the most divergent strain of the cluster in terms of biochemical reactions (e.g. oxidase-negative, production of brown pigment on tyrosine-supplemented media).

DFA

Isolate Turin I no. 1T and the 41 other members of the 'Turin' cluster gave a 3 + to 4 + fluorescence with L. spiritensis serogroup 1 antisera. Antisera developed against the Turin I no. 1T isolate gave a 4 + fluorescence with the homologous strain and 3 + with the L. spiritensis type strain. The cross-reaction could not be abolished by absorption with L. spiritensis.

Cellular fatty acids and ubiquinone composition

The Turin I no. 1T isolate and members of this group contain the 14-methylpentadecanoic acid (16:0) which accounts for more than 20% of the fatty acid composition; this is followed by 10–20% 12-methyltetradecanoate (a15:0), cis-9-hexadecenoate (16:1) and hexadecanoate (16:0). The branched-chain fatty acid 14-methylhexadecanoate (a17:0) accounts for 10% and the i14:0 for 3% only; minor quantities of 15:1, heptadecanoate (17:0) and octadecanoate (18:0) were found. In comparison, the Madrid IV no. 1 isolate, the most divergent strain of this cluster, had less a15:0 (1%) and more a17:0 (21%). The ubiquinone profiles of 'Turin' cluster isolates were dominated by the presence of quinone Q-12. Ubiquinones Q-11 and Q-13 each accounted for approximately 20% of the amount of Q-12.

Protein profiling

Protein profiles revealed 90–95% homology for the 11 isolates of cluster 'Turin' tested and a maximum of 86% homology with L. rubrilucens, 83% with L. erythra and 79% with L. spiritensis.

RAPD analysis

Strains of the cluster 'Turin' showed a unique pattern by RAPD analysis that differed from those obtained for all the 41 other Legionella species tested (data not shown).

Sequencing of the 16S rRNA genes

The sequence of the 16S rRNA gene of the strain Turin I no. 1T (GenBank no. AF037597) was 99.10% identical to sequence X73398 of L. rubrilucens, which was the closest sequence, 99.03% identical to sequence Z32643 of L. rubrilucens, 98.44% identical to sequence M36027 of L. erythra serogroup 1 and only 95.8% identical to the 16S rRNA gene of L. spiritensis serogroup 1. The sequence was less than 95% related to each of the 16S rDNA sequences of the LLAP strains (including L. lytica).

DNA hybridization

3H-labelled DNA from strain Turin I no. 1T was hybridized with unlabelled DNAs from type strains of the other Legionella species and three strains of the same cluster (Table 2). Table 2 shows that there was ≥ 78% relatedness between each of the four strains of the cluster 'Turin' with a maximum of 2.5 °C divergence in thermal stability of the hybrids. In optimal reactions performed at 60 °C, labelled DNA from the strain Turin I no. 1T was 64% related to the type strain of L. rubrilucens. The divergence in thermal stability of this hybrid was 7.5 °C. In the reciprocal reaction, the level of DNA relatedness between the type strain of L. rubrilucens and strain Turin I no. 1T fell to 54%. The DNA relatedness obtained with Madrid IV no. 1, the most divergent strain of the cluster 'Turin', was 56% when it was hybridized to labelled DNA from L. rubrilucens.

G+C content

The G+C content of strain Turin I no. 1T was 46 mol% by each of the two methods used.
DISCUSSION

The red autofluorescence exhibited by the majority of the isolates of the ‘Turin’ cluster under UV light did not affirm the match with the phenotypic characteristics of *L. spiritensis* with which the cluster appeared to show a potentially strong antigenic relationship. Further biochemical characterization of the ‘Turin’ cluster isolates revealed that a marked difference existed between the isolates and *L. spiritensis*, *L. rubrilucens* and *L. erythra*, in that the isolates of the cluster did not produce the brown pigment on tyrosine-supplemented media. The major fatty acids of the type strain, Turin I no. 1T, comprised those with 16 carbon atoms, which is the case for the red autofluorescent species and *L. spiritensis*; however, the isolates of the ‘Turin’ cluster had more n16:0 than *L. erythra*, *L. rubrilucens* and *L. spiritensis*, and less i16:0 than *L. rubrilucens*. Since the differentiation of all these species by fatty acid composition varies according to growth medium, this feature would appear to be more useful for ascribing unknown isolates to groups of species (Wilkinson et al., 1990). The ubiquinone composition of the ‘Turin’ cluster clearly differentiated it from *L. spiritensis*, which contains ubiquinone Q-13 as major ubiquinone and not quinone Q-12.

Sequencing of the 16S rRNA gene has proved to be a useful tool for assigning an unidentified isolate to the nearest species. Sequence homologies of this gene between the two described red autofluorescent *Legionella* species (*L. rubrilucens* and *L. erythra*) were 99.4% (Hooke et al., 1996). Comparison of the 16S rRNA gene sequence of the type strain, Turin I no. 1T, with those of the *L. rubrilucens* and *L. erythra* species, revealed high similarities (99.1 and 98.4% respectively). This raised the possibility that strain Turin I no. 1T was a subspecies or a new serogroup of *L. rubrilucens*. DNA hybridization ruled out this possibility by showing that strain Turin I no. 1T constitutes a distinct species. It should be noted that DNA hybridization was not possible with the five LLAP groups (since total DNA was not available), but their 16S rDNA sequence had less than 95% homology with Turin I no. 1T making it very unlikely that strain Turin I no. 1T belonged to any of the LLAP groups (Stackebrandt & Goebel, 1994).

Recently, the RAPD technique has been used for the identification of a newly described clinical strain, *L. parisiiens*. This approach showed that species-specific profiles could be obtained for the bluish-white autofluorescent *Legionella* species (Lo Presti et al., 1997). By applying the RAPD technique to the red autofluorescent species and to the ‘Turin’ cluster, a specific profile for the newly proposed species was produced. This specific profile yielded no common bands with the species-specific profile of *L. rubrilucens*, indicating that the ‘Turin’ cluster was a homogeneous group differing in genotype from *L. rubrilucens*.

Ultimately, the genetic definition of a species for the ‘Turin’ cluster (Wayne et al., 1987) was fulfilled by the data obtained from whole DNA–DNA hybridization studies, which manifested a homogeneous group with more than 70% DNA relatedness between the four tested strains and less than 5 °C difference between them in thermal stability (Table 2). Moreover, this cluster has less than 70% relatedness at the DNA level to the closest species, *L. rubrilucens*, and the thermal stability of the hybrid of these two species differs by more than 7.5 °C. Consequently, both results confirm that the ‘Turin’ cluster forms the 43rd *Legionella* species. Thus, the confidence attained in differentiating this new species according to phenotypes from other *Legionella* species (Table 3) presents the opportunity to formalize the name (Benson et al., 1996; Ursing et al., 1995).

**Description of Legionella taurinensis** sp. nov.

*Legionella taurinensis* (tau.ri.nen’sis. M.L. adj. taurinensis pertaining to Turin, Italy, whose classical Latin name was Augusta Taurinorum)

This bacterium is a Gram-negative rod with a single polar flagellum. It gives positive reactions for catalase, oxidase, gelatinase and the presence of β-lactamase. Negative reactions are recorded for urease, nitrate reduction, acid production from D-glucose, browning of tyrosine agar and hippurate hydrolysis. Some of the strains autofluoresce red (at 365 nm). DFA results exhibit strong cross-reaction with *L. spiritensis*. This species has ubiquinone Q-12 as the major component. All the strains of this species have been isolated from water samples throughout Europe. The type strain of

<table>
<thead>
<tr>
<th>Legionella species</th>
<th>Oxidase</th>
<th>Tyrosine</th>
<th>Fluorescence under UV light</th>
<th>DFA Major ubiquinone</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. taurinensis</em></td>
<td>+</td>
<td>−</td>
<td>−/Red (68%)</td>
<td><em>L. spiritensis</em> serogroup 1 Q-12</td>
</tr>
<tr>
<td><em>L. spiritensis</em> serogroup 1</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td><em>L. spiritensis</em> serogroup 1 Q-13</td>
</tr>
<tr>
<td><em>L. spiritensis</em> serogroup 2</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td><em>L. spiritensis</em> serogroup 2 Q-13</td>
</tr>
<tr>
<td><em>L. rubrilucens</em></td>
<td>−</td>
<td>+</td>
<td>Red</td>
<td><em>L. rubrilucens</em> Q-12</td>
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<tr>
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<td>Variable</td>
<td>+</td>
<td>Red</td>
<td><em>L. rubrilucens</em> Q-12</td>
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</tbody>
</table>
L. taurinensis is Turin I no. 1\(^T\) (= ATCC 700508\(^T\)), which was isolated from a humidifier in the city of Turin (Italy). It has a G+C content of 46 mol%.

ACKNOWLEDGEMENTS

We are grateful to Maddalena Castellani Pastoris (Istituto Superiore di Sanita, Roma, Italy) and Hans G. Trüper (University of Bonn, Bonn, Germany) for their help in designating the name of the new species, and to Morris Goldner (University of Laval, Canada) for editing the manuscript. We are also grateful to D. Blanc (CHU Vaudois, Lausanne, Switzerland), V. Drasar (Czech National Legionella Reference Laboratory, Vyskov, Czech Republic), M. Marques (Hospital of Santa Cruz, Carnaxide, Portugal) and C. Pelaz (Laboratorio de Legionella, Majadahonda, Spain) who provided us with some of the strains used in this study.

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Legionella taurinensis sp. nov.


