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Problems Associated with Identification of *Legionella* Species from the Environment and Isolation of Six Possible New Species

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Following investigation of an outbreak of legionellosis in South Australia, numerous *Legionella*-like organisms were isolated from water samples. Because of the limited number of commercially available direct fluorescent-antibody reagents and the cross-reactions found with some reagents, non-*pneumophila* legionellae proved to be difficult to identify and these isolates were stored at −70°C for later study. Latex agglutination reagents for *Legionella pneumophila* and *Legionella anisa* developed by the Institute of Medical and Veterinary Science, Adelaide, Australia, were found to be useful as rapid screening aids. Autofluorescence was useful for placing isolates into broad groups. Cellular fatty acid analysis, ubiquinone analysis, and DNA hybridization techniques were necessary to provide definitive identification. The species which were isolated most frequently were *L. pneumophila*, followed by *L. anisa*, *Legionella jamestowniensis*, *Legionella quinlivanii*, *Legionella rubrilucens*, *Legionella spiritensis*, and a single isolate each of *Legionella erythra*, *Legionella jordanis*, *Legionella birminghamensis*, and *Legionella cincinnatiensis*. In addition, 10 isolates were found by DNA hybridization studies to be unrelated to any of the 26 currently known species, representing what we believe to be 6 possible new species.

In January 1986 an outbreak of legionellosis due to *Legionella pneumophila* serogroup 1 occurred in Adelaide, Australia. During the investigation of this outbreak, a large number of *Legionella*-like organisms were isolated from various water sources, mainly in the metropolitan area. Prior to the outbreak, this laboratory (the Institute of Medical and Veterinary Science, Adelaide, Australia) had gained some experience in the use of direct fluorescent-antibody (DFA) reagents provided by the Centers for Disease Control, Atlanta, Ga., for identification of clinical isolates of legionellae. Since most isolates were *L. pneumophila* serogroup 1, little difficulty had been encountered in their identification. However, with the sudden increase in environmental sampling and isolation of many non-*pneumophila* legionellae, the limitations of this technique soon became apparent. Cross-reactions between serogroups of *L. pneumophila* and between species are a well-known problem (6, 7, 15, 16). These cross-reactions and the limited availability of commercial DFA reagents make DFA techniques unsatisfactory for species identification. The use of DFA staining as a screening method for *L. pneumophila* was also found to be too time consuming and costly for the large number of organisms submitted for identification. This necessitated the development of latex slide agglutination reagents for the most common isolates, namely *L. pneumophila* and *Legionella anisa*. However, difficulty was still encountered with other non-*pneumophila* isolates, and most of these isolates were stored at −70°C for further study. This report describes the methods we used for the identification of more than 600 isolates of *Legionella*-like organisms from the South Australian environment collected over a 2-year period. Descriptions are also provided for 6 possible new species which appear to be unrelated to any of the 26 currently known species (1, 20).

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MATERIALS AND METHODS

Sources of isolates. Type strains of the 26 currently described species (1, 20) were obtained from the American Type Culture Collection (ATCC), Rockville, Md. The majority of environmental isolates were obtained from water samples processed at this institute between January 1986 and December 1987. Water samples were submitted from a variety of sources, including cooling towers and air conditioning units in buildings located in the Adelaide, Australia, metropolitan area. Approximately 140 isolates were also forwarded from the State Water Laboratory for identification. Many of these isolates were obtained from the reticulated water supply. Small numbers of isolates included in the study were received from other hospital laboratories in Adelaide, Australia.

Isolation from water. Water samples submitted to this Institute were examined by the following method during the period in which the isolates studied were collected. Samples were concentrated 10-fold by centrifugation at 3,000 × *g* for 20 min. A 0.1-ml sample was spread over the surface of a buffered charcoal-yeast extract (BCYE) agar plate containing BMPAα selective supplement (Oxoid Ltd., Basingstoke, United Kingdom). The remainder of the concentrated sample was decontaminated by the addition of 9 volumes of HCL-KCL buffer, pH 2.2 (2), and left for 5 min before plating onto BMPAα medium. A further 1 ml of the original sample was diluted 1/10 in 0.1% peptone water, and 0.1 ml was inoculated onto a BMPAα plate. From January 1988, BCYE agar containing MWY selective supplement (Oxoid) was used in addition to the BMPAα plate for the decontaminated sample.

Plates were placed in plastic bags, incubated at 35°C, and examined for *Legionella* colonies, using a plate microscope, after 4 days and again after 7 days of incubation. Colonies resembling *Legionella* were subcultured to blood agar and BCYE agar, with and without growth supplement containing

cysteine and ferric pyrophosphate. Those organisms which grew only on BCYE agar with supplement but not on the other two media were retained for further study.

DFA tests. Each isolate was tested against either one or both of Genetic Systems polyvalent *L. pneumophila* reagent and Zeus Technologies, Inc. polyvalent *L. pneumophila* serogroups 1 through 6. Other reagents used for testing where appropriate included Centers for Disease Control reagents for *Legionella bozemanii*, *Legionella dumoffii*, *Legionella gormanii*, *Legionella jordanis*, *Legionella oakridgensis*, *Legionella longbeachae* serogroups 1 and 2 and Zeus Technologies, Inc. reagent for *Legionella micdadei*. Tests were performed according to the instructions of the manufacturer by using organisms suspended in 1% formalin and placed on Teflon-coated multiwell slides.

Latex slide agglutination reagents. Antiserum for the preparation of the latex reagents was produced by immunizing New Zealand White rabbits with a series of intravenous injections of heat-killed antigen preparations. The resultant immune sera were coated onto latex particles (0.8 μm; Sigma Chemical Co., St. Louis, Mo.) by using the method described by Severin (13). Three different latex reagents were prepared. The first reagent, a *L. pneumophila* serogroup 1 reagent, was prepared by using the ATCC strains representing subgroups 1a, 1b, and 1c of serogroup 1 (17). The second reagent, a polyvalent reagent directed against *L. pneumophila* serogroups 2 through 14, was produced by pooling portions of the individual serogroup-specific antisera prepared by using the ATCC type strains of each serogroup (20). The third reagent was prepared by using the ATCC type strain of *L. anisa*.

Analysis of cellular fatty acids. Four methods of fatty acid extraction were compared for their yield of both straight-chain and branched-chain acids, and the following method, which is a modification of the Folch procedure (4), was found to give the highest yield. Isolates were grown for 3 to 4 days on BCYE agar plates at 35°C. Organisms were removed from the surface of one or two plates, depending on the density of growth, and suspended in 0.5 ml of water. Cellular fatty acids were extracted by shaking with 10 ml of 2:1 chloroform-methanol for 5 min. After partition with 2 ml of water, the bottom (chloroform) layer was removed to a clean tube and evaporated to dryness under nitrogen. The extracts were transesterified by the addition of 5 ml of 1.5% H_2SO_4 in methanol at 75°C for 3 h or 70°C overnight. After cooling to room temperature, 1 ml of water was added and methyl esters were extracted twice with 2 ml of *n*-hexane. The hexane extracts were concentrated under nitrogen to approximately 0.5-ml volumes and stored in small screw-cap vials at −20°C.

Extracts were analyzed by using a series 8500 DANI gas chromatograph fitted with a programmed temperature vaporizing (PTV) injector, flame ionization detector, and a DANI ALS 3940 autosampler. Samples (1 μl) of the extracts were analyzed on a capillary column (25 m by 0.22 mm) (SGE Pty Ltd., Ringwood, Vic., Australia) coated with 0.25 μm of BP-1 stationary phase, using hydrogen as the carrier gas. The PTV injector was programmed to operate in the solvent split mode. Chromatograms were recorded and analyzed by using Nelson Analytical series 3000 Chromatography Data System software. Fatty acid peaks were identified by comparison of relative retention times with a standard bacterial fatty acid mix (Supelco, Bellefonte, Pa., product no. 4-7080). Where necessary, further information on peak identity was obtained by capillary column gas chromatography-mass spectometry by using a 5988A/HP 1000 computer system (Hewlett-Packard Co., Palo Alto, Calif.). Material eluting from the column was ionized by electron impact and scanned in the range of *m/z* 40 to *m/z* 400. The displayed spectra were compared with library data or with those obtained from authentic compounds.

Ubiquinone analysis. Ubiquinones were extracted by the method of Moss and Guerrant (12), with the following modifications. Isolates were grown on two BCYE agar plates for 3 to 4 days. Growth was removed by scraping with a bent glass rod, using approximately 5 ml of distilled water, and it was placed in Teflon-lined screw-cap glass tubes. The tubes were centrifuged at 1,200 × *g* for 20 min to pellet the organisms and remove the water prior to the extraction procedure. Following extraction, the residues were suspended in 0.1 ml of methanol.

Samples were analyzed by high-performance liquid chromatography with a modular system (ICI Australia Operations Pty Ltd. Scientific Instruments Div, Melbourne, Australia) comprising three LC1500 pumps controlled by a model 50 Programmer; a AS2000 Autosampler; a TC19000 Column Temperature Controller set at 37°C, containing an ICI 10-μm particle size Spherisorb ODS2 reverse-phase C18 column (4.6 mm by 25 cm); and two Knauer Variable Wavelength Spectrophotometers set at 275 and 248 nm, respectively. Data acquisition and analysis was by Nelson Analytical series 3000 chromatography software. Ubiquinones were separated by using a three-phase solvent gradient of methanol-isopropanol-water at a flow rate of 1 ml/min. The initial solvent composition was 75:20:5 and was changed in a linear fashion with time to 80:20:0 at 2 min and then to 20:80:0 at 12 min. This composition was then held for 7 min. Because of the difficulty experienced with accurately pumping solvent proportions below 5%, the gradient was achieved by pumping the starting proportions as a single solvent mix. Ubiquinones were detected at 275 nm. Peaks were said to be ubiquinones if the peak height at 275 nm was significantly greater than at 248 nm (12). The chain lengths were determined by a comparison of the retention times with that of commercial standards Q6, Q9, and Q10, supplied by Sigma Chemical Co.

DNA relatedness studies. Membrane filter (dot-blot) hybridizations were performed by the method of Steele et al. (14) to screen isolates for relatedness to standard ATCC *Legionella* species. The purified DNA of selected unknown isolates was labeled and hybridized with filters onto which culture suspensions of each ATCC *Legionella* species had been dotted, denatured, and baked. Whole genomal DNA was extracted by a modification of the method of Fennell (8) from isolates grown for 3 to 4 days on BCYE medium. Growth from two plates was suspended in 4 ml of TE buffer (10 mM Tris hydrochloride and 1 mM EDTA, pH 8.0). The incubation times for each of the lysing steps was increased to 1 h, and the proteinase K was omitted. The RNase step was also omitted from the procedure. The probe DNA was labeled with [α-^{32}P]dCTP by nick translation (11) to an approximate activity of 28 μCi/μg.

Studies on the quantitative DNA relatedness of the isolates were performed essentially by the method of Brenner et al. (3), with the following modifications. Hybridization reactions were performed in total volumes of 200 μl of 0.28 M phosphate buffer containing 20 μg of test DNA and approximately 5 ng of labeled-probe DNA. The hybridizations were carried out overnight at the stringent temperature of 75°C or at 60°C. The following day the reaction mixture was adjusted to 0.14 M phosphate buffer with water and 1 ml of hydroxylapatite suspension (DNA grade Bio-Gel HTP; Bio-Rad

TABLE 1. Identifications of environmental *Legionella* isolates

Species or group	No. identified
L. anisa	204
L. pneumophila serogroup 1	211
L. pneumophila other serogroups	105
L. jamestowniensis	53
L. rubrilucens	26
L. quinlivanii	17
L. spiritensis	2
L. erythra	1
L. jordanis	1
L. birminghamensis	1
L. cincinnatiensis	1
Unidentified	10

TABLE 2. Grouping of *Legionella* species by fatty acid profile

Group[a]	Species
I (major *i*16:0 or *n*16:1)	*L. cincinnatiensis*
	L. longbeachae
	L. pneumophila
	L. sainthelensi
	L. santicrusis
	L. spiritensis (*i*16:1; *a*17:1 present)
II (major *a*15:0; *i*16:0>*a*17:0; *cyc*17:0 present)	*L. anisa*
	L. bozemanii
	L. cherrii
	L. dumoffii
	L. gormanii
	L. parisiensis
	L. steigerwaltii
III (major *a*15:0 and *a*17:0>*i*16:0)	*L. hackeliae*
	L. israelensis
	L. jamestowniensis
	L. jordanis
	L. maceachernii (*a*17:1 present)
	L. micdadei
	L. wadsworthii
	Legionella species E
IV (major *n*16:1; significant *a*15:0 and *a*17:0)	*L. birminghamensis*
	L. erythra
	L. feeleii
	L. quinlivanii
	L. rubrilucens
	Legionella species A
	Legionella species C
V (major *i*16:0 and *cyc*17:0; significant *n*18:0)	*L. oakridgensis*
	Legionella species B

[a] Fatty acid nomenclature: the number before the colon indicates the carbon chain length and the number after the colon indicates the number of double bonds; *a*, methyl branch chain at the *anteiso* carbon atom; *i*, methyl branch at the *iso* carbon atom; *n*, straight chain; *cyc*, cyclopropane fatty acid.

Laboratories, Richmond, Calif.) was added; it was mixed well and then incubated for 5 min at the temperature of hybridization. The hydroxylapatite was pelleted at 2,000 × *g* for 30 s, and the supernatant was aspirated off into scintillation vials. The hydroxylapatite was washed twice more with 2-ml portions of 0.14 M phosphate buffer to remove all single-stranded DNA, and the respective supernatants were pooled. Double-stranded DNA was eluted from the hydroxylapatite by using 0.4 M phosphate buffer, and again washings were collected into scintillation vials. Radioactivity in both of the supernatant pools was estimated by Cerenkov counting (5) by using a MINAXI Tri-Carb 4000 Series scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). Control reactions containing only labeled DNA (both heat denatured and undenatured) were included with each batch. The hydroxylapatite-binding values for the denatured controls were subtracted from the test reaction-binding values before normalization. The relative binding ratio was calculated as the percentage of DNA bound in the heterologous reaction divided by the percentage of DNA bound in the homologous reaction, expressed as a percentage.

RESULTS

A total of 632 isolates were classified into the groups or species shown in Table 1 by utilizing various combinations of DFA staining, cellular fatty acid and ubiquinone analysis, DNA hybridization, and latex agglutination, as described below. The 10 unidentified isolates did not conform to recognized species and were grouped into six possible new species, designated A to F. Isolates of *L. pneumophila* and *L. anisa* which were identified later in the study by latex agglutination only are not included in this table.

Each isolate was tested for its ability to autofluoresce under long-wave UV light. This characteristic has been reported to be useful in separating the legionellae into groups (7, 18, 20). In our experience, on our media, we were able to reliably demonstrate blue-white fluorescence but the red fluorescence of *Legionella erythra* and *Legionella rubrilucens* was not always apparent. The *Legionella jamestowniensis* isolates, along with the type culture, all consistently gave a strong yellow-green fluorescence on our media. Several other species appeared to give a dull yellow-green fluorescence, but this was not a consistent finding.

Cellular fatty acid analysis and determination of ubiquinone content were also useful, not only for confirmation of the genus *Legionella* but also for enabling isolates to be placed into broad groups (10, 19). Table 2 shows the grouping by cellular fatty acid content we obtained by using type cultures of the 26 currently described *Legionella* species. Group I, consisting of six *Legionella* species, was characterized by major amounts of the fatty acids *i*16:0 and *n*16:1, with lesser amounts of *a*15:0 and *a*17:0. Cyclopropane 17:0 was absent or present in only trace amounts. Group II consisted of the seven blue-white species which had *a*15:0 as the dominant fatty acid, with lesser amounts of the 16-carbon acids and significant *cyc*17:0. Group III species were characterized by having both *a*15:0 and *a*17:0 as major acids, with lesser amounts of 16-carbon acids. *cyc*-17:0 was also present, but in smaller amounts than in Group II species. Group IV species were similar to Group I except that *a*15:0, *i*16:0, *n*16:1, and *a*17:0 were found in nearly equal amounts. Individual species characteristics such as the presence of *a*17:1 in *Legionella spiritensis*, *L. micdadei*, and *Legionella maceachernii*, and *i*16:1 in *Legionella feelei*, *L. pneumophila*, and *L. spiritensis* were apparent within these groupings. *L. oakridgensis* had a unique pattern of fatty acids with only trace amounts of 15-carbon acids and large amounts of *cyc*17:0 and *n*18:0. This pattern is shared only by our species

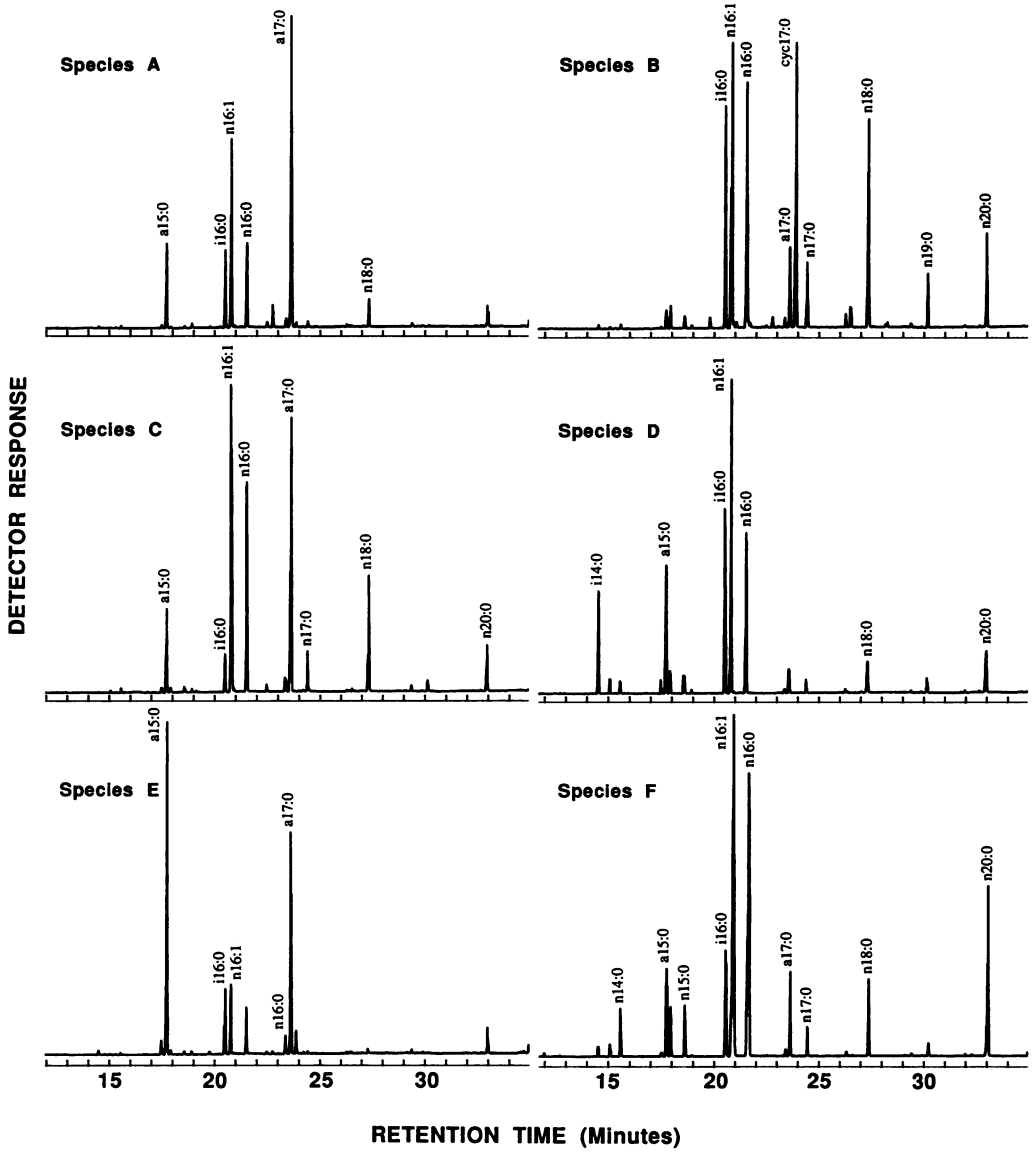

FIG. 1. Fatty acid profiles of the six possible new species A to F. For fatty acid designations, see the footnote to Table 2.

B isolate. Figure 1 shows the fatty acid chromatograms of the six possible new species. Species A and C have profiles similar to those of Group IV isolates, species E is similar to Group III, and species D and F each have a unique fatty acid profile. Studies with several of our wild strains of *L. pneumophila*, *L. anisa*, *Legionella quinlivanii*, and *L. rubrilucens* suggest that the fatty acid profiles are highly reproducible, since there was very little strain-to-strain variation.

Similarly, species could be grouped according to their ubiquinone content (Table 3; Fig. 2). Six groupings were found, designated A to F. Group A species contained a major amount of Q12 and much smaller amounts of Q11 and Q13. *L. erythra* and *L. rubrilucens* extracts consistently contained quantities of Q7 and could be subgrouped together. Group B, which was the largest group and contains all of the blue-white fluorescent species, was characterized by major amounts of Q10, Q11, and Q12 and smaller quantities of Q9 and Q13. Group C species characteristically contained Q10 as its major ubiquinone. However, both species in this group could be separated by the differing amounts of other ubiquinones. Species B was placed in this group, being indistinguishable from *L. oakridgensis*. Group E included species containing a major peak of Q13. Four of these also contained substantial amounts of Q12, and in some extracts Q12 was present in greater amount. Species E belongs to this group. *L. feeleii* is similar to Group D species, containing major amounts of Q13. However, it differs in also containing substantial amounts of Q14, so it has been placed on its own in Group E. The two new species C and F appeared to be unique, containing major amounts of Q11, and were placed in a new Group F.

For definitive identification, the DFA test with commercially available conjugates was of little use and possibly misleading due to the number of significant cross-reactions between serogroups of *L. pneumophila* and between species. In the case of *L. pneumophila*, we found the polyvalent DFA conjugates to be specific but lacking in sensitivity. Of the 316 *L. pneumophila* isolates that we identified, only 214 gave a strong reaction by using either or both of the Genetic Systems and Zeus Technologies polyvalent *L. pneumophila*

TABLE 3. *Legionella* ubiquinone profiles

Species name	Ubiquinone content[a]									Isolate/ extract[b]
	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14	Q15	
Group A										
L. birminghamensis	0	1	3	5	42	100	11	0	0	1/1
L. erythra	8–40	0	0–5	5–16	25–37	100	13–17	0	0	2/4
L. israelensis	0	0–5	0–3	4–10	16–41	100	23–62	0	0	1/2
L. pneumophila	0	1	1	2–3	12–16	100	42–50	2–3	0	7/7
L. quinlivanii	0–2	0–2	1–3	1–4	32–50	100	6–9	0	0	11/11
L. rubrilucens	7–100	0–11	0–4	1–11	18–39	83–100	7–13	0	0	13/13
Species A	0	0	0	2–3	22–25	100	10–13	0	0	1/2
Species D	0	1–5	2–4	2–4	29–47	100	16–30	0–3	0	5/6
Group B										
L. anisa	0–T	2–7	16–46	51–100	65–81	62–100	5–14	0–T	0	18/18
L. bozemanii	0–6	4–19	15–62	59–100	59–75	67–100	7–17	0	0	5/7
L. cherrii	0	3–9	12–56	30–100	50–91	95–100	10–12	0	0	1/3
L. cincinnatiensis	T–6	7–34	27–65	81–100	49–80	41–100	1–3	0	0	2/4
L. dumoffii	0–3	1	2–16	10–43	67–69	100	1–12	0	0	1/2
L. gormanii	0–2	4–6	28–42	72–100	66–81	62–100	3–14	0	0	1/3
L. longbeachae	0–6	6–28	41–56	100	51–94	39–66	2–6	0	0	7/7
L. parisiensis	0	4–7	15–50	59–100	62–76	62–100	7–14	0	0	1/5
L. sainthelensi	0	6–10	63–67	100	79–83	48–51	T–2	0	0	1/3
L. santicrusis	0–3	5–11	46–60	94–100	81–100	53–76	1–4	0	0	1/3
L. steigerwaltii	0	2–20	35–52	80–100	76–81	84–100	10–12	0	0	1/3
Group C										
L. oakridgensis	0–T	1–3	23–26	100	8–11	0–T	0	0	0	1/2
L. wadsworthii	1–6	3–15	39–51	100	44–56	18–35	2–4	0	0	1/3
Species B	0	T–2	9–14	100	10–13	0–T	0	0	0	1/3
Group D										
L. hackeliae	0	0	0	0–7	4–23	40–100	64–100	7–11	0	1/3
L. jamestowniensis	0–T	0–1	0–T	2–4	7–15	52–100	72–100	4–10	0	22/22
L. jordanis	0	0	0–1	T–13	3–10	65–100	88–100	2	0	2/3
L. maceachernii	0	0	0	2	15	7	100	24	0	1/1
L. micdadei	0	0–1	0–1	1–7	3–21	41–82	100	2–6	0	2/5
L. spiritensis	0	0	0	0	1–3	12–25	100	10–12	0	2/4
Species E	0	0	0–1	0–2	1–16	12–31	100	9–31	0	1/3
Group E (*L. feeleii*)	0	2–9	0–2	0–1	2–17	2–14	97–100	52–100	1–4	2/6
Group F										
Species C	0–T	0–3	5–7	59–71	100	20–22	2	0	0	1/2
Species F	0–1	0	0–10	0–50	100	18–19	0–3	0	0	1/2

[a] Normalized peak area percents, i.e., expressed as a percentage of the major peak. Range indicates variation among isolates and/or extractions.
[b] Total number of isolates tested/total number of extracts analyzed.

reagents. The remaining isolates were identified as *L. pneumophila* by dot-blot DNA hybridization only. The existence of significant cross-reactions with isolates of *L. anisa* and DFA conjugates for *L. micdadei* and *L. longbeachae* serogroup 2 initially led to the misidentification of these organisms. The isolates were later confirmed as *L. anisa* by DNA hybridization and latex agglutination tests. Difficulty was also experienced with cross-reactions among serogroups of *L. pneumophila*, particularly between serogroups 4, 5, 8, and 10. We therefore decided not to attempt to serogroup *L. pneumophila* isolates which did not react with the serogroup 1 reagent.

In order to reduce the workload in the laboratory, latex slide agglutination reagents were developed for the two most commonly isolated species, *L. pneumophila* and *L. anisa*, thereby providing a rapid method of presumptive identification. The *L. pneumophila* reagents (a monovalent serogroup 1 and polyvalent serogroups 2 through 14) were very specific and sensitive, allowing identification of even those strains which gave either a weak or no reaction with the DFA conjugates. These strains were confirmed as *L. pneumophila* by dot-blot DNA hybridization. The *L. anisa* reagent was shown to cross-react with *L. bozemanii* serogroup 2 and *L. longbeachae* serogroup 2 type cultures. However, *L. longbeachae* could be distinguished from *L. anisa* by its lack of blue-white autofluorescence under long-wave UV light and from *L. bozemanii* by its ability to grow on *Legionella* blood agar (18). Several of the *L. anisa* isolates were confirmed by quantitative DNA hybridization studies, showing >85% DNA relatedness to the ATCC type strain at the stringent temperature for reassociation, 75°C. Levels of relatedness of the other blue-white autofluorescent species to the *L. anisa* type strain ranged from 5 to 38%.

The remaining species were identified by using a combination of fatty acid and ubiquinone profiles and dot-blot DNA hybridization with ATCC type culture DNA probes. There were 10 isolates which could not be identified by these methods and which we believe to represent six new species. None of these isolates were found to react with any of the type cultures in DNA hybridization studies. These have

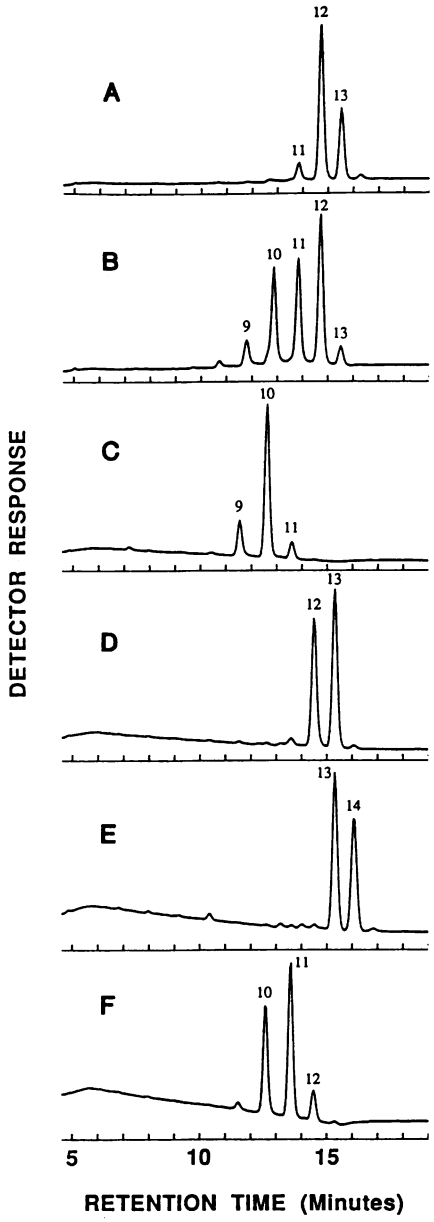

FIG. 2. Example ubiquinone profiles of the six groups A to F. Numbers above the peaks refer to the number of isoprene units of each ubiquinone.

been labeled species A through F, and their characteristics are summarized in Table 4. A number of these isolates were found to be very slow growing, and fatty acid and ubiquinone extractions were performed when sufficient growth could be obtained, usually at about 5 to 7 days.

Species A, B, C, E, and F are represented by a single isolate each. Species D is represented by five isolates obtained from three different water samples. DNA probes were made from two of the isolates in this group (no. 499 and 532), and hybridization by dot-blot was shown with each of the five isolates but not with any of the type cultures or other new species. Quantitative DNA hybridization studies showed that the five isolates were 76 to 100% interrelated. A weak cross-reaction did occur with dot-blot hybridization between the species B isolate and *L. oakridgensis*. In view of the similarity between the fatty acid profile and ubiquinone pattern of this isolate and those of *L. oakridgensis*, quantitative DNA hybridization was carried out between the two organisms. The species B isolate showed only 12% DNA relatedness at 75°C, thus indicating that they are indeed separate species.

TABLE 4. Summary of characteristics of six possible new *Legionella* species

Species designation	Isolate code no.	Fatty acid profile group	Ubiquinone profile group	Hybridization of the following isolates with ^{32}P-labeled DNA probes (dot-blot hybridization)						
				36	86	449	499	532	594	636
A	36	IV	A	+	−	−	−	−	−	−
B	86	V	C	−	+	−	−	−	−	−
C	449	IV	F	−	−	+	−	−	−	−
D	498	New(1)	A	−	−	−	+	+	−	−
D	499	New(1)	A	−	−	−	+	+	−	−
D	500	New(1)	A	−	−	−	+	+	−	−
D	532	New(1)	A	−	−	−	+	+	−	−
D	533	New(1)	A	−	−	−	+	+	−	−
E	594	III	D	−	−	−	−	−	+	−
F	636	New(2)	F	−	−	−	−	−	−	+

The *L. jamestowniensis* group of organisms is currently under study and appears by quantitative DNA hybridization to be a heterogeneous group. All isolates autofluoresce yellow-green under long-wave UV, and all react strongly with the ATCC type culture of *L. jamestowniensis* in the dot-blot hybridization assay. However, a latex reagent recently prepared to the type culture does not show agglutination with all of the isolates in this group. Restriction-fragment-length polymorphism analysis of six of these isolates has shown patterns consistent with but distinct from the type strain of *L. jamestowniensis* (T. G. Harrison, personal communication).

DISCUSSION

The results of our identification of a large number of stored isolates of legionellae indicate the range of *Legionella* species present in the South Australian environment. Because of the selective manner in which isolates were chosen for study, the proportions of species represented in Table 1 do not necessarily reflect the distribution of legionellae in the environment.

The use of DFA, only, as a rapid method for identification of legionellae to the species level can be misleading, due to the cross-reactions that exist between certain conjugates. DFA reagents for the identification of *L. pneumophila* serogroup 1 were found to be specific but lacking in sensitivity.

For rapid presumptive identification of the most commonly isolated species, the latex slide agglutination reagents were found to be very useful. Although these reagents share the same problems of serological cross-reactions between species as do DFA conjugates, they are easier to prepare and more convenient to use. The *L. pneumophila* serogroup 1 reagent was also found to be more sensitive than the Zeus Technologies DFA conjugate.

The technique of dot-blot DNA hybridization was a useful screening method for the identification of many of the species. However, in cases where species are very closely related, such as the blue-white fluorescent group and the red fluorescent species *L. erythra* and *L. rubrilucens*, significant cross-reactions were seen to occur. For reliable species identification within these groups, it was necessary to use the quantitative DNA hybridization method.

The grouping of *Legionella* isolates by fatty acid and ubiquinone analysis was found to simplify the process of species identification by restricting the number of DNA probes that had to be tested to give definitive species identification. The fatty acid groupings are very similar to those suggested recently by Wait (19) but were arrived at by using a different extraction technique and different gas chromatography conditions, indicating that these groupings are fairly reproducible. Individual chromatograms were found to differ from those of Wait mainly in the relative content of the higher carbon chain length fatty acids. This is probably a reflection of the difference in injection technique between a hot split injection as used by Wait, which discriminates against the higher-boiling-point compounds, and the PTV injector, which is reported to eliminate this discrimination (9). Despite these differences, the groupings were similar, with the exception of *Legionella israelensis* and *Legionella hackeliae*, which we tend to group with *L. micdadei* (Table 2).

A recent publication by Lambert and Moss (10) has also suggested the placing of 23 *Legionella* species into three groups according to the most abundant cellular fatty acids. This grouping differs slightly from ours and that of Wait in that unlike Lambert and Moss, we were unable to separate *L. bozemanii* and *L. dumoffii* from the other blue-white fluorescent species (our fatty acid group II). The 16C group of Lambert and Moss is equivalent to the combination of our Groups I and IV. These authors also suggest that further differentiation is possible in many cases, allowing tentative species identification. Our findings would support this view, although we would caution that laboratories undertaking similar studies should form their own library of chromatograms for comparison, since variations in extraction procedure and chromatographic conditions may lead to slight differences in the profiles. However, once established, the technique would appear to give highly reproducible results.

Our ubiquinone patterns were similar to those of Lambert and Moss (10). Some differences were noted, but there are also minor quantitative differences in the patterns published by Wait (19). We have chosen to group *L. israelensis* in Group A, for in our hands too little Q13 is present to place it in Group D. We cannot explain the presence of Q7 in the *L. erythra* and *L. rubrilucens* isolates. This finding, which has not been reported by other workers, was reproducible for all isolates and is a reliable marker for these two species. Further work, including mass spectrometry, is being carried out to confirm the identity of this peak. We found some variation in the relative amounts of Q12 and Q13 in extracts of *L. jamestowniensis*. This variation is consistent with the latex agglutination findings and further indicates that this species may be a heterogeneous group.

A combination of the techniques described above has enabled us to define six possible new species from the South Australian environment. These strains are the subject of further study.

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