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Identification of *Legionella* Species by Use of an Oligonucleotide Array▽†

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The genus *Legionella* contains a diverse group of motile, asaccharolytic, nutritionally fastidious gram-negative rods. *Legionella pneumophila* is the most important human pathogen, followed by *L. micdadei*, *L. longbeachae*, *L. dumoffii*, and other rare species. Accurate identification of *Legionella* spp. other than *L. pneumophila* is difficult because of biochemical inertness and phenotypic identity of different species. The feasibility of using an oligonucleotide array for identification of 18 species of *Legionella* was evaluated in this study. The method consisted of PCR amplification of the macrophage infectivity potentiator *mip* gene, followed by hybridization of the digoxigenin-labeled PCR products to a panel of 30 oligonucleotide probes (16- to 24-mers) immobilized on a nylon membrane. A collection of 144 target strains (strains we aimed to identify) and 50 nontarget strains (44 species) were analyzed by the array. Both test sensitivity (144/144 strains) and specificity (50/50 strains) of the array were 100%. The whole procedure for identification of *Legionella* species by the array can be finished within a working day, starting from isolated colonies. It was concluded that species identification of clinically relevant *Legionella* spp. by the array method is very reliable and can be used as an accurate alternative to conventional or other molecular methods for identification of *Legionella* spp.

The genus *Legionella* currently contains 50 validly named species (http://www.dsmz.de/bactnom/bactname.htm), and among them, 20 have been found to be human pathogens (6, 10). Legionnaires' disease (LD) is caused mainly by inhalation of aerosols generated from water sources contaminated with *Legionella* spp. (6, 40). While most species of *Legionella* are normal environmental flora, many are implicated in opportunistic infections in immunocompromised patients (14). Pulmonary infections caused by *Legionella* may be subclinical or severe (27), and the fatality rate can approach 50% in immunocompromised patients (49).

Legionella pneumophila accounts for about 85 to 90% of cases of LD (6, 26, 49). Other *Legionella* spp. implicated in human infections include *L. micdadei*, *L. longbeachae*, *L. dumoffii*, and some less encountered species, such as *L. anisa*, *L. bozemanae*, *L. feeleii*, and *L. wadsworthii* (49). *L. pneumophila* is normally identified by immunofluorescent-antibody assay. A specific FDA-cleared fluorescein isothiocyanate-labeled monoclonal antibody (Bio-Rad, Hercules, CA) for all serogroups of *L. pneumophila* and fluorescein isothiocyanate-labeled polyclonal antisera specific for *L. pneumophila* serogroup 1 (m-TECH, Atlanta, GA) are commercially available (6). Accurate identification of *Legionella* spp. other than *L. pneumophila* and *L. pneumophila* serogroup 1 can be quite difficult due to serological cross-reactivities between serogroups and species, biochemical inertness, and phenotypic identity of different species (6). *Legionella* isolates which fail to react with *L. pneumophila* antibodies are recommended to be identified by public health or reference laboratories (6). Antigen detection in urine specimens is also commonly used in hospitals for diagnosing infection caused by *L. pneumophila* (46).

Molecular approaches have been developed to provide more rapid and accurate identification of *Legionella* spp. These methods include PCR (20, 25, 34), gene probe hybridization (24, 41), restriction fragment length polymorphism analysis (21, 38), and sequence analysis of the rRNA gene (47) and the macrophage infectivity potentiator gene *mip* (35, 41). Since diagnostic delay may result in increased mortality for patients with LD (15), real-time PCR assay has been a focus of many studies in recent years (5, 13, 14, 17, 19, 34, 36, 41, 48). However, with real-time PCR assay, only *L. pneumophila* and a very limited number of *Legionella* spp. can be detected or identified.

Recently, DNA array technology has been applied to identify a wide variety of bacteria that are difficult to be differentiated by phenotypic traits or whose identification may take a long time (12, 31, 43). This study aimed to develop an oligonucleotide array based on *mip* gene sequences to identify 18 species of *Legionella* that have been found to cause human infections in the literature (10).

MATERIALS AND METHODS

Bacterial strains. A collection of 52 reference strains and 92 clinical isolates were used as target strains (species we aimed to identify) of the array (Table 1). Reference strains were obtained from the American Type Culture Col-

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TABLE 1. Reference strains and clinical isolates used in this study

Species	Reference strain(s)[a]	No. of clinical isolates[c]	Total no. of strains
L. anisa	ATCC 35292[T], ATCC 35290, ATCC 35291	7	10
L. birminghamensis	ATCC 43702[T], ATCC 700709	0	2
L. bozemanii	ATCC 33217[T], ATCC 35545, CCUG 31569, CCUG 48836	2	6
L. cincinnatiensis	ATCC 43753[T]	0	1
L. dumoffii	ATCC 33279[T], CCUG 47789	0	2
L. feeleii	ATCC 35072[T], ATCC 35849, ATCC 700513, ATCC 700514	0	4
L. gormanii	ATCC 33297[T], ATCC 43769	0	2
L. hackeliae	ATCC 35250[T], ATCC 35999	0	2
L. jordanis	ATCC 33623[T], ATCC 700762	0	2
L. lansingensis	ATCC 49751[T]	0	1
L. longbeachae	ATCC 33462[T], ATCC 33484, CCUG 28612	1	4
L. maceachernii	ATCC 35300[T]	0	1
L. micdadei	ATCC 33218[T]	0	1
L. oakridgensis	ATCC 33761[T], ATCC 700515, ATCC 700516	0	3
L. pneumophila[b]	ATCC 33152[T], ATCC 33154, ATCC 33155, ATCC 33156[T], ATCC 33215, ATCC 33216, ATCC 33823, ATCC 35096, ATCC 35251, ATCC 35289, ATCC 43109, ATCC 43130, ATCC 43283, ATCC 43290, ATCC 43703, ATCC 43736	82	98
L. sainthelensi	ATCC 35248[T], ATCC 49322, ATCC 700517	0	3
L. tucsonensis	ATCC 49180[T]	0	1
L. wadsworthii	ATCC 33877[T]	0	1
Total	52	92	144

[a] ATCC, American Type Culture Collection, Manassas, VA; CCUG, Culture Collection, University of Göteborg, Sweden.
[b] ATCC 33156[T], ATCC 33216, and ATCC 35251 are strains of *L. pneumophila* subsp. *fraseri*. Others are strains of *L. pneumophila* subsp. *pneumophila* comprising 12 serogroups.
[c] Non-*L. pneumophila* clinical strains were identified by *mip* gene sequence analysis.

lection (ATCC), Manassas, VA, the Bioresources Collection and Research Center (Hsichu, Taiwan), and Culture Collection, University of Göteborg, Sweden. Clinical isolates were obtained from the Centers for Disease Control (Taipei, Taiwan) and the Super Laboratory (Taipei, Taiwan). Clinical isolates were isolated from respiratory specimens and identified as *L. pneumophila* (82 strains) by standard techniques (42) or as other *Legionella* species (10 strains) by *mip* gene sequence analysis (35). A total of 50 nontarget strains (44 species) were used for specificity testing of the array (see Table S1 in the supplemental material). Buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutaric acid was used for culture of *Legionella* spp., while sheep blood agar was used to cultivate non-*Legionella* strains. All plates were incubated at 35°C for 24 to 72 h.

DNA preparation. One to several colonies of pure cultures were suspended in an aliquot (50 μl) of sterilized water, heated at 100°C for 15 min in a heating block, and centrifuged in a microcentrifuge (6,000 × *g*, 10 min) (28). The supernatant containing bacterial DNA was stored at −20°C for further use.

Design of species-specific oligonucleotide probes. Oligonucleotide probes (16- to 24-mers) were designed from the *mip* gene to identify the *Legionella* spp. listed in Table 1. One or multiple probes were designed to identify a single *Legionella* species, depending on the availability of divergent sequences in the *mip* regions (Table 2). Multiple sequence alignment of the *mip* fragments was performed by using the software Vector NTI (Invitrogen Corporation, Carlsbad, CA), and areas displaying low intraspecies and high interspecies sequence divergences were used for probe synthesis. The designed probes were checked for self-annealing, secondary structure, internal repeats, and GC content by using the software Vector NTI (Invitrogen Corporation). In addition, the designed probes were screened against the databases of the National Center for Biotechnology Information for homology with other bacterial sequences, using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Fifteen, 10, or 5 thymine bases were added to probes that had a melting temperature (T_m) of ≤40°C, ≤45°C, or >45°C, respectively (3) (Table 2). The positive control probe was designed from a conserved region in the *mip* region for all *Legionella* species. An irrelevant 20-mer oligonucleotide labeled with a digoxigenin (DIG) molecule at its 5′ end (5′-DIG-GGGTTYCCCCRTTCRGAAAT-3′; Y = C or T and R = A or G) was used as a position marker of hybridization (Fig. 1 and 2).

Preparation of oligonucleotide arrays. The arrays contained 42 dots (6 by 7 dots), including 29 dots for species identification, 3 dots for the negative controls (probe code NC; tracking dye only), 1 dot for the positive control (probe code PC), and 9 dots (probe code P) for the position markers (Fig. 1). The oligonucleotide probes were diluted 1:1 (final concentration, 10 μM) with a tracking dye solution, drawn into wells of 96-well microtiter plates, and spotted onto positively charged nylon membranes (Roche, Mannheim, Germany) as described previously (43). The arrays were fabricated with an automatic arrayer (model SR-A300; Ezspot, Taipei, Taiwan) by use of a solid pin (400 μm in diameter).

Amplification of the *mip* gene. The *mip* gene was amplified by PCR with a pair of degenerate primers, MIPF (5′-GGGRATTVTTTATGAAGATGARAYTGG-3′) and MIPR (5′-DIG-GGGTTYCCCCRTTCRGAAAT-3′) (R = A or G, V = A, C, or G). The reverse primer MIPR was labeled with a DIG molecule at the 5′ end. The conditions used for PCR were the same as those described previously (35). The presence of the PCR product was checked by 2% agarose gel electrophoresis.

Array hybridization. All reagents except for buffers were included in each DIG nucleic acid detection kit (Roche). Unless indicated otherwise, the hybridization procedures were carried out at room temperature in an oven with a shaking speed of 60 rpm. Each array was prehybridized for 2 h with 1 ml of hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% [wt/vol] blocking reagent, 0.1% *N*-laurylsarcosine, 0.02% sodium dodecyl sulfate) in an individual well of a 24-well cell culture plate. The DIG-labeled PCR product amplified from a strain was heated in a 100°C heating block for 5 min and immediately cooled on an ice bath. Ten microliters of the denatured PCR product of the test organism was diluted with 0.3 ml of hybridization solution and added to each well. Hybridization was carried out at 45°C for 90 min. After removal of the nonhybridized PCR products, the array was washed four times (3 min each) in 1 ml of 0.25× SSC–0.1% sodium dodecyl sulfate, followed by incubation for 1 h with 1 ml of blocking solution (1% [wt/vol] blocking reagent dissolved in maleic acid buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]). After removal of the blocking solution, 0.3 ml of alkaline phosphatase-conjugated anti-DIG antibodies (diluted 1:2,500 in blocking solution) was added to each well and incubated for 1 h. The array was washed three times (15 min each) in 1 ml of washing solution (0.3% [vol/vol] Tween 20 in maleic acid buffer), followed by washing in 1 ml of detection buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 9.5) for 5 min. Finally, 0.2 ml of alkaline phosphatase substrate (stock solution of nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate diluted 1:50 in detection buffer) was placed on each array and incubated at 37°C without shaking. Color development was visible between 30 min and 1 h after the start of the reaction. The hybridized spots (400 μm in diameter) could be read by the naked eye. The images of the hybridization patterns were captured and processed by a scanner (PowerLook 3000; UMAX, Taipei, Taiwan). A strain was

TABLE 2. Oligonucleotide probes used in this study

Species	Probe	Sequence (5′–3′)[a]	Probe length (mer)	T_m (°C)	Location[b]	GenBank accession no.
L. anisa	Lagtw[c]	CCTTCAGACTTAGCTTAT(T)	18	35.1	1072–1089	U91607
	Lan	GGCGTAACGGTACTGCC(T)	17	48.5	832–848	U91607
L. birminghamensis	Lbi1	CAGCGGACTCCAGTACA(T)	17	43.6	412–428	U91608
	Lbi2	AACTGGCAGCGGTGCAA(T)	17	53.1	439–455	U91608
L. bozemanii	Lbo	(T)CCTTCAGATTTAGCGTA	18	39.6	1070–1087	U91609
L. cincinnatiensis	Lci	AGGTAAGTCAGACACTGTAA(T)	20	37.9	855–874	U91636
L. dumoffii	Ldu1	AAGAGAATAAAGCAAAAGGC(T)	20	45.5	747–766	U91637
	Ldu2	CAGGTTCAGGCGCTAAGCC(T)	19	54.9	846–864	U91637
L. feeleii	Lfe1[d]	AAAATAATCCAGGCTGCTA(T)	19	44.1	778–796	U92205
	Lfe2	AAGCGTTCATGAGCCAGA(T)	18	48.6	713–730	U92205
L. gormanii	Lagtw[c]	CCTTCAGACTTAGCTTAT(T)	18	35.1	1072–1089	U91607
	Lgo1	CAGCGCAGAGTTTAACAAGA(T)	20	48.4	613–632	U91638
	Lgo2	GTACAGGCAGTAAACCAG(T)	20	46.5	739–758	U91638
L. hackeliae	Lha	AGGGTGACGGCGCTAA(T)	16	49.3	690–705	U92207
L. jordanis	Ljo	AAGCATTCTTAAACGCAAAC(T)	20	46.9	767–786	U92209
L. lansingensis	Lla1	GTTACTCACAGAGCAGCAAA(T)	20	45.3	630–649	U92210
	Lla2	AAACGCAGCAACGCCTACT(T)	19	52.6	447–465	U92210
L. longbeachae	Ll	ACTGGTACCTTGATTGATG(T)	19	41.5	1004–1022	X83036
L. maceachernii	Lma1	ACAATAAGGCAAAAGGAG(T)	18	41.6	680–697	U92211
	Lma2	TCATTGAGCGCGGTGAT(T)	17	50.1	767–783	U92211
L. micdadei	Lmi1	AGCTTTCCTTAACGAAAA(T)	18	41.9	1039–1056	S62141
	Lmi2	CACCGGCAAGCTGATTG(T)	17	50.3	1165–1181	S62141
L. oakridgensis	Loa1	AATGGTTCAAGGGTTGCA(T)	18	48.2	284–301	U92214
	Loa2	CTGGCTCCGTTTGGGA(T)	18	49.4	685–700	U92214
L. pneumophila	Lp1	AAACAAGCCAGGCGTT(T)	16	45.6	329–344	AF022334
	Lp2	CAATTGGCTTTAACCGAACAA(T)	21	52.2	189–208	AF022334
L. sainthelensi	Lsai	ACTGGTGCGAAACCCG(T)	16	49.3	853–868	U91219
L. tucsonensis	Lagtw[c]	CCTTCAGACTTAGCTTAT(T)	18	35.1	1072–1089	U91607
	Ltu	(T)TCAAAATCCGGCGTAGT	17	45.8	804–820	U92224
L. wadsworthii	Lagtw[c]	CCTTCAGACTTAGCTTAT(T)	18	35.1	1072–1089	U91607
	Lwad1	CAGTAAGACAGATACTGTTACTGT(T)	24	42.2	740–756	U92225
	Lwad2	AGGCGATTCATTCTTA(T)	16	36.6	638–653	U92225
Positive control	PC	CARGTNATHCCNGGNTGGACHGA(T)	23	60.5	510–532	AF022334

[a] (T), additional bases of thymine (T) were added to the 5′ or 3′ end of the probe. Fifteen, 10, or 5 thymine bases were added to probes that had a T_m of ≤40°C, ≤45°C, or >45°C, respectively. The positive control probe contained 15 thymine bases at the 3′ end.
[b] The location of the probe is indicated by the nucleotide numbers of the *mip* gene, unless otherwise indicated.
[c] Group-specific probe.
[d] A single mismatch base was intentionally incorporated into the probe to avoid cross-hybridization by other *Legionella* spp.

identified as one of the 18 *Legionella* species listed in Table 1 when both the positive control probe and the probe (or all probes) specified for that species were hybridized (Table 2).

Detection limit of *L. pneumophila* in spiked urine samples. *L. pneumophila* ATCC 33152 was used to determine the detection limit of the oligonucleotide array. A clean-catch urine sample obtained from a healthy person was spiked with *L. pneumophila* ATCC 33152 to a concentration of 10^8 CFU/ml. The DNA of the spiked urine sample was extracted (28) and serially diluted 10-fold with a carrier DNA (10 ng/μl) extracted from *Escherichia coli* TW1 by the boiling method (28). After PCR amplification of the diluted DNA, the amplicon was hybridized to the oligonucleotide array.

	1	2	3	4	5	6	7
A	PC	Lagtw	Lan	Lbi1	Lbi2	Lbo	P
B	Lci	Ldu1	Ldu2	Lfe1	Lfe2	Lgo1	P
C	Lgo2	Lha	Ljo	Ll	Lla1	Lla2	NC
D	Lma1	Lma2	Lmi1	Lmi2	Loa1	Loa2	P
E	Lp1	Lp2	Lsai	Ltu	Lwad1	Lwad2	P
F	P	P	P	NC	P	P	NC

FIG. 1. Layout of oligonucleotide probes on the array. Probes coded "NC" were negative controls (tracking dye only). The probe coded "P" was an irrelevant 20-mer oligonucleotide labeled with a DIG molecule at its 5′ end and was used as a position marker. The corresponding sequences of all probes are listed in Table 2.

RESULTS

Probe development. In the beginning of this study, one to six probes (data not shown) were designed for identification of each species, and a total of 70 probes were synthesized to identify the 18 *Legionella* species listed in Table 1. Through extensive hybridization screening, many probes cross-reacted with heterologous species or produced no hybridization signals with homologous species. Finally, 30 probes were selected for fabrication of the array (Fig. 1). Among the 30 probes, 28 were species specific, 1 was group specific, and 1 was a positive control that could hybridize with all *Legionella* spp. *L. anisa*, *L. gormanii*, *L. tucsonensis*, and *L. wadsworthii* shared a group-specific probe (code Lagtw) due to high *mip* sequence similarities among these species. However, each of the four species had its own species-specific probe(s) and could be differentiated from the others (Table 2). An individual species was identified by one, two, or three probes, depending on the availability of divergent sequences in the *mip* gene. For example, *L. bozemanii* was identified by a single probe (code Lbo), while *L. birminghamensis* and *L. gormanii* were identified by two (codes Lbi1 and Lbi2) and three probes (codes Lagtw, Lgo1, and Lgo2), respectively (Table 2).

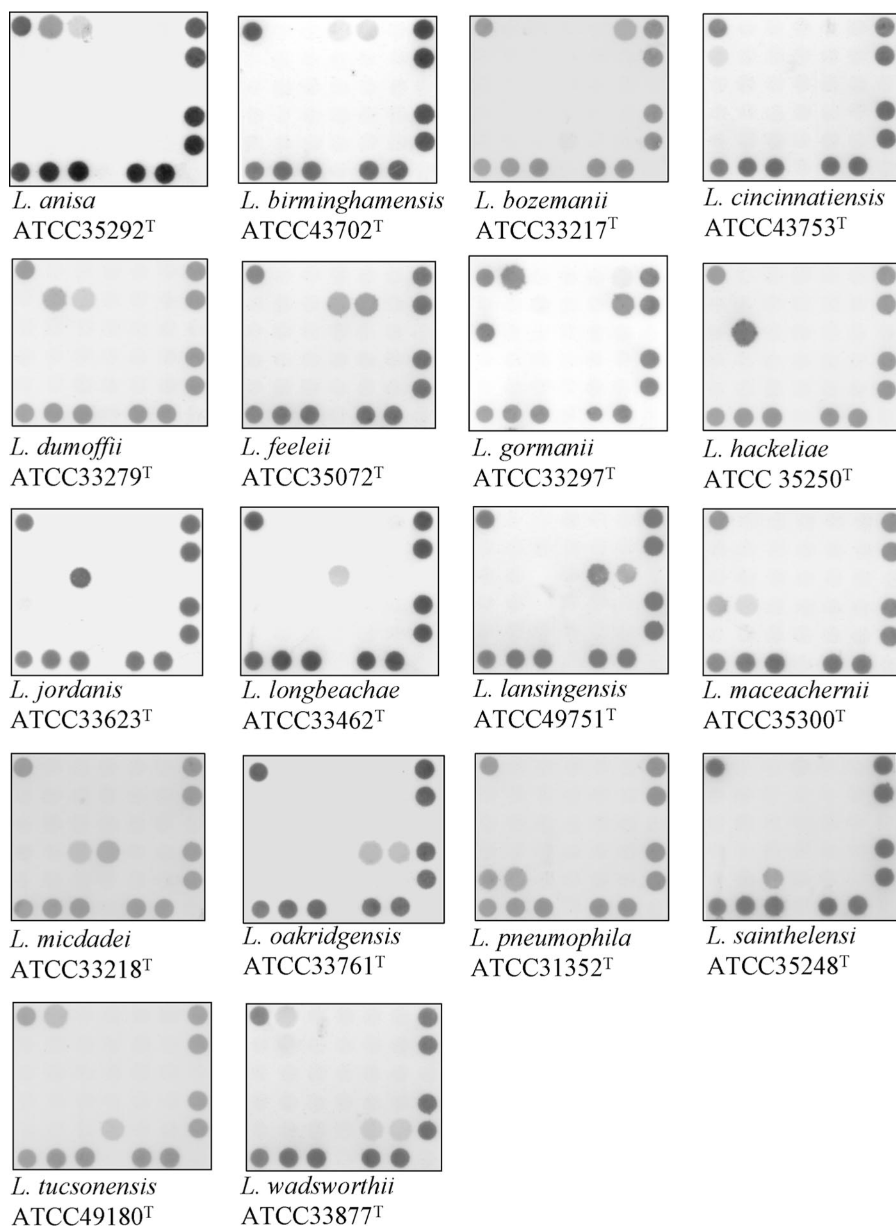

FIG. 2. Hybridization results for type strains of 18 *Legionella* species. The corresponding probes hybridized on the arrays are indicated in Fig. 1, and the corresponding sequences of the hybridized probes are shown in Table 2.

Identification of *Legionella* strains. Of 52 target reference strains representing 18 *Legionella* species, all strains hybridized to the positive control probe and their species-specific probes. In other words, all strains were correctly identified by the array. The hybridized spots (400 μm in diameter), appearing blue on a white nylon membrane, could easily be read by the naked eye. The hybridization patterns of 18 type strains representing the 18 species are shown in Fig. 2. In addition, a total of 92 clinical isolates, including 82 strains of *L. pneumophila* and 10 strains of other *Legionella* species, were analyzed, and all strains were correctly identified by array hybridization. Therefore, the test sensitivity of the array was 100% (144/144 strains). All reference strains and clinical isolates were also tested in a blinded fashion, and the same hybridization results were obtained. In addition, reproducible results were obtained for all target strains after repeat testing.

Hybridization of nontarget strains. Of 50 nontarget strains (44 species), including five *Legionella* species (see Table S1 in the supplemental material), no strain hybridized to any probe on the array, resulting in a test specificity of 100% (50/50 strains). *Pseudomonas aeruginosa*, which is a common mimic of *Legionella* colonies on buffered charcoal yeast extract plates (6), produced a negative hybridization reaction, although the bacterium exhibits no growth dependence on L-cysteine. In addition, *Staphylococcus aureus*, which can cross-react with *L. pneumophila*-specific monoclonal antibody (6), also produced a negative hybridization reaction (see Table S1 in the supplemental material).

Detection limit of the array. Serial 10-fold dilutions of the DNA of *L. pneumophila* ATCC 33152 were used to determine the detection limit of the microorganism in urine. The present method was able to detect the microorganism at a concentration of 25 CFU per assay (data not shown). No hybridization signal was observed for the nonspiked urine sample.

DISCUSSION

The biochemical data for legionellae other than *L. pneumophila* are very limited (10), although some laboratories have described methods for identifying putative *Legionella* isolates to the genus or species level by using phenotypic characteristics (11, 44). Currently, immunological methods are the most widely used techniques for identification of *L. pneumophila* and *L. pneumophila* serogroup 1 (6, 42). Antibodies to *L. pneumophila* serogroup 1 are quite specific, but otherwise cross-reactions exist, making immunological identification only presumptive (6). Cross-reactions have been experienced between different species and serogroups even when monovalent antisera were used (1, 6, 8, 32, 37). Adsorbed antisera may have the ability to avoid cross-reactivity, but these antisera are only available in some research laboratories and are not commercially available (2, 6).

At present, there is still a low level of clinical awareness regarding LD 30 years after it was first reported. Large, focal outbreaks of LD continue to occur worldwide (10). Most diagnostic tests currently used are directed at the species that causes most human cases of legionellosis, *L. pneumophila* serogroup 1. For this reason, information on the incidence of human respiratory disease attributable to other *Legionella* species is lacking (10). An accurate identification method, such as the array proposed in this study, is a prelude to revealing the epidemiology of non-*L. pneumophila Legionella* species that may cause human infections.

In this study, an individual species was identified by either one, two, or three probes, depending on the availability of divergent sequences in the *mip* region. The advantage of using multiple probes is the increase in specificity, since the chance of an irrelevant strain hybridizing to all probes designed for a species is very low. However, the use of multiple probes to identify a species may potentially decrease sensitivity due to the possibility of a single nucleotide polymorphism that occurs in strains at the region used for probe design. The successful design of species-specific probes was based on the known *mip* sequences in public databases. Multiple sequence alignment (interspecies and intraspecies) plays an essential role in finding regions that can be used for probe design.

The two probes (Lp1 and Lp2) used to identify *L. pneumophila* were species specific rather than serogroup or subspecies specific. Among the 16 strains of *L. pneumophila* obtained from the ATCC (Table 1), 3 strains (ATCC 33156, ATCC 33216, and ATCC 35251) were *L. pneumophila* subsp. *fraseri*, while the remaining 13 strains were *L. pneumophila* subsp. *pneumophila*. The 13 strains of *L. pneumophila* subsp. *pneumophila* covered serogroups 1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, and 14 (http://www.atcc.org/). Regardless of serogroup and subspecies, all 16 strains were identified as *L. pneumophila* by the array.

Due to the inadequateness of conventional identification methods, molecular techniques have been developed to provide more rapid and accurate alternatives for identification of *Legionella* spp. Some PCR tests that have been developed for legionellae target random DNA sequences (39), the 16S rRNA gene (20, 22, 29), the 5S rRNA gene (23, 24), and *mip* genes (4, 14, 24, 35, 41, 48). *Legionella* DNA has been detected in a variety of clinical specimens, including respiratory secretions, pharyngeal swabs, nasopharyngeal swabs, urine, serum, and peripheral blood mononuclear cells (10, 16, 18, 20, 30, 33). Sequence analysis of the *mip* gene was successfully used to differentiate *Legionella* spp. (35, 41), and identification through sequence data comparison is available on the Internet (http://www.ewgli.org/). After analyzing the *mip* genes of 17 clinical and environmental *Legionella* isolates, Bumbaugh et al. (4) found that the *mip* gene is highly conserved in each species of *Legionella* and is a good target for molecular diagnosis. Stølhaug et al. (41) observed that the *mip* gene sequence discriminates more reliably between *Legionella* spp. than does the partial (386 bp) 16S rRNA gene sequence. For this reason, the *mip* gene was selected as a target for identification of *Legionella* species in this study. For several *Legionella* species, a single reference strain was used in this study (Table 1); this was due to the limited availability of strains of these species in public culture collections.

The identification of legionellae to the species level is usually not of clinical significance, but it may have significant public health and scientific importance (6). LD caused by *Legionella* spp. other than *L. pneumophila* may have less of a response to erythromycin treatment (9), but whether accurate identification of the *Legionella* spp. causing infection would have a positive impact on patient management is debatable, especially since erythromycin is currently not used for treating severe LD (7).

The oligonucleotide probes (16- to 24-mers) used in this study were relatively short, and the T_m values of these probes varied to a large degree (35.1 to 54.9°C). Many probes even had T_m values lower than the hybridization temperature (45°C) (Table 2). Although some species produced relatively weak hybridization, clear signals were obtained for all 18 species tested (Fig. 2). Volokhov et al. (45) also reported the successful use of probes having T_m values significantly lower than the hybridization temperature for identification of *Listeria* species. The addition of multiple thymine bases to the 3′ end (or 5′ end) of an oligonucleotide can improve the hybridization signal of a probe, probably due to the increased binding of the probe to the nylon membrane, and thus an increased hybridization intensity (3).

In conclusion, an oligonucleotide array based on *mip* sequences was developed to identify 18 species of *Legionella* that have been reported to cause human infections (6, 10). With a high sensitivity and specificity, the array technique is an accurate tool for differentiating *Legionella* species that may be encountered in clinical settings. The current array utilizes a standardized protocol encompassing DNA extraction, PCR amplification, and hybridization of PCR products to probes on the array.

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