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Evaluation of a Commercial Gene Probe for Identification of *Legionella* Cultures

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Confirmation of a culture as *Legionella* when it is unreactive with available serologic reagents involves tests that are impractical in most clinical laboratories. A nucleic acid probe that hybridizes only to members of the genus *Legionella* was recently prepared for marketing by Gen-Probe, Inc., San Diego, Calif. We tested 215 *Legionella* strains, representing 22 species, and 84 non-*Legionella* strains, representing 17 bacterial genera, with the Gen-Probe kit. All but four *Legionella* strains (*L. bozemanii*, <2% of total) and no heterologous strains gave positive test results. We conclude that the *Legionella* gene probe is a valuable addition to existing diagnostic tests for *Legionella* organisms.

The laboratory diagnosis of *Legionella* infections relies on isolation of the organism with subsequent serologic identification, demonstration of the bacterium in tissue or body fluids by the direct immunofluorescence assay, detection of antigenuria, or detection of an antibody response to infection (H. W. Wilkinson, Clin. Immunol. Newsl. 5:96–99, 1984).

The direct immunofluorescence assay is advantageous when cultures cannot be obtained and also provides rapid test results. However, false-positive results due to cross-reactive antigens in morphologically compatible bacteria can occur (3, 6), and this test should probably be considered only a presumptive test. Antigenuria can be measured with the enzyme-linked immunosorbent assay or radioimmunoassay (8), but these tests are not yet available outside research centers, and the efficacy of the test in detecting antigens other than *L. pneumophila* serogroup 1 is unknown. Serology is used as a retrospective diagnostic aid when serum is the only specimen available; however, currently used serologic tests are neither rapid nor specific (10). Isolation has become the most definitive and sensitive technique when appropriate specimens are available for culturing (2).

When cultures can be obtained, they are identified to species and serogroup with the slide agglutination test or direct immunofluorescence assay (1, 9). The problem of testing each specimen with 33 antisera is partially obviated by using pooled reagents. However, *Legionella*-like organisms of uncharacterized species or serogroups can give negative serologic test results. Gas-liquid chromatographic and DNA hybridization studies are then required to confirm their identity. The latter tests are impractical in most laboratories.

Nucleic acid probes for *L. pneumophila* and for the *Legionella* genus were recently described by Grimont et al. (4) and Kohne et al. (5), respectively. Preliminary findings suggested that the latter probe detected all known *Legionella* species and hybridized with no nucleic acids from heterologous microorganisms. If it was standardized for clinical laboratory use, a *Legionella* probe could be valuable in detecting *Legionella* cells in a variety of specimens and could rapidly confirm the presence of *Legionella*-like organisms when serologic tests are negative. Since the preliminary report, the gene probe of Kohne et al. has been modified and produced commercially by Gen-Probe, Inc., San Diego, Calif. The purpose of this study was to evaluate its use in identifying *Legionella* cultures.

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MATERIALS AND METHODS

Bacterial strains. The gene probe was tested on 215 *Legionella* strains, including the type strains of all species and serogroups, and 84 bacterial strains other than *Legionella*, all from Centers for Disease Control stock culture collections. The legionellae included 119 clinical isolates and 76 environmental isolates. Twenty-two *Legionella* species (9) and 17 heterologous genera representing 31 species were included. *Legionella* strains were identified in our laboratory by the direct immunofluorescence assay or slide agglutination test procedure (1, 9). Non-*Legionella* bacterial strains were supplied by Robert E. Weaver, Richard R. Facklam, and Caroline O'Hara of the Centers for Disease Control.

Preparation of test samples. Two agar plates of the appropriate medium were streaked for confluent growth of each strain: buffered charcoal-yeast extract agar for *Legionella* species and *Bordetella pertussis* and heart infusion or Trypticase (BBL Microbiology Systems, Cockesville, Md.) soy agar supplemented with 5% rabbit blood for the remaining bacteria. The *Legionella* strains were harvested in 2.0 ml of distilled water after 48 h of incubation at 35°C, diluted to a density corresponding to a McFarland standard of 2, and

TABLE 1. Gene probe analysis of *Legionella* and non-*Legionella* strains by percent-bound and ratio methods

Strains and analytical method	Hybridization			
	Range	Mean	SD	CV
Legionella (n = 215)				
Percent bound[a]	4.0%–72.0%	40.7%	12.8%	31.56
Ratio[b]	0.13–1.90	1.13	0.39	34.47
Heterologous (n = 84)				
Percent bound[a]	−0.3%–3.0%	0.7%	0.9%	123.55
Ratio[b]	−0.01–0.10	0.02	0.03	126.39

[a] [(Mean counts per minute of duplicate test samples − counts per minute of negative control)/counts per minute in 100 μl of probe] × 100.

[b] Percent probe bound by test sample/percent bound by *L. pneumophila* strain Philadelphia 1.

TABLE 2. Gene probe analysis of *Legionella* strains by species

Legionella species	No. of strains[a]			Probe bound (%)			No. of strains positive/total no. of strains
	Clin	Env	Unk	Range	Mean	SD	
L. anisa		8		22–37	32.1	4.6	8/8
L. bozemanii[b]	10			4–38	13.2	9.1	6/10[c]
L. cherrii		5		27–49	34.2	8.1	5/5
L. dumoffii	3	2	1	19–38	29.7	6.5	6/6
L. erythra		3	1	32–44	35.3	5.1	4/4
L. feeleii[b]	3	1	1	11–47	29.6	11.9	5/5
L. gormanii		1		44	NA[d]	NA	1/1
L. hackeliae[b]	2			26–36	31.0	5.0	2/2
L. jamestowniensis		1	1	18–40	29.0	11.0	2/2
L. jordanis	1	2		20–38	29.3	7.4	3/3
L. longbeachae[b]	10		2	21–49	31.8	8.4	12/12
L. maceachernii	1	1		15–35	25.0	10.0	2/2
L. micdadei	7			16–39	27.3	8.3	7/7
L. oakridgensis		4		22–32	25.5	3.8	4/4
L. parisiensis		1		33	NA	NA	1/1
L. pneumophila[e]	79	38	9	27–72	48.6	7.0	126/126
L. rubrilucens		2	5	26–47	32.9	6.4	7/7
L. sainthelensi		3		20–46	30.8	11.4	3/3
L. santicrucis		1		42	NA	NA	1/1
L. spiritensis		2		33–44	38.5	5.5	2/2
L. steigerwaltii		1		55	NA	NA	1/1
L. wadsworthii	3			18–36	27.3	7.4	3/3

[a] Clin, Clinical isolate; Env, environmental isolate; Unk, isolate from unknown source.
[b] Serogroups 1 and 2.
[c] Two of the four strains that gave negative results were positive when tests were repeated.
[d] NA, Nonapplicable.
[e] Serogroups 1 to 10 and "species 1" of Selander et al. (8a).

stored at 4°C. The heterologous bacteria were harvested and diluted as above after 24 h of incubation at 35°C but were tested immediately to avoid premature cell lysis.

Gene probe procedure. The *Legionella* Confirmation Kit, provided by Gen-Probe, Inc., consisted of a probe solution (Reagent I) containing ^{125}I-labeled DNA homologous to *Legionella* rRNA, a separation solution (Reagent II), and a wash solution (Reagent III). The protocol provided with the kit was followed for all assays: 100 μl of Reagent I was added to 100 μl of bacterial suspension. The vial containing the mixture was incubated in a 72°C water bath for 2 h. Reagent II (5 ml) was added to the vial, the contents of which were thoroughly mixed, and then the vial was incubated in a 72°C water bath for 5 min. The DNA-RNA duplex was pelleted by centrifugation, and 5 ml of Reagent III was added to the pellet. After another centrifugation step, the supernatant fluid was discarded. Radioactivity in the pellet was counted in a gamma counter (Isodyne, Automatic Gamma System; Searle Analytic, Inc., Des Plaines, Ill.), and the data were captured on an IBM-PC (IBM Corp., Boca Raton, Fla.). The percent hybridization was calculated by subtracting the counts per minute of a negative control (tube containing probe alone) from the mean counts per minute of duplicate test samples, dividing by the total counts per minute in 100

TABLE 3. Gene probe analysis of *L. pneumophila* and "species 1" strains

L. pneumophila serogroup	No. of strains[a]			Probe bound (%)			No. of strains positive/total no. of strains
	Clin	Env	Unk	Range	Mean	SD	
1	29	2		36–55	47.5	4.7	31/31
2	8			49–59	53.1	3.2	8/8
3	3	2	1	41–71	52.3	9.8	6/6
4[b]	9	2	2	38–61	48.0	7.1	13/13
5[b]	6	2	1	39–64	47.4	7.4	9/9
6	6	18	1	44–72	49.7	5.5	25/25
7		1		50	NA[c]	NA	1/1
8	2	1		34–66	45.3	14.6	3/3
9	4	2		46–67	53.3	6.5	6/6
10	2			31–38	34.5	3.5	2/2
1 and 4		1		51	NA	NA	1/1
4 and 5[b]	2	6	2	45–56	50.5	3.9	10/10
Lansing 3[b]	2		2	27–49	39.3	8.8	4/4
New	6	1		32–57	48.1	7.2	7/7

[a] Clin, Clinical isolate; Env, environmental isolate; Unk, unknown source.
[b] Serogroups 4 and 5 include strains of *L. pneumophila* and "species 1" of Selander et al. (8a) that cannot be differentiated serologically with currently available antisera. Lansing 3 denotes a serologically distinct serovar in "species 1."
[c] NA, Nonapplicable.

μl of probe, and multiplying by 100 (percent-bound method). According to the Gen-Probe protocol, ≥10% hybridization was considered positive. Data were also analyzed by dividing the value (percent bound) of the test sample by the value of *L. pneumophila* strain Philadelphia 1, which was used as a positive control in each assay (ratio method). Statistical analyses were done on an IBM PC with the Lotus 1-2-3 software package (Lotus Development Corp., Cambridge, Mass.).

RESULTS

The mean percent hybridization of the probe to *L. pneumophila* serogroup 1 strain Philadelphia 1 for 12 consecutive assays was 35.9% (range, 30.0 to 60.0%; standard deviation, 7.8%; coefficient of variation [CV], 21.61). The apparent high degree of variation was due to the 60.0% value obtained on day 1, as shown by analysis of the last 11 consecutive assays (mean, 33.7%; range, 30.0 to 39.0%; standard deviation, 2.9%; CV, 8.49). The results of data analysis by the percent-bound method and the ratio method are shown in Table 1. If greater than or equal to two standard deviations above the maximum value obtained in testing non-*Legionella* bacteria was considered positive, all but one *Legionella* strain was positive by the percent-bound (≥4.8%) or ratio (≥0.16) method. If ≥10% was used as the cutoff level for a positive test result, four *Legionella* strains were negative and no heterologous bacterial strains were positive.

The four *Legionella* strains that gave negative results were *L. bozemanii* isolates (Table 2). Two of the strains gave positive results when they were retested: a serogroup 1 strain, which initially bound 8% of the probe, bound 11% when it was retested; and a serogroup 2 strain, which initially bound 9%, bound 10% when it was retested. Each of the remaining *Legionella* strains was positive on initial tests. The mean percent bound by the 22 *Legionella* species ranged from 13.2% for *L. bozemanii* to 48.6% for *L. pneumophila*. Within the latter species, the mean percent bound by individual serogroups ranged from 34.5% for serogroup 10 to 53.3% for serogroup 9 (Table 3). No appreciable difference was found in binding by clinical and environmental isolates of *L. pneumophila* or of all the *Legionella* species tested (Table 4). The maximum binding by non-*Legionella* bacteria was 3.0%, the value obtained for one strain each of *Alcaligenes faecalis*, *Neisseria meningitidis*, and *Pseudomonas cepacia* (Table 5).

DISCUSSION

The results of this study confirmed the usefulness of the Gen-Probe *Legionella* gene probe assay. That our tests showed hybridization values frequently less than 50% was probably because we chose not to normalize our values to the maximum percent-bound value shown by *L. pneumophila* serogroup 1 strains, as was done previously (5). The values of 12 consecutive assays of strain Philadelphia 1 ranged from 30 to 60% of probe bound. However, less variability was estimated by omitting the 60% value obtained on day 1 (range, 30 to 39%; CV, 8.49). To compensate for any possible variability, we calculated the ratio of the mean of duplicate test samples to the mean of duplicate Philadelphia 1 samples tested concomitantly. One *Legionella* strain (*L. bozemanii*) gave a ratio value and a percent-bound value within two standard deviations above the maximum hybridization by heterologous strains. All other *Legionella* strains, representing 22 species, showed greater than or equal to two standard deviations above the maximum value obtained in testing 84 non-*Legionella* strains. Because the Gen-Probe protocol recommends conservatively that 10% be the cutoff value for a positive test result, an interpretation with which four *L. bozemanii* strains were negative, we recommend that values less than 10 but ≥5% be deemed suggestive of *Legionella* organisms. Alternatively, the procedure could be further modified to enhance lysis of recalcitrant *L. bozemanii* strains, because inefficient lysis of the cells could have been the cause of minimal probe binding due to the inaccessibility of cellular rRNA (James Hogan, personal communication). However, subsequent tests of the *L. bozemanii* strains at Gen-Probe, Inc., suggested that all of these strains gave positive test results if they were tested on

TABLE 4. Gene probe analysis of *Legionella* strains by source

Strains and source of isolates (*n*)	% Probe bound		
	Range	Mean	SD
All *Legionella*			
Clinical (119)	4–72	40.2	13.6
Environmental (76)	19–71	42.4	11.6
L. pneumophila			
Clinical (80)	31–72	47.8	6.7
Environmental (37)	34–71	50.9	6.8

TABLE 5. Non-*Legionella* bacteria tested with *Legionella* gene probe

Organism	No. of strains tested	Probe bound (%)
Acinetobacter lwoffi	1	0.4
Alcaligenes faecalis	1	3.0
Bordetella bronchiseptica	2	2.0
B. pertussis	2	2.0
Enterobacter aerogenes	4	−0.3–0.1
Escherichia coli	1	2.0
E. fergusonii	1	−0.2
E. hermannii	1	0
Haemophilus influenzae serotypes a to f	8	0.3–2.0
Klebsiella oxytoca	3	−0.1–(−0.2)
K. pneumoniae	3	−0.2–0.7
Kluyvera ascorbata	1	0
Neisseria meningitidis serogroups A, B, C, L, W135, X, Y, Z	9	1.0–3.0
Providencia rettgeri	1	0.2
P. stuartii	1	0.4
Pseudomonas acidovorans	1	0.7
P. aeruginosa	4	−0.3–0.2
P. cepacia	1	3.0
P. diminuta	1	0.1
P. maltophilia	1	0.3
P. paucimobilis	1	0.2
P. testosteroni	1	0.2
Serratia marcescens	4	0–0.2
Shigella flexneri	1	0
S. sonnei	1	0.2
Staphylococcus aureus	4	−0.2–0.3
S. saprophyticus	1	−0.1
Streptococcus groups A to D, G	9	0.4–0.7
S. pneumoniae types 1, 4, 6A, 6B, 9N, 14, 18C, 23F	13	−0.3–0.9
Yersinia enterocolitica	1	0.9
Y. pseudotuberculosis	1	0.7

the same day that they were resuspended in water (James Hogan, personal communication). Although practical with isolated cultures, fresh suspensions may not mimic the milieu in which *Legionella* cells are found in vivo or in environmental samples. In either situation, controls in each assay should include whole-cell *Legionella* and non-*Legionella* strains to assure that all components of the procedure, including the lysing agent, are functioning properly.

The distribution of *Legionella* species and serogroups in our evaluation may not have accurately represented their distribution in clinical or environmental specimens, because our objective was to test adequate numbers of strains in all the species if they were available. *L. bozemanii* accounted for 4.7% of the total number of *Legionella* strains selected for this study, whereas in clinical specimens, this species may be <3% of the total (7). Therefore, the sensitivity of the assay on isolated cultures may exceed 98%. Based on our data, the specificity was 100%. The test is rapid (3 h or less) and is independent of phenotypic characteristics other than cell envelope integrity of the organisms. The disadvantage of the probe is its radioactive label. The relatively short half-life of ^{125}I, 60 days, limits the probe reagent to a short shelf life. Nevertheless, gene probe analysis should be valuable in the *Legionella* diagnostic laboratory, especially if further studies show that it can be used to detect *Legionella* cells in primary specimens, such as sputum and lung tissue.

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LITERATURE CITED

1. **Cherry, W. B., B. Pittman, P. P. Harris, G. A. Hebert, B. M. Thomason, L. Thacker, and R. E. Weaver.** 1978. Detection of Legionnaires disease bacteria by direct immunofluorescent staining. J. Clin. Microbiol. **8**:329–338.
2. **Edelstein, P. H.** 1983. Culture diagnosis of *Legionella* infections. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. Reihe A **255**:96–101.
3. **Edelstein, P. H., R. M. McKinney, R. D. Meyer, M. A. C. Edelstein, D. J. Krause, and S. M. Finegold.** 1980. Immunologic diagnosis of Legionnaires' disease: cross-reactions with anaerobic and microaerophilic organisms and infections caused by them. J. Infect. Dis. **141**:652–655.
4. **Grimont, P. A. D., F. Grimont, N. Desplaces, and P. Tchen.** 1985. DNA probe specific for *Legionella pneumophila*. J. Clin. Microbiol. **21**:431–437.
5. **Kohne, D. E., A. G. Steigerwalt, and D. J. Brenner.** 1984. Nucleic acid probe specific for members of the genus *Legionella*, p. 107–108. *In* C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowski (ed.), Legionella. Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
6. **Orrison, L. H., W. F. Bibb, W. B. Cherry, and L. Thacker.** 1983. Determination of antigenic relationships among legionellae and non-legionellae by direct fluorescent-antibody and immunodiffusion tests. J. Clin. Microbiol. **17**:332–337.
7. **Reingold, A. L., B. M. Thomason, B. J. Brake, L. Thacker, H. W. Wilkinson, and J. N. Kuritsky.** 1984. Legionella pneumonia in the United States: the distribution of serogroups and species causing human illness. J. Infect. Dis. **149**:819.
8. **Sathapatayavongs, B., R. B. Kohler, L. J. Wheat, A. White, W. C. Winn, Jr., J. C. Girod, and P. H. Edelstein.** 1982. Rapid diagnosis of Legionnaires' disease by urinary antigen detection. Am. J. Med. **72**:576–582.

8a. **Selander, R. K., R. M. McKinney, T. S. Whittam, W. F. Bibb, D. J. Brenner, F. S. Nolte, and P. E. Pattison.** 1985. Genetic structure of populations of *Legionella pneumophila*. J. Bacteriol. **163**:1021–1037.

9. **Thacker, W. L., B. B. Plikaytis, and H. W. Wilkinson.** 1985. Identification of 22 *Legionella* species and 33 serogroups with the slide agglutination test. J. Clin. Microbiol. **21**:779–782.
10. **Wilkinson, H. W., A. L. Reingold, B. J. Brake, D. L. McGiboney, G. W. Gorman, and C. V. Broome.** 1983. Reactivity of serum from patients with suspected legionellosis against 29 antigens of Legionellaceae and *Legionella*-like organisms by indirect immunofluorescence assay. J. Infect Dis. **147**:23–31.