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Retrospective Study of Gen-Probe Rapid Diagnostic System for Detection of Legionellae in Frozen Clinical Respiratory Tract Samples

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The Gen-Probe Rapid Diagnostic System for legionellae, which uses ^{125}I-labeled cDNA directed against the rRNAs of legionellae, was evaluated for its ability to detect members of the genus by using clinical specimens which had been frozen at −70°C for 2 to 8 years. Culture and direct immunofluorescence (DFA) results obtained at the time of specimen collection were used to categorize samples. The specimens tested were 112 samples culture positive for legionellae and 230 samples negative on culture and DFA tests. They were tested in a blinded and randomized fashion. Results were expressed in terms of the ratio of counts per minute of the sample to the counts per minute of the provided negative control. A ratio of greater than or equal to 4.0 was picked for optimal specificity. Of the 112 previously positive specimens, 63 (57%) were positive by the probe assay, and of the 230 previously negative samples, 228 (99.1%) were negative. The 51 discrepant specimens were reexamined by culture and DFA testing if adequate amounts remained; this was possible for 34 specimens. On repeat culture, 22 of 33 previously culture-positive samples yielded legionellae and 11 were negative. Ten of the positive repeat cultures yielded two or fewer colonies per plate. One probe-positive but previously culture-negative sample was overgrown by contaminants on repeat culture. Reanalysis of data after exclusion of the 17 unavailable, 11 repeat culture-negative, and 1 unevaluable specimen gave a probe sensitivity of 74% and specificity of 100%. The Gen-Probe test is therefore specific and is of useful sensitivity.

Previous investigations of the Gen-Probe DNA probe for legionellae have determined that it correctly detects all named *Legionella* species taken from culture plates and that it does not react with any of a large number of nonlegionellae tested (3, 6). An assay kit for direct use of the probe with clinical samples has now been developed. The Gen-Probe Rapid Diagnostic System for *Legionella* spp. contains cDNA labeled with ^{125}I, which specifically hybridizes to the rRNAs of all named *Legionella* species. The clinical sample is homogenized, and the bacterial cell wall is lysed to release nucleic acids into solution. The cDNA probe is added to the specimen, and the reaction mixture is heated to promote specific hybridization of the probe to the rRNAs of legionellae. Unreacted cDNA is removed from the reaction mixture by addition of a hydroxyapatite suspension, which binds only double-stranded nucleic acids under the assay conditions. The hydroxyapatite is pelleted by centrifugation, and the unreacted cDNA is discarded with the supernatant. The pellet is washed, and the remaining (hybridized) cDNA is quantitated with a gamma counter.

This study was designed to determine the ability of the probe kit to specifically detect legionellae in clinical specimens. Because freshly collected culture-positive specimens were not available in sufficient numbers to perform a prospective study, we performed a retrospective analysis of frozen specimens, the majority of which had been frozen in small volumes for more than 5 years. The small volume of the specimens precluded performing repeat culture and direct immunofluorescence (DFA) tests, so that original culture and DFA results, performed 2 to 8 years previously, were used to classify the specimens.

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MATERIALS AND METHODS

Clinical specimens. A total of 112 clinical specimens collected from 64 patients and previously demonstrated to be culture positive for legionellae were used as positive samples (see Table 1). A total of 230 negative specimens were selected; these were age and specimen type matched to positive samples if possible. A negative sample was defined as one that was previously demonstrated to be negative for legionellae on culture and by DFA testing (2). All samples were submitted from patients suspected of having Legionnaires disease and had been frozen without specific preservative at −70°C for 2 to 8 years.

During the period of specimen collection and testing, different laboratory methods were used for culture and DFA staining (1, 2, 4). Buffered charcoal-yeast extract medium supplemented with α-ketoglutaric acid (BCYE) was used almost from the start, and various selective media were added to the culture process. By 1982, two different selective

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media were being used, BCYE supplemented with cefamandole, polymyxin B, and anisomycin (BMPA) and BCYE supplemented with glycine, vancomycin, polymyxin B, and anisomycin (MWY) (2). In addition, an acid-wash pretreatment of samples was used for plating all samples (2). Briefly, one portion of the sample was diluted (1:10) in tryptic soy broth (Difco Laboratories, Detroit, Mich.) and one portion was diluted (1:10) in a KCL-HCL solution (pH 2.2). The sample portion diluted in tryptic soy broth was plated directly (0.1 ml) onto a nonselective plate (BCYE) and onto each of the two selective plates (BMPA and MWY). The portion diluted in the acid solution was incubated at room temperature for 4 min and then plated in identical fashion onto each of the three media. Plates were incubated at 35°C in a humidified air incubator and examined daily for up to 2 weeks. Legionellae were identified on the basis of culture, biochemical, and immunological characteristics (2). Direct immunofluorescence testing was performed initially only with anti-*Legionella pneumophila* serogroup 1 and 4 antisera but was later expanded to include other species and serogroups.

Assay method. All 342 clinical samples were randomized by using a computer-generated random-number list. Technicians performing the assay had no knowledge of the true identity of the samples. Just before use, specimens were thawed at room temperature (20°C), and the volume of sample was estimated by comparison with standards. Solid samples were first ground in an all-glass tissue grinder with about 100 μl of Mueller-Hinton broth. One-tenth volume of solubilizer was added, and the sample was briefly vortexed and allowed to stand at room temperature for 15 min. If samples were not liquid at this stage, the process was repeated with an additional 0.1 volume of solubilizer. A 100-μl portion of the sample was then added to a gamma counting tube which contained 100 μl of lysing solution and approximately 200 μl of glass beads. After centrifugation for about 1 min, the tubes were placed in a sonicator bath for 15 min. Probe solution (2.0 ml) was then added to each tube. The tubes were vortexed briefly and then immersed in a 72°C water bath for 1 h. Separation suspension (2.3 ml) was added to each tube, and after incubation at 72°C for 5 min, the sample was pelleted by centrifugation for 2 min. The supernatant was discarded, and 4 ml of wash solution was added. The pellet was thoroughly suspended by vortex mixing, and the tube was incubated at 72°C for 5 min. The pellet was again sedimented by centrifugation for 5 min, the supernatant was discarded, and the radioactivity in each tube was measured in a gamma counter for 5 min. Positive and negative control solutions were included with each run. An adjustment was made for nonspecific background counts by subtracting the mean counts per minute in three tubes containing separation suspension from raw measurements. The ratio of corrected counts per minute of the sample to that of the negative control was used as a numerical index of probe hybridization.

Equipment. Centrifugation was performed with a Dynac tabletop centrifuge (Clay Adams, Towson, Md.) set at top speed (about 1,000 × *g*). Sonication was performed with a model 12 bath sonicator (Branson Sonic Power Co., Danbury, Conn.) half filled with boiled, degassed, distilled water at 72°C. Gamma 5500 (Beckman Instruments, Fullerton, Inc., Calif.) and Berthold LB2103 (Westchem; San Diego, Calif.) multicrystal gamma counters were used interchangeably; both had nearly identical counting efficiencies, 79%, as determined by the use of a sealed, calibrated ^{125}I source. The counting efficiency of the gamma counter was determined daily by using a calibrated sealed-source ^{125}I standard (Westchem).

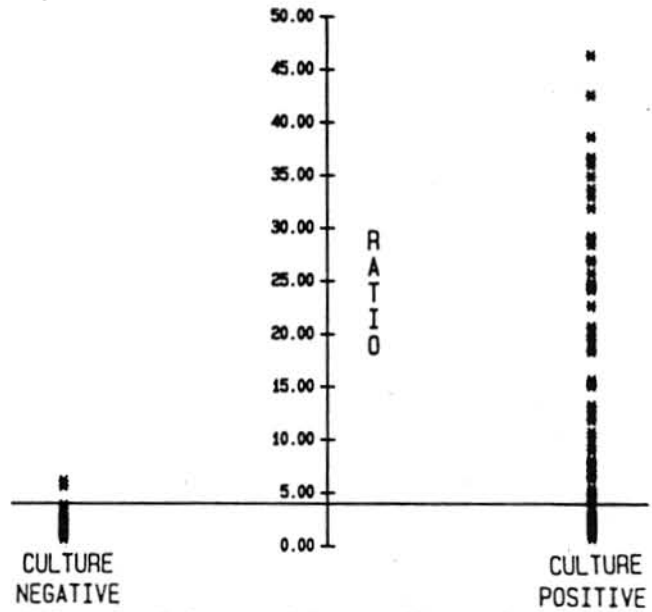

FIG. 1. Gen-Probe test ratio for each of 230 samples negative for *Legionella* species on culture and DFA testing and for each of 112 samples positive for *Legionella* species on culture. The horizontal axis is at a ratio of 4.0, the breakpoint value for a positive test; samples shown on or above this axis are positive in the Gen-Probe DNA probe assay.

Evaluation of discrepancies. All specimens yielding discrepant results were recultured, if additional frozen material was available, on BCYE, BMPA, and MWY, with and without acid-wash pretreatment as described above. If adequate sample was available after culturing, DFA testing was performed with antibody directed against the species and serogroup previously isolated; conjugates for *L. micdadei* and *L. longbeachae* were obtained from the Centers for Disease Control, Atlanta, Ga., conjugate for *L. pneumophila* was obtained from Genetic Systems Corp., Seattle, Wash., and conjugate for *L. wadsworthii* was made at the Veterans Administration Wadsworth Medical Center (2). Technicians performing the repeat culture and DFA assays were aware of previous results.

RESULTS

Overall results. As a group, culture-positive samples could be distinguished easily from culture-negative ones (Fig. 1). The mean ratio of culture-positive samples was 10.7 with a median of 5.1, range of 0.8 to 46.3, and 99% confidence intervals of 7.8 to 13.6. These values differed significantly from the culture-negative sample mean value of 1.5, median of 1.3, range of 0.7 to 6.1, and 99% confidence intervals of 1.3 to 1.6 ($P < 0.0005$; Student nonpaired one-sided t test).

A ratio of greater than or equal to 4.0 was selected as the lower limit for a test to be considered positive, after evaluation of all test results. This breakpoint was selected to optimize the specificity of the test.

Of the 112 originally culture-positive specimens, 63 were positive by the Gen-Probe assay; all but 2 of the 230 culture-negative specimens were correctly identified as negative. Thus the overall test sensitivity and specificity were

TABLE 1. Probe test results according to type of specimen, analyzed by individual specimen

Source	Before specimen exclusion[a]		After specimen exclusion[b]	
	Sensitivity[c]	Specificity[d]	Sensitivity[c]	Specificity[d]
Sputum	25/38 (66)	112/113 (99)	25/32 (78)	112/112 (100)
Lung	21/25 (85)	54/54 (100)	21/22 (95)	54/54 (100)
TTA[e]	3/21 (14)	40/41 (98)	3/7 (43)	40/40 (100)
Tracheal[f]	11/18 (59)	5/5 (100)	11/18 (61)	5/5 (100)
Bronchoscopy	2/4 (50)	8/8 (100)	2/3 (67)	8/8 (100)
Pleural fluid	0/3 (0)	9/9 (100)	0/2 (0)	9/9 (100)
Miscellaneous organs[g]	0/3 (0)	–[h]	0/3 (0)	–

[a] Analysis of data with all specimens tested.
[b] Analysis of data after excluding 29 discrepant specimens that were negative on repeat culture (11 specimens), unavailable for reculture (17 specimens), or overgrown on reculture (1 specimen). See text.
[c] Results are expressed as the number of specimens that were probe positive and culture positive for legionellae/number of specimens that were culture positive. Numbers in parentheses are percentages.
[d] Results are expressed as the number of specimens that were probe negative and culture negative for legionellae/number of specimens that were culture negative. Numbers in parentheses are percentages.
[e] TTA, Transtracheal aspirate.
[f] Tracheal aspirate via an endotracheal tube or nasal catheter.
[g] All autopsy tissues: liver, spleen, and tricuspid heart valve.
[h] –, There were no culture-negative specimens.

56.3% and 99.1%, respectively, when compared with the results of culture tests originally performed on these specimens at the time of collection. Analysis by patient rather than by specimen type yielded a sensitivity of 60% and the same specificity.

Twenty-five tests were performed in an average total time of 3 h, including incubation times. The actual hands-on time required to test a single specimen was estimated to be about 20 min.

Analysis of discrepant samples. Of the 49 discrepant samples that were originally culture positive, 33 were available for reculture to detect legionellae. Of these, 22 were positive; relatively low numbers of *Legionella* colonies were present on these plates. The average maximum number of *Legionella* colonies per plate was 10, with a range of 1 to 100 and median of 5; 45% of specimens had a maximum of two or fewer colonies per plate. Repeat DFA testing of the 22 repeat culture-positive samples showed that 9 were positive; 10 had been positive previously. Seven of the nine samples that were positive by repeat DFA testing contained two to five bacilli per smear, and two contained 25 to 100 bacilli per smear. The 11 repeat-culture-negative specimens were examined by DFA; all but 3 were negative for the species and serogroup isolated previously.

One of the two discrepant specimens that were originally culture negative was available for reculture for legionellae; all six selective and nonselective plates were overgrown by enteric-type organisms. DFA testing for *L. pneumophila* (all serogroups) was negative. Examination of the original culture results for this specimen showed that there was no significant growth on BMPA, but all other plates were overgrown by enteric-type bacteria.

Recalculation of the probe assay sensitivity after excluding the 11 repeat-culture-negative samples gave a sensitivity of 62% if analyzed by specimen type and 71% if analyzed by patient. When the 16 samples unavailable for reculture were also excluded from analysis, the sensitivity was 74% if analyzed by specimen and 81% if analyzed by patient. When the same samples, less the three repeat-culture-negative, DFA-positive samples, were excluded from analysis, the sensitivity of the probe assay was 83% for DFA-positive, culture-positive samples and 28% for DFA-negative, culture-positive samples. This contrasts with preexclusion sensitivities of 71% for DFA-positive samples and 17% for DFA-negative samples. When the two probe-positive repeat-culture-indeterminate samples were excluded from analysis, the specificity of the probe test changed from 99.1% before exclusion to 100% after exclusion.

When the results were examined by specimen type, differences in test performance were observed (Tables 1 and 2). Transtracheal aspirates, pleural fluids, and autopsy organs gave poor results with the probe assay before specimen exclusion. After specimen exclusion, these differences were not significant.

Analysis of results by *Legionella* species causing infection, before specimen exclusion, showed that 59 of 100 samples positive for *L. pneumophila* were positive by the probe assay, as were 2 of 8 *L. longbeachae*-positive samples, 1 of 2 *L. micdadei*-positive samples, 1 of 1 *L. dumoffii*-positive sample, and 0 of 1 *L. wadsworthii*-positive sample. After sample exclusion, these numbers were 59 of 78 for *L. pneumophila*, 2 of 5 for *L. longbeachae*, 1 of 1 for *L. micdadei* and *L. dumoffii*, and 0 of 0 for *L. wadsworthii*.

DISCUSSION

This study shows that the Gen-Probe kit for detecting legionellae in clinical samples is specific and sensitive enough to be used for laboratory diagnosis of Legionnaires disease. Owing to the retrospective nature of this study and to the nature of specimen handling and storage, the results obtained can, at best, define the minimum performance of the DNA probe assay.

Since posttest exclusion of some specimens from analysis significantly affects the interpretation of these results, it is worthwhile to detail the rationale for so doing. Precise handling procedures for samples were not recorded at the time of original freezing. Some samples were obtained in very small amounts and had to be diluted before processing or freezing; this was especially true of transtracheal aspirate samples (45% of repeat-culture-negative samples). Thus it is possible that samples originally containing very small numbers of legionellae no longer contained them on retesting

TABLE 2. Probe sensitivity according to type of specimen, analyzed by individual patient

Source	Sensitivity before specimen exclusion[a]	Sensitivity after specimen exclusion[a]
Sputum	13/24 (54)	13/19 (68)
Lung	22/25 (85)	22/23 (96)
TTA[b]	4/20 (20)	4/6 (67)
Tracheal[c]	7/7 (100)	7/7 (100)
Bronchoscopy	2/4 (50)	2/3 (67)
Pleural fluid	0/3 (0)	0/2 (0)
Miscellaneous organs[d]	0/3 (0)	0/3 (0)

[a] Specimen exclusion criteria are given in Table 1, footnote *b*. Results are expressed as number of patients with specimens from specified site that were probe positive and culture positive for legionellae/number of patients with specimens from specified site that were culture positive for legionellae. Numbers in parentheses are percentages.
[b] TTA, Transtracheal aspirate.
[c] Tracheal aspirate via an endotracheal tube or nasal catheter.
[d] All autopsy tissues: liver, spleen, and tricuspid heart valve.

because of the combined possibilities of dilution effect and sampling error, as well as a partial or complete loss of viable organisms while the samples were frozen. It therefore seems correct to exclude these repeat-culture-negative samples from analysis. A similar argument can be made for the 16 samples unavailable for reculture, in that their culture positivity at the time of the DNA probe assay could not be confirmed.

Whether to exclude from analysis the two probe-positive, repeat-culture-indeterminate samples is problematic. No clinical or other laboratory evidence exists for the diagnosis of Legionnaires disease in these patients, but this does not exclude that possibility. The patient whose sputum sample overgrew all plates on reculture had clinically diagnosed aspiration pneumonia, which responded promptly to penicillin and gentamicin therapy. Serum collected from the patient over a 24-day period showed no significant titer rise for *L. pneumophila* serogroups 1 to 7, *L. longbeachae* serogroups 1 and 2, *L. dumoffii*, *L. micdadei*, *L. bozemanii* serogroup 1, *L. gormanii*, *L. jordanis*, and *L. oakridgensis*. Two other sputum samples from the same patient taken during the same period were negative on DFA testing and culture; these other clinical specimens did not overgrow the selective culture plates. The second patient developed pulmonary infiltrates after blood transfusion; the infiltrates responded promptly to diuretic therapy alone. A transtracheal aspiration was performed prior to diuretic therapy to aid in the differential diagnosis of pneumonia versus pulmonary edema. The transtracheal aspirate, which was the specimen tested in the DNA probe assay, had no growth of any organism on any selective or nonselective medium inoculated for detection of legionellae. The DFA examination was negative for *L. pneumophila* serogroups 1 to 4.

The efficiency of the probe assay for testing clinical specimens containing *Legionella* species other than *L. pneumophila* could not be thoroughly studied. The probe does not hybridize equally well to all *Legionella* species and, in fact, hybridizes most completely with *L. pneumophila* and less so with other *Legionella* species (3, 6). In a previous study, several strains each of *L. micdadei* and *L. longbeachae* hybridized to the probe about 75% as completely as did *L. pneumophila* (6). Thus, the probe assay may fail to detect some *Legionella* species other than *L. pneumophila*, if they are present in low numbers in clinical samples.

Some types of specimens gave apparently poor results with the probe assay, in particular transtracheal aspirates, pleural fluids, and extrapulmonary organ specimens. The low apparent sensitivity noted with these samples may be an artifact. For example, it is possible that legionellae are less well preserved in these types of specimens or that prior dilution or processing may have adversely affected organism survival. Another possibility is that these samples generally contain very small numbers of legionellae, below the lower limit of detection of the probe assay. Data reanalysis after exclusion of some samples supports the possibility of artifactual results, since differences in test performance by specimen type were not significantly different. However, more information is needed before the true sensitivity of the probe assay is known for these types of specimens. Exclusion of transtracheal aspirates, pleural fluids, and extrapulmonary organ specimens from testing may be indicated until further trials document the efficiency of the probe assay with these types of specimens.

The sensitivity of the probe assay was measured against culture and DFA tests as performed in a research laboratory devoted to Legionnaires disease diagnostic studies. DFA testing in this laboratory is more sensitive (60 to 75%) than that reported from other laboratories (25 to 60%) (1, 4, 7). Also, the specimen plating methods used in this laboratory involved several different selective and nonselective media prepared in the laboratory rather than purchased, which probably enhances the sensitivity of culture. Therefore, the performance of the probe assay assessed in this trial was measured against standards which could be lower in routine laboratories. In such a case, the probe assay would compare much more favorably with culture and DFA diagnosis.

Where does this place the use of probe assay in clinical microbiology laboratories? Substitution of this test for DFA testing would seem to be justified by the data presented here. The probe assay demands considerably less expertise for test interpretation, since it yields a numerical result rather than requiring subjective judgements of organism morphology and fluorescence intensity. The hands-on time required for a technician to perform the probe test is similar to that required for DFA testing of single specimens, and the time required for testing of multiple samples is considerably less for the probe assay than for DFA testing. Use of the probe assay would be most advantageous in laboratories in which large-volume testing for *L. pneumophila* is performed because the test can be run most economically in batches. Laboratories in which little testing for *L. pneumophila* is performed might benefit by use of the probe assay if they do not have skilled immunofluorescence microscopists. Workers in laboratories in regions where pneumonia is commonly caused by non-*L. pneumophila Legionella* species could still benefit from use of the probe assay but would have to exercise caution over the uncertain sensitivity of the probe assay for these other species.

Because Legionnaires disease is generally a low-prevalence disease, only positive tests, including DFA and culture, have diagnostic meaning, since a negative test only slightly decreases the a posteriori likelihood of disease (1, 4, 5, 7). For this reason, multiple test procedures should be used, and culture should be performed for all samples negative by probe assay, DFA assay, or both, which would represent the vast majority of samples, except during a major epidemic of Legionnaires disease. Culture confirmation of positive probe assay results would be desirable in many cases for epidemiologic reasons.

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