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Direct Immunofluorescent Detection of *Legionella pneumophila* in Respiratory Specimens

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Respiratory secretions from patients with clinically suspected Legionnaires pneumonia were examined by direct immunofluorescent tests at the Medical Center Hospital of Vermont and at the Center for Disease Control. No fluorescent bacteria were found by either laboratory in eight specimens from eight patients who were seronegative. Twenty specimens were obtained from seven patients who had serologically confirmed Legionnaires disease. Four of the seven cases were identified at the Medical Center Hospital of Vermont, and six of the seven were identified at the Center for Disease Control. Of 20 specimens, 8 were positive at the Center for Disease Control (six or more bacilli per slide), and 7 specimens were suspicious (one to five bacilli per slide); at the Medical Center Hospital of Vermont, 4 of 20 specimens were positive, and 2 were suspicious. The inclusion of a rhodamine-conjugated counterstain at the Center for Disease Control facilitated the examination and may have improved the sensitivity. Smears from transtracheal aspirates, bronchoscopic aspirates, transcutaneous lung aspirates, pleural fluids, and tracheal aspirate-expectorated sputum produced positive results. Several specimens contained fluorescing bacilli when stained for serogroup 2 as well as serogroup 1, perhaps reflecting the presence of cross-reacting antigens in vivo.

In the 1976 Philadelphia outbreak, Legionnaires disease (LD) was diagnosed by isolating the bacterium from postmortem lung tissue or by demonstrating high, stable antibody titers or fourfold rises in antibody levels in surviving patients (13). *Legionella pneumophila* has been proposed as a new genus and species (2); four serogroups have been recognized among isolates from human infections (14). Culture of the agent will remain an important diagnostic tool, but the fastidious nature of the bacterium makes recovery from clinical specimens more difficult. For specimens that contain an indigenous microflora, a selective medium must be developed. Serological diagnosis is, of necessity, retrospective. A rapid and specific method for detecting the bacterium is needed to facilitate the diagnosis of LD.

The test that has been evaluated most thoroughly is direct immunofluorescence (DIF). This technique was employed by Cherry et al. for the demonstration of bacteria in Formalin-fixed lung tissue and a few selected clinical specimens (5). A retrospective evaluation of respiratory secretions that were collected during the 1977 Burlington outbreak demonstrated high specificity for *L. pneumophila* (3). The sensitivity appeared low (6 of 69 smears positive, 5 of 21 patients identified). The smears had been stored under suboptimal conditions, however, and up to 6 months had elapsed before they were evaluated.

The present investigation evaluates the effectiveness of DIF tests on respiratory specimens that were processed specifically for the identification of *L. pneumophila* and examined within 1 month of collection. The source of the specimens was the same hospital at which the previous retrospective evaluation had been performed, and the same investigator (W.B.C.) at the Center for Disease Control (CDC) evaluated the smears in the two series. In addition, the results of the reference laboratory at CDC are compared with the results obtained in the diagnostic microbiology laboratory at the Medical Center Hospital of Vermont (MCHV). Immunofluorescent identification of group A beta-hemolytic streptococci and fluorochrome detection of mycobacteria are used routinely in the hospital laboratory, but immunofluorescent identification of *L. pneumophila* had not been attempted previously.

MATERIALS AND METHODS

From June through October 1978, patients with pneumonia compatible with LD were identified by one of us (R.O.F.). Multiple smears were prepared from respiratory secretions (expectorated sputa, endotra-

cheal aspirates, transtracheal aspirates, bronchoscopic washings or aspirates, lung aspirates, or pleural fluid). The secretions were smeared within etched rings on clean glass slides, air dried, heat fixed, and immersed in 10% Formalin for 10 min. Patients were included in the study if at least two smears of one specimen were available and if an acute serum for antibody determination was obtained.

Smears were stored at room temperature and examined by DIF at MCHV within a few days of preparation. Coded companion smears were examined at CDC by an observer (W.B.C.) who did not know the code. The CDC evaluation usually was completed within 1 month after the specimen was prepared. Immunofluorescent staining was performed as previously described (3). Briefly, two demarcated areas of each smear, one with immune conjugate and the other with preimmune conjugate, were incubated in a moist chamber for 20 min at room temperature. Slides were rinsed and soaked twice in phosphate-buffered saline for 5 min, rinsed in distilled water, air dried, and mounted in buffered glycerol (pH 9.0). Each smear was fixed and stained separately, and each was rinsed in a separate container to minimize the transfer of bacteria from one smear to another. At CDC, smears were examined on a Leitz Ortholux microscope with a 200-W mercury arc lamp and incident light. The KP 490 exciter filter was combined with a BG 23 red-absorbing filter and a K480 edge filter. The Ploem illuminator contained the TK510 dichroic mirror and K510 suppression filter. Smears were scanned with a 63× oil immersion objective and examined more closely with the 100× objective. At MCHV, smears were examined on a binocular Zeiss incident-light fluorescent microscope with a 100-W tungsten-halogen lamp and the standard Zeiss fluorescein isothiocyanate (FITC) filter set (no. 48-77-09, transmittance at 450 to 490 nm). Smears were scanned with a 40× oil immersion lens followed by a 100× oil immersion lens. All smears were examined for at least 5 min before a negative result was recorded. If fluorescent bacilli were demonstrated with the specific conjugate, a smear stained with preimmune rabbit serum conjugated to FITC was examined.

The antisera employed at both CDC and MCHV were as follows: (i) hyperimmune rabbit antisera to the Knoxville 1 strain (serogroup 1) of *L. pneumophila* which had been conjugated to FITC and (ii) a preimmune rabbit serum-FITC conjugate. The immunoglobulin was prepared and coupled to FITC at CDC as previously described (4). The only difference in the serogroup 1 reagents used in the two laboratories was that a rhodamine-conjugated normal rabbit serum counterstain was used at CDC as a diluent for the FITC conjugates (4). In addition, FITC-conjugated rabbit antiglobulins to the other serogroups of *L. pneumophila* (serogroup 2, Togus 1 strain; serogroup 3, Bloomington 2 strain; and serogroup 4, Los Angeles 1 strain) were employed at CDC as the reagents became available.

Smears were considered positive if six or more fluorescent bacilli that were compatible morphologically with *L. pneumophila* were demonstrated with the specific conjugate and no fluorescent bacteria were demonstrated with the preimmune serum. If five or fewer bacilli were demonstrated, the smear was considered suspicious. The presence of fluorescent debris was recorded, but was not considered a positive or suspicious result.

Smears from most specimens were examined by Gram stain; the counterstain consisted of 0.5% basic fuchsine in a 2.5% safranine solution. The presence of polymorphonuclear neutrophils and alveolar macrophages was recorded. Transtracheal aspirates, specimens from lung parenchyma, pleural fluids, and fiberoptic bronchoscopy aspirates were inoculated onto commercial chocolate agar with a gonococcal (GC) agar base (GIBCO Diagnostics) and onto Feeley-Gorman agar, modified by substituting GC agar for Mueller-Hinton agar (8). Plates were incubated in a candle jar at 35°C for 10 days. Each batch of medium was tested for the ability to support growth of the Burlington 1 strain of *L. pneumophila*, which was isolated during the 1977 Vermont outbreak.

Sera were stored at −35°C until a convalescent specimen was received. Antibody levels were measured at the Vermont Public Health Laboratories by the indirect fluorescent-antibody test (13), with reagents supplied by CDC.

Cases were defined by a fourfold or greater rise in indirect fluorescent-antibody titer (seroconversion), a single indirect fluorescent-antibody titer of ≥1:256 in a patient who had a compatible clinical illness, isolation of the bacterium, or demonstration of *L. pneumophila* by DIF staining of postmortem lung tissue. Clinical aspects of these cases have been reported previously (10).

RESULTS

Seven patients with LD were included in the study. From these patients, 20 specimens (transtracheal aspirate, 6; bronchoscopy washing or aspirate, 3; lung aspirate, 4; pleural fluid, 3; and endotracheal aspirate or expectorated sputum, 4) were examined by DIF. The diagnosis of LD was confirmed by seroconversion in five patients, seroconversion plus portmortem DIF identification of *L. pneumophila* in the lung in one patient, and a single titer of 1:512 in one patient.

Respiratory secretions from eight patients who did not develop antibody to *L. pneumophila* were examined. One specimen of transtracheal aspirate, lung biopsy, pleural fluid, or bronchial washings from each of the eight patients was negative for *L. pneumophila* by DIF both at CDC and at MCHV.

The results of DIF tests on the specimens from the patients with Legionnaires pneumonia are shown in Table 1. Of 20 specimens, 6 were positive or suspicious at MCHV; 15 of the 20 smears were positive or suspicious at CDC. All four smears that were negative or suspicious at MCHV and positive at CDC were prepared from tracheal aspirates or expectorated sputum and contained moderate to large amounts of fluores-

TABLE 1. *Comparison of DIF results at CDC and MCHV on 20 respiratory specimens from patients with LD*[a]

MCHV results	CDC results		
	Positive	Suspicious	Negative
Positive	④	0	0
Suspicious	1	①	0
Negative	3	6	⑤

[a] Positive, Six or more specifically staining bacilli per slide; suspicious, one to five specifically staining bacilli per slide; negative, no fluorescent rods seen. Circled numbers indicate agreement between CDC and MCHV.

cent debris. Three of the six smears recorded as negative at MCHV and suspicious at CDC contained faintly fluorescing bacteria in small numbers (fewer than five bacilli per slide). No smears were considered positive or suspicious at MCHV and negative at CDC.

Specimens from six of the seven patients with serologically documented LD were positive at CDC, and four of the cases were identified at MCHV. If only those patients from whom more than one specimen was obtained are considered, the MCHV evaluation identified four of five patients, whereas the CDC evaluation identified all five.

Cellular evidence of lower respiratory origin (alveolar macrophages) or of the inflammatory character of the specimen (moderate numbers of polymorphonuclear neutrophils) did not correlate well with the immunofluorescent results (Table 2), although the more inflammatory specimens tended to have larger numbers of bacteria present. The number of sputum samples was too small to be analyzed separately; the only two sputa on which Gram stains were available contained both polymorphonuclear neutrophils and alveolar macrophages.

The effect of therapy on the detection of *L. pneumophila* could not be evaluated systematically because serial specimens were not available on most patients. One patient who had a dual infection with *L. pneumophila* and *Klebsiella pneumoniae* developed fever and new pulmonary infiltrates twice after the institution of antimicrobial therapy (10). A pleural fluid and transtracheal aspirate obtained 3 days after the onset of symptoms and before the institution of erythromycin therapy were both positive for *L. pneumophila*. At 10 days after the institution of erythromycin therapy, a pleural fluid and lung aspirate contained four and two *L. pneumophila* bacteria per smear, respectively. After 2 weeks, at the time of a second clinical relapse, a transtracheal aspirate and lung aspirate contained four weakly fluorescent bacteria each, and a pleural fluid was negative. Sequential evaluation of sputum specimens on future patients may provide additional information about the clearance of bacteria after the institution of antimicrobial therapy.

Results of immunofluorescent examination of each specimen type are shown in Table 3. Specifically stained bacilli were demonstrated in each specimen type. The results with transtracheal aspirates and lung aspirates may have been adversely affected by sampling error. In some patients, these specimens were obtained because sputum was not produced or because symptoms had recurred after erythromycin therapy had been instituted (10).

Although six specimens which contained more than five *L. pneumophila*-like bacteria per smear by DIF were cultured for *L. pneumophila*, no isolates of this organism were obtained.

Eighteen specimens also were examined with an FITC-conjugated antiserum to the Togus 1 strain (serogroup 2) of *L. pneumophila*. Two specimens which were positive with the Knoxville conjugate were also positive with the Togus conjugate; one specimen was classified as suspicious with both conjugates. In each instance, another specimen from that patient was positive

TABLE 2. *Results of DIF tests in relation to inflammatory nature of specimen*[a]

Presence of alveolar macrophages and PMN	Smear results		
	Positive	Suspicious	Negative
Alveolar macrophages or moderate PMN or both present	5	4	4
No alveolar macrophages; few or no PMN	0	3	1

[a] See Table 1, footnote *a*, and the text for definitions. Gram-stained smears were not available on three positive specimens. PMN, Polymorphonuclear neutrophils.

TABLE 3. *Comparison of DIF identification of L. pneumophila by specimen type (CDC results)*

Type of specimens	No. of specimens	Smear results[a]		
		Positive	Suspicious	Negative
Transtracheal aspirate	6	2	2	2
Bronchoscopy	3	2	0	1
Lung aspirate	4	1	2	1
Pleural fluid	3	1	1	1
Tracheal aspirate or sputum	4	2	2	0

[a] See Table 1, footnote *a*, for definitions.

with the Knoxville conjugate only. Two specimens were examined with conjugates directed against the Bloomington 2 (serogroup 3) and Los Angeles 1 (serogroup 4) isolates. One transtracheal aspirate contained fewer than five bacilli with the Knoxville conjugate, but was negative with all the other serogroups. Two postmortem lung scrapings from the same patient, however, contained many fluorescent bacilli when stained with conjugates directed against both the Knoxville 1 and Los Angeles 1 isolates.

DISCUSSION

DIF has played an important role in the investigation of LD from the outset. The application of the technique to clinical respiratory specimens was validated in a retrospective study of the 1977 Burlington, Vt., outbreak (3). The specificity of the immunological reaction was excellent; there were no false-positive results on 64 specimens from 47 patients who did not have LD. A more limited number of specimens from seronegative patients in the present study was also negative. Additional evidence for the specificity of immunofluorescence has been provided by the isolation of *L. pneumophila* in pure culture from a transtracheal aspirate in which the bacterium had been demonstrated by DIF (12). After completion of the present study, *L. pneumophila* was isolated at MCHV from respiratory specimens from three patients in whom the diagnosis of LD had been made by DIF (Winn and Casey, unpublished observations).

Two patients in whom *L. pneumophila* was demonstrated by DIF have not developed specific antibody at least 6 weeks after the onset of illness or had Legionnaires pneumonia confirmed by postmortem examination. Neither patient was hospitalized during the present study. A 48-year-old woman, who had systemic lupus erythematosus and had received 20 mg of prednisone per day for 5 months, was transferred to MCHV with a clinical diagnosis of LD. She responded to erythromycin therapy. A single positive smear of sputum was stained at MCHV, and the interpretation was corroborated at CDC. The sputum culture yielded a few mixed bacterial types, and there was no growth of gram-negative bacilli on MacConkey agar. Sera obtained as late as 1 year after the onset of illness failed to show antibody by the indirect fluorescent-antibody technique. The second patient was a 60-year-old man with a history of alcohol abuse, who had been treated with oral erythromycin for possible LD. On admission to MCHV, intravenous erythromycin and cefazolin therapies were instituted for a left-upper-lobe pneumonia. The clinical diagnosis was probable LD, and he responded to antimicrobial therapy. A sputum specimen and a transtracheal aspirate contained *L. pneumophila* by DIF. No bacteria were seen by Gram stain in the transtracheal aspirate, and no bacteria were isolated. At 6 weeks after the onset of illness, no antibody to serogroups 1 through 4 of *L. pneumophila* was detected.

Recently, a third patient, who was classified as suspicious by DIF (three specifically fluorescing bacteria per smear), has failed to develop serum antibody to *L. pneumophila* 6 weeks after the onset of fever, sore throat, and pulmonary infiltrates. Sputum cultures yielded only a few mixed bacterial flora.

Particular attention must be paid to future cases in which DIF, particularly when applied to sputum, is not corroborated by rises in serum antibody levels. The possibility of nasopharyngeal carriage of *L. pneumophila* has not been investigated adequately. The contamination of laboratory water by nonviable *L. pneumophila* bacteria from environmental sources is another potential source of false-positive DIF results. On the other hand, a small percentage of epidemiologically defined cases in the original outbreak never developed detectable antibody (9). Early erythromycin therapy may diminish the antibody response (11).

In a retrospective study of smears from respiratory specimens, the sensitivity of DIF was low: 6 of 69 smears were positive, and 5 of 21 patients with LD were identified (3). The smears had been heat fixed and stored at room temperature for as long as 6 months. In contrast, 8 of 20 coded specimens were identified as positive, and an additional 7 of the 20 specimens were considered suspicious in the present prospective study. Six of the seven patients with LD were identified, and all five patients from whom more than one specimen was obtained were detected. One investigator (W.B.C.) examined the smears in both studies.

More patients and more specimens were identified as positive at CDC than at MCHV. Several factors may play a role in the discrepancy, including greater experience with the technique at CDC and a conservative approach to interpretation of the smears at MCHV during the early phase of the study. Specimens that contain fewer than five bacilli per smear never will be easy to identify. A very likely explanation for the difference is the rhodamine counterstain which was incorporated into the FITC conjugate at CDC. Without the counterstain, many specimens have intense background fluorescence, which makes interpretation more difficult and may mask specifically fluorescing bacteria. After the rhodamine counterstain was incorporated into the

FITC conjugate at MCHV, all five cases of LD diagnosed by culture or serology were identified by DIF (Winn and Casey, unpublished observations).

The analysis of results by specimen type is potentially biased because more invasive techniques might be reserved for difficult diagnostic situations in which expectorated specimens were inadequate. It is apparent, nevertheless, that expectorated sputa or tracheal aspirates are potentially useful diagnostic specimens. In those patients who are producing sputum, a more invasive procedure may be avoided. The inflammatory character of the smear was not a good predictor of smear result, but too few expectorated specimens were available to analyze.

The failure to isolate the bacterium from specimens that contained *L. pneumophila* is disappointing. The media in use adequately supported the growth of a stock strain. Isolation attempts were made from specimens that contained both large and small numbers of bacteria. The use of charcoal-yeast extract agar may facilitate the isolation of *L. pneumophila* in the future (7).

The staining of bacteria in a few specimens by FITC antiglobulin to the Togus 1 strain (serogroup 2), as well as by FITC antiglobulin to the Knoxville 1 strain (serogroup 1), is of interest. Although *L. pneumophila* was not isolated from these cases, three of four strains which have been isolated from other patients at MCHV have been members of serogroup 1. A dual infection is possible, but an immunological cross-reaction is more likely. In the cases from which the Togus 1 (15) and the Los Angeles 1 (serogroup 4) (6) strains were isolated, positive immunofluorescence with a conjugate directed against the Knoxville 1 strain was observed on at least one occasion. It is possible that species-specific antigens, which are expressed in human infections, are masked after in vitro cultivation.

The value of immunofluorescence for the diagnosis of LD is established. The applicability of the technique to expectorated sputum may obviate the need for more invasive diagnostic procedures in some patients. Production of polyvalent antisera will facilitate the examination of clinical specimens for the presence of all four serogroups. The procedure will be difficult to adapt to routine screening of expectorated sputum. Conservation of resources may be achieved, however, by limiting the DIF examination to patients with clinical features which are suggestive of Legionnaires pneumonia (1, 11).

Although a negative result on a single specimen does not eliminate the possibility of LD, an acceptable level of sensitivity can be achieved by an examination of multiple specimens. The immunological specificity of the reaction appears to be good, and most positive results have been confirmed by other means. The evaluation of the sensitivity and specificity of DIF for the diagnosis of Legionnaires pneumonia should be continued, particularly where the incidence of the disease is high.

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