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Detection of Legionnaires Disease Bacteria by Direct Immunofluorescent Staining

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Antisera and fluorescein isothiocyanate conjugates prepared for five strains of the Legionnaires bacteria were tested in both homologous and heterologous staining reactions with 10 isolates of the organism from patients in seven geographic areas. The strains were related but not identical as judged by the results of direct immunofluorescence staining. The conjugates were successfully used to detect Legionnaires disease bacteria in Formalin-fixed lung scrapings, in histological sections, and in fresh lung tissue obtained at biopsy or autopsy. In addition, the labeled antibodies are valuable for staining suspected cultures of the bacterium and for searching for the source of these organisms in soil, water, and other environmental niches. The reagents are highly specific for detecting the Legionnaires organism in clinical specimens.

The clinical, pathological, and epidemiological features of Legionnaires disease were detailed in previous publications (5, 7, 12). McDade et al. (12) first demonstrated the pathogen in the yolk sacs of embryonated eggs that had been inoculated with macerated tissue from guinea pigs injected with infected human lung tissue obtained at autopsy. They used indirect immunofluorescence (IF) to obtain convincing evidence that these organisms were the cause of the disease. A high titer (1:128) against the Legionnaires bacteria in a single serum specimen or a fourfold rise (to 1:64 or more) in the titer of convalescent-phase serum relative to acute-phase serum indicated that infection had occurred (12). One of us (R.E.W.) isolated the bacterium on nonliving media. Yolk sac isolates were cultured on Mueller-Hinton agar supplemented with 1% hemoglobin and 1% (vol/vol) IsoVitaleX (BBL) enrichment. Isolation of the organism from clinical material by passage through guinea pigs and embryonated eggs is a slow and tedious process, although a very sensitive one (12). Direct isolation on the laboratory media that are presently available is uncertain because a large inoculum is required, and significant growth does not usually occur for several days. The indirect IF test is valuable for seroepidemiological surveys and for retrospective diagnosis of the disease, but does not fulfill the critical need for a rapid and specific diagnostic test that can guide the physician in management of the infection. This unmet need was the stimulus for developing the direct IF test described in this paper.

MATERIALS AND METHODS

Cultures. The following strains of the Legionnaires bacteria were used in this study.

Philadelphia 1, 2, 3, and 4, isolated by McDade and Shepard from the lungs of four persons with fatal Legionnaires disease or Broad Street pneumonia contracted in Philadelphia, Pa., during July and August 1976 (12). The organisms were isolated by passage of lung tissue through guinea pigs and into the yolk sac of embryonated eggs.

Pontiac, isolated by McDade from the frozen lung tissue of a sentinel guinea pig that had been exposed to the interior environment of the Oakland County, Mich., Department of Health during an outbreak of a mild respiratory disease among the occupants of that building in 1968 (1). The tissue had been maintained at −70°C since that time. The five strains listed above were later grown in pure culture on enriched Mueller-Hinton medium by one of us (R.E.W.).

Knoxville, isolated by McDade on enriched Mueller-Hinton medium after guinea pig and egg passage of tissue from the lung of a patient who died of the disease in Knoxville, Tenn., in February 1977.

Albuquerque, isolated by McDade on enriched Mueller-Hinton agar following guinea pig and egg passage of lung tissue from a patient who died of pneumonia in New Mexico in November 1977.

Flint 1, isolated directly from pleural fluid inoculated onto enriched chocolate agar. It was submitted for identification by Morris Dumoff.

Bellingham, isolated on enriched chocolate agar by Beth Hayhow at St. Joseph's Hospital in Bellingham, Wash. It was submitted for identification by M. McDowell of the Washington State Laboratory. The source of the culture was pleural fluid from a patient with bronchopneumonia.

Burlington, isolated directly on enriched Mueller-Hinton agar by one of us (W.B.C.) from the lung of a

patient who died of pneumonia during the epidemic in Vermont in the summer of 1977 (4).

All cultures (374) of non-Legionnaires bacteria were obtained from stocks or were freshly isolated reference cultures.

Note. Enriched Mueller-Hinton agar was Mueller-Hinton agar (BBL or Difco) to which was added 1% dehydrated hemoglobin and either 1 or 2% IsoVitaleX (BBL), or in which 0.05% cysteine hydrochloride was substituted for the IsoVitaleX. Enriched chocolate agar was one of the commercial GC base media enriched with heated blood and IsoVitaleX.

Safety procedures. All work with living cultures of Legionnaires disease bacteria or with clinical specimens known or suspected of containing living bacteria was performed in a biological safety cabinet under Class 2 conditions (2). These included geographic isolation and controlled access, negative air pressure, protective equipment, and disinfection of the work area and of materials leaving that area.

Antigen preparation. Immunogens were prepared with the following cultures: Philadelphia 1 and 2, Flint 1, Pontiac, and Knoxville. Mueller-Hinton agar plates supplemented with 1% hemoglobin and 1% (vol/vol) IsoVitaleX (BioQuest Laboratories, Cockeysville, Md.) were inoculated heavily using sterile swabs to scrape cells from young cultures of the above strains. The plates were incubated in a candle extinction jar for 72 h at 35 to 37°C. The growth from three to five plates was collected and suspended in approximately 3.0 ml of 0.85% NaCl solution containing 0.5% Formalin (37% formaldehyde). The suspensions were refrigerated overnight and tested for viability by adding several drops to a fresh plate of the growth medium. No growth was observed with any strain. However, preliminary data from others suggest that 0.5% Formalin may not kill all Legionnaires bacteria (personal communication, G. Gorman and J. McDade). Smears from the cell suspensions were tested for purity with Gram staining. The cells were centrifuged, resuspended in the same menstruum, and concentrated to give a turbidity reading of 18 IU, relative to that obtained with an international substandard of Pyrex glass beads (11). At this concentration, the suspension contained approximately 2×10^9 bacteria cells per ml, estimated from the cell concentration of a no. 7 McFarland standard for bacteria of comparable size.

Immunization. Young adult New Zealand white rabbits were immunized according to two injection schedules. The intravenous injection schedule is shown in Table 1. Note that the concentration of the antigen was doubled to 36 to 40 IU on day 24 of injection and used at that concentration for the remainder of the schedule. Boosters were given on days 54 and 56.

The other immunization method consisted of an injection into the "footpad" of the rabbit followed by an intramuscular booster. The Formalin-saline suspension of whole cells at a density of 18 IU was mixed with an equal amount of Freund complete adjuvant (Difco) and emulsified by shaking. One milliliter of this mixture was injected into each front foot. The animals were bled on day 35 to monitor antibody response. On day 42, each animal was given 2.0 ml of the above antigen intramuscularly in the hip. Serum was harvested on days 49 and 56.

TABLE 1. *Protocol for intravenous immunization of rabbits with antigens[a] of Legionnaires disease bacteria*

Day of injection	Vol of antigen at (concn)[b]
1	0.1 (18–20)
3	0.2 (18–20)
5	0.5 (18–20)
7	0.75 (18–20)
9	1.0 (18–20)
11	2.0 (18–20)
13	3.0 (18–20)
15	4.0 (18–20)
17	5.0 (18–20)
24, 26, 28, 35, 37, 39, 41	3.0 (36–40)
54, 56	5.0 (36–40)

[a] Cell suspensions in 0.85% NaCl containing 0.5% Formalin. Main bleedings were on days 34, 48, and 63.

[b] Volume is expressed in milliliters, concentration in international units (IU) of turbidity. Total volume injected per rabbit (of 36–40 IU concentration or equivalent volume of 18–20 IU concentration), 39.3 ml.

Preimmunization serum for labeling with fluorescein isothiocyanate (FITC) was obtained from each animal and served as a control to assess the specificity of the staining reaction.

Preparation of conjugates. Immunization progress was followed by preparing whole serum conjugates as McKinney et al. prescribed (13). This is a rapid preliminary method for determining relative IF titers and consists of reacting a constant amount of FITC with 1.0 ml of whole serum and separating the unreacted dye from the labeled protein on a Sephadex G-25 or G-50 column. When the direct IF staining titers appeared to be high enough (1:16 to 1:64), serum was obtained from the rabbits, fractionated by $(NH_4)_2SO_4$, and labeled with FITC. If these trial conjugates had satisfactory titers (3+ to 4+ at dilutions of 1:40 or more), several successive bleedings were done, and the serum was pooled for preparation of the final product.

Sera were fractionated by three successive precipitations in 35% saturated $(NH_4)_2SO_4$ (final concentration in the mixture) (9). The globulin fraction was labeled with FITC certified by the Biological Stain Commission to be of 99% purity (International Biologicals, Inc., Melbourne, Florida), based on its labeling efficiency for bovine serum albumin (6).

The amount of FITC used for labeling was calculated to produce fluorescein-to-protein ratios of approximately 25 to 30 μg of FITC per mg of protein (13). The protein content of most conjugates was adjusted to approximately 10 mg of protein per ml was measured by the method of Gornall et al. (8).

The fractionation and labeling procedures used were those detailed by Hebert et al. (9).

Testing of conjugates. All conjugates were tested with their homologous antigens and with the heterologous antigens of all available Legionnaires cultures. Antigens consisted of suspensions of cells in 0.85%

NaCl containing 1% Formalin. The cells were harvested from cultures incubated on enriched Mueller-Hinton medium at 35°C for 72 h. All cell suspensions were adjusted to a turbidity approximating that of a McFarland no. 1 standard. Twofold dilutions of the immune conjugates were made in 0.01 M phosphate (pH 7.1) containing 0.1% NaN_3 as a preservative. The initial dilution was 1:20. Conjugates of preimmune serum were tested at a single dilution only (1:20), although the protein level of two conjugates, Flint and Knoxville (Table 2), was less than 10 mg/ml. All reagents were stored in the refrigerator at 4°C. The titer of the conjugates was defined as the end point dilution factor of the highest dilution giving at least 3+ staining. The working dilution was defined as the dilution factor of one doubling dilution less than that of the titer. Observations were made by incident illumination with a 50-W halogen lamp utilizing the Ploem illuminating system on a Leitz Dialux microscope. The primary filters were 2-KP 490; the dichroic beam-splitting mirror, TK 510, was coupled with a built-in K 515 suppression filter to form "filter system H for wide-band, blue-light excitation"; the ocular filter was K 510.

All conjugates were analyzed by physicochemical characterization using the methods of Hebert et al. (9). These included protein and FITC measurements for determination of the fluorescein-to-protein ratio and cellulose acetate strip electrophoresis for quantitation of the immunoglobulin.

Application of conjugates to the detection of Legionnaires bacteria. Detection in scrapings of Formalin-fixed tissue. At least one area of the lung or other tissue was tested. In the lung, areas of dense consolidation were selected. Each block of tissue was transferred to a sterile petri dish. A sharp scalpel was used to cut through these areas and expose new tissue faces. While the tissue was held with forceps, a scalpel was used to scrape off a fine puree of tissue particles. (The lung tissue of victims of Legionnaires disease is usually quite friable.) The tissue particles were smeared with the scalpel blade onto two 1.5-cm-diameter circular areas on a microscope slide. The smears were air dried and heat fixed. One smear was stained with the working dilution of the Legionnaires conjugate and another with preimmune conjugate of approximately the same fluorescein-to-protein ratio and protein content. Smears were stained for 20 min at room temperature in a moist chamber, quickly rinsed in phosphate-buffered saline (0.01 M phosphate buffer, pH 7.6, containing 0.85% NaCl), and immersed in a container of phosphate-buffered saline for 5 min. They were then rinsed with distilled water, air dried, and mounted with a cover slip and a drop of glycerol buffered at pH 8.0 to 9.0 (10).

The stained preparations were examined first with the ×10 objective of the fluorescence microscope and then under oil immersion at ×1,250.

Detection in fresh or fresh-frozen tissue (autopsy or biopsy). If the tissue was to be cultured, this was done before the impression smears were made. All fresh or fresh-frozen tissue was processed in a safety cabinet. Sterile instruments were used to hold and to cut a fresh face of tissue and to press and squeeze the tissue first onto the surface of an agar plate for culture and then against a clean slide, making at least two smears. If the tissue was very moist, it was blotted on sterile gauze before touching it to the slide. Smears were air dried and fixed for 10 min by covering them with 10% neutral Formalin and placing them in a moist chamber to retard evaporation of the Formalin. The smears were rinsed in distilled water, air dried, gently heat fixed, and stained as described above for tissue scrapings.

Homogenates of fresh lung tissue were prepared by the method described by McDade et al. (12). Smears were processed as described above for the tissue imprints.

Detection in tissue sections. Sections from paraffin blocks cut as thin as possible (4 μm or less) were fixed for approximately 15 min at 58 to 60°C and freed from paraffin by two passages through xylol followed by two passages each through absolute ethanol, 95% ethanol, and water. They were stained as described above.

Detection in exudates from the lungs. Sputum, transtracheal aspirates, bronchial washings, pleural fluids, or other specimens from the lower respiratory tract were examined for Legionnaires bacteria when suitable material was available. Rather thick smears were prepared and processed as outlined above.

RESULTS

Characteristics of the conjugates. The characteristics of the conjugates for the Legionnaires bacteria are shown in Table 2. The results on final bleedings were obtained from conjugates prepared from a pool of two or more specimens taken near the end of the immunization period. Results on trial bleedings were obtained from conjugates of earlier single bleedings taken during the course of immunization.

The immunoglobulin content in the immune conjugates ranged from 84 to 93%; that in the preimmune, from 65 to 72%. Fluorescein-to-protein ratios ranged from 19 to 33 μg/mg of protein. The protein content of immune conjugates varied between 3 and 12 mg/ml, and that of the preimmune conjugates ranged between 4 and 10 mg/ml. Direct staining titers on the conjugates from the trial and final bleedings varied from 1:8 to 1:1,280, being considerably higher in some of the earlier bleedings than in the final. This range probably reflects the effects of the timing of some of the final bleedings and of the pooling of the serum from several bleedings.

Homologous and heterologous staining titers of conjugates. In Table 3, homologous and heterologous titers are shown for six conjugates tested against 10 isolates of the Legionnaires bacterium. The Bellingham strain can be differentiated from the other nine strains on the basis of its generally weak reactions with the conjugates tested. Nevertheless, it stained well enough (3+ fluorescence) to permit its detection by the Knoxville conjugate at a high dilution

TABLE 2. *Characteristics of conjugates prepared for five strains of Legionnaires bacteria by two routes of inoculation*

Strain	Rabbit no.	Injection route	Bleeding no.	Percent gamma[a]	F/P ratio[b]	Protein (mg/ml)	Homologous 3+ titer[c]
Philadelphia 1	338–39	i.v.[f]	1[d]	ND[e]	28	10	
			3	92	33	8	64
			Finals	85	29	10	20
Philadelphia 2	341	i.v.	1	ND	27	10	
			3	90	19	3	8[g]
			Finals	90	28	12	320
Philadelphia 2	344	Footpad	1	ND	30	10	
			3	93	20	5	32[g]
			Finals	88	26	10	320
Flint 1	342–43	i.v.	1	65	32	4	
			3	91	31	11	128
			Finals	88	25	8	160
Pontiac	348	Footpad	1	ND	28	10	
			2	84	29	10	1,280
			Finals	87	29	9	640
Knoxville	350	Footpad	1	72	32	4	
			2	90	25	10	320
			Finals	91	28	11	160

[a] Calculated from the cellulose acetate strip electrophoresis profile. Percent gamma is the ratio of the area under the gamma curve to the total area under the protein profile (9).

[b] The ratio of FITC, determined by reference to a fluorescein diacetate standard, to total protein, determined by the biuret method (9).

[c] End point dilution factor of highest dilution giving 3+ homologous staining.

[d] Bleeding no. 1, Preimmunization bleeding. All preimmune conjugates were negative when tested.

[e] ND, Not done.

[f] i.v., Intravenous injection.

[g] If titers were adjusted to correspond to 10 mg of protein per ml, they would be approximately 32 and 64, respectively.

TABLE 3. *Homologous and heterologous titers of six Legionnaires strain conjugates*

Strain[a]	Conjugate titers[b] of:					
	Phil. 1 (i.v.)	Flint (i.v.)	Phil. 2 (i.v.)	Phil. 2 (fp)	Pontiac (fp)	Knoxville (fp)
Phil. 1	20	80	40	320	320	320
Phil. 2	40	160	320	320	320	320
Phil. 3	<20[c]	160	80	320	640	320
Phil. 4	<20[c]	160	160	320	320	160
Flint 1	20	160	80	320	160	320
Pontiac	20	160	40	320	640	640
Knoxville	40	40	40	320	80	160
Bellingham	20	20	<20[c]	20	<20[c]	160
Burlington	<20[c]	40	<20[c]	320	320	640
Albuquerque	<20[c]	160	160	640	640	160

[a] Suspension of cells in 0.85% NaCl containing 1% Formalin; turbidity = McFarland no. 1 standard. Phil., Philadelphia strain.

[b] End point dilution factor of highest dilutions giving 3+ or greater staining reactions, iv., Intravenous inoculation; fp, footpad inoculation.

[c] End point dilution factor of lowest dilutions tested which did not give a 3+ staining reaction.

(1:160) and by three other conjugates at a low dilution (1:20) (Table 3). It is evident that Philadelphia 1 was the weakest conjugate tested, although a conjugate from an earlier bleeding had given a homologous titer of 1:64 (Table 2).

Table 3 shows that the conjugates giving the highest titers against heterologous antigens were those obtained from the animals receiving "foot-

pad" and intramuscular injections.

Preimmune conjugates comparable in every other way to the immune conjugates usually gave no staining of any antigens at the 1:20 dilution. Weak staining (+− to 1+) of all cells was observed occasionally, and strong (3+) staining of a few cells was seen rarely.

Detection in scrapings of Formalin-fixed tissues, tissue sections, and fresh tissue imprints of homogenates. The direct IF staining procedure was used to study specimens of lung tissue from 143 patients with suspected Legionnaires disease. The conjugate for the Knoxville strain was diluted 1:80 in phosphate-buffered saline at pH 7.6 and used for the examinations. The tissues consisted of wet Formalin-fixed lung, paraffin-embedded lung, and either fresh or fresh-frozen lung. Most of the specimens examined were scrapings of Formalin-fixed lung, followed in order by tissue sections and by imprints or homogenates of unfixed fresh lung. The criteria for determining IF positivity of lung specimens were those shown in Table 4, except that early in our experience with direct IF staining a few specimens were reported positive when less than 25 Legionnaires bacteria were seen per smear. In fluid specimens from the lower respiratory tract, however, the Legionnaires bacteria are seldom numerous. Thus, the presence of five or more brightly stained bacteria per smear, with morphology typical of the Legionnaires bacteria, is considered a positive result with these specimens.

Approximately two-thirds of the patients whose tissues were positive by direct IF staining, and about half of those whose tissues were negative, were involved in outbreaks of Legionnaires disease in either Philadelphia, Pa., in 1976 (7), or Columbus, Ohio (3), Burlington, Vt. (4), or Kingsport, Tenn., in 1977 (4).

The criteria for determining tissue positivity (John A. Blackmon, personal communication) were based (i) on the examination of hematoxylin and eosin-stained sections for tissue pathology that was distinctive for Legionnaires disease and (ii) on the detection of Legionnaires-like bacteria in sections stained by the Dieterle silver impregnation method (5).

In Table 5 the results of IF tests on a series of Legionnaires disease tissues are compared with the pathological evaluation of the same specimens.

In Fig. 1 the Legionnaires bacteria (Knoxville strain) are seen in a smear of scrapings of Formalin-fixed lung stained with the IF conjugate. The organisms in deparaffined sections of lung tissue from the same patient are shown in Fig. 2. The enormous number of bacteria in the lung tissue is a striking feature of Legionnaires disease. Frequently, from 50 to several hundred bacteria per oil immersion field are present in lung tissue from patients dying of the disease. Chains or filaments of more than two bacterial cells are seldom observed in tissues. The organisms are found both intracellularly and extracellularly but, as demonstrated by comparing Fig. 1 and 2, they are more easily visualized in the lung scrapings than in sections.

The appearance of IF-stained pure cultures of the Knoxville strain of the Legionnaires bacterium is shown in Fig. 3 and 4. The smear photographed in Fig. 3 was from a young (48-h) culture; that in Fig. 4 was from an older culture (5 day) on a medium of somewhat different composition. The bacteria in Fig. 4 are typical of those usually seen in IF-stained culture smears; those in Fig. 3 are more like the tissue-phase organisms, although perhaps somewhat larger.

TABLE 4. *Criteria for reporting results of direct IF tests for Legionnaires bacteria in lung tissues and fluids*

Result	Report
>50 strongly fluorescing bacteria per field[a, b]	IF + (many)
2–50 strongly fluorescing bacteria per field[a, b]	IF + (moderate)
>25 strongly fluorescing bacteria per smear,[a, b] but >1 strongly fluorescing bacterium per field[a, b]	IF + (few)
<25 strongly fluorescing bacteria per smear[a, b]	Report numbers only
0 strongly fluorescing bacteria per smear[a, b]	IF −
>5 strongly fluorescing bacteria per smear[b, c]	IF +; report numbers

[a] Includes scrapings of Formalin-fixed tissues, fresh tissue imprints and homogenates, and histological sections.

[b] Oil immersion objective (×1,250).

[c] Applicable only to fluid specimens from the lower respiratory tract because Legionnaires bacteria are seldom numerous in these specimens.

Detection in exudates from the lungs. Twenty-five respiratory tract specimens received before 11 November 1977, and rather poorly documented as to site of origin or method of collection, were examined by direct IF staining. Only two specimens were positive. One of these was from a patient whose lung biopsy section was judged questionable for Legionnaires disease by the pathologist. By the indirect test the patient had a significant titer rise from 1:32 to 1:1,024 by the criteria of McDade et al. (12). The other patient also had a significant titer, but no pathological data were available.

TABLE 5. *Comparison of results of IF tests and pathological[a] examination of Legionnaires disease tissues[b]*

Test category	No. of specimens (patients)
IF +; pathology +	38[c]
IF +; pathology −	5[d]
IF +; pathology ?	2
IF −; pathology +	4[e]
IF −; pathology ?	10
IF −; pathology −	22

[a] Refers to the pathologist's diagnosis based on examination of the tissues by hematoxylin and eosin staining and by the Dieterle silver stain.

[b] Lung scrapings, homogenates, and sections from autopsy or biopsy specimens.

[c] Including patients from whose tissues six of the strains discussed in this paper were isolated.

[d] Tissue examined by the pathologist may have been from a different area of the lung.

[e] Three of these four specimens were fixed in Zenker mercury solution. Tissue pathology was compatible with Legionnaires disease, but the Dieterle stains and direct IF tests were negative possibly because of the fixative. One of these patients had an indirect If titer of 1:1,024. Sixty-two additional specimens (13 IF +; 49 IF −) were not examined by the pathologist.

For none of the 23 IF-negative specimens were any histological data available. However, 12 of the 23 patients had significant indirect IF titers. Three patients were serologically negative, and specimens from eight others were either not available or inappropriate for testing.

Two pleural fluids were positive by direct IF staining. No other confirmatory data were available from these patients. Pleural fluids from five patients were negative. One of these patients had a significant indirect test titer, but no tissue was examined. No data were available on the other four patients with suspected Legionnaires disease.

Specificity and reliability of direct IF staining reactions. The preimmune sera of rabbits used in our studies did not contain significant antibody reactive with Legionnaires antigens.

To obtain data on the specificity of the conjugates and to search for possible serological relationships, we screened smears of 374 pure

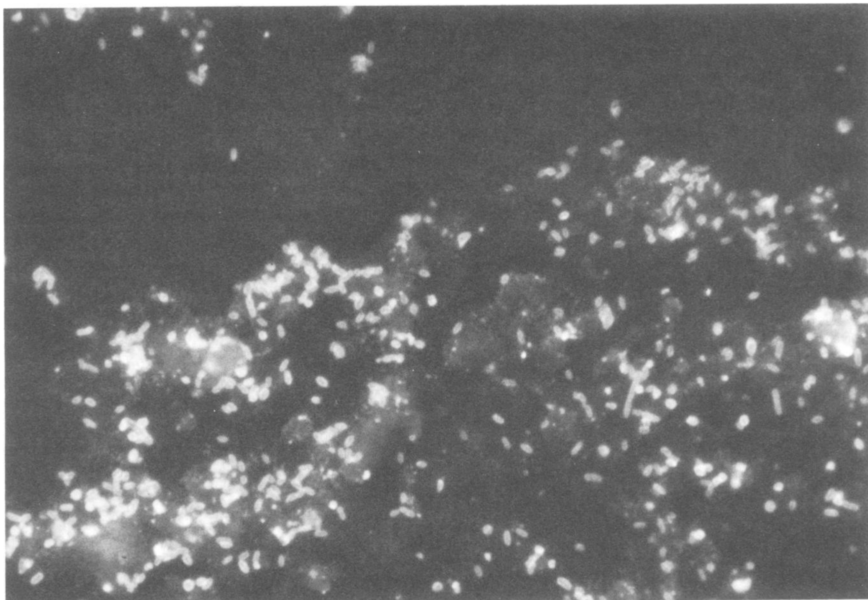

FIG. 1. *Smear of scrapings of Formalin-fixed lung tissue of a patient who died of Legionnaires disease in Knoxville, stained with a 1:80 dilution of the homologous conjugate. Photographed on 135-mm Ektachrome 200, EPD film. Magnification, ×400.*

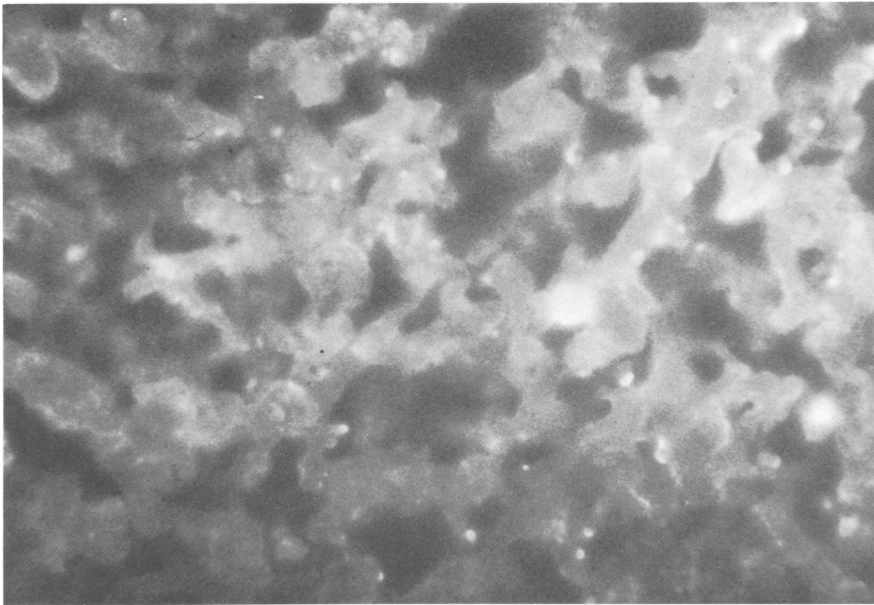

FIG. 2. *Histological section of Formalin-fixed and paraffin-embedded lung of patient from Knoxville, stained and photographed as in Fig. 1. Note relative difficulty of visualization of the bacteria in comparison to smear of lung scrapings.*

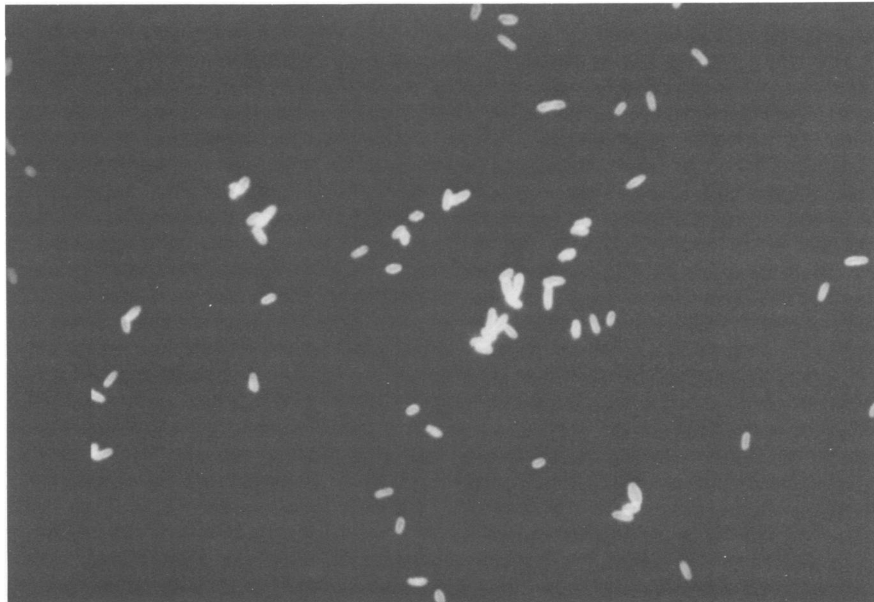

FIG. 3. *Smear of 48-h culture of Knoxville strain of Legionnaires bacteria, stained with a 1:80 dilution of homologous conjugate. Culture was grown in modified Mueller-Hinton medium and suspended in phosphate-buffered saline containing 1% Formalin. Photographed as in Fig. 1. Note that these cells resemble those seen in tissue (Fig. 2).*

cultures of bacteria by staining them with the working dilution (1:80) of the Knoxville conjugate. The cultures represented 25 known genera and 59 known species (Table 6). Also included were 54 unidentified cultures. No significant staining of any of these cultures was noted.

In general, the results of direct IF staining reactions for detecting Legionnaires bacteria in

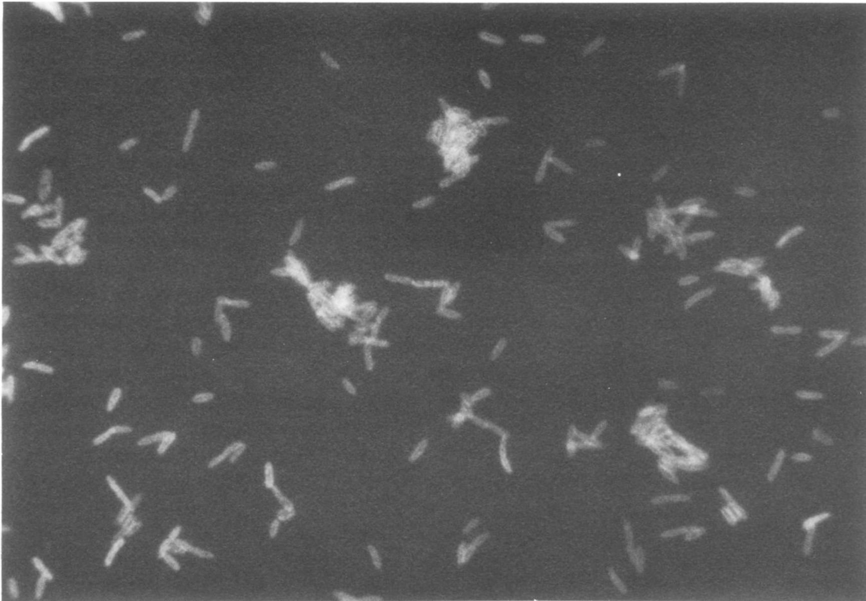

FIG. 4. *Smear of 5-day-old culture of Knoxville Legionnaires strain grown on a different modification of Mueller-Hinton medium. Stained and photographed as in Fig. 1.*

tissues correspond to the pathologist's assessment of the tissue appearance and the results of applying the Dieterle silver impregnation stain (Table 5).

In another series of examinations of Formalin-fixed lung tissues from 13 pneumonia patients, we found that 10 tissues were positive by direct IF tests. Because of the high percentage of positive results, we sought confirmation. A pathologist examined sections of these tissues and reported that his diagnosis agreed with the IF result for the eight specimens which we observed to contain "many," "moderate," or "few" fluorescent organisms per field (×100 oil immersion objective). Of the other two specimens that we found to be positive, one was from a patient with pneumococcal pneumonia with bacteremia, but only six cells typical of Legionnaires bacteria were seen in the entire smear. In the other positive specimen we saw 6 to 10 typical organisms per smear. The pathologist rated this specimen as questionable and the former specimen as negative for Legionnaires disease. Later, scrapings from a second block of Formalin-fixed tissue from the questionable specimen were strongly positive by direct IF staining.

An additional experiment was performed in which the pathologist selected a group of 10 Formalin-fixed lung specimens from patients with pneumonia or pulmonary infection. The specimens were coded and submitted for direct IF examination for Legionnaires disease bacteria. The Legionnaires bacteria were correctly detected in three specimens; all others were negative. The pathological assessment and the IF test results agreed in all 10 specimens.

TABLE 6. *Pure cultures (374) of bacteria of 25 genera and 59 species giving negative results when examined for relationship to Legionnaires bacterium by direct IF staining*[a]

Bacteria (no. of species)	No. of cultures
Achromobacter (1)	7
Acinetobacter calcoaceticus; 31 serotypes	56
Actinobacillus actinomycetem-comitans	3
Agrobacterium radiobacter	5
Alcaligenes (3)	11
Bacteroides ochraceus	4
Bordetella (3)	8
Brucella (1)	2
Campylobacter fetus	2
Cardiobacterium hominis	1
Corynebacterium (3)	5
Flavobacterium (3)	7
Francisella tularensis	5
Haemophilus influenzae	6
Klebsiella pneumoniae; 30 capsular types	30
Lactobacillus (7)	11
Moraxella (1)	4
Mycobacterium rhodochrous	1
Neisseria (2)	5
Pasteurella (1)	1
Pseudomonas (18)	88
Serratia (1)	1
Streptococcus pneumoniae; 30 capsular types	45
Vibrio (3)	10
Yersinia pestis	2
Unknown	54

[a] The diagnostic dilution (1:80) of the Knoxville conjugate was used to examine these cultures. All were negative.

DISCUSSION

Conjugates prepared from sera raised in rabbits by injection of Formalin-treated cells were valuable in detecting the Legionnaires bacteria in both Formalinized and fresh autopsy and biopsy lung tissue and in cultures.

We had assumed that cells of the Legionnaires bacteria might be quite toxic to rabbits because of the frequency of shock seen in human victims (7), but saw no evidence of toxic reactions. Treating the cells with 0.5% Formalin may have detoxified them.

In immunizing rabbits with depot antigens for the Legionnaires bacteria we recommend giving the inoculation intracutaneously rather than into the "footpad." The former route results in less trauma to the animals and yields conjugates of comparable titers (Richard George, personal communication).

Because of the extremely large number of Legionnaires bacteria present in the lung tissues of many victims of the disease, each specimen must be processed separately to prevent cross-contamination of tissues by "wash over" of stained organisms from a positive to a negative smear. The degree to which "wash over" is a problem can be estimated by examining known negative control smears appropriately spaced on a slide with known IF-positive smears. Also, the microbiologist has no way of knowing whether the tissue he examines was contaminated before he received it. For these reasons we do not classify tissues containing less than 25 well-stained organisms per smear (3- to 5-min search) as positive or negative (Table 4). The clinician must make the diagnosis on the basis of all the available clinical, epidemiological, pathological, serological, and microbiological data.

The results that have been presented indicate good agreement between the direct IF tests and the pathologist's diagnosis based on the tissue reaction and the Dieterle silver impregnation stain. When disagreement occurred, it was likely to be with lung specimens in which very few Legionnaires bacteria were seen either with the Dieterle stain or with IF tests or when the pathologist was uncertain about the tissue reaction. In these specimens the results depend to a large extent on the particular area of the organ that is selected for study. When Legionnaires disease is suspected, it is desirable to select gross tissue for study in consultation with a pathologist. Even if the initial IF test results are negative, the pathologist should examine the tissue microscopically, because appropriate areas for further study by IF staining may be found.

The serological specificity of the IF stain when compared with the Dieterle stain is clearly advantageous. Additional data may be obtained from indirect IF tests for antibody in the sera of patients with evidence of Legionnaires disease. We have not presented serological data in our report because appropriate sera were available from only a few of the patients. Relevance of indirect IF titers to the results of direct IF examinations of tissues or lower respiratory tract specimens from Legionnaires disease patients depends upon factors such as the timing, method, and type of specimen collection, treatment regimen, and severity of the disease process.

The Legionnaires bacteria were readily stained in imprints of fresh tissue. At times, however, they were not as easily discernible in the imprints as they were in Formalin-fixed scrapings of the same tissue. In imprints the organisms may be surrounded by an envelope that tends to obscure their outline; this did not occur in the Formalin-fixed tissue. Also, scraping the tissue appeared to liberate intracellular bacteria from macrophages and polymorphonuclear cells.

Smears to be examined for Legionnaires bacteria by IF staining should not be fixed with Kirkpatrick fixative because it depresses the staining reaction. We have not used acetone to fix this organism, although it has been used successfully by others (12).

In embedded and deparaffined tissue sections, the organisms were shrunken in size and obscured to some extent by the thickness and complexity of the section, but they were highly fluorescent and easily seen if present in moderate numbers. The integrity of the antigenic surface was maintained throughout the histological processing.

Only a small number of fluid specimens from the lower respiratory tract of people suspected of having Legionnaires disease have been available for study. Results indicate that generally very few Legionnaires organisms are found in these specimens. Thus, the method and timing of specimen collection may prove to be the critical factors in successfully demonstrating these bacteria by direct IF staining. Research is needed to evaluate the use of mucolytic agents to liquefy and homogenize the specimens, to devise appropriate methods for concentrating the organisms that are present, and to study the use of counterstains for improving visualization of fluorescent Legionnaires bacteria. Since the organism grows slowly and is difficult to isolate, direct and indirect IF staining are currently the only diagnostic tests available for rapid and specific diagnosis.

In reasonably extensive testing of pure cultures of known and of unidentified bacteria associated with humans we encountered only one organism, a strain of *Pseudomonas fluorescens*, that can confound the diagnosis of Legionnaires disease by direct IF staining. This organism was actively growing in a solution of commercial 1:5,000 Evans blue which contained 3% bovine serum albumin and Merthiolate at a dilution of 1:10,000. It was brilliantly and specifically stained by the diagnostic dilution (1:80) of the conjugate for the Knoxville Legionnaires strain and to a lesser extent by conjugates for the other four strains. In stained cultures the cells were considerably wider and somewhat longer than those of the Legionnaires disease bacteria. Twenty-two additional cultures of this species were examined, but none fluoresced. Thus, care in handling IF reagents and in the interpretation of results is essential, because we do not know the frequency with which such serologically related bacteria may occur. Preliminary data indicate that organisms serologically identical to or related to the Legionnaires bacterium are common in soils and soil animals from several geographic locations. None of these organisms has been isolated or identified.

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