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Evaluation of the Merifluor-*Legionella* Immunofluorescent Reagent for Identifying and Detecting 21 *Legionella* Species

PAUL H. EDELSTEIN[1,2]* AND MARTHA A. C. EDELSTEIN[1]

Departments of Pathology and Laboratory Medicine[1] and Medicine,[2] University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-4283

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We evaluated a 33-valent polyclonal indirect immunofluorescent-reagent kit (Merifluor-*Legionella*; Meridian Diagnostics Inc., Cincinnati, Ohio) made for the detection of *Legionella* species by testing bacterial isolates, seeded sputum, and negative sputum samples. Use of the reagent according to the directions of the manufacturer gave false-negative staining of homologous culture isolates due to a prozone phenomenon; this was solved by diluting test strain suspensions. After this change in testing protocol was made, the reagent gave bright fluorescent staining with 31 of the 33 *Legionella* strains with which it supposedly reacts. Strongly reacting *Legionella* strains included the type strains of *L. pneumophila* serogroups 1 to 10, *L. longbeachae* serogroups 1 and 2, and serogroup 1 of *L. anisa*, *L. bozemanii*, *L. cherrii*, *L. dumoffii*, *L. gormanii*, *L. hackeliae*, *L. jamestowniensis*, *L. jordanis*, *L. maceachernii*, *L. micdadei*, *L. oakridgensis*, *L. rubrilucens*, *L. sainthelensi*, *L. spiritensis*, *L. steigerwaltii*, and *L. wadsworthii*. Type strains of *L. erythra* and *L. feeleii* fluoresced only dimly with the reagent. Of 10 non-*Legionella* bacteria known to cross-stain with other polyvalent antisera, 5 also cross-reacted with the Merifluor reagent; these included 3 *Bacteroides fragilis* and 2 *Pseudomonas fluorescens* strains. The lower limit of detection of *L. pneumophila* serogroup 1 in seeded sputum was about 5×10^4 to 5×10^5 cells per ml. None of 21 randomly collected sputum specimens tested contained fluorescing legionellalike organisms, but 6 specimens did contain brightly fluorescing bacteria atypical in morphology for *Legionella* species. The Merifluor-*Legionella* kit appears to perform as well as other polyclonal immunofluorescent reagents used for detection of *Legionella* species. Because of the cross-reactions observed, which are common to all polyclonal reagents, utilization of this reagent for either bacterial identification or detection must be performed in combination with culture.

The identification of legionellae isolated from culture plates and detection of legionellae in clinical specimens are difficult because of the phenotypic heterogeneity of this genus, now composed of at least 33 species and 48 serogroups (8, 11). It is necessary to use multiple pools of antisera for the immunologic identification and detection of more than one species or serogroup, a process which can be difficult for the routine clinical microbiology laboratory.

The use of pooled antisera for identification and detection of legionellae in lung specimens was first studied by Brown and colleagues; however, these investigators did not determine the specificity of this reagent for the examination of sputum samples, nor did they look for cross-staining bacteria (2). A commercial product (Merifluor-*Legionella* kit; Meridian Diagnostics Inc., Cincinnati, Ohio) based on this concept is now available and contains a pool of rabbit antisera which reacts with 33 different *Legionella* antigens. A total of 21 *Legionella* species is putatively detected with the reagent, including *L. pneumophila* serogroups 1 to 10 and *L. longbeachae* serogroups 1 and 2.

We evaluated this product to determine its sensitivity and specificity in detecting legionellae and non-*Legionella* bacteria grown on culture plates and also determined its potential utility in screening sputum samples for the presence of legionellae.

MATERIALS AND METHODS

Bacteria. All *Legionella* strains used were grown on buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutarate (BCYEα), at 35°C in a humidified air incubator for 1 to 3 days, depending on growth rate (4, 5). Non-*Legionella* aerobic bacteria were grown on 5% sheep blood Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.) and incubated identically. Obligately anaerobic non-*Legionella* bacteria were grown in an anaerobic glove box on brucella blood agar at 37°C for 1 to 3 days. Bacteria were harvested into 10% neutral Formalin and stored at 5°C until used in the assay. The identities of bacteria used are shown in Table 1. Bacteria studied were obtained from various investigators at the Centers for Disease Control, Atlanta, Ga.; from the American Type Culture Collection, Rockville, Md.; from the Genetic Systems Corporation; and by the investigators themselves. The specific strains used were those listed by the manufacturer as being reactive in the assay. All legionellae used in the study have been characterized extensively by immunologic reactions, cellular fatty acid analysis, cellular ubiquinone composition, and other identification methods (5). The nonlegionellae used have also been well characterized (9, 10).

Detection of bacteria by immunofluorescence. The Merifluor kit was used as described by the manufacturer, with one exception. Rather than putting in each slide well 10 μl of a bacterial suspension equivalent in turbidity to a no. 1 McFarland barium sulfate standard, we used a 1:100 dilution of such a suspension; we found in pilot studies that use of the denser suspension resulted in false-negative tests due to a prozone reaction. The kit uses an indirect-immunofluorescence method to detect bacteria. Both a non-*Legionella*-reactive primary antibody and a positive-control antigen suspension are supplied for use as negative and positive controls, respectively. Both of these controls, as well as a negative-control antigen suspension (*Escherichia coli*), were

* Corresponding author.

TABLE 1. *Legionella* bacteria tested with Merifluor-*Legionella* test

Species	Serogroup	Strain	Fluorescence intensity[a]
L. anisa	1	WA316C3	3+
L. bozemanii	1	WIGA	4+
L. cherrii	1	ORW	3+
L. dumoffii	1	TEXKL	4+
	1	NY23	3+
L. erythra	1	SE32AC8	2+[b]
L. feeleii	1	WO44C	2+[b]
L. gormanii	1	LS13	3+
L. hackeliae	1	Lansing 2	3+
L. jamestowniensis	1	JA26G1E2	3+
L. jordanis	1	BL540	3+
L. longbeachae	1	Long Beach 4	4+
	2	Tucker 1	3+
L. maceachernii	1	PX1G2E2	4+
L. micdadei	1	TATLOCK	4+
L. oakridgensis	1	OR10	3+
L. parisiensis	1	PF209CC2	4+
L. pneumophila	1	Philadelphia 1	4+
	1	Knoxville 1	4+
	1	Bellingham 1	3+
	2	Togus 1	3+
	3	Bloomington 2	4+
	4	Los Angeles 1	3+
	5	Dallas 1E	3+
	6	Chicago 2	3+
	7	Chicago 8	3+
	8	Concord 3	3+
	9	IN23G1C2	3+
	10	Leiden 1	4+
L. rubrilucens	1	WA270C2	3+
L. sainthelensi	1	MSH4	3+
L. spiritensis	1	MSH9	4+
L. steigerwaltii	1	SC18C9	3+
L. wadsworthii	1	WA81-716A	3+

[a] 4+, Maximum-brightness yellow-green fluorescence; 1+, barely visible fluorescence.
[b] Repeat testing with a different lot number kit yielded an identical result.

used in all runs. Two different lots of the kit were tested with all *Legionella* strains giving less than a 3+ reaction. The specimen or bacterial suspension is fixed to a microscope slide, after which a primary multivalent rabbit antiserum is added. After incubation for 30 min, the nonbound primary antibody is rinsed off with buffer. After drying, fluorescein-conjugated goat anti-rabbit serum is added. The slide is then incubated for another 30-min period, washed with buffer, and dried. A cover slip is then mounted with supplied buffered glycerol solution, and the slide is read with a microscope, using UV light illumination. Microscopy was performed with a Diaphot microscope (Nikon) equipped with epi-illumination, an HBO 100-W mercury bulb, a B1A filter set, 12.5× oculars, a 40× objective, and a 1.25× magnification module (total magnification, ×635). All slides were read independently by two different observers, one of whom had no knowledge of the identity of the test bacteria on the slides. A scale of 1+ to 4+ was used to grade the fluorescence intensity of bacterial staining, where 1+ represents barely visible fluorescence and 4+ represents maximum brightness of fluorescence.

The Merifluor kit was tested in several ways. It was tested with the *Legionella* bacteria with which it putatively reacts, as well as with non-*Legionella* bacteria known to cross-react with other *Legionella* antisera. A sputum sample seeded with known amounts of *L. pneumophila* serogroup 1 was tested to determine the analytical sensitivity of the assay. Finally, several randomly chosen sputum specimens were tested to determine whether nonspecific staining occurred.

Determination of analytical sensitivity. A randomly picked purulent sputum sample was seeded with known amounts of 1% Formalin-fixed *L. pneumophila* serogroup 1 strain Philadelphia 1. The bacterial concentration of the Formalin suspension of the *L. pneumophila* test strain was determined microscopically by use of a Petroff-Hausser counting chamber (3). Tenfold dilutions of the bacterial suspension were made in 1% Formalin and added in known amounts to aliquots of the sputum sample. Known volumes of the seeded samples were added to slides, stained, and examined for fluorescing bacteria, as described above.

Examination of randomly selected sputum samples. To detect nonspecific staining, 21 grossly purulent sputum specimens were randomly selected from sputum samples sent to the clinical microbiology laboratory for culture. Clinical information about the patients from whom the samples were collected was not obtained. However, because this laboratory documents only about one case of Legionnaires disease per year, the likelihood that any one specimen was from a patient with Legionnaires disease is exceptionally low. This low prevalence is based on extensive culture and antigen detection testing for *Legionella* species being performed on the majority of lower respiratory tract specimens from immunosuppressed patients with pneumonia.

RESULTS

The Merifluor kit stained all 33 *Legionella* strains tested. All controls gave expected test results, and both slide readers agreed on the interpretation of each slide. Thirty-one of the strains fluoresced 3+ to 4+ in intensity, and two strains fluoresced 2+ in intensity (Table 1). Repeat staining of the two dimly fluorescing strains gave identical results, even after making fresh suspensions of the bacteria from new cultures and after using a new and different lot of the kit.

Of the 10 non-*Legionella* bacteria tested, 5 fluoresced brightly (3+ to 4+) with the Merifluor kit; none of these 5 reacted with the supplied negative-control antibody (Table 2). One of the five nonreactive bacteria fluoresced dimly (2+) when stained with the negative-control antibody.

The lower limit of detection of *L. pneumophila* serogroup 1 in seeded sputum was between 5×10^4 and 5×10^5 bacteria per ml. Multiple brightly fluorescing legionellalike organisms were seen at the higher bacterial concentration, and only a single brightly fluorescing legionellalike organism was seen at the lower bacterial concentration.

None of 21 randomly selected sputum samples tested contained fluorescing legionellalike organisms when stained with the Merifluor kit. However, two samples contained brightly fluorescing streptococcuslike organisms, which stained with both the negative control and legionella-specific antibodies, and four contained brightly fluorescing, irregularly staining, very large bacilli atypical of *Legionella* species which stained only with the legionella-specific antibody.

DISCUSSION

Our evaluation of the Merifluor kit showed that it performs similarly to other polyclonal-antibody-based immunofluorescent reagents for detection of *Legionella* species, in terms of its specificity and analytical sensitivity (6). The failure of the kit to brightly stain two homologous *Legionella* species may marginally limit its overall potential usefulness.

TABLE 2. Non-*Legionella* bacteria tested with Merifluor-*Legionella* test

Strain[a]	Fluorescence intensity[b]
Bacteroides fragilis	
ATCC 43936	4+
ATCC 43937	4+
ATCC 43935	4+
Pseudomonas fluorescens	
ATCC 49271	4+
ATCC 49270	3+
Pseudomonas aeruginosa	
F1850	Neg
ATCC 49266	Neg
ATCC 49267	Neg
ATCC 49268	Neg
ATCC 49269	Neg[c]

[a] Reference 7 describes the *B. fragilis* strains, and reference 10 describes the remainder of the strains.

[b] See Table 1, footnote *a*, for definitions of fluorescence intensity. All but one of the strains tested were unreactive with the negative-control antibody. Neg, Negative reaction.

[c] Fluorescence intensity of 2+ when stained with the negative-control antibody.

Cross-staining reactions with some *Pseudomonas* and *Bacteroides fragilis* strains is well known for polyclonal, but not monoclonal, *L. pneumophila* antibodies (3, 6, 7, 10). These reactions are generally serogroup specific and seem to be variable for different polyclonal antibodies. Since known cross-reacting organisms were chosen for testing, it is not unusual that 50% of them reacted with the Merifluor kit. Since these cross-reacting organisms appear morphologically similar, if not identical, to legionellae, it is always possible for false-positive reactions to occur, especially when examining clinical specimens. Because these cross-reactions are not detected with the negative control antibody, the inexperienced user might falsely assume that all such reactions are true-positive ones.

An accurate estimate of the true clinical specificity of this reagent is limited by the small number of presumptively negative sputum specimens examined. However, the test appears to be grossly specific for the examination of sputum, as long as the microscopist is familiar with the morphology of *Legionella* species in clinical specimens. The brightly fluorescent bacteria visualized in 6 of the 21 negative sputum specimens examined were so atypical in morphology for *Legionella* species that no reasonably trained microscopist would confuse them with *Legionella* species. As with other polyclonal antibodies used for immunofluorescence microscopic identification and detection of legionellae, cross-staining organisms with morphology typical of *Legionella* species will occasionally be observed with the Merifluor product and result in false-positive tests. The frequency with which such false-positive tests will occur is unanswered by this study. From this limited trial, it seems that use of the negative-control antibody adds little to the specificity of the test, as cross-reacting bacteria were not correctly identified.

Our estimate of the analytical sensitivity of the test kit is about the same as that previously determined for monoclonal antibodies directed against *L. dumoffii* or *L. micdadei* and for a polyclonal antibody directed against *L. pneumophila*, all of which had lower limits of detection of about 5×10^4 *Legionella* per ml (1, 3). It is likely that the *Legionella* species strains reacting less strongly with the Merifluor kit would be more difficult to detect in low numbers than was the *L. pneumophila* serogroup 1 strain that was tested, hence reducing the clinical sensitivity of the test under some circumstances.

The failure of the Merifluor kit to brightly stain homologous organisms may marginally limit its usefulness, in the form of reduced clinical sensitivity as discussed above. Since we tested those *Legionella* strains with which the antibody supposedly reacts, strain variation is an unlikely explanation of our discrepant results.

The greatest utility of this test may be in identifying putative *Legionella* isolates to the genus level. As with other *Legionella* polyclonal antibodies, any isolate tested must be checked for L-cysteine growth dependence or misidentification may occur. Since use of the Merifluor reagent identifies organisms only to the genus level, its use for isolate identification will not add significantly to the information obtained by using simple growth dependence tests. The only possible advantage of this approach would be to obtain rapid results, which would have to be regarded as presumptive until the growth dependence tests were completed.

Direct testing of clinical specimens can be performed with the Merifluor kit. However, users will have to keep in mind the possibility of false-positive reactions because of cross-staining bacteria present in the specimen or in buffers used in the test, especially when testing sputum samples. Since many of these cross-staining organisms can be differentiated neither by morphologic criteria nor by use of the negative-control antibody, a positive test should be regarded as presumptive pending culture confirmation. Extensive prospective studies of this kit are needed to determine its true clinical specificity, which will likely be significantly higher for the examination of lung biopsy specimens than it will be for the examination of sputum.

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