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Detection of Cell-Associated or Soluble Antigens of *Legionella pneumophila* Serogroups 1 to 6, *Legionella bozemanii*, *Legionella dumoffii*, *Legionella gormanii*, and *Legionella micdadei* by Staphylococcal Coagglutination Tests

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Current methods used for the detection of whole-cell isolates of *Legionella* or for the detection of *Legionella* soluble antigens are technically impractical for many clinical laboratories. The purpose of this study was to explore practical alternatives. The results showed that whole cell isolates of *Legionella pneumophila* serogroups 1 to 6, *Legionella bozemanii*, *Legionella dumoffii*, *Legionella gormanii*, and *Legionella micdadei* were identified specifically by a simple slide agglutination test or slide coagglutination test in which the reagent antisera are first bound to staphylococcal protein A. Soluble antigens were also identified specifically by the slide coagglutination test and by a sandwich immunofluorescence assay. The latter test may be useful in detecting antigen in body fluids of patients with legionellosis or in environmental samples.

Previously, we showed that a slide agglutination test (SAT) with hyperimmune rabbit sera could be used to determine the serogroup of isolates of *Legionella pneumophila* and *Legionella*-like organisms (14). Formalinized whole-cell suspensions of *L. pneumophila* serogroups 1 to 4 and two *Legionella*-like organism strains, WIGA and MI-15, gave strong homologous SAT reactions and no cross-reactions with group-specific antisera. Since then, strains WIGA and MI-15 have been placed in the species *Legionella bozemanii* (2), and two additional *L. pneumophila* serogroups and three new *Legionella* species have been found, for a total of 10 serogroups: *L. pneumophila* serogroups 1 to 6 (5, 9, 10), *L. bozemanii* (2), *Legionella dumoffii* (strains NY-23 and TEX-KL; 2), *Legionella gormanii* (strain LS-13; 11), and *Legionella micdadei* (strains PI-1 [PPA], HEBA, and TATLOCK; 6) (*Legionella pittsburgensis* [12]). Preliminary evidence, based on the finding that seroconversions among paired sera from patients with suspected Legionnaires disease occurred against indirect immunofluorescence assay antigens of the new species, suggests that, like *L. pneumophila*, these new species may cause a significant number of infections annually (13). Accordingly, we now describe modifications in the SAT which allow the detection of isolated whole cells of the recently described *Legionella* species and also detection of group-specific soluble antigens of all legionellae. In addition, we describe a sensitive microscopic test for soluble antigen. In both tests, rabbit antisera are first allowed to bind via the Fc region of the immunoglobulin molecule to protein A sites on *Staphylococcus aureus* Cowan I cells (8). In the first test, the staphylococci agglutinate when homologous antigen is present. In the second test, the staphylococci are attached to the microscope slide and thus allow fixation of the soluble test components.

MATERIALS AND METHODS

Antisera. Rabbit antisera for *L. pneumophila* serogroups 1 to 4 and *L. bozemanii* were prepared with vaccine strains Philadelphia 1, Togus 1, Bloomington 2, Los Angeles 1, and WIGA, respectively, as described previously (14). Antisera for *L. pneumophila* serogroup 5 (Cambridge 2 vaccine) and *L. micdadei* (HEBA vaccine) were generously donated by Robert Benson, Biological Products Division, Centers for Disease Control. Antisera against *L. dumoffii*, *L. pneumophila* serogroup 6, and *L. gormanii* (vaccine strains NY-23, Chicago 2, and LS-13, respectively) were prepared by modifying the immunization procedure slightly because of the reported toxicity of some of these strains when they were injected intravenously (Roger M. McKinney, personal communication). Briefly, these modifications consisted of weekly injections of 1.0 ml of each heat-killed vaccine (30 min at 100°C, adjusted to a McFarland no. 6 standard in sterile phosphate-buffered saline, 0.01 M, pH 7.2 [PBS]) subcutaneously once and intramuscularly four times. Two weeks after the last intramuscular injection, 0.5 ml of vaccine was given intravenously. Two booster injections of 1.0 ml each were given intramus-

cularly 2 and 3 weeks later. Indirect immunofluorescence assay titers of ≥4,096 were obtained in week 4 of immunization with fluorescein isothiocyanate-conjugated antirabbit immunoglobulin (15). By week 7, the sera had titers of ≥32,000 and were used undiluted in the SAT, or were first bound to staphylococcal protein A as a coagglutination reagent for a slide coagglutination test (coSAT).

Coagglutination reagents. The staphylococcal protein A reagent was prepared by modifications of previously described methods (4, 7, 8). A suspension of an overnight growth of *S. aureus*, Cowan I strain, in 1 liter of Trypticase soy broth was packed by centrifugation and was then washed twice in 150 ml of PBS. The cells were suspended in 150 ml of 0.5% Formalin in PBS and allowed to sit at room temperature for 3 h. They were then centrifuged, washed three times in 150 ml of PBS, and resuspended in PBS (10% suspension, vol/vol; approximately 30 ml). The suspension was held in an 80°C water bath for 5 min, with constant swirling, and was then placed in an ice bath for 5 min. After washing three more times in 30 ml of PBS, the cells were resuspended in PBS to a final concentration of 10%. Alternatively, the commercially available Pansorbin (Calbiochem-Behring Corp., La Jolla, Calif.), which had previously been diluted 1:2 in PBS, was used as the source of staphylococcal cells. The optimal dilution of each antiserum (from 1:5 to 1:200) was added to an equal volume of staphylococcal suspension. Reagents were stable for at least 6 months at 4°C.

Preparation of antigens. Antigens for the SAT or the coSAT were prepared by suspending the growth on a charcoal-yeast extract agar slant of each *Legionella* strain (listed in Table 1) in 2.5 ml of 10% (vol/vol) neutral Formalin, as described previously (14). Soluble antigens for the coSAT were extracted from whole cells of each strain by suspending and transferring the growth from a charcoal-yeast extract agar slant in 2.0 ml of distilled water into a screw-cap test tube (13 by 100 mm) and by heating the tubed suspension for 15 min in a boiling-water bath. The extract was then clarified by centrifugation and filtration.

Agglutination tests. The SAT was performed by mixing 1 drop (approximately 0.05 ml) of antiserum with 1 drop of formalinized whole-cell suspension on a slide, which was then rocked gently for 15 s (14). The coSAT was performed in the same way, except that coagglutination reagents were substituted for undiluted antisera, and it was used to test either formalinized whole cells or soluble antigens. A positive test was defined as macroscopically visible agglutination within 15 s.

SIA for soluble antigen. For the microscopic sandwich immunofluorescence assay (SIA), 1 drop (approximately 0.05 ml) of a 1:100 dilution of Pansorbin in PBS was placed in each well of a 12-well fluorescent antibody microscope slide (Cel-Line Associates, Inc., Minolta, N.J.). After excess moisture was aspirated from the wells, the staphylococcal cells were allowed to air dry and then were gently heat-fixed to the slides. Rabbit antisera were diluted 1:200 (*L. pneumophila* serogroup 5 antiserum) or 1:100 (all others) in PBS. One drop of the appropriate antiserum was placed in each well bearing fixed staphylococci. The slides were incubated in a moist chamber at 37°C for 30 min. They were then rinsed quickly in PBS and placed in a PBS bath for 10 min. The slides were next blotted dry, and 1 drop of a 1:100 dilution of the soluble antigen extract was placed in each well. After another 30-min incubation and 10-min wash step, 1 drop of direct immunofluorescence conjugate with the same serological specificity as the antiserum on the well was added. Each conjugate was used at its direct immunofluorescence assay working dilution (9). After another 30-min incubation and 10-min wash step, the slides were blotted dry, mounted with buffered glycerol (pH 9.0), and examined on a Leitz Dialux 20 fluorescence microscope, equipped with an HBO-100 mercury incident light source, the Leitz I-cube filter system, 40× dry objective, and 6.3× binoculars. A positive test was defined as one in which the staphylococcal cells showed ≥3+ fluorescence intensity, which was an indication that soluble antigen had bound to the antiserum on the slide and then to the added conjugate. A schematic diagram of the SIA method is shown in Fig. 1.

RESULTS AND DISCUSSION

Representative examples of the three agglutination tests are shown in Fig. 2: identification of *L. micdadei* whole cells in the SAT and in the coSAT (A and B, respectively) and detection of *L. micdadei* soluble antigen in the coSAT (C). At optimal dilutions of antisera and coagglutination reagents (determined by checkerboard titrations), all tests were species specific and, for *L. pneumophila*, were also serogroup specific, as shown by the absence of agglutination with heterologous antisera (SAT) or with heterologous coagglutination reagents (coSAT). Similarly, soluble antigen from each species or serogroup was specifically detected with the homologous antiserum and conjugate in the SIA (not shown). Comparative endpoint titrations with *L. pneumophila* serogroup 1 and *L. mic-*

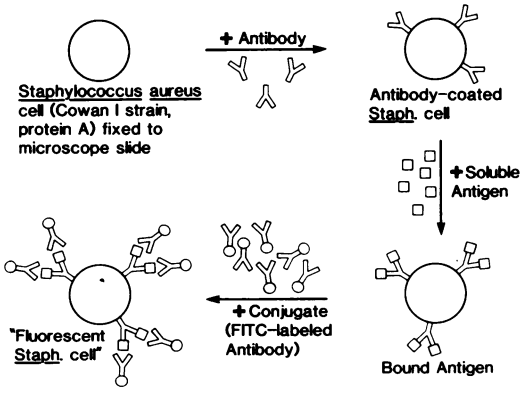

FIG. 1. *Schematic diagram of SIA for Legionella soluble antigens.*

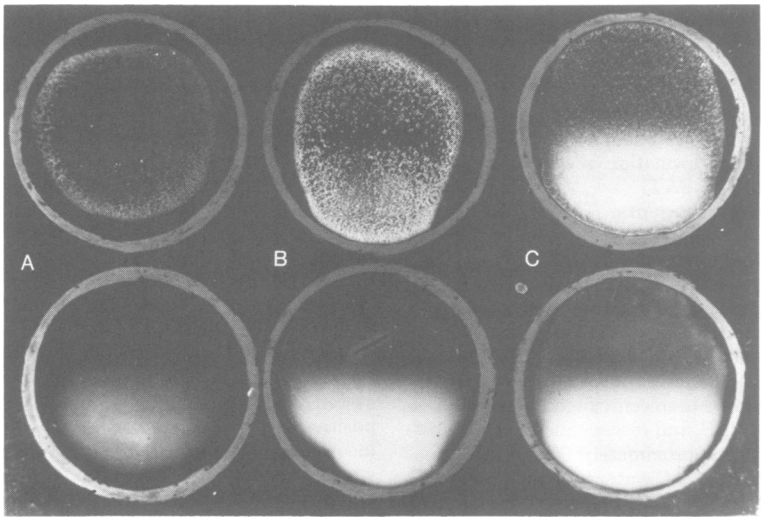

FIG. 2. *Detection of L. micdadei (strain HEBA) whole cells with the SAT (A) or coSAT (B) and L. micdadei (HEBA) soluble antigen with the coSAT (C). Antisera were L. micdadei (top wells, showing homologous agglutination) and L. bozemanii (bottom wells, control tests).*

TABLE 1. *Detection of Legionella antigens with the SAT (cell-associated), coSAT (cell-associated and soluble), or SIA (soluble)*

Legionella antigen			Positive test[a] obtained with antisera for:										
Species	Sero-group	Strains tested	*L. pneumophila* serogroup:						*L. bozemanii*	*L. dumoffii*	*L. gormanii*	*L. micdadei*	NRS[b]
			1	2	3	4	5	6					
L. pneumophila	1	Philadelphia 1	+	−	−	−	−	−	−	−	−	−	−
L. pneumophila	2	Togus 1	−	+	−	−	−	−	−	−	−	−	−
L. pneumophila	3	Bloomington 2	−	−	+	−	−	−	−	−	−	−	−
L. pneumophila	4	Los Angeles 1	−	−	−	+	−	−	−	−	−	−	−
L. pneumophila	5	Cambridge 2	−	−	−	−	+	−	−	−	−	−	−
L. pneumophila	6	Chicago 2	−	−	−	−	−	+	−	−	−	−	−
L. bozemanii	1	WIGA, MI-15, Arizona 1	−	−	−	−	−	−	+	−	−	−	−
L. dumoffii	1	NY-23, Tex-KL	−	−	−	−	−	−	−	+	−	−	−
L. gormanii	1	LS-13	−	−	−	−	−	−	−	−	+	−	−
L. micdadei	1	PI-1 (PPA), TATLOCK, HEBA	−	−	−	−	−	−	−	−	−	+	−

[a] Positive defined as macroscopically visible agglutination within 15 s for SAT and coSAT, or ≥3+ fluorescence intensity for SIA.

[b] NRS, Normal rabbit serum.

dadei soluble antigens showed that the SIA was approximately 100-fold more sensitive than the coSAT in detecting soluble antigen. Table 1 provides a summary of all the experiments in which complete agreement with the previously determined direct immunofluorescence test results was obtained in testing whole cells in the SAT or coSAT and extracted antigens in the coSAT or SIA. The direct immunofluorescence test has been the basis for the serological differentiation of all *Legionella* groups and species defined so far (2, 3, 5, 6, 9–11).

This study provides evidence that the SAT is a simple alternative to the direct immunofluorescence test in serogrouping *L. pneumophila* isolates and identifying the more recently discovered species: *L. bozemanii*, *L. dumoffii*, *L. gormanii*, and *L. micdadei*. The antisera used in the test can be diluted from 5- to 200-fold by preparing them as coagglutination reagents with staphylococcal cells bearing protein A. The coSAT can then be used either to test whole

cells or to detect *Legionella* soluble antigens. If greater sensitivity is required, the SIA provides an alternative test for soluble antigen. The same conjugates can be used for direct immunofluorescence detection of whole cells and SIA detection of soluble antigen.

Additional studies will be required to determine whether the coSAT or SIA can detect antigen in environmental samples or in body fluids of patients with legionellosis and thus provide a simpler test for early diagnosis than either the enzyme-linked immunosorbent assay (1) or radioimmunoassay (R. B. Kohler, S. Zimmerman, S. Allen, L. J. Wheat, P. H. Edelstein, and A. White, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 503, 1980). Preliminary data with urine specimens obtained from normal individuals and seeded with soluble antigens suggest that nonspecific agglutination in the coSAT can be removed by absorbing the concentrated urine with *S. aureus* Cowan I cells (5:1, 1 min, room temperature) or by first heating the urine for 10 min in a boiling-water bath, either before or after concentration. The coSAT then showed strong homologous reactions and no cross-reactions. Prior treatment of urine was not required for the SIA, which also has the advantage of greater sensitivity.

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