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Identification of 22 *Legionella* Species and 33 Serogroups with the Slide Agglutination Test

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We used the slide agglutination test to determine the serologic relationships of 22 *Legionella* spp. representing 33 serogroups. Antisera prepared against 14 of the *Legionella* spp. contained cross-reactive antibodies (1+ or greater) at their working dilutions. Numerous cross-reactions were observed for the blue-white fluorescing *Legionella* spp. With only three exceptions in the latter group, cross-reactive antibodies were removed by absorption, thereby producing serogroup-specific antisera. For screening tests or for identification only to the genus level, nine polyvalent antiserum pools were prepared. Routine use of slide agglutination test reagents should expand the number of *Legionella* spp. that can be identified in the clinical laboratory and, at the same time, provide a simpler, less costly test procedure.

Currently, 22 *Legionella* spp. have been differentiated by DNA hybridization (5–9, 11, 13, 16–18, 20, 25, 26). Four of these contain two or more serogroups: *L. pneumophila* (1, 3, 12, 15, 21, 23, 24), *L. bozemanii* (28), *L. longbeachae* (2), and *L. feeleii* (W. L. Thacker, H. W. Wilkinson, B. B. Plikaytis, A. G. Steigerwalt, W. R. Mayberry, C. W. Moss, and D. J. Brenner, submitted for publication). Serological data have not yet been reported for 11 of the 22 species (6, 16).

In previous studies (29, 31), Wilkinson et al. showed that the slide agglutination test (SAT) can replace the direct immunofluorescence assay as a means of identifying *Legionella* isolates. In this report, we used the SAT to examine the antigenic relationships of the 22 *Legionella* spp. We also prepared *Legionella* antiserum pools and serogroup-specific antisera for the identification of *Legionella* isolates belonging to the 33 constituent serogroups.

MATERIALS AND METHODS

Strains. The *Legionella* strains used to prepare antisera for use in the SAT are listed in Table 1. The same strains were used to prepare positive control antigens for use in the SAT.

Antiserum preparation. Antisera were prepared with whole-cell vaccines by methods described previously (29). Briefly, the 2-day growth from five buffered charcoal-yeast extract (BCYE) agar plates (15 by 100 mm) was suspended in 30 ml of phosphate-buffered saline (pH 7.6) containing 1% Formalin. The suspension was held overnight at room temperature to kill the bacteria, filtered through sterile gauze, and then centrifuged at 2,000 × *g* for 15 min. The cell pellet was suspended in phosphate-buffered saline containing 0.5% Formalin and adjusted to 40 IU of turbidity (19).

A 2-ml portion of a suspension containing equal volumes of cells and incomplete Freund adjuvant (Sigma Chemical Co., St. Louis, Mo.) was injected intracutaneously in approximately 20 sites along the shaved back of a young adult New Zealand White rabbit. Usually two rabbits were inoculated with each antigen because some of the antigens were toxic when injected in high concentrations. On day 31, the rabbits were injected with 2 ml of the above suspension divided into two deep-muscle injections in the hindquarters. On days 38 and 45, the rabbits were injected intramuscularly with 2 ml of cell suspension without adjuvant. On days 52 and 59, 50 ml of blood was taken from an ear artery, and then 2 ml of cell suspension was injected intramuscularly. On day 66, the rabbits were exsanguinated.

Antiserum titers were determined by preparing twofold dilutions in phosphate-buffered saline (pH 7.6). The serum titer was the highest dilution which gave 4+ agglutination of homologous antigen within 30 s. This dilution of antiserum was used as the working dilution and was tested against all of the *Legionella* antigens. Working dilutions of antisera were stable for at least 6 months when stored at 4°C.

SAT. The SAT has been described previously (29). Briefly, the growth of each strain from a BCYE agar slant (18 by 150 mm) was suspended in, depending on the growth obtained, 2.0 to 3.0 ml of 10% (vol/vol) neutral Formalin and heated in a boiling-water bath for 15 min. The SAT was performed by mixing 1 drop (approximately 0.025 ml) of antigen with 1 drop of antiserum on a glass slide (50 by 75 mm) and then rocking the slide for 30 s. The reactions were scored on a scale from 1+ (barely visible) to 4+ (strong agglutination).

Absorption of antisera. Cross-reactive antibodies were removed, if possible, by absorption with the cross-reacting *Legionella* strain or strains. Cells for absorption were prepared as described previously (29). The cells were mixed at a 1:5 ratio (vol/vol) with undiluted antiserum, incubated for 2 h at 37°C, and then kept at 4°C overnight. The cells were removed by centrifugation at 2,000 × *g* for 15 min at room temperature, and the antiserum was tested for residual cross-reactivity. If cross-reactive antibodies were still present, the absorption procedure was repeated. If the titer remained at ≥1:16, absorption of the antiserum was continued until it contained only homologous agglutinating antibodies at its working dilution.

RESULTS

Preparation and absorption of antisera. The results of testing the 33 *Legionella* antisera with homologous and heterologous SAT antigens are presented in Table 1. Numerous cross-reactions were observed, most notably for the 10

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TABLE 1. Preparation of *Legionella* antisera for the SAT

Immunizing antigen		SAT titer[a]	Cross-reactions	Absorption of antisera		
Species (serogroup)	Strain			Absorbing antigen	SAT titer	Residual cross-reactions
L. pneumophila						
1	Philadelphia 1	1:32				
2	Togus 1	1:8				
3	Bloomington 2	1:32	*L. pneumophila* gp 2 (1+)[b]	*L. pneumophila* gp 2 (1×)[c]	1:16	
			L. pneumophila gp 6 (4+)	*L. pneumophila* gp 6 (1×)		
4	Los Angeles 1	1:16	*L. pneumophila* gp 8 (1+)	*L. pneumophila* gp 8 (1×)	1:8	
5	Dallas 1	1:64				
6	Chicago 2	1:16				
7	Chicago 8	1:64				
8	Concord 3	1:64	*L. pneumophila* gp 5 (1+)	*L. pneumophila* gp 5 (1×)	1:16	
9	IN-23-G1-C2	1:64	*L. pneumophila* gp 1 (1+)	*L. pneumophila* gp 1 (1×)	1:32	
L. dumoffii 1	NY-23	1:64	*L. hackeliae* (1+)	*L. hackeliae* (1×)	1:64	
L. longbeachae 1	Long Beach 4	1:32	*L. santicrucis* (4+)	*L. santicrucis* (3×)	1:16	
			L. sainthelensi (3+)			
L. longbeachae 2	Tucker 1	1:16	*L. anisa* (4+)	*L. anisa* (1×)	1:16	
			L. bozemanii gp 2 (4+)	*L. jordanis* (1×)		
			L. jordanis (1+)	*L. longbeachae* gp 1 (1×)		
			L. longbeachae gp 1 (1+)			
L. bozemanii 1	WIGA	1:64	*L. jordanis* (4+)	*L. jordanis* (3×)	1:2	*L. bozemanii* gp 2 (4+)
			L. anisa (3+)	*L. bozemanii* gp 2 (1×)		
			L. bozemanii gp 2 (3+)			
L. bozemanii 2	Toronto 3	1:32	*L. anisa* (4+)	*L. anisa* (1×)	1:4	*L. pariseinsis* (4+)[d]
				L. bozemanii gp 1[d] (1×)		
L. gormanii 1	LS-13	1:8				
L. micdadei 1	TATLOCK	1:16	*L. anisa* (3+)	*L. anisa* (1×)	1:8	
L. wadsworthii 1	81-716	1:32				
L. oakridgensis 1	Oak Ridge 10	1:16	*L. sainthelensi* (4+)	*L. sainthelensi* (2×)	1:8	
L. feeleii 1	WO-44C-C3	1:32				
L. feeleii 2	691-WI-H	1:16				
L. sainthelensi 1	Mt. St. Helens 4	1:64				
L. jordanis 1	BL-540	1:32	*L. anisa* (3+)	*L. bozemanii* gp 1 (1×)	1:8	
			L. bozemanii gp 2 (1+)			
			L. bozemanii gp 1 (1+)			
L. anisa 1	WA-316-C3	1:32	*L. jordanis* (4+)	*L. jordanis* (1×)	1:16	
			L. bozemanii gp 2 (3+)	*L. bozemanii* gp 2 (1+)		
L. spiritensis 1	Mt. St. Helens 9	1:64				
L. hackeliae 1	Lansing 2	1:64				
L. maceachernii 1	PX-1-G2-E2	1:16	*L. anisa* (2+)	*L. anisa* (1×)	1:16	
L. jamestowniensis 1	JA-26-G1-E2	1:16	*L. anisa* (4+)	*L. anisa* (1×)	1:16	
L. santicrucis 1	SC-63-C7	1:64	*L. sainthelensi* (1+)	*L. sainthelensi* (1×)	1:64	
L. cherrii 1	ORW	1:64	*L. steigerwaltii* (4+)	*L. steigerwaltii* (2×)	1:8	
L. steigerwaltii 1	SC-18-C9	1:32	*L. cherrii* (4+)	*L. cherrii* (3×)	Undiluted	
L. parisiensis 1	PF-209C-C2	1:32	*L. bozemanii* gp 1 (4+)	*L. jordanis* (2×)	1:8	*L. bozemanii* gp 1 (4+)
			L. bozemanii gp 2 (4+)	*L. bozemanii* gp 1 (1×)		*L. bozemanii* gp 2 (4+)
			L. jordanis (4+)			
			L. anisa (4+)			
L. rubrilucens 1	WA-270A-C2	1:32				
L. erythra 1	SE-32A-C8	1:32				

[a] Highest dilution giving 4+ agglutination.
[b] Intensity of cross-reactions observed by using working dilution of antisera.
[c] Number of times undiluted antiserum was absorbed.
[d] Cross-reactions that were apparent at the 1:4 dilution but not at the 1:32 dilution.

recently described *Legionella* spp. and members of the blue-white fluorescing *Legionella* group.

All cross-reactions were removed by absorption with the appropriate heterologous strain or strains, except for three antisera: *L. bozemanii* serogroups 1 and 2 and *L. parisiensis*. Extensive absorption lowered the homologous titers of these antisera two- to fourfold without removing the cross-reactive antibodies. Therefore, antisera to these members of the blue-white fluorescing group could not be made serogroup specific. In addition, *L. cherrii* and *L. steigerwaltii*, also members of the blue-white fluorescing group, were closely related to each other serologically. Removal of *L. cherrii* reactivity from *L. steigerwaltii* antiserum was accomplished only after three absorptions, and then the homologous titer of *L. cherrii* antiserum was reduced from 1:32 to neat.

Preparation of pooled antisera. To conserve absorbed antisera and provide a rapid screening test for *Legionella* isolates, we combined unabsorbed antisera, each at its predetermined working dilution, into nine pools (Table 2). To include antibodies of the major subtypes of *L. pneumophila* serogroup 1 (22), pool I consisted of antisera to strains Philadelphia 1 and Olda. The remaining eight pools were formed to minimize cross-reactivity between pools while maximizing the cross-reactivity of the constituent antisera within each pool. Four of the pools reacted only with homologous antigens, two of the pools reacted with only one

heterologous antigen, and the remaining three pools each reacted with two or three heterologous antigens (Table 2).

DISCUSSION

The data presented in this paper showed numerous cross-reactions in antisera prepared to diverse *Legionella* spp. Included were slide-agglutinating and direct immunofluorescence antibodies for species reagents that appeared to be specific until they were tested against 11 newly characterized *Legionella* spp. Isolates identified previously as *L. bozemanii*, *L. jordanis*, *L. oakridgensis*, *L. dumoffii*, *L. longbeachae*, or *L. micdadei* may have been misidentified, since antisera to these species were cross-reactive. Furthermore, antisera to 7 of the 11 new species reacted with heterologous antigens. Most of the cross-reactive antibodies could be removed by absorption, thereby producing monospecific slide-agglutinating reagents. However, three antisera remained cross-reactive after absorption: *L. bozemanii* serogroups 1 and 2 and *L. parisiensis*. These organisms display blue-white fluorescence under long-wave UV light and cannot be differentiated by currently used biochemical tests. Therefore, definitive identification depends on DNA hybridization, a test beyond the capabilities of most clinical laboratories.

TABLE 2. Slide agglutination reactions of *Legionella* antigens with polyvalent antiserum pools

Legionella antigen and serogroup	Antiserum pool								
	I	II	III	IV	V	VI	VII	VIII	IX
L. pneumophila									
1	4+[a]	–[b]	–	–	–	–	–	–	–
2	–	4+	–	–	–	–	–	–	–
3	–	4+	–	–	–	–	–	–	–
6	–	4+	–	–	–	–	–	–	–
9	–	4+		–	–	–	–	–	–
4	–	–	4+	–	–	–	–	–	–
5	–	–	4+	–	–	–	–	–	–
7	–	–	4+	–	–	–	–	–	–
8	–	–	4+	–	–	–	–	–	–
L. micdadei 1	–		–	4+	–	–	–	–	–
L. maceachernii 1	–	–	–	4+	–	–	–	–	–
L. gormanii 1	–	–	–	4+	–	–	–	–	–
L. wadsworthii 1	–	–	–	4+	–	–	–	–	–
L. spiritensis 1	–	–	–	–	4+	–	–	–	–
L. rubrilucens 1	–	–	–	–	4+	–	–	–	–
L. erythra 1	–	–	–	–	4+	–	–	–	–
L. feeleii 1	–	–	–	–	4+	–	–	–	–
L. feeleii 2	–	–	–	–	–	4+	–	–	–
L. hackeliae 1	–	–	–	–	–	4+	–	–	–
L. oakridgensis 1	–	–	–	–	–	4+	–	–	–
L. dumoffii 1	–	–	–	–	–	4+	–	–	–
L. cherrii 1	–	–	–	–	–	–	4+	–	–
L. steigerwaltii 1	–	–	–	–	–	–	4+	–	–
L. jamestowniensis 1	–	–	–	–	–	–	4+	–	–
L. longbeachae 1	–	–	–	–	–	–	4+	–	–
L. longbeachae 2	–	–	–	–	–	–	–	4+	–
L. parisiensis 1	–	–	–	–	–	–	–	4+	–
L. bozemanii 1	–	–	–	–	–	–	–	4+	1+
L. bozemanii 2	–	–	–	–	–	–	–	4+	3+
L. jordanis 1	–	–	–	–	–	–	–	4+	4+
L. santicrucis 1	–	–	–	–	–	–	4+	–	4+
L. sainthelensi 1	–	–	–	–	–	4+	3+	–	4+
L. anisa 1	–	–	–	3+	–	–	4+	4+	4+

[a] Positive reactions scored on a scale from 1+ (barely visible) to 4+ (strong agglutination). Blocks indicate homologous reactions.
[b] –, Negative reaction.

With an expanding list of *Legionella* spp. and serogroups to identify, the clinical laboratorian now needs a simplified, reliable test that can affect treatment decisions. We recommend plating the specimen on BCYE agar, BCYE agar without cysteine, blood agar, and for contaminated specimens, semiselective BCYE agar. Pretreating the specimen with acid or heat may also be a beneficial selective procedure (4, 14). A gram-negative bacillus that grows on BCYE agar but not on blood agar or BCYE agar without cysteine can be identified as a member of the *Legionella* genus with slide-agglutinating antiserum pools. Therapy so far has been the same for all species for which information is available. Therefore, monospecific antisera could be reserved for occasions when it is necessary to identify the *Legionella* sp. to the species and serogroup level.

If a culture cannot be obtained and it is necessary to stain tissues or respiratory secretions with direct immunofluorescence assay conjugates, a broadly reactive antiserum pool could also be used to prepare the conjugate. A reagent such as this should be tested extensively with bacteria representing heterologous genera, since this has been done only for conjugates to *L. pneumophila* serogroup 1 (10). Alternatively, DFA conjugates could be used only for the *Legionella* spp. and serogroups that are the most prevalent in human infection: *L. pneumophila* serogroups 1 and 6 and *L. micdadei* (27, 30).

The antiserum pools listed in Table 2 will undoubtedly change as additional *Legionella* spp. and serogroups are found and may change with different antiserum lots. Homologous and heterologous antibody responses will vary in individual immunized rabbits, as will the relative success of absorptions in removing cross-reactive antibodies. Furthermore, antisera that appear to be specific when tested against currently known *Legionella* spp. may cross-react with antigens of species not yet discovered.

Past experience has shown that the routine use of reagents prepared to species that were first identified in environmental samples usually results in the identification of the species in clinical specimens. We and others have identified the following *Legionella* spp. in specimens sent to the Centers for Disease Control for reference diagnosis: *L. pneumophila*, *L. bozemanii*, *L. dumoffii*, *L. micdadei*, *L. longbeachae*, *L. jordanis*, *L. wadsworthii*, *L. feeleii*, and more recently, *L. hackeliae*, *L. rubrilucens*, *L. maceachernii*, and *L. parisiensis*. Further use of the new reagents and reexamination of specimens previously tested with reagents now known to be cross-reactive should provide additional information on the pathogenicity of the *Legionella* spp.

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