INFECTION AND IMMUNITY, June 1995, p. 2180–2184 Vol. 63, No. 6
0019-9567/95/$04.00+0

Cross-Reacting Lipopolysaccharide Antigens in *Legionella pneumophila* Serogroups 1 to 14

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Received 25 May 1994/Returned for modification 14 September 1994/Accepted 8 March 1995

Immunological cross-reactions among *Legionella* species were investigated with sonicated, proteinase K-digested cell lysates. The antigens separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were either analyzed for lipopolysaccharides (LPSs) by silver staining or transferred to nitrocellulose membranes for serological characterization with rabbit antibodies directed against *Legionella pneumophila* serogroups 1 and 5. When antiserum prepared against serogroup 5 was used to probe the LPSs from *L. pneumophila* serogroups 1 to 14, the antibodies recognized a common epitope harbored by all *L. pneumophila* serogroups but not by other *Legionella* species or by the gram-negative bacteria tested as controls. Hence, the serogroup 5 antiserum correctly identified all serogroups of *L. pneumophila* tested in the LPS immunoblot assay. Moreover, the silver-stained profiles of the isolated LPSs revealed characteristic patterns allowing the identification of the individual serogroups of *L. pneumophila*.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a gram-negative bacterium that multiplies intracellularly in blood monocytes (13) and human alveolar macrophages (17). In the past, several groups investigated constituents of the outer membrane with the aim to identify genus- and/or species-specific antigens that might be of relevance as immunological reagents for the diagnosis of *Legionella* infections. Among the well-characterized antigens, the major outer membrane protein with a molecular mass of 29 kDa (3, 8, 11) and the 24-kDa surface protein Mip (11), which are present in all *L. pneumophila* serogroups, have been investigated. In addition, Sampson et al. (23) found that 14- and 25-kDa protein antigens were present only in *L. pneumophila* strains, whereas antigens with molecular masses of 58, 79, and 154 kDa were detected in all *Legionella* species investigated. Antigens with molecular masses of 44 and 97 kDa were found in *L. pneumophila* and non-*L. pneumophila* strains.

The *Legionella* cell envelope has special characteristics and a unique lipopolysaccharide (LPS) structure which differs completely from those of the typical LPS cell wall constituents of the members of the family *Enterobacteriaceae*. Gabay and Horwitz (9) found that the LPS of *L. pneumophila* could not be extracted by the phenol-water procedure (27), and when *Legionella* LPS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a strikingly atypical band pattern was obtained. Chemical analysis showed that the LPS of *L. pneumophila* has a unique fatty acid composition characterized by a large amount of branched-chain fatty acids and by the absence of hydroxy-fatty acids generally associated with lipid A (19, 24). Several serological studies revealed that the LPS was the serogroup-specific antigen which is responsible for the diversity of serogroups 1 to 14 of *L. pneumophila* (18, 19). In previous studies, Ciesielski et al. (5) produced monkey antisera directed against *L. pneumophila* serogroups 1 to 6 which recognized only homologous purified LPS and not the LPSs of the five heterologous serogroups. Nolte et al. (18) also demonstrated that rabbit antisera to *L. pneumophila* serogroups 1, 2, 4, and 6 reacted only with the LPS from the homologous serogroup. However, Barthe et al. (2) described a common epitope on the LPS of *L. pneumophila* that was recognized by a monoclonal antibody.

With respect to the conflicting results published earlier (2, 5, 18), it was the aim of this study to investigate the electrophoretic properties of the LPSs of *L. pneumophila* serogroups 1 to 14. Special attention was focused on the extent of cross-reactivity of LPS antigens among these serogroups.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Legionella* species used in this study were obtained from the American Type Culture Collection (Rockville, Md.) or were isolated from clinical specimens (Tables 1 and 2). Organisms were grown in yeast extract broth containing 0.1% alpha-ketoglutaric acid, 0.04% cysteine, and 0.0125% PP_i at 35°C for 48 h and plated thereafter on buffered charcoal-yeast extract agar with 0.1% alpha-ketoglutaric acid (7). Subsequently, the plates were incubated in a moist atmosphere at 35°C for 72 h.

Preparation of LPS. Organisms grown on solid medium (Tables 1 and 2) were harvested by suspending colonies from the agar plates in 2.5 ml of sterile distilled water. The suspension was placed in a boiling water bath for 30 min and then sonicated on ice at 70 W for 1 min (Sonifier B-12; Branson Sonic Power Company, Danbury, Conn.). The sonicated suspension (0.1 ml) was solubilized in 0.1 ml of lysing buffer containing 4% (wt/vol) SDS, 10% (vol/vol) 2-mercaptoethanol, 20% (vol/vol) glycerol, 0.625 M Tris (pH 6.8), and bromphenol blue. Lysates were heated at 100°C for 10 min and digested with proteinase K as described previously (12). Briefly, after being boiled, the cell lysates were incubated at 60°C for 60 min with 75 μg of proteinase K to digest 750 μg of protein. Thereafter, LPS was precipitated by incubating the mixture with 2 volumes of a solution of 0.375 M $MgCl_2$ in 95% ethanol overnight at −20°C (6). The pellet was collected by centrifugation at 1,000 × *g* for 30 min at 4°C in an Eppendorf centrifuge, suspended in 0.1 ml of distilled water, and stored at −20°C.

SDS-PAGE. LPS preparations from *L. pneumophila* serogroups 1 to 14 and commercial LPS preparations from *Pseudomonas aeruginosa* serotype 10 (Habs), *Escherichia coli* serotype O111:B4, and *Salmonella minnesota* (Sigma Chemical Co., St. Louis, Mo.) were analyzed by SDS-PAGE in slab gels (80 by 120 by 1 mm; T = 12%; C = 2.6%) with the discontinuous buffer system described by Laemmli (15). Phosphorylase (M_r, 95,000), bovine serum albumin (BSA) (M_r, 68,000), fumarase (M_r, 49,000), carboanhydrase (M_r, 30,000), and lysozyme (M_r, 14,000), purchased from Boehringer (Mannheim, Federal Republic of Germany [FRG]), were used as standard proteins. Commercial LPS preparations (2 μg) from the above-mentioned species as well as 5 μl of extracted LPSs of *Legionella* species were each mixed with 1 volume of sample buffer containing 2% (wt/vol) SDS, 10% (wt/vol) saccharose, 5% (vol/vol) 2-mercaptoethanol, 62 mM Tris (pH 6.8), and 0.003% bromphenol blue. The mixtures were boiled for 5 min. Samples were electrophoresed at a constant current of 60 mA per gel until the tracking dye was approximately 1 cm from the bottom of the gel. After electrophoresis, the gels were fixed in 40% (vol/vol) ethanol–5% (vol/vol) acetic acid and stained for LPS with silver nitrate by the method of Tsai and Frasch (26).

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TABLE 1. Strains used in this study

Species	Strain designation	ATCC[a] no.	Serogroup
L. pneumophila	Philadelphia-1	33152	1
L. pneumophila	Knoxville	33153	1
L. pneumophila	Allentown	43106	1
L. pneumophila	Benidorm	43108	1
L. pneumophila	Olda	43109	1
L. pneumophila	Oxford 4032E	43110	1
L. pneumophila	France 4811	43112	1
L. pneumophila	Camperdown	43113	1
L. pneumophila	Togus-1	33154	2
L. pneumophila	Bloomington 2	33155	3
L. pneumophila	Los Angeles-1	33156	4
L. pneumophila	Dallas 1E	33216	5
L. pneumophila	Chicago 2	33215	6
L. pneumophila	Chicago 8	33823	7
L. pneumophila	Concord 3	35096	8
L. pneumophila	IN-23	35289	9
L. pneumophila	Leiden 1	43283	10
L. pneumophila	797/PA/H	43130	11
L. pneumophila	570-CO-H	43290	12
L. pneumophila	82 A 3105	43736	13
L. pneumophila	1169-MN-H	43703	14
L. bozemanii	1 WIGA	33217	1
L. gormanii	LS-13	33297	
L. micdadei	Tatlock	33218	

[a] ATCC, American Type Culture Collection.

Immunoblotting. For the Western blot (immunoblot) procedure, 5 μl of extracted LPSs from *L. pneumophila* serogroups 1 to 14 (diluted 1:500 [serogroup 1 strains], 1:100 [serogroup 5], or 1:10 [serogroups 2 to 4 and 6 to 14] and from *Legionella bozemanii*, *Legionella gormanii*, and *Legionella micdadei* (diluted 1:5) were electrophoresed in a 12% acrylamide gel as described above. Electrophoretic transfer to nitrocellulose sheets (Schleicher & Schuell GmbH, Dassel, FRG) was performed at 0.8 A and 8°C for 2.5 h in the Transfor-System (Pharmacia LKB GmbH, Freiburg, FRG) with the transfer buffer described by Towbin et al. (25). Thereafter, the sheets were placed in a sealed plastic box with blocking buffer (phosphate-buffered saline [PBS] [pH 7.4] containing 3% [wt/vol] BSA) and incubated at 8°C for 3 h to block remaining binding sites. The sheets were rinsed in PBS and then incubated with rabbit antiserum directed against *L. pneumophila* serogroup 1 or 5, diluted 1:500 in blocking buffer containing 0.05% (wt/vol) Tween 20, at 8°C overnight. The sheets were then washed five times with 50 mM Tris-buffered saline (pH 7.5) containing 50 mM EDTA and 0.05% Tween 20 for 10 min each and finally were incubated at 8°C for 3 h with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G diluted 1:500 in blocking buffer with 0.05% Tween 20. Thereafter, the immunoblots were developed in a freshly prepared substrate solution containing 50 mg of 3,3′-diaminobenzidine (Fluka AG, Neu-Ulm, FRG) and 0.1 ml of H_2O_2 (30%) in 100 ml of 50 mM ammonium-citrate buffer, pH 5.0. The reaction was terminated by washing the strips with distilled water.

Preparation of antisera. Antisera to whole cells of *L. pneumophila* serogroups 1 and 5 were prepared in rabbits as previously described (14) by using American Type Culture Collection strains Philadelphia-1 and Dallas 1E (Table 1).

Determination of protein. Protein was measured as described by Peterson (21) with BSA as a standard.

TABLE 2. *L. pneumophila* strains isolated from clinical specimens[a]

Serogroup	No. of isolates	Strain designation(s)
1	2	L 12, L 13
2	2	L 20, L 21
4	1	L 43
6	1	L 62
8	2	L 87, L 88
12	1	L 129

[a] From the collection of laboratory strains of the Robert Koch-Institute, Berlin, Germany.

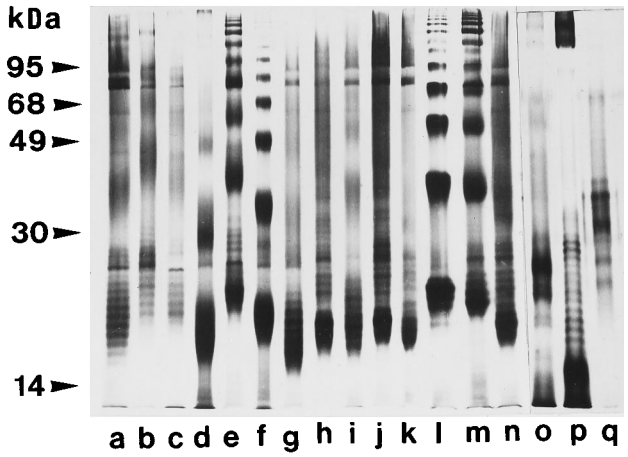

FIG. 1. Silver stain of *Legionella* LPSs on a 12% polyacrylamide gel. Lane a, *L. pneumophila* serogroup 1 (strain Philadelphia-1); lane b, *L. pneumophila* serogroup 2 (strain Togus-1); lane c, *L. pneumophila* serogroup 3 (strain Bloomington 2); lane d, *L. pneumophila* serogroup 4 (strain Los Angeles-1); lane e, *L. pneumophila* serogroup 5 (strain Dallas 1E); lane f, *L. pneumophila* serogroup 6 (strain Chicago 2); lane g, *L. pneumophila* serogroup 7 (strain Chicago 8); lane h, *L. pneumophila* serogroup 8 (strain Concord 3); lane i, *L. pneumophila* serogroup 9 (strain IN-23); lane j, *L. pneumophila* serogroup 10 (strain Leiden 1); lane k, *L. pneumophila* serogroup 11 (strain 797/PA/H); lane l, *L. pneumophila* serogroup 12 (strain 570-CO-H); lane m, *L. pneumophila* serogroup 13 (strain 82 A 3105); lane n, *L. pneumophila* serogroup 14 (strain 1169-MN-H); lane o, *L. bozemanii* (strain 1 WIGA); lane p, *L. gormanii* (strain LS-13); lane q, *L. micdadei* (strain Tatlock). The arrowheads indicate the positions of molecular mass markers.

RESULTS

Electrophoretic and immunological characterization of LPS. For the isolation of smooth-type LPS, the hot phenol-water extraction procedure developed by Westphal and Jann (27) is commonly used. However, several investigators have reported that the LPS of *L. pneumophila* could not be extracted by this procedure (4, 5, 22). We therefore prepared the LPSs of *Legionella* spp. by proteinase K digestion as described by Hitchcock and Brown (12).

The isolated LPS preparations were subjected to SDS-PAGE and visualized by silver staining according to the method of Tsai and Frasch (26), which preferentially stains the LPS. In order to detect possible protein contamination of the LPS extract, the gel was stained with Coomassie blue. The stained gel (not shown) revealed a protein of 19 kDa common to all *L. pneumophila* serogroups and described earlier as being associated with peptidoglycan (16). Additionally, a protein band of 25 or 24 kDa was stained in the LPS preparations from *L. pneumophila* serogroup 1 and *L. micdadei*. A few additional bands in the molecular mass range of 60 to ≥100 kDa were slightly stained in the preparations of *L. pneumophila* serogroups 5, 6, 12, and 13.

As shown in Fig. 1, the LPSs of *L. pneumophila* serogroups 1 to 14 displayed a typical ladder-like pattern with regularly spaced bands representing LPS molecules with different numbers of repeating units in their O side chains (10, 20). The electrophoretic patterns of LPSs from *L. pneumophila* serogroups 1 to 14 were different from each other, with the exception of serogroups 2 and 3, as well as serogroups 9 and 11, which had similar profiles (Fig. 1, lanes b, c, i, and k). Serogroups 4, 5, 6, 12, and 13 showed unique profiles with widely separated intensely stained bands. It is obvious from Fig. 1 that the prominent LPS bands as well as the ladder-like profiles are spread over the molecular mass range of 14 to 100 kDa. Compared with the clear resolution of the LPS bands of gram-

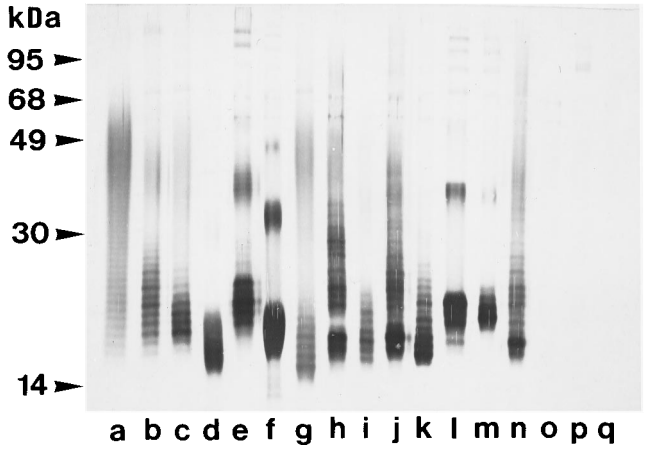

FIG. 2. Immunoblot analysis of *Legionella* LPSs with rabbit antibodies directed against *L. pneumophila* serogroup 5 (strain Dallas 1E). Lane a, *L. pneumophila* serogroup 1 (strain Philadelphia-1); lane b, *L. pneumophila* serogroup 2 (strain Togus-1); lane c, *L. pneumophila* serogroup 3 (strain Bloomington 2); lane d, *L. pneumophila* serogroup 4 (strain Los Angeles-1); lane e, *L. pneumophila* serogroup 5 (strain Dallas 1E); lane f, *L. pneumophila* serogroup 6 (strain Chicago 2); lane g, *L. pneumophila* serogroup 7 (strain Chicago 8); lane h, *L. pneumophila* serogroup 8 (strain Concord 3); lane i, *L. pneumophila* serogroup 9 (strain IN-23); lane j, *L. pneumophila* serogroup 10 (strain Leiden 1); lane k, *L. pneumophila* serogroup 11 (strain 797/PA/H); lane l, *L. pneumophila* serogroup 12 (strain 570-CO-H); lane m, *L. pneumophila* serogroup 13 (strain 82 A 3105); lane n, *L. pneumophila* serogroup 14 (strain 1169-MN-H); lane o, *L. bozemanii* (strain 1 WIGA); lane p, *L. gormanii* (strain LS-13); lane q, *L. micdadei* (strain Tatlock). The arrowheads indicate the positions of molecular mass markers.

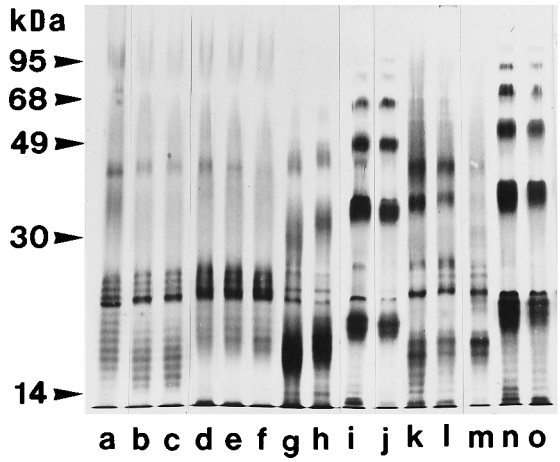

FIG. 3. Silver stain of the LPSs of *L. pneumophila* clinical isolates and reference strains on a 12% polyacrylamide gel. Lanes a to c, *L. pneumophila* serogroup 1 (strains Philadelphia-1, L 12, and L 13); lanes d to f, *L. pneumophila* serogroup 2 (strains Togus-1, L 20, and L 21); lanes g and h, *L. pneumophila* serogroup 4 (strains Los Angeles-1 and L 43); lanes i and j, *L. pneumophila* serogroup 6 (strains Chicago-2 and L 62); lanes k to m, *L. pneumophila* serogroup 8 (strains Concord 3, L 87, and L 88); lanes n and o, *L. pneumophila* serogroup 12 (strains 570-CO-H and L 129). The arrowheads indicate the positions of molecular mass markers.

negative bacteria (see Fig. 4A, lanes i to k), it is more difficult to resolve the LPSs of *L. pneumophila* serogroups 1 to 14 into ladder-like structures because of the more densely packed bands. In this context the amount of LPS from an individual serogroup applied to the gel is important for resolution and reproducibility and lies within a narrow concentration range.

The antigens used for LPS analysis for Fig. 1 were diluted as described in Materials and Methods and subjected to Western blotting to investigate their serological activities with rabbit antibodies directed to *L. pneumophila* serogroup 5. The blot indicated that the LPSs of *L. pneumophila* serogroups 1 to 14 reacted with antibodies raised against *L. pneumophila* serogroup 5 (Fig. 2). However, no cross-reactions with the LPSs of *L. bozemanii*, *L. gormanii*, and *L. micdadei*, tested as controls, were observed (Fig. 2, lanes o to q). The data showed that the LPSs of *L. pneumophila* serogroups 1 to 14 share at least one common epitope that is predominantly exposed within the LPS of serogroup 5. In contrast, under the given experimental conditions, the serogroup 1 antiserum recognized the homologous purified LPS and LPSs of serogroups 3, 7, and 12 but not the LPSs of the other heterologous serogroups and species (data not shown).

While Fig. 1 depicts the LPS profiles of *L. pneumophila* reference strains (serogroups 1 to 14; see also Table 1), Fig. 3 shows the profiles of six reference strains together with those of nine clinical isolates serotyped in our laboratory (Table 2). It can be seen in Fig. 3 that the LPS profiles of the same serogroups are possibly identical or reveal at least a high degree of relatedness. The immunoblot analysis with the serogroup 5 antiserum (not shown) of the LPS patterns of the strains depicted in Fig. 3 showed the same result as described for Fig. 2. Thus, it can be concluded from our experiments that the *L. pneumophila* strains investigated can be typed by LPS electrophoresis and silver staining or by immunoblotting, using in the latter case the cross-reacting serogroup 5 antiserum.

Additionally, the LPS pattern of *L. pneumophila* Philadelphia-1 was analyzed and compared with those of the other serogroup 1 subtypes, i.e., Knoxville, Allentown, Benidorm, Olda, Oxford 4032E, France 4811, and Camperdown. The LPS banding pattern in silver-stained gels showed the typical ladder-like structure in the molecular mass range of 14 to 25 kDa (Fig. 4A, lanes a to h). In the range of 25 to 30 kDa, the LPS profile is less clearly resolved (Fig. 4A, lanes b, d, f, and h). In contrast, the typical LPS banding pattern is precisely visible in the immunoblot for all subtypes of *L. pneumophila* serogroup 1 (Fig. 4B, lanes a to h). In addition, Fig. 4B demonstrates that a nearly homogeneous cross-reacting LPS profile is detected with the antiserum raised against *L. pneumophila* serogroup 5. In contrast, the gel profiles of commercial LPS preparations of *E. coli*, *P. aeruginosa*, and *S. minnesota* (Fig. 4A, lanes i to k) differed markedly from the profiles obtained with the LPSs of either serogroup 1 subtypes or the other *Legionella* species and exhibited no cross-reactivity with the serogroup 5 antiserum (Fig. 4B, lanes i to k).

DISCUSSION

The aim of numerous studies in the past was to characterize cross-reactive *Legionella* antigens with possible diagnostic potential. For this purpose several *Legionella* antigen preparations were investigated with antisera raised in different animal species and with various immunization schedules (1, 2, 5). Interest was also focused on *Legionella* LPS in an attempt to isolate and characterize these substances from different *Legionella* species. Hence, several investigators tried to isolate *Legionella* LPS by the classical phenol-water procedure (27), which is suitable for the extraction of smooth-type LPS from gram-negative bacteria. Gabay and Horwitz (9) had noted that the LPS of *L. pneumophila* was not found in the water phase of a phenol-water extract. Petitjean et al. (22) reported that only a very small amount of LPS of Philadelphia-1 was recovered from the water phase. To overcome this obstacle, we followed the extraction protocol of Darveau and Hancock (6), using

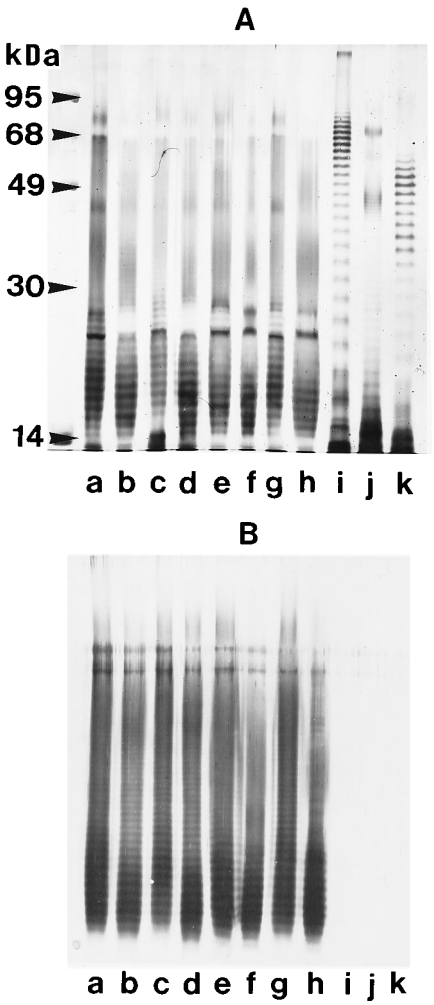

FIG. 4. (A) Silver stain of LPSs of eight strains of *L. pneumophila* serogroup 1 and three other gram-negative bacteria on a 12% polyacrylamide gel. (B) The same samples diluted 1:500 (*L. pneumophila* strains) or 1:10 (gram-negative organisms) were transfered to a nitrocellulose membrane for immunoblot analysis with rabbit antibodies directed against *L. pneumophila* serogroup 5. Lanes a, strain Philadelphia-1; lanes b, strain Knoxville; lanes c, strain Allentown; lanes d, strain Benidorm; lanes e, strain Olda; lanes f, strain Oxford 4032E; lanes g, strain France 4811; lanes h, strain Camperdown; lanes i, *S. minnesota*; lanes j, *P. aeruginosa* serotype 10 (Habs); lanes k, *E. coli* serotype O111:B4. The arrowheads indicate the positions of molecular mass markers.

cold ethanol for extraction of LPS from the proteinase K-digested cell lysates of the *Legionella* strains investigated.

Our electrophoretic analysis of *Legionella* LPS revealed multiple bands representing species with different molecular masses due to the differences in the number of repeating units of their O side chains. The LPSs of *L. pneumophila* serogroups 1 to 14 showed tight ladder-like patterns, with the exception of those of serogroups 4, 5, 6, 12, and 13, which showed widely separated bands comparable to those seen earlier by Zanen-Lim and Zanen (28, 29) for serogroups 6 and 12. Nolte et al. (18) investigated SDS-PAGE proteinase K-digested cell lysates of *L. pneumophila* serogroups 1 to 8. In contrast to our observation, they noticed a considerable similarity in the banding pattern, except for strain Chicago 2.

However, our studies demonstrated that the LPS profiles of *L. pneumophila* serogroups 1 to 14, as well as those of *L. bozemanii*, *L. gormanii*, and *L. micdadei*, were substantially different from each other. Unlike those reported by Nolte et al. (18), our experiments demonstrated that after LPS electrophoresis and silver staining, the banding patterns of homologous serogroups showed a high degree of similarity, thus allowing the recognition and identification of the serogroup of the initially serotyped clinical isolates included in this study.

Previous serological studies have shown that the serogroup specificity of *L. pneumophila* is located in the LPS portion of the bacterial cell surface (5, 18, 19). Nolte et al. (18) investigated the serological reactivities of *L. pneumophila* serogroups 1 to 8 with a rabbit antiserum prepared against serogroup 1 (Knoxville 1) and demonstrated that the antiserum recognized only the LPS from the serogroup 1 strain. Ciesielski et al. (5) studied the LPSs of *L. pneumophila* serogroups 1 to 6 in immunoblot assays and found that the monkey antisera which they used reacted only with the homologous strain.

Our work, however, revealed that a rabbit antiserum directed against *L. pneumophila* serogroup 5 contained cross-reacting antibodies which recognized the LPSs of *L. pneumophila* serogroups 1 to 14. The serogroup 5 antiserum, however, failed to react with the LPSs of the non-*L. pneumophila* species and did also not react with the LPSs of gram-negative bacteria.

Hence, it may be concluded from our study that polyclonal rabbit antibodies raised against serogroup 5 antigens are useful in the identification of all serogroups of *L. pneumophila*. Moreover, *L. pneumophila* serogroups 1 to 14 can be typed by means of their specific LPS banding pattern either in silver-stained gels or by immunoblotting. LPS typing, however, is valuable only for the identification of individual serogroups (1 to 14); it cannot differentiate subtypes within a given serogroup. Thus, *L. pneumophila* serogroup 1 subtypes (such as Philadelphia-1, Knoxville, Allentown, Benidorm, Olda, Oxford 4032E, France 4811, and Camperdown) could be distinguished neither by silver staining nor by the immunoblot assay because of their similar LPS profiles.

ACKNOWLEDGMENTS

We thank Rita Sprengel for manuscript preparation and Christa-Maria Schmidt and Susanne Enders for technical assistance.

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