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Identification of *Legionella* Species by Lipopolysaccharide Antigen Pattern

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Electrophoretic analysis of lipopolysaccharide (LPS) extracts from 430 previously serotyped *Legionella* isolates and 28 American Type Culture Collection (ATCC) non-*Legionella pneumophila Legionella* reference strains representing different *Legionella* species and serogroups has been performed. LPS was prepared from *Legionella* suspensions by sonication and proteinase K digestion. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis, LPS bands were either stained with silver nitrate or transferred onto a nitrocellulose membrane and detected with rabbit antibodies raised against *L. pneumophila* serogroup 5, which was known to cross-react with *L. pneumophila* serogroups 1 to 14. Silver staining revealed that each of the 28 ATCC non-*L. pneumophila Legionella* strains possessed an individual and characteristic LPS banding pattern. The LPS profile was defined by the molecular weight of the visualized bands and/or the individual ladder-like LPS pattern. It was demonstrated by immunoblotting that non-*L. pneumophila Legionella* strains did not react with the serogroup 5 antiserum, thus allowing for the differentiation between *L. pneumophila* and non-*L. pneumophila* species.

Legionella spp., the causative agents of Legionnaires' disease are easily differentiated from other bacterial species by classical microbiological techniques. However, the identification of the 56 serogroups within the 38 species by serogrouping is still unreliable due to considerable cross-reactivity between individual serogroups and species. At present, several procedures such as gene probe, fatty acid, ubiquinone, and membrane protein analyses (7, 8, 10, 16) are used in parallel to overcome this drawback. However, these laborious or sophisticated typing procedures are still infrequently used outside reference laboratories. On the other hand, serogrouping, which is more commonly used, frequently gives unsatisfactory results. Furthermore, cross-reactions have been observed not only among serogroups and species of legionellae (2, 12, 13) but also between legionellae and some other gram-negative bacteria (3, 21).

Given the successful application of lipopolysaccharide (LPS) typing to other bacterial species (1, 17, 18, 20), it seemed appropriate to evaluate LPS typing for the identification of *Legionella* species and serogroups. However, one must consider the fact that the chemical nature of the *Legionella* LPS is significantly different from those of the LPSs of other gram-negative bacteria (16, 19, 25). We therefore investigated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) the LPS profiles of 28 American Type Culture Collection (ATCC) strains representing most known *Legionella* species and serogroups. Silver-stained LPS profiles were analyzed and compared with the aim of identifying individual LPS profiles. The ATCC type strain profiles of non-*Legionella pneumophila Legionella* strains together with those of ATCC *L. pneumophila* strains investigated recently (12) were then used in a blinded study to identify 430 wild-type strains of clinical and environmental origins serogrouped previously.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Legionella* reference strains listed in Table 1 were obtained from ATCC (Rockville, Md.). Serogrouped *Legionella* strains were kindly supplied by N. Bornstein, Centre National de Référence des Légionella, Lyon, France; C. M. Bourgon, Centro National de Mikrobiologia, Virologia e Immunologia Santarias, Madrid, Spain; M. Castellani Pastoris, Istituto Superiore di Sanità, Rome, Italy; A. Cheresky, Special Pathogens Laboratory, NZ Communicable Disease Centre, Porirua, New Zealand; V. Drašar, National Legionella Reference Laboratory, Vyškov, Czech Republic; D. Groothuis, Rijksinstituut voor de Volksgezondheit en Milieuhygiene, Bilthoven, The Netherlands; T. Harrison, Public Health Laboratory Services, London, United Kingdom; P. C. Lück, Universitätsklinikum Carl Gustav Carus, Dresden, Germany; M. Tully, Centre for Applied Microbiology & Research, Salisbury, United Kingdom; and G. Wewalka, Bundesstaatliche Bakteriologische und Serologische Untersuchungsanstalt, Vienna, Austria. 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 were subsequently grown on buffered charcoal-yeast extract agar with 0.1% alpha-ketoglutaric acid (6). The plates were then incubated in a moist atmosphere at 35°C for 72 h.

LPS preparation. The cells were harvested from plates in 2.5 ml of sterile distilled water, heated to 100°C 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 mixed with 1 volume 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. The lysates were then boiled for 10 min. Protein (1.5 to 2.0 mg) was digested by the addition of proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) by the method of Hitchcock and Brown (11) at a ratio of 1 μg of proteinase K to 10 μg of protein. The samples were incubated at 60°C for 60 min. LPS was precipitated by adding 2 volumes of a solution of 0.375 M $MgCl_2$ in 95% ethanol overnight at −20°C (4). 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 were analyzed by SDS-PAGE in slab gels (80 by 120 by 1 mm; concentration of polyacrylamide = 12%; degree of polymer cross-link = 2.6%) with the buffer system described by Laemmli (15). Phosphorylase (M_r, 95,000), bovine serum albumin (M_r, 68,000), fumarase (M_r, 49,000), carboanhydrase (M_r, 30,000), and lysozyme (M_r, 14,000), purchased from Boehringer (Mannheim), were used for calibration. Samples of 5 μl of extracted LPSs were 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, and the mixture was then heated to 100°C for 5 min. Electrophoresis was performed with 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 (23).

RESULTS

Electrophoretic characterization of the LPS of non-*L. pneumophila Legionella* species. As demonstrated previously (9, 19), *Legionella* LPS cannot be extracted by the classical phenol-water extraction method. Therefore, the LPSs of 28 *Legionella* species (ATCC strains) were prepared by proteinase K digestion followed by ethanol precipitation of the digested bacterial

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TABLE 1. Reference strains used in this study

Species	Strain designation	ATCC strain no.	Serogroup
L. anisa	WA-316-C3	35292	
L. birminghamensis	1407-AL-H	43702	
L. bozemanii	WIGA	33217	1
L. bozemanii	Toronto-3	35545	2
L. brunensis	441-1	43878	
L. cherii	ORW	35252	
L. cincinnatiensis	72-OH-H	43753	
L. dumoffii	NY-23	33279	
L. erythra	SE-32A-C8	35303	
L. feeleii	WO-44C	35072	1
L. feeleii	691-WI-H	35849	2
L. gormanii	LS-13	33297	
L. hackeliae	Lansing-2	35250	1
L. hackeliae		35999	2
L. israelensis	Bercovier-4	43119	
L. jamestowniensis	JA-26-G1-E2	35298	
L. jordanis	BL-540	33623	
L. longbeachae	Long Beach-4	33462	1
L. longbeachae	Tucker-1	33484	2
L. maceachernii	PX-1-G2-E2	35300	
L. micdadei	TATLOCK	33218	
L. moravica	316-36	43877	
L. oakridgensis	OR-10	3376	
L. rubrilucens	WA-270A-C2	35304	
L. sainthelensi	Mt. St. Helens-4	35248	
L. spiritensis	Mt. St. Helens-9	35249	
L. steigerwaltii	SC-19-C9	35302	
L. wadsworthii	81-716A	33877	

suspension. SDS-PAGE and silver staining of the 28 different LPS extracts revealed an individual LPS profile for each of the strains investigated (Fig. 1 and 2). While individual ladder-like structures were observed for most serogroups of *L. pneumophila*, with the exceptions of serogroups 4, 6, 12, and 13 (12), non-*L. pneumophila Legionella* strains differed in this respect since only 4 of 28 species had ladder-like LPS profiles (Fig. 1, lanes i and l, and Fig. 2, lanes j and l). Interestingly, the LPS

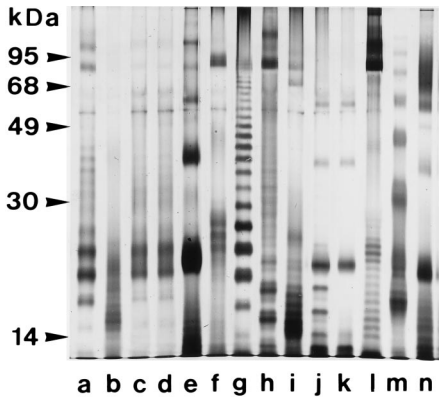

FIG. 1. Silver stain of the LPSs of *Legionella* reference strains on a 12% polyacrylamide gel. Lane a, *L. anisa* WA-316-C3; lane b, *L. birminghamensis* 1407-AL-H; lanes c and d, *L. bozemanii* serogroups 1 and 2 respectively (strains WIGA and Toronto-3, respectively); lane e, *L. brunensis* 441-1; lane f, *L. cherii* ORW; lane g, *L. cincinnatiensis* 72-OH-H; lane h, *L. dumoffii* NY-23; lane i, *L. erythra* SE-32A-C8; lanes j and k, *L. feeleii* serogroups 1 and 2, respectively (strains WO-44C and 691-WI-H, respectively); lane l, *L. gormanii* LS-13; lane m, *L. hackeliae* Lansing-2 serogroup 1; lane n, *L. hackeliae* serogroup 2. The arrowheads indicate the positions of the molecular mass markers.

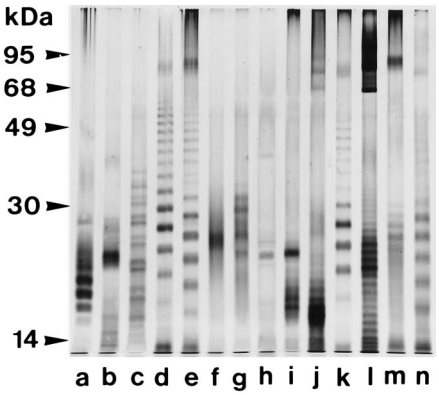

FIG. 2. Silver stain of the LPSs of *Legionella* reference strains on a 12% polyacrylamide gel. Lane a, *L. israelensis* Bercovier-4; lane b, *L. jamestowniensis* JA-26-G1-E2; lane c, *L. jordanis* BL-540; lanes d and e, *L. longbeachae* serogroups 1 and 2, respectively (strains Long Beach-4 and Tucker-1, respectively); lane f, *L. maceachernii* PX-1-G2-E2; lane g, *L. micdadei* TATLOCK; lane h, *L. moravica* 316-36; lane i, *L. oakridgensis* OR-10; lane j, *L. rubrilucens* WA-270A-C2; lane k, *L. sainthelensi* Mt. St. Helens-4; lane l, *L. spiritensis* Mt. St. Helens-9; lane m, *L. steigerwaltii* SC-19-C9; lane n, *L. wadsworthii* 81-716A. The arrowheads indicate the positions of the molecular mass markers.

profiles of the non-*L. pneumophila Legionella* strains showed a large number of broader bands, representing different molecular species, also varying in density and intensity. Clusters of bands were spread over a narrow range of molecular masses of 14 to 30 kDa (Fig. 1, lanes a to d, and Fig. 2, lanes a, b, f, i, and m) as well as over an area of 14 to 95 kDa (Fig. 1, lanes g, h, and l, and Fig. 2, lanes d, e, k, and n). The representative LPS profile of *L. jordanis* was characterized by seven double bands regularly distributed over the range of 14 to 40 kDa (Fig. 2, lane c). While serogroups 1 and 2 of *L. feeleii* and *L. hackeliae* both displayed characteristic profiles readily distinguishable from each other (Fig. 1, lanes k and l and lanes m and n, respectively), the LPS profiles of *L. bozemanii* serogroups 1 and 2 (Fig. 1, lanes c and d, respectively) were indistinguishable. The numbers of bands visualized for *L. longbeachae* serogroups 1 and 2 were identical, but a shift of individual bands to lower molecular masses of 16 to 40 kDa was observed (Fig. 2, lanes d and e).

Typing of legionellae by LPS profiles. It has been shown earlier that antibodies against *L. pneumophila* serogroup 5 raised in rabbits cross-reacted with *L. pneumophila* serogroups 1 to 14 (12). In contrast, none of the 28 non-*L. pneumophila Legionella* strains tested reacted with the serogroup 5 antiserum (data not shown). Therefore, the serogroup 5 antiserum can discriminate between *L. pneumophila* and non-*L. pneumophila* species. We have recently shown that *L. pneumophila* serogroups 1 to 14 can be typed according to their individual LPS profiles after SDS-PAGE and silver staining (12). The same procedure was applied in this study to identify non-*L. pneumophila* and *L. pneumophila* wild-type strains by their characteristic LPS profiles. Thus, 430 clinical and environmental isolates, provided by one New Zealand and nine European reference laboratories, were investigated, together with 28 ATCC reference strains.

Figures 1 and 2 depict the LPS profiles of the ATCC reference strains of non-*L. pneumophila Legionella* strains. For illustration, the profiles of 11 clinical and environmental isolates of non-*L. pneumophila Legionella* strains have been compared with those of the corresponding ATCC reference strains (Fig. 3). It can be seen from Fig. 3 that the LPS profiles of the same

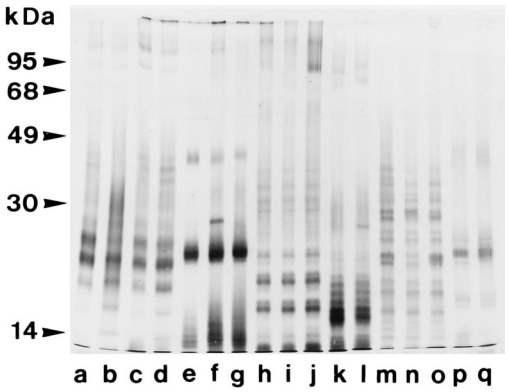

FIG. 3. Silver stain of the LPSs of *Legionella* isolates and reference strains on a 12% polyacrylamide gel. Lane a, *L. anisa* WA-316-C3; lane b, *L. anisa* isolate from the Czech Republic; lanes c and d, *L. anisa* isolates from Germany; lane e, *L. brunensis* 441-1; lanes f and g, *L. brunensis* isolates from the Czech Republic; lane h, *L. dumoffii* NY-23; lane i, *L. dumoffii* isolate from the Czech Republic; lane j, *L. dumoffii* isolate from New Zealand; lane k, *L. erythra* SE-32A-C8; lane l, *L. erythra* isolate from the Czech Republic; lane m, *L. jordanis* BL-540; lanes n and o, *L. jordanis* isolates from the Czech Republic; lane p, *L. moravica* 316-36; lane q, *L. moravica* isolate from the Czech Republic. The arrowheads indicate the positions of the molecular mass markers.

species were identical or at least revealed a high degree of relatedness.

The results of the LPS typing of 430 strains investigated in this study are summarized in Table 2. It can be seen that of 246 serogrouped *L. pneumophila* serogroup 1 strains, 239 were confirmed to be serogroup 1 strains by LPS typing. Disagreement between typing methods was more pronounced for *L. pneumophila* serogroup 2 to 10 strains. The identities of only 44% of the *L. pneumophila* serogroup 3, 5, 6, 8, and 10 strains were confirmed by LPS typing. The concordance for non-*L. pneumophila Legionella* strains was more satisfactory since the LPS typing results for only 7 of 40 strains were in disagreement with the serogrouping results.

TABLE 2. Comparison of serotyping with LPS profile typing of *Legionella* species

Strain and serogroup	No. of strains serotyped	No. of strains identified by LPS profile typing	
		Serotype confirmed	Serotype not confirmed
L. pneumophila			
1	246	239	7
2	9	4	5
3	18	9	9
4	21	11	10
5	31	8	23
6	31	14	17
7	2	0	2
8	10	7	3
9	1	0	1
10	10	6	4
12	9	9	0
13	1	1	0
14	1	1	0
Non-*L. pneumophila* legionellae	40	33	7

DISCUSSION

Although a variety of identification and typing procedures have been developed (5, 7, 22), typing results are often in disagreement. Of the various typing procedures, the slide agglutination test (22) and the direct immunofluorescence assay (24) with specific rabbit antisera have found wide application, although cross-reactions within the genus *Legionella*, as well as with other gram-negative rods, have been reported (2, 3, 12, 13, 24). While fatty acid and ubiquinone analyses by gas-liquid chromatography and high-performance liquid chromatography (7, 16) allow for the separation of legionellae from other bacterial species, these methods fail to differentiate legionellae down to the species level.

It has been shown earlier by Hitchcock and Brown (11) that differences in the LPS compositions of *Salmonella* species can be detected after electrophoresis by silver staining. This has been also found to be true for the LPS isolated from most other gram-negative bacteria (1, 14, 17, 20). Moreover, Palva and Mäkelä (18) demonstrated that the individual differences in the LPS banding patterns after SDS-PAGE were due to the different numbers of repeating units in the O-site chain of the LPS molecule. Hence, it has been suggested that isolates of one bacterial species that exhibit different LPS profiles in SDS-PAGE are likely to possess serologically distinct O antigens.

We found, with very few exceptions, that the LPS profiles of the majority of the *Legionella* wild-type strains matched well the profiles of the respective ATCC reference strains. However, some strains were found to have an LPS profile which differed from the LPS profile of the respective ATCC reference strain due to the presence or absence of a particular LPS band. These minor variations were ignored, and the strains were allocated to a serogroup according to the dominating banding pattern (i.e., *L. brunensis*, Fig. 3, lane f). It is not clear whether minor changes in the LPS concentration are responsible for the apparent presence or absence of individual bands. Genotyping may provide further information for the correct taxonomic allocation of the strains in question.

Although some minor drawbacks are still encountered with the LPS typing procedure described here, the method can be performed with good reproducibility due to both the stability of the LPS molecule and the simplicity of the test itself.

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