Antigenic Homogeneity among Legionella pneumophila Serogroups 1 to 6 Evaluated by Crossed Immunoelectrophoresis

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The objective of this study was to investigate the antigenic profiles of Legionella pneumophila serogroups 1 to 6 and antigenic relatedness among individual serogroups. A reference precipitate pattern which had 71 anodic- and 6 cathodic-migrating antigens was established with a pooled antigen preparation of sonicated L. pneumophila cells of serogroups 1 to 6 against purified homologous rabbit antibody. Sixteen antigens made heat stable by boiling for 10 min were found, of which one could be demonstrated as being the common antigen of gram-negative bacteria and another could be shown as being the serogroup-specific antigen of L. pneumophila. Precipitin patterns similar in overall appearance and precipitin numbers were demonstrated for each serogroup by testing antigen preparations of individual serogroups against reference system antibody. Crossed-line experiments revealed <5 non-cross-reacting antigens among serogroups, with resulting matching coefficients (ratio of the number of cross-reactive to total antigens) all above 0.91. The matching coefficients found when testing antigen preparations from L. pneumophila serogroups 7 and 8 were 0.93 and 0.99, respectively. By contrast, the matching coefficients of Tatlockia micdadei, Fluoribacter bozemanae, Fluoribacter dumoffii, and Fluoribacter gormanii was 0.39, 0.45, 0.39, and 0.41, respectively. Comparison of precipitate patterns and experimental results between the previously described monovalent L. pneumophila serogroup 1 system and the polyvalent crossed immunoelectrophoresis system with L. pneumophila serogroups 1 to 6 substantiated the antigenic homogeneity of L. pneumophila and the significant difference in antigenic composition between this taxon and other members of the family Legionellaceae. In addition, reproducibility of the antigenic profile of L. pneumophila by crossed immunoelectrophoresis was demonstrated.

At present, 21 species within the family Legionellaceae, isolated both from human and environmental sources, have been reported (5), 4 of which include multiple serogroups (Legionella pneumophila, Legionella bozemanii, Legionella longbeachae, and Legionella feelei). Classification of these organisms is based on phenotypic characteristics such as growth requirements, biochemical reactions, pigment production, cellular fatty acid composition, ubiquinone content, serological tests, and deoxyribonucleic acid homology studies.

Many investigators prefer to include all Legionella organisms in a single genus, Garity et al., however, have suggested classification of Legionella micdadei and Legionella bozemanii in two different genera, Tatlockia and Fluoribacter, respectively (16). The increasing number of new Legionella species and serogroups and the heavy dependence on serological techniques to diagnose legionellosis underline the importance of a thorough understanding of the complex antigenic structure of these organisms.

In our previous publication, the antigenic profile of L. pneumophila serogroup 1 investigated by crossed immunoelectrophoresis (XIE) was described (9). The purposes of the present study were to extend antigenic characterization of L. pneumophila by XIE to other serogroups and to quantify the degree of antigenic similarity (matching coefficients) among serogroups when tested in a polyvalent (6 serogroups) XIE reference system. In addition, the reproducibility of the XIE technique for characterization of antigenically complex organisms was tested.

MATERIALS AND METHODS

Bacterial strains. L. pneumophila serogroups 1 (Knoxville 1 and Philadelphia 1) to 6 used for this study were ATCC strains 33153, 33152, 33154, 33155, 33156, 33216, and 33215, respectively. Serogroups 7 and 8 were kindly provided by Jacquelyn S. Sampson, Centers for Disease Control, Atlanta, Ga. Other ATCC strains used were Tatlockia (Legionella) micdadei 33218, Fluoribacter (Legionella) bozemanii 33217, Fluoribacter dumoffii 33279, and Fluoribacter gormanii 33297. A clinical isolate of L. pneumophila serogroup 1 was obtained from A. Friis-Møller, State Serum Institute, Copenhagen, Denmark, and was designated LplK-9422.

Antigen preparation. The organisms were grown on buffered charcoal yeast extract medium (GIBCO Diagnostics, Madison, Wis.) and harvested and sonicated as described previously (9). Colloidal concentrations of each antigen were calculated after the refractive index was measured with human immunoglobulin as a standard. The concentration range for individual serogroup antigen preparations was 18.7 to 42.6 mg/ml. The antigen preparations were designated LplK (Knoxville 1), LplP (Philadelphia 1) and Lp2 to Lp8 for the respective serogroups. The reference antigen, designated Lp1-6, was produced by pooling equal amounts of antigens LplK and Lp2 to Lp6 and was diluted to a concentration of 15 mg/ml. All antigen preparations were stored at −20°C.

Heat-stable antigens were produced by submerging 1-ml vials of the antigen preparations in boiling water for 10 min. The colloidal concentration of the boiled sonicates was 60 to 74% of the unboiled antigen preparations as measured by refractometry.

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Antibody production. Ten adult New Zealand White rabbits were each immunized intracutaneously with 100 μl of Lp1-6 reference antigen in incomplete adjuvant as previously described (9). Antisera harvested in months 9 to 17 were pooled for the study described here. Antibody was isolated and purified by the method of Harboe and Ingild (19). This antibody was designated Lp1-6Ab and had a concentration of 20.8 mg/ml.

Monospecific antiserum against the *Escherichia coli* common antigen was produced by immunizing two adult New Zealand White rabbits subcutaneously every other week with 200 μl each of *E. coli* common antigen, prepared by the precipitin excision method (18), in an equal volume of incomplete adjuvant. These rabbits were bled weekly, and sera from weeks 8 and 12 were used (43 μl/cm²) in intermediate gels. Serum samples obtained from the rabbits before immunization were used as preimmune serum controls.

Monoclonal antibody against *L. pneumophila* serogroup 1 (7 9C3-R3) was kindly provided by Ian Watkins, University of Oxford, England.

XIE was performed as described by Svendsen et al. (33) with equipment, agarose, and buffer similar to those of our previous study (9). A fixed antigen amount of 60 μg (4 μl of Lp1-6 antigen) was used in all experiments with unheated antigen preparations. Boiled antigen preparations, however, were tested with volumes equal to those of the unboiled preparations.

All experiments were performed with an intermediate gel containing either saline (100 μl of a 0.154 M solution), Lp1-6 reference antibody (in increasing amounts from 14 to 57 μl/cm²), or antigen in crossed-line immunoelectrophoresis (XLIE) experiments (in increasing amounts from 213 to 851 μg/cm²). Second dimension electrophoresis was performed with Lp1-6Ab in the back gel (14 μl/cm²) in all experiments. The pressing and drying of the gels followed by staining with Coomassie brilliant blue R were performed as described by Svendsen et al. (33).

Matching coefficients. Matching coefficients were used to express the degree of antigenic homogeneity between Lp1-6 and other serogroups and species of legionellae. This parameter has been used in several other serological studies (10, 12, 13, 23, 32). We defined it as follows: matching coefficient = number of cross-reactive antigens/average number of antigens demonstrable in a single organism in the reference system.

The denominator of this equation is usually the total number of antigens routinely found in a XIE reference system. While this value is appropriate with XIE reference systems containing few serovars, in a polyvalent system such as ours, no individual component organism may contain all reference system antigens. Thus, 55.8, the average of the number of antigens found by XIE of individual *L. pneumophila* serogroups, was used for all matching coefficient calculations.
FIG. 2. Comparison of XIE precipitin patterns produced when each individual *L. pneumophila* serogroup was electrophoresed against Lp1-6 polyvalent reference antibody. (A) Serogroup 1 Knoxville 1 strain (Lp1Ag); (B) serogroup 2 (Lp2Ag); (C) serogroup 3 (Lp3Ag); (D) serogroup 4 (Lp4Ag); (E) serogroup 5 (Lp5Ag); (F) serogroup 6 (Lp6Ag). Panels A through D reflect 60 μg of antigen in the first dimension and 300 μl of Lp1-6Ab in the back gel, with saline (NaCl) in the intermediate gel. All 6 precipitin patterns were similar quantitatively (51 to 57 precipitins) and qualitatively except for the notable absence of antigen 2 in Lp1, Lp4, and Lp5.

**Terminology.** Terminology guidelines for serotaxonomic studies involving immunodiffusion and immunoelectrophoresis as reported by Chaparas et al. were followed (7).

**RESULTS**

**Reference system.** In the Lp1-6 XIE reference system (Fig. 1), 71 anodic- and 6 cathodic-migrating antigens could be demonstrated. Only the anodic-migrating antigens were numbered and studied further. Routinely, reference gels produced 58 peaks. The standard deviations of peak numbers within and among experiments were ±2.7 and ±3.6 peaks, respectively (10 determinations).

The overall appearance of the reference system was similar to the previously published Lp1P reference system (9), with a tall fast-migrating antigen (no. 1) and a group of slower-migrating antigens. The number of total anodic-migrating antigens was 82 in Lp1P versus 71 in Lp1-6, and the number of routinely counted antigens was 51 in Lp1P and 58 in Lp1-6. It was shown that all Lp1P antigens were represented in the Lp1-6 system but not vice versa, as peaks 2 and 61 in the Lp1-6 system did not cross-react with antigens of Lp1P.

Antigen 66 in the Lp1-6 system had a morphology, mobility, and staining intensity very similar to the common antigen of gram-negative bacteria in the Lp1P system (also designated no. 66). That antigen 66 represented the common antigen could be demonstrated by using rabbit antiserum against the common antigen isolated from *E. coli* in the intermediate gel, which resulted in a reaction of identity with antigen 66.
Further experiments, described below, suggested that antigen 61 represented the serogroup-specific antigen for each serogroup in the Lpl-6 pattern. This precipitate was situated just below and left of the common antigen, was ill-defined, and had a peculiar morphology indicative of a complex and electrophoretically heterogeneous antigen: a short, sharp peak with a long right leg stretching out into a flat, distinct bow.

**Individual serogroups.** XIE of the individual serogroups, Lp1K, Lp2, Lp3, Lp4, Lp5, and Lp6 produced 51, 55, 57, 58, 57, and 57 precipitates, respectively (each value is the mean of three determinations). The overall appearance of each individual serogroup precipitate pattern was similar to that of the reference pattern, although slight differences in precipitate pattern heights were found (Fig. 2). Antigen 2 could be routinely demonstrated only in serogroups 2, 3, and 6. Antigen 61 in the Lpl-6 reference system was demonstrated in each individual serogroup. Serogroups Lp1K and Lp1P were tested with monoclonal antibody against Lp1P in the intermediate gel to demonstrate the serogroup-specificity of this antigen. The monoclonal antibody produced a reaction of identity with antigen 61, suggesting that it represented the serogroup-specific antigen (Fig. 3). The monoclonal antibody did not precipitate antigens in other serogroups.

The number of non-cross-reacting antigens among each serogroup was established by XLIE (Table 1). In serogroups 1, 2, 3, and 6, precipitin 61 plus 0 to 3 additional precipitates were non-cross-reacting. With serogroup 4 in the first dimension, however, a serogroup-specific antigen could not always be demonstrated (100% one-way cross-reactivity when tested against Lp3, Lp5, and Lp6.

**Heat-stable antigens.** In the Lpl-6 reference system, 16 antigens were stable to boiling for 10 min. In crossed-line experiments, antigen 66 in the unheated Lpl-6 showed only partial identity with the boiled preparation. However, with rabbit antiserum against *E. coli* common antigen, the presence of the common antigen in the boiled sonicates was clearly demonstrated. In individual serogroups, 11 to 14 heat-stable antigens were found, among which was the serogroup-specific antigen.

**Antigenic homology.** XIE of *L. pneumophila* serogroups, 7, 8, and a clinical isolate of Lp1 against the reference system antibody gave 52, 55, and 56 precipitates with corresponding matching coefficients of 0.93, 0.99, and 1.00,

<table>
<thead>
<tr>
<th>L. pneumophila serogroup in first dimension</th>
<th>No. of precipitates in control gel</th>
<th>No. of antigens placed in intermediate gel for serogroup:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lp1K</td>
<td>51</td>
<td>Lp1K Lp2 Lp3 Lp4 Lp5 Lp6</td>
</tr>
<tr>
<td>Lp2</td>
<td>55</td>
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<td>57</td>
<td>2 2 4 3 1</td>
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<td>Lp4</td>
<td>58</td>
<td>1 1 0 0 0 0</td>
</tr>
<tr>
<td>Lp5</td>
<td>57</td>
<td>2 2 0 1 1</td>
</tr>
<tr>
<td>Lp6</td>
<td>57</td>
<td>4 3 1 4 3</td>
</tr>
</tbody>
</table>
respective. Sonicates prepared from T. micdadei, F. bozemanae, F. dumoffii, and F. gormanii tested against reference antibody showed 22, 25, 22, and 23 precipitates, respectively, giving matching coefficients of 0.39, 0.45, 0.39, and 0.41, respectively.

**DISCUSSION**

The polyvalent XIE reference system for L. pneumophila serogroups 1 to 6 described in the present paper was developed independently from the L. pneumophila serogroup 1 system described previously (9). These two XIE systems used different antigens: Philadelphia 1 strain in the L. pneumophila serogroup 1 system and the Knoxville 1 strain of serogroup 1 plus serogroups 2 to 6 in the polyvalent system (Lp1-6). They also used different pools of antibody, each produced by immunization of 10 rabbits with sonicates of live cells, followed by purification of immunoglobulins G and A. Considering these differences, the qualitative as well as quantitative similarities between the precipitate patterns of the two XIE systems were remarkable. Høshøj previously demonstrated reproducibility between antigen batches and antibody pools with a single *Pseudomonas aeruginosa* XIE reference system (21, 22). His work confirmed the findings of Axelsen, who, working with *Candida albicans*, laid the foundation for the standardization of the XIE technique (1). Our studies again demonstrated reproducibility of the XIE technique for analysis of antigenically complex organisms. It should be noted that standardized procedures must be adhered to strictly.

Each serogroup of *L. pneumophila* had a precipitate identifiable as the serogroup-specific antigen. The precipitate (no. 61) pattern was similar in position and morphology in four of six serogroups tested but was electrophoretically faster and more bell-shaped in serogroups 4 and 5. Monoclonal antibody to the serogroup-specific antigen of Lp1 Philadelphia 1 precipitated only antigen 61 of serogroup 1 and no antigens in any of the other five serogroups tested.

The definitive number of cross-reacting antigens among serogroups of *L. pneumophila* was determined in two-way cross-reaction studies with XLIE (Table 1). The numbers of non-cross-reacting antigens among serogroups were often not identical in reciprocal cross-reaction tests. This occurred when a given serogroup had no antigen(s) present in the cross-reacting serogroup.

Precipitate counts for the six individual serogroups in the reference system ranged from 51 to 58, with a mean of 55.8 ± a standard deviation of 2.6. With this mean as the denominator in the equation defined above, the matching coefficients for *L. pneumophila* serogroups 7 and 8 plus a clinical isolate from a patient in Copenhagen (serogroup 1) were 0.93, 0.99, and 1.00, respectively. Thus, the polyvalent Lp1-6 antibody recognized antigens common to all serogroups of *L. pneumophila*, although only six of them had been used in the immunogen for reference antibody production. Based on cross-reaction experiments among serogroups 1 to 6, only one to four additional antigens may potentially be found in other *L. pneumophila* serogroups. The antigenic homogeneity (as defined by matching coefficients) of *L. pneumophila* serogroups was ≥0.91. By contrast, the matching coefficients of *T. micdadei*, *F. bozemanae*, *F. dumoffii*, and *F. gormanii* were 0.39, 0.45, 0.39, and 0.41, respectively. These findings are in complete agreement with our previous studies involving the Lp1P XIE reference system (10) and with those of Joly and Kenny (25).

Because antigenic homogeneity of the first eight serogroups of *L. pneumophila* was so high (≥0.91), it is easy to explain cross-reactivity among serogroups in serological tests. Although boiling of the Lp1-6 sonicates reduced from 58 to 16 the number of antigens recognized by the polyspecific antibody, these heat-stable antigens included the serogroup-specific antigen plus the common antigen of gram-negative bacteria and many other antigens common to multiple *L. pneumophila* serogroups.

Serological homogeneity of *L. pneumophila* has been studied by indirect (39) and direct (8, 30, 34) immunofluorescence and agglutination techniques (17, 24, 34, 35) with cross-absorption of antibody titers as the criterion of antigenic similarity. Antisera for such studies were prepared with Formalin or heat-killed *L. pneumophila* cells as immunogens. Although antibodies to multiple antigens probably participated in the net antibody titer measured, immunofluorescence and agglutination assays could not distinguish the specific antigens which participated in these serological tests. By XIE, up to 58 *L. pneumophila* antigens, unaltered by Formalin or heat treatment, were evaluated simultaneously. XLIE permitted in situ absorption of cross-reacting antibodies and visualization of the particular antigens which cross-react. With these techniques, *L. pneumophila* serogroups 1 to 8 were shown to share ≥91% of their antigens. These findings correlate well with the homogeneity of *L. pneumophila* phenotypic characteristics (2, 3, 6, 15, 20, 31, 36, 37), outer membrane protein profiles (11, 27), cellular fatty acid composition (28, 29), carbohydrate components (14, 38), isoprenoid quinone content (26), and deoxyribonucleic acid hybridization (4, 5).

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**LITERATURE CITED**

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