Antigenic Heterogeneity Among Legionella, Fluoribacter, and Tatlockia Species Analyzed by Crossed Immunoelectrophoresis

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Crossed immunoelectrophoresis (XIE) reference systems were established for Fluoribacter (Legionella) (containing Fluoribacter bozemanae, Fluoribacter dumofii, and Fluoribacter gormanii) and for Tatlockia (Legionella) micdadei. The Fluoribacter and Tatlockia XIE reference systems contained 54 and 72 anode-migrating antigens, respectively. These two systems, together with the previously described polyvalent Legionella pneumophila (serogroups 1 through 6) XIE reference system, were used to study the cross-reactivities of antigens from organisms comprising the three proposed genera in the family Legionellaceae. Antigenic homology was expressed as the matching coefficient (MC), the ratio of the number of cross-reactive antigens to the total number of antigens. The MCs for individual L. pneumophila serogroups when the polyvalent L. pneumophila antibody was used were 0.98 ± 0.05, which was significantly higher than the MCs determined by using Fluoribacter or Tatlockia antibodies (0.50 ± 0.13) (P < 0.001). The MCs for the three species of Fluoribacter when polyvalent Fluoribacter antibody was used were 0.93 ± 0.10, which was also significantly higher than the MCs when heterologous antibodies were used (0.40 ± 0.04) (P < 0.001). The MCs for T. micdadei with the two heterologous antibody preparations were similar to each other (0.32 and 0.46) and to all other heterologous MCs among members of the Legionellaceae. The MCs for organisms representing three other families of bacteria were 0.16 ± 0.04 in all three XIE reference systems and were significantly lower than the MCs among members of the Legionellaceae (P < 0.001). When a priori criteria for MC interpretation established in previous serotaxonomic studies of other bacterial species by XIE were used, our results from studies on the antigenic relationships among Legionella, Fluoribacter, and Tatlockia supported the proposal that there are multiple genera in the family Legionellaceae.

Antigenic analysis of proteins or peptides has been used to study phylogenetic relationships among both procaryotes and eucaryotes (11, 18, 44). In most studies, purified enzymes and monospecific polyclonal antisera have been used. These studies have demonstrated that protein antigens do not cross-react unless they are homologous and isofunctional and have a common phylogenetic origin (11, 42–44, 52), that the degree of cross-reaction is detectable until 30 to 40% of the amino acid sequence is substituted (43, 44), and that there is a linear correlation between immunological distance and percentage of deoxyribonucleic acid-ribonucleic acid homology down to homology values of 45 to 50% (4).

Crossed immunoelectrophoresis techniques are ideal for simultaneous evaluation of cross-reactions among multiple (40 to 90) protein antigens from different organisms. These techniques have been applied to serotaxonomic studies of Pseudomonas aeruginosa (28, 29), Neisseria meningitidis (25, 26), Haemophilus influenzae (47), Bordetella pertussis (24, 32), Salmonella typhi (16, 17), Staphylococcus aureus (46), and Staphylococcus epidermidis (15).

We previously reported one-way cross-reaction studies in which a Legionella pneumophila serogroup 1 cross immunoelectrophoresis reference system was used (13). Our findings were similar to those of Jolly and Kenny (33), who, also by using crossed immunoelectrophoresis, showed that there was a high degree of antigenic homogeneity among L. pneumophila serogroups 1 through 4 (27 of 31 antigens in common) and that only five antigens of Tatlockia micdadei cross-reacted with antibodies to L. pneumophila. Consequently, in 1981 we adopted the nomenclature validly published by Brown et al. (9, 21) and initiated production of crossed immunoelectrophoresis reference systems to demonstrate antigenic profiles for Fluoribacter and Tatlockia, as well as L. pneumophila (serogroups 1 through 6), as previously described (2), by using the type cultures available at that time. Using these three crossed immunoelectrophoresis systems, we quantitated the degrees of antigenic homology among the three genera.

We recognize the controversy concerning legionella nomenclature, but use the genus designations Fluoribacter and Tatlockia because our original hypothesis, upon which this study was founded, was that these organisms are sufficiently antigenically dissimilar to warrant classification as distinct genera in the family Legionellaceae.

MATERIALS AND METHODS

Bacterial strains. The strains of L. pneumophila serogroups 1 through 6 used in this study were strains ATCC 33153, ATCC 33154, ATCC 33155, ATCC 33156, ATCC 33216, and ATCC 33215, respectively. The Fluoribacter (Legionella) bozemanae serogroup 1, Fluoribacter dumofii, Fluoribacter gormanii, and Tatlockia (Legionella) micdadei strains used were strains ATCC 33217, ATCC 33279, ATCC 33297, and ATCC 33218, respectively. The Escherichia coli, Pasteurella multocida, and Pseudomonas aeruginosa strains used were clinical isolates from the culture collection of our laboratory.

Antigens prepared from these organisms and the homologous antibodies were designated as follows: Lp1, Lp2, Lp3, Lp4, Lp5, and Lp6 for L. pneumophila serogroups 1 through 6, respectively; Lp1-6 for the pool of all six L. pneumophila serogroups Fb for F. bozemanae; Fd for F. dumofii; Fg for

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F. gormanii; Fbdg for the pool of all three Fluoribacter species; and Tm for T. micaedai.

Antigen and antibody production and the crossed immunoelectrophoresis reference system for Lpl-6 have been described previously (2).

Antigen preparations. All organisms were cultivated on buffered charcoal yeast extract agar (GIBCO Diagnostics, Madison, Wis.) incubated at 37°C. The organisms were harvested by gently scraping the agar surface with a sterile bent glass rod, and then the cells were suspended in sterile distilled water. Antigen preparations were made by sonication (three times for 45 s) followed by high-speed centrifugation (20,000 × g for 60 min at 4°C) as described previously (2, 12). The colloidal concentration of each antigen preparation was determined by refractometry, using human immunoglobulin as a standard (12). Concentrations ranged from 13.3 to 29.3 mg/ml.

The pooled Lpl-6 reference antigen preparation has been described previously (2). A pooled reference antigen for Fluoribacter species (Fbdg) was prepared by using equal amounts (by weight) of all of the individual species antigen preparations. For T. micaedai, the type strain was used alone as the reference antigen. Identical antigen preparations were used for both rabbit immunizations and immunoelectrophoresis.

Reference antibody production. Ten adult New Zealand White rabbits were used for Fluoribacter reference antibody production, and five rabbits were used for T. micaedai reference antibody production. At each immunization, 100 μl of the antigen preparation was homogenized with 100 μl of incomplete adjuvant and injected intracutaneously at multiple sites on the back of the rabbit. The immunization and bleeding schedules were similar to those reported previously (12, 22). Antiserum for each group of rabbits harvested from months 5 to 17 were pooled, and the immunoglobulins were purified by using the method of Harboe and Ingild (22). The immunoglobulin concentrations were 20.8, 29.3, and 30.4 mg/ml for the Lpl-6, Fbdg, and Tm reference antibodies, respectively.

Crossed immunoelectrophoretic techniques. All immunoelectrophoretic procedures were performed as described previously (2, 12, 13, 29, 48) by using glass plates (5 by 7 cm), 1% agarose (Litex LSM, batch 812; Accurate Chemical and Scientific Corp., Westbury, N.Y.), and barbital buffer (pH 8.6; ionic strength, 0.1). First-dimension electrophoresis was performed at 10 V/cm until 2 μl of bromothymol blue-labeled human albumin marker migrated exactly 35 mm. Second-dimension electrophoresis was conducted at 2 V/cm for 18 to 20 h. During all electrophoretic procedures a constant temperature of 12°C was maintained by a recirculating cooler.

The following three crossed immunoelectrophoretic techniques were used: (i) crossed immunoelectrophoresis with a blank intermediate gel containing saline (100 μl of 0.154 M NaCl), referred to below as XIE (30); (ii) crossed immunoelectrophoresis with an intermediate gel containing antigen preparations at varying concentrations (this technique has been called crossed-line immunoelectrophoresis), referred to as XLIE (35); (iii) crossed immunoelectrophoresis with antibody-containing intermediate gels, referred to as XIE-Ab (1).

For all three techniques, a standard amount of antigen preparation (60 μg) was placed in the first-dimension well, and a standard amount of reference antibody (300 μl) was mixed with the agarose used to pour the second-dimension or back gel (final concentration, 14.3 μl/cm²).

MCs. Matching coefficients (MCs) were used to express the degrees of antigenic relatedness among species and genera in the family Legionellaceae. This numerical measure of antigenic similarity has been employed in several other serological studies (13, 15, 17, 27, 47). Each MC was calculated as a mean of four independent determinations, and all precipitation line quantitations were done without knowledge of the antigen or antibody reagents employed. The t test was used for pairwise comparison of mean MC values. A comprehensive review of the taxonomic application of crossed immunoelectrophoresis has recently been published (31).

Terminology. The terminology guidelines described by Chaparas et al. (10) for serotaxonomic studies in which immunodiffusion and immunoelectrophoresis are used were adhered to. The antibodies which cross-react with antigens from different bacterial isolates are those which bind specifically to one or more similar epitopes on the antigens. Antigens carrying such (similar) epitopes were designated cross-reactive antigens.

RESULTS

The Legionella (Lpl-6) XIE reference system has been described previously (2). With this system 58 precipitation lines were routinely demonstrated.

The Fluoribacter (Fbdg) XIE reference system (Fig. 1) had 54 precipitates, and the Tatlockia (Tm) XIE reference system (Fig. 2) had 72.

Cross-reacting antigens between genera were identified and quantitated by using three crossed immunoelectrophoretic techniques (XLIE, XIE-Ab, and XIE) with heterologous antigen preparations in the first-dimension gel.

All three techniques produced comparable results, but XIE in which heterologous antigen and antibody preparations were used, counting all precipitate lines as cross-reactive (Fig. 3), provided the easiest-to-read and most reproducible results. To confirm that all precipitate lines produced by XIE represented truly cross-reactive antigens, a control XLIE gel was run with the same combination of heterologous antigen and antibody preparations but with the reference antigen, homologous with the antibody, in the intermediate gel. In every instance, 100% of the antigens were absorbed in situ, proving their cross-reactivity (Fig. 3D). XLIE was also used to evaluate cross-reactivity among closely related species, i.e., among L. pneumophila serogroups as previously described (2) and among Fluoribacter species (Table 1).

The MCs for the antigens of Legionella, Fluoribacter, and Tatlockia were calculated by using the number of cross-reactive antigens counted by XIE, as shown in Fig. 3, and are listed in Table 2. The MCs for individual L. pneumophila serogroups when the polyvalent L. pneumophila antibody was used were 0.98 ± 0.05, which was significantly higher than the MCs determined with Fluoribacter or Tatlockia antibodies (0.50 ± 0.13) (P < 0.001). The MCs for the three species of Fluoribacter when polyvalent Fluoribacter antibody was used were 0.93 ± 0.10, which was also significantly higher than the MCs obtained when heterologous antibodies were used (0.40 ± 0.04) (P < 0.001). The MCs for T. micaedai with the two heterologous antibody preparations were similar to each other, 0.32 and 0.46, and to all other heterologous MCs among members of the Legionellaceae. The MCs for organisms representing three other families of bacteria were all <0.22 in all three XIE reference systems and were significantly lower than the MCs among members of the Legionellaceae (P < 0.001).
FIG. 1. *Floribacter* XIE reference system. A pooled antigen preparation (60 μg) containing equal amounts of *F. bozemanae*, *F. dumoffii*, and *F. gormanii* was placed in the first-dimension well (Fbdg Ag). Saline (NaCl) was placed in the intermediate gel. Purified immunoglobulins (300 μl; 14.3 μl/cm²) containing antibodies to the pooled *Floribacter* antigen were incorporated in the back gel (Fbdg Ab). In the first-dimension electrophoresis the anode was to the right. In the second-dimension electrophoresis the anode was at the top. The horizontal bar shows the migration of a human albumin marker in the first dimension. The gel was stained with Coomassie brilliant blue R. A total of 54 anode-migrating precipitates are numbered.

FIG. 2. *Tatlockia* XIE reference system. The first-dimension well contained 60 μg of *T. micdadei* antigen (Tm Ag). Saline (NaCl) was placed in the intermediate gel. Homologous purified antibodies (Tm Ab) were incorporated in the back gel (300 μl; 14.3 μl/cm²). For technical details see the legend to Fig. 1. A total of 72 anode-migrating precipitates are numbered.
FIG. 3. Crossed immunoelectrophoresis in which heterologous antigens were used in the first dimension. (A through D) A 300-μl amount of *L. pneumophila* antibody (Lp1-6 Ab) was in the back gel. (A) Control with 60 μg of homologous antigen (Lp1-6 Ag) in the first dimension and saline in the intermediate gel, showing 60 reference system precipitates (peaks). (B) *Fluoribacter* antigen (60 μg of Fbdg antigen [Fbdg Ag]) in the first dimension and saline in the intermediate gel. The *L. pneumophila* antibody precipitated 35 antigens. (C) *Tutlockia* antigen (60 μg of Tm antigen [Tm Ag]) in the first dimension and saline in the intermediate gel. The *L. pneumophila* antibody precipitated 27 antigens. (D) XLIE control for gel B. This gel contained the same first-dimension antigen (60 μg of Fbdg Ag) and back gel antibody (300 μl of Lp1-6 Ab) as gel B but had 50 μl of Lp1-6 Ag in the intermediate gel. This gel confirmed that all Fbdg antigens precipitated by Lp1-6 antibody do, in fact, cross-react with Lp1-6 antigens by the formation of lines of identity (arrows).

**TABLE 1.** Number of non-cross-reacting antigens among *F. bozemanae*, *F. dumoffii*, and *F. gormanii* determined by XLIE

<table>
<thead>
<tr>
<th>Antigen prepn in first dimension</th>
<th>No. of precipitates in control XIE gel</th>
<th>No. of non-cross-reacting antigens with the following antigen prepn in the intermediate gel:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>F. bozemanae</em></td>
</tr>
<tr>
<td><em>F. bozemanae</em></td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td><em>F. dumoffii</em></td>
<td>36</td>
<td>8</td>
</tr>
<tr>
<td><em>F. gormanii</em></td>
<td>36</td>
<td>8</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Genetic and biochemical data (3, 5–9, 20, 21, 23, 42, 49, 50), ubiquinone patterns (34), fatty acid analysis data (38–41), peptide profiles (14, 36), and carbohydrate compositions (19, 51) for members of the family *Legionellaceae* have been reported and are nicely summarized in a recent publication by Fox et al. (19). These data are relative measures of similarities and differences among the taxa studied and have been interpreted as evidence either that all legionellae cluster as members of a single genus (6, 7) or that subgroups within the *Legionellaceae* occur which are sufficiently unique to warrant designation as separate genera (9, 19, 21, 36, 50).

Such interpretations are primarily governed by the body of data which one chooses to use as a standard. For example, if a family of bacteria with a high degree of phylogenetic relationship, such as the *Enterobacteriaceae*, is used as a standard, then legionellae appear to be a relatively diverse group of organisms. On the other hand, comparison of legionellae with families having much broader and deeper phylogenetic groupings, such as the *Pseudomonadaeae*, makes legionellae appear relatively homogeneous.

XIE studies on the antigens of *Pseudomonas aeruginosa* by Højby in 1975 (28) revealed a high level of antigenic homogeneity within the species and established methods for reproducibly quantifying the number of cross-reactive anti-
gens between bacterial species by using quantitative immunoelectrophoresis techniques. A number of subsequent XIE studies on other bacterial species indicated a close agreement between the degree of antigenic homogeneity among bacterial species and their taxonomic relatedness as defined by more traditional methods (12, 16, 17, 26, 28, 32, 46, 47). For example, of six *Neisseria* species investigated, all had MCs of 0.79 with *N. meningitidis* (26). In contrast, 13 *Pseudomonas* species tested, all had MCs of 0.16 with the *Pseudomonas aeruginosa* reference system, whereas different serotypes of *Pseudomonas aeruginosa* had MCs of 0.97 (28). Members of the *Enterobacteriaceae* had MCs of 0.70 with *Salmonella typhi* (17). Bordetella pertussis and *Bordetella bronchiseptica* had MCs of 0.91 with *B. pertussis*, whereas *Brucella*, members of the *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *N. meningitidis*, and *H. influenzae* had MCs of 0.05 (32). Based on these studies, together with our previously published studies on XIE analysis of *L. pneumophila* (2), we defined, a priori, the following general criteria for taxonomic interpretation of antigenic relationships between organisms as defined by MCs: serovars or subtypes within a species have MCs of 0.90; species of the same genus have MCs of 0.70 to 0.90; genera within a family have MCs of 0.25 to 0.70; and distantly related taxa have MCs of 0.25.

In the present study we developed XIE reference systems for *Fluoribacter* and *Tatlockia* which demonstrated antigenic profiles containing 54 and 72 anode-migrating antigens, respectively. When these two systems, together with the previously described polyvalent *L. pneumophila* serogroup 1 through 6 system (2), were used, reciprocal cross-reaction studies demonstrated that MCs among *L. pneumophila* serogroups were 0.98 ± 0.05. MCs among *Fluoribacter* species were 0.86 ± 0.07 (based on the XLIIE data in Table 1), and MCs among the genera *Legionella*, *Fluoribacter*, and *Tatlockia* were 0.46 ± 0.12. By contrast, MCs for bacteria representing three other families were 0.16 ± 0.04, similar to previous findings (13). Thus, the antigenic homogeneity among serogroups of *L. pneumophila* was significantly higher than that found among *Fluoribacter* species (*P < 0.001). Antigen sharing among *Fluoribacter* species was significantly greater than antigen sharing among the three proposed genera of the *Legionellaceae* (*P < 0.001), and all stains of legionellae tested had significantly more antigens in common than they did with representatives of three other families of bacteria (*P < 0.001).

Our results illustrate the potential of XIE techniques as serotaxonomic tools, agree with proposed MC cut-off limits, and support the proposal that there are multiple genera in the family *Legionellaceae*, which is consistent with reports on the genetic distance between species of *Legionella* as determined by studies of 16S ribosomal ribonucleic acid (37) and deoxyribonucleic acid homology (6, 7, 9, 21). In addition, the relatively low degree of antigen sharing found among *L. pneumophila*, *Fluoribacter sp.*, and *T. micdadei* has practical implications for development of polyspecies-specific serodiagnostic tests.

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**LITERATURE CITED**


