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# Subtyping of *Legionella pneumophila* Serogroup 1 Isolates by Monoclonal Antibody and Plasmid Techniques

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**A group of environmental and clinical *Legionella pneumophila* serogroup 1 isolates was subtyped by monoclonal antibody dot immunoblotting and plasmid analysis. Monoclonal antibody analysis defined seven subtypes within three major groups. Plasmid analysis (including restriction endonuclease digestion) revealed 10 subtypes. By combining plasmid and monoclonal techniques, all 16 strains were shown to be distinct. Plasmid profiles and monoclonal antibody reactivities of selected strains were stable despite serial passage (>100 times). No plasmid-associated antigen was defined by this panel of monoclonal antibodies. The observed dissociation of plasmid profiles and monoclonal antibody reactivity patterns suggests that accurate epidemiologic typing of *L. pneumophila* serogroup 1 strains will require use of both techniques.**

Monoclonal antibody typing and plasmid analysis have been used in epidemiologic investigations of sporadic, epidemic, and endemic Legionnaires disease (1, 3–5, 7, 8, 10–12, 15–19, 21, 24, 26). These techniques have identified *Legionella pneumophila* strains isolated from nosocomial Legionnaires disease cases and strains found in the environment (in potable water supplies or nearby air-conditioning cooling towers). In cases in which more than one potential source of *L. pneumophila* dissemination was shown to be contaminated with organisms of the same serogroup, these methods enabled investigators to identify the most probable sources of the epidemic *L. pneumophila* strain. Outbreak control efforts, then, were directed toward decontamination of specific environmental reservoirs (8, 10, 12, 19, 24).

Plasmid analysis and monoclonal antibody typing have also been used to define subtypes within *L. pneumophila* serogroup 1 which may have different biological properties. Investigators have presented evidence that certain *L. pneumophila* serogroup 1 subtypes are more virulent than others (4, 21, 26), are better suited to persistence in the environment (4, 7, 26), or tend to segregate into specific environmental reservoirs within a defined geographic region (11, 15, 19, 21). Some data have also suggested that plasmid content is related to surface antigens of *L. pneumophila* serogroup 1 strains (4, 21). Although *L. pneumophila* serogroup 1 subtypes have displayed phenotypic differences, the clinical significance and the exact roles of the various plasmids and antigens in these biologic and ecologic differences have yet to be defined.

To better understand the utility of plasmid and monoclonal antibody techniques in epidemiologic investigations of Legionnaires disease and to further investigate the relationship of plasmid content and surface antigen composition, we examined a group of environmental and clinical *L. pneumophila* serogroup 1 isolates. These strains were subtyped according to their reactivities with a panel of six unique monoclonal antibodies and according to the results of plasmid analysis with restriction endonuclease fingerprinting.

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## MATERIALS AND METHODS

**Bacterial strains.** Clinical-1, Clinical-2, and Clinical-3 were *L. pneumophila* serogroup 1 strains cultured from community-acquired cases of Legionnaires disease diagnosed at the Ohio State University Hospitals, Columbus, Ohio (6). *L. pneumophila* serogroup 1 strains UH-1, UH-2, RH-1, and RH-2 were recovered from the potable water systems of the Ohio State University Hospitals, as well as from nosocomial Legionnaires disease cases associated with this institution (15). *L. pneumophila* serogroup 1 strains UPH, SLH, and CH-1 were environmental strains cultured from the potable water system of the Ohio State University Hospitals (UPH and SLH) or a community hospital (CH-1) in Columbus, Ohio. *L. pneumophila* serogroup 1 strain Pittsburgh-1 was kindly provided by Victor Yu. *Legionella bozemanii* WIGA and *L. pneumophila* serogroup 1 strains Albuquerque-1, Detroit-8, Knoxville-1, Bellingham-1, Longbeach-3, and Johannesburg-1E were kindly provided by James Barbaree of the Centers for Disease Control, Atlanta, Ga. Organisms were grown on buffered charcoal-yeast extract (BCYE) agar (GIBCO Diagnostics, Madison, Wis.) and identified by characteristic colonial morphology and direct fluorescent-antibody staining with reagents supplied by the Centers for Disease Control. Legionellae were analyzed after 48 to 72 h of growth on BCYE agar. Isolates were stored at −80°C in 50% glycerol–50% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Strains from Columbus, Ohio, were passed at most 3 or 4 times on BCYE agar before analysis. Strains from elsewhere were of uncertain passage history.

**Monoclonal antibodies.** A panel of eight unique agglutinating monoclonal antibodies was produced by immunizing BALB/c mice with Formalin-killed *L. pneumophila* serogroup 1 organisms as previously described (1, 20). Table 1 displays the designations of the monoclonal antibodies and the source and plasmid content of each immunizing strain.

**Dot Immunoblotting.** A suspension of live *L. pneumophila* serogroup 1 organisms (3 μl of a McFarland 5 opacity standard suspension in sterile water) was spotted on mixed cellulose-ester filters (Metricel; Gelman Sciences, Inc., Ann

TABLE 1. Plasmid content and sources of immunizing *L. pneumophila* serogroup 1 strains for production of monoclonal antibodies

| Monoclonal antibody[a] | Immunizing strain | Source | Plasmid content |
|---|---|---|---|
| 81 | Knoxville-1 | Centers for Disease Control | |
| 149, 75 | RH-1 | Environment, Columbus, Ohio | 45 and 85[b] MDa |
| 369, 103, 380 | CH-1 | Environment, Columbus, Ohio | 85 MDa |
| 252, 26 | UPH | Environment, Columbus, Ohio | 35 MDa |

[a] Numbers are designations of hybridoma media used in immunologic studies.
[b] The 85-MDa plasmids were identical by *Eco*RI restriction.

Arbor, Mich.), and the filters were dried overnight at 35°C. Spotted filters were then incubated, with mild agitation, for 1 h at room temperature in 5 ml of 3% bovine serum albumin (RIA grade; Sigma Chemical Co., St. Louis, Mo.) in 0.01 M phosphate-buffered saline (pH 7.2). Next, filters were incubated for 1 h at room temperature with 3 ml of monoclonal antibody media derived from a hybridoma cell suspension containing approximately $10^5$ cells per ml. Filters were then washed by four 10-min incubations at room temperature in 5 ml of phosphate-buffered saline. Color was developed by incubating the filters with peroxidase-labeled, affinity-purified antibody to mouse anti-immunoglobulin G and -immunoglobulin M (heavy and light chains) goat and human sera absorbed (Kirkegaard and Perry, Gaithersburg, Md.) at a 1/1,000 dilution in 1% bovine serum albumin in phosphate-buffered saline. Another wash with 4 to 5 ml of phosphate-buffered saline was followed by the addition of 1 to 3 ml of 0.06% 4-chloro-1-naphthol (Sigma) and 0.012% hydrogen peroxide solution. Color was allowed to develop in a 5-min incubation at room temperature, and the reaction was stopped by washing filters liberally with distilled water. Results were scored as positive (a definite blue color on the spot), negative (lack of color on the spot, which was identical to control spots), or weak (a faint outline of the spot). The negative controls were similar spots of a suspension of live *L. bozemanii* WIGA cells and spotted filters incubated with RPMI-1640 medium (Whittaker M.A. Bioproducts, Walkersville, Md.) containing 15% fetal bovine serum rather than with monoclonal antibody media.

**Plasmid analysis.** Plasmid analysis was conducted by the methods of Kado and Liu (13), Tompkins et al. (25), and Shlaes and Currie (23). Legionellae were grown on BCYE plates and harvested at 48 to 72 h of growth. Crude phenol-chloroform-extracted lysates were further purified by sequential extraction with chloroform-isoamyl alcohol (20/1) and chloroform, salt precipitation with cold 4 M sodium acetate, and precipitation with an equal volume of isopropanol or 2 volumes of 95% ethanol. Plasmid DNA precipitates were digested with restriction enzyme *Eco*RI (Bethesda Research Laboratories, Gaithersburg, Md.) under conditions recommended by the manufacturer. Plasmid preparations and restriction digests were then electrophoresed on agarose gels, and DNA was visualized with ethidium bromide stain under UV light. Molecular weights of plasmids and restriction fragments were determined by comparison with plasmids of *L. bozemanii* WIGA (2) and *Hin*dIII restriction fragments of lambda phage DNA (Bethesda Research Laboratories), respectively.

**Serial-passage experiments.** Strains UH-2, RH-1, and CH-1 were serially passed on BCYE agar over 100 times. Each passage consisted of a 72-h growth. Plasmid content and monoclonal antibody reactivity were examined at 1- to 2-week intervals. In this series of experiments, monoclonal antibody reactivity was examined by microagglutination assay, as previously described (1, 20), rather than by dot immunoblotting.

## RESULTS AND DISCUSSION

Sixteen *L. pneumophila* serogroup 1 isolates were subtyped by monoclonal antibody dot immunoblotting (with a six-antibody panel) and plasmid analysis. Results are summarized in Table 2. Among these 16 strains, three major groups were recognized by their reactions to monoclonal antibodies 81 and 149. Group 1 was 81 positive and 149 negative, group 2 was 81 negative and 149 positive, and group 3 was 81 negative and 149 negative. None was found to be both 81 and 149 positive. If weak reactions are interpreted as different from negative or positive reactions, there were seven subgroups of serogroup 1 defined by the monoclonal antibody typing alone. Plasmid typing alone revealed 10 subtypes with and 7 subtypes without restriction endonuclease digestion. Combined plasmid (with *Eco*RI digestion) and monoclonal antibody analysis was able to distinguish each of these 16 strains from each other. If weak reactions to monoclonal antibodies were considered either positive or negative, the same number of isolates could have been distinguished by combined analysis.

Plasmid profiles and monoclonal antibody reactivities (by microagglutination) of strains RH-1, UH-2, and CH-1 were not altered by more than 100 serial passages on BCYE agar. For strain UH-2, microagglutination differed from dot immunoblotting only in reaction to antibody 380; dot immunoblotting revealed a weak positive, and microagglutination was

TABLE 2. Subtype pattern of *L. pneumophila* serogroup 1 strains

| Group and strain | Reaction[a] to monoclonal antibody: | | | | | | Plasmid (MDa) |
|---|---|---|---|---|---|---|---|
| | 81 | 149 | 75 | 103 | 380 | 26 | |
| Group 1 | | | | | | | |
| UH-1 | + | − | + | − | W | + | |
| UH-2 | + | − | + | − | W | + | 45[b] |
| Clinical-1 | + | − | + | − | W | + | 85[c] |
| Albuquerque-1 | + | − | + | W | + | + | 85[c] |
| Clinical-2 | + | − | + | W | W | + | 35[d] |
| Clinical-3 | + | − | + | − | W | − | 35[d] |
| Group 2 | | | | | | | |
| Bellingham-1 | − | + | + | + | + | − | |
| RH-2 | − | + | + | + | + | − | 12 and 45 |
| RH-1 | − | + | + | + | + | − | 45 and 85[c] |
| Detroit-8 | − | + | + | + | + | − | 85[d] |
| Pittsburgh-1 | − | + | + | + | + | − | 85[c] |
| Group 3 | | | | | | | |
| CH-1 | − | − | + | + | + | + | 85[c] |
| UPH | − | − | + | + | + | + | 35[d] |
| Longbeach-3 | − | − | + | + | + | + | 90[e] |
| Johannesburg-1E | − | − | + | + | + | + | 90 |
| SLH | − | − | + | + | − | + | |

[a] +, Positive; −, negative; W, weak.
[b] The 45-MDa plasmids were identical by *Eco*RI restriction.
[c] Designated 85-MDa plasmids were identical by *Eco*RI restriction.
[d] Designated plasmids were different from plasmids of same molecular weight by *Eco*RI restriction.
[e] The 90-MDa plasmids were very similar (see text) by *Eco*RI restriction.

clearly negative. For strain RH-1, microagglutination was negative with antibody 103, but dot immunoblotting gave a clearly positive reaction. Strain CH-1 displayed the same pattern regardless of the reactivity assay. As noted above, the dot immunoblotting assay was not evaluated on the serially passed isolates.

By *Eco*RI restriction, the 85-megadalton (MDa) plasmid common to strains Clinical-1, Albuquerque-1, Pittsburgh-1, CH-1, and RH-1 appeared to be identical. This was also true of the 45-MDa plasmid found in strains UH-2, RH-1, and RH-2. The 90-MDa plasmids of strains Johannesburg-1E and Longbeach-3 were quite similar, with 15 fragments in common. Unique to Longbeach-3 were two fragments in the 2- to 2.5-kilobase-pair range. Strain Johannesburg-1E lacked these two small fragments but had a distinctive fragment of approximately 15 kilobase pairs. The 85-MDa plasmid of strain Detroit-8, the 35-MDa plasmid of strain UPH, the 35-MDa plasmid of strain Clinical-2, and the 35-MDa plasmid of strain Clinical-3 were unique by *Eco*RI analysis and unrelated to plasmids of similar size. In general, plasmids were cut into 10 to 15 fragments in a molecular weight range of approximately 2 to 23 kilobase pairs by restriction enzyme *Eco*RI. These fragments were well resolved on 1.2% agarose gels, resulting in easy recognition of different restriction patterns.

Plasmidless strains were plasmidless on five or more occasions. Strains Bellingham-1 and UH-1 were plasmidless by the method of Kado and Liu (13) as well as by other alkaline lysis methods (23, 25). Plasmidless strains were noted in all major groups. No plasmid-associated antigen was defined by our panel of monoclonal antibodies. The blotting of spotted filters with monoclonal antibody media 369 and 252, additional unique antibodies derived from these mouse immunizations and cell fusions, did not either add to the discrimination of these strains or define any plasmid-associated antigens (data not shown).

The dot immunoblot assay used in monoclonal antibody typing is similar to methods used to screen hybridoma clones for monoclonal antibody production (9). It is also similar to a method for the detection and quantitation of *L. pneumophila* described by Barbaree et al. (J. M. Barbaree, W. T. Martin, J. C. Feeley, P. L. Garbe, and L. E. Markowitz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q6, p. 205). In other published studies of monoclonal antibody typing of legionellae, an indirect fluorescent-antibody technique has been used (7, 16, 26). We did not compare these two techniques, but we discriminated between strains by using approximately the same amount of antigen that was used in the indirect fluorescent-antibody assays. Watkins (26) used 10 μl of a $10^8$-CFU/ml suspension of legionellae on the slide. We used 3 μl of a McFarland 5 suspension, roughly $15 \times 10^8$ CFU/ml (14). Most spots were clearly positive or negative, although some variable reactivity was noted in group 1. The dot immunoblot method would seem to be no less quantitative than the indirect fluorescent-antibody test and somewhat cheaper to conduct since it requires no fluorescence microscope. On the negative side, it required about 3 ml of monoclonal antibody medium to cover the filters in a petri dish. Because of the ready availability of monoclonal antibody reagents, this medium requirement may be less of a problem. Also, it may well be that smaller amounts of medium may be sufficient to conduct the assay (9).

In addition to identifying the same plasmids among different monoclonal antibody subtypes, we identified strains of very similar or identical monoclonal antibody type and plasmid content from widely different geographic origins. These findings suggest that certain *L. pneumophila* serogroup 1 strains, as well as plasmids, have been disseminated worldwide. It also suggests that plasmid transfer in the local aquatic microenvironment may be less important in the determination of the plasmid content of *L. pneumophila* strains, unless the plasmid content of other components of the potable water flora has been given the same broad geographic dissemination. It is of interest that a survey of the plasmid content of five nonfermentative, gram-negative bacilli from the same faucet from which strain RH-2 was isolated revealed no plasmids of the same sizes (W. E. Maher, unpublished observations).

Our combined subtyping system appeared to be based on stable markers and was able to define many subtypes within serogroup 1. Whether the identity of strains as determined by this or any other typing scheme implies clonal identity remains to be proved by additional epidemiologic data. Examination of the origins of some of our test strains, though, does yield some insight into the biologic significance of our subtypes. For example, strains UH-1 and Clinical-1 had identical monoclonal antibody types but quite different plasmid types. Clinical-1 was a community-acquired strain from a nursing home Legionnaires disease case in Columbus, Ohio; UH-1 was the predominant strain in the potable water system of the main university hospital and was responsible for 29 of 36 culture-positive nosocomial Legionnaires disease cases from that building (15; W. E. Maher, M. F. Para, and J. F. Plouffe, unpublished results). Strains UH-1 and UPH, isogenic by the sensitive enzyme isotyping techniques of Selander et al. (22), differed in plasmid content and reactivity with three of six of our monoclonal antibodies. We have previously demonstrated (15) that strains UH-1 and UPH have segregated into different buildings of the same medical center. Each building has the same main water supply but separate recirculating hot water systems.

There is also evidence that plasmid analysis would enhance the discrimination of other monoclonal antibody panels. Selander et al. (22) reported monoclonal antibody typing of two strains included in the present study, i.e., RH-1 and Bellingham-1. These strains were identical by the monoclonal antibody panel (McKinney's) used in that study, as they were by our own panel. It was only by plasmid analysis, as reported above, and the multilocus genotyping system of Selander et al. that the two strains were distinguished. In the study of Edelstein et al. (7), there was a correlation of certain plasmid types with certain monoclonal antibody reactivity patterns and multilocus genotypes, but different plasmid patterns were observed within the subtypes defined by these techniques. It would appear that future epidemiologic studies of Legionnaires disease should use both plasmid and monoclonal techniques.

Our panel of monoclonal antibodies failed to identify a plasmid-associated antigen. Because our monoclonal antibodies have been shown to recognize endotoxin epitopes (M. F. Para, W. E. Maher, and J. F. Plouffe, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 156, 1984), this result was not unexpected. Definition of a plasmid-associated antigen may require probing strains with other existing monoclonal antibody panels or with a new group of monoclonal antibody reagents directed at a totally different class of antigens. Despite epidemiologic data presented by Plouffe et al. (21) and Brown et al. (3) suggesting decreased virulence of plasmid-bearing strains, the function of these plasmids remains cryptic. The presence of similar plasmids in strains from widely separated geographic regions suggests similar ecology of legionellae in

different parts of the world and similar environmental stresses in these habitats.

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