Plasmid and Surface Antigen Markers of Endemic and Epidemic *Legionella pneumophila* Strains

A. BROWN,^{1,2,3*} R. M. VICKERS,³ E. M. ELDER,³ M. LEMA,^{1,3} AND G. M. GARRITY³

William Jennings Bryan Dorn Veterans' Hospital,^{1*} and Department of Medicine, University of South Carolina School of Medicine,² Columbia, South Carolina 29201, and Veterans Administration Medical Center, Pittsburgh, Pennsylvania 15140³

Received 8 February 1982/Accepted 30 April 1982

Environmental and clinical isolates of *Legionella pneumophila* obtained from the Pittsburgh Veterans Administration Medical Center were studied for the presence of plasmids and for unique surface antigens. The majority of environmental isolates contained a single 80-megadalton plasmid. After an epidemic of nosocomial Legionnaires disease subsided in the spring of 1981, plasmid-bearing environmental isolates persisted in the environment. Whereas *L. pneumophila* could not be reisolated from most sites with plasmidless isolates. During this epidemic the attack rate was highest on wards with plasmidless isolates. All clinical isolates were plasmidless. Strains were serotyped by the indirect immunofluorescence method with serum from a single immunized rat which was used both without absorption and after absorption with various plasmid-bearing and plasmidless isolates. These studies suggested that a plasmid-associated surface antigen was present and that the most common plasmidless environmental serotype was similar to the epidemic clinical serotype.

Plasmids and bacterial surface antigens have been used to identify and characterize epidemic strains of organisms involved in outbreaks of disease (3, 11). Although plasmids of unknown function in some strains of *Legionella pneumophila* and other *Legionella*-like organisms have been described (1, 8, 10), they have not been studied as epidemiological markers or in relation to disease production. Similarly, although six serogroups of *L. pneumophila* have been recognized (9) and some antigenic subtypes have been described (6, 17), the strains involved in outbreaks have not been studied for unique antigenic markers. Our data suggest that: (i) plasmid and surface antigenic markers may be useful in studying outbreaks of Legionnaires disease; (ii) although strains with a single unique plasmid were more prevalent and persistent in our hospital environment, only plasmidless strains were associated with disease; and (iii) unique surface antigens may be associated with plasmid-bearing strains.

MATERIALS AND METHODS

Bacterial media and strains. Bacteria were grown on buffered charcoal-yeast extract agar (12) for plasmid and antigen studies. Stocks were stored in 50% calf serum-50% tryptic soy broth at -20°C. Plasmid and antigenic properties were stable for at least 9 to 12 months under these conditions of storage. The primary isolation and identification techniques used have been described previously (5, 6).

Environmental isolates were obtained at the Pitts-

FIG. 1. Agarose (0.7%) gel with examples of the plasmids of *Legionellaceae*. Lanes: 1, *L. pneumophila* Atlanta 4; 2, *L. pneumophila* PUH 6; 3, *L. pneumophila* Atlanta 1; 4, *F. bozemanii* WIGA; 5, *L. pneumophila* SICU 5; 6, *L. pneumophila* HWT-3.

TABLE 1. Influence of the presence or absence of a plasmid in environmental ward isolates on the attack rate during the 1981 outbreak of Legionnaires disease

Type of isolate found in ward	No. of cases	No. of discharges ^a	Attack rate ^b	Relative risk ^c
With plasmid	1	205	4.9	0.21
Without plasmid	6	112	53.6	2.4
Both ^d	2	162	12.3	0.54
Not sampled at time of outbreak	6	181	33.3	1.5
All wards	15	660	22.7	1.0

^a During the 5-week period the outbreak was observed.

^b Number of cases per 1,000 discharges.

^c Attack rate compared with that of all wards.

^d Three wards contained both plasmid-containing and plasmidless isolates. No cases were acquired on one of these wards. On one ward the site from which a plasmidless isolate was obtained was in the room of the patient who acquired Legionnaires disease on that ward.

burgh Veterans Administration (VA) Medical Center as part of an ongoing environmental survey of 50 plumbing fixtures in 18 hospital areas as previously described (15). Isolates were also obtained from the hot and cold water tanks of these hospitals. These sites included patient care areas and nonpatient areas. Samples were obtained from shower heads, shower mixing valves, spigots, hot and cold shut-off valves, hot and cold supply lines, and both water and sediment from the hot and cold tanks. The interior surfaces of the plumbing fixtures were vigorously scrubbed with Dacron-tipped swabs. These swabs and water samples obtained before and after swabbing were cultured.

Clinical isolates of *L. pneumophila* were obtained from 23 of the 60 patients with Legionnaires disease seen at the Pittsburgh VA Medical Center. A total of 11 of the 23 isolates were obtained from 15 nosocomial cases occurring during a 5-week period beginning 30 March 1981.

Plasmid extraction and detection. Plasmid extraction and electrophoresis were performed by using a modification of the procedure of Kado and Liu (7). Briefly, two 10- μ l loops of bacteria were harvested from buffered charcoal-yeast extract agar after 3 days of growth at 37°C. The bacteria were suspended in 1 ml of running buffer (40 mM Tris, 2 mM disodium EDTA, adjusted to pH 7.9 with glacial acetic acid); then 2 ml of lysis buffer (3% sodium dodecyl sulfate, 50 mM Tris, adjusted to pH 12.6 with 2 N NaOH) was added, and the solution was gently mixed by inverting the tube 12 times. After 20 min at 65°C, 2 volumes of phenol-chloroform (1:1, vol/vol) was added. The solution was again gently mixed and centrifuged for 10 min at 10,000 rpm in a Sorvall RC2-B centrifuge with an SS-34 rotor. A polyethylene Pasteur pipette with a large opening was used to remove the relatively non-viscous portion of the supernatant, which contained the plasmid(s). A 40- μ l portion of this supernatant was mixed with 10 μ l of running buffer containing 0.07% bromophenol blue, 0.7% sodium dodecyl sulfate, and 33% glycerol, and 25 μ l of this mixture was subjected to horizontal slab gel electrophoresis at 145 V for 2.5 h in 0.7% agarose. Molecular weight markers for each run included extracts of the plasmid-containing *L. pneumophila* isolate Atlanta 1 (1), the *Fluoribacter bozemanii* isolate WIGA (1, 10), *Escherichia coli* strains containing the plasmids pBR322 (2) and NTP 1 (14), and a *Pseudomonas aeruginosa* isolate contain-

ing the plasmid R68.45 (13). Running buffer was recirculated to minimize polarization. The gel was then stained for 45 min in a solution of 0.5 μ g of ethidium bromide per ml, and DNA bands were visualized with short-wave UV light.

Detection of unique surface antigens. Antigenic differences among strains of *L. pneumophila* were distinguished by antiserum absorption studies. Sera of rats surviving 10 days after intratracheal infection with the

TABLE 2. Strains used for antigenic subtyping

Isolate	Source ^a	Plasmid ^b
55	Human (MAL)	—
58	Human (FOS)	—
59	Human (CAP)	—
60	Human (RHU)	—
61	Human (CAL)	—
65	Human (BLR)	—
66	Human (SMA)	—
45	Environment (11ES)	—
82	Environment (7W)	—
89	Environment (9E-39)	—
120	Environment (MICU-1 isolate no. 2)	—
121	Environment (7W-LS)	—
129	Environment (HWT 1/2)	—
B4-3	Rat infected with isolate 129	—
42	Environment (MICU-1 isolate no. 1)	+
116	Environment (HWT 3)	+
D7-4	Rat infected with isolate 116	+
D6-1	Rat infected with isolate 116	+
71	Environment (1C-MH)	+
88	Environment (11EJ)	+
99	Environment (9E-30)	+
87	Control serogroup 5-reacting environmental isolate	—
Knox	Human (Knoxville 1) ^c	—

^a Strain designations are given within parentheses.

^b —, Plasmidless; +, plasmid containing.

^c Obtained from the Centers for Disease Control, Atlanta.

TABLE 3. Antigenic subtyping of clinical, environmental, and rat-passaged isolates^a

Serum used for absorption reaction ^b	Staining intensity of following isolate:						
	Clinical						
	55	58	59	60	61	65	66
None	+	+	+	+	+	+	+
116	—	—	—	—	—	—	—
D7-4	—	—	—	—	—	—	—
71	+	+	—	+	+	—	+
121	—	—	—	—	—	—	—
B4-3	—	—	—	—	—	—	—
Knox	—	—	—	—	—	—	—
60	—	—	—	—	—	—	—
61	—	—	—	—	—	NT ^d	NT

environmental isolate HWT-3 (plasmid containing), the environmental isolate HWT 1/2 (plasmidless), or the clinical isolate CAR (plasmidless) were tested for homologous antibody by indirect immunofluorescence (IFA), and those sera with IFA titers of $\geq 1:160$ were used in absorption studies. Sera from uninfected rats were used as negative controls.

The immune rat sera were absorbed with heat-inactivated organisms by mixing equal volumes of serum and packed bacterial cells as previously described (6). This suspension was incubated at 37°C for 1 h and then at 4°C overnight. The suspension was then centrifuged to pack the bacteria, and the filtered supernatant was used in IFA tests.

The IFA procedure of Wilkinson et al. (16) was modified as follows. Antigen-coated slides were prepared with 20- μ l drops of a suspension in 1% buffered formalin of various environmental and clinical *L. pneumophila* isolates taken from 72-h-old buffered charcoal-yeast extract agar cultures. The turbidity of these suspensions had been adjusted to that of a McFarland no. 1 standard. Smears were air dried and heat fixed. Absorbed and unabsorbed rat sera were tested undiluted, and specific antigen-antibody complexes were detected with an fluorescein isothiocyanate-labeled rabbit anti-rat immunoglobulin G conjugate (Miles Laboratories, Inc., Elkhart, Ind.). Smears were examined by epifluorescence, and the intensity of fluorescence was graded and recorded as previously described (16).

RESULTS

Figure 1 shows a sample agarose gel displaying the various plasmids detected in representative members of *Legionellaceae*. Atlanta 4 contained a 23-megadalton (Mdal) plasmid (lane 1) which has not previously been described. PUH6, an environmental isolate from a neighboring hospital, contained a single, 37-Mdal plasmid (lane 2). The previously described (1) 62-Mdal plasmid in Atlanta 1 and the 35-, 40-, 45-, and 80-Mdal plasmids (1) of *F. bozemanii* (5) (*Legionella bozemanii*) WIGA can be seen in lanes 3 and 4, respectively, however, the bands representing the two smaller plasmids in WIGA are barely resolved on these gels. The remaining

lanes (5 and 6) show examples of the 80-Mdal plasmid from two of the plasmid-containing environmental isolates from the Pittsburgh VA Medical Center. Environmental strains from this hospital either contained this plasmid or contained no plasmid at all. Not only were these plasmids uniform in size, but their identity was confirmed by the consistent patterns of fragmentation by the endonucleases *Bgl*II and *Kpn*I (data not shown).

A total of 55 sites within the plumbing system of the Pittsburgh VA Medical Center were sampled on different surveys, and 17 of these sites were sampled on several occasions. Of these, 38 sites were positive for *L. pneumophila* on one or more occasions. The 38 positive sites included multiple sites on 10 of 13 wards, the 3 (2 single and 1 tandem-double) hot water tanks, 1 laboratory, 2 clinic areas, and 3 office areas. From these positive sites, 62 serogroup 1 *L. pneumophila* isolates were obtained; of these, 49 were available for testing, representing 32 of the 38 positive sites. A total of 30 isolates from 20 of the 32 sites contained the single 80-Mdal plasmid; these plasmid-containing isolates were found on 7 of the 10 wards from which *L. pneumophila* was isolated. A total of 13 sites, including sites on 5 of the wards studied, contained plasmidless isolates. Three wards contained both plasmid-containing and plasmidless isolates, although most isolates obtained from these units contained the 80-Mdal plasmid. None of the 23 isolates obtained from patients at the Pittsburgh VA Medical Center contained a plasmid.

During a 5-week period beginning 30 March 1981, a nosocomial outbreak of Legionnaires disease involved 15 patients at the Pittsburgh VA Medical Center; 11 of these patients had respiratory tract cultures positive for *L. pneumophila*. Three culture-positive community-acquired cases were also seen during this period. Coincident with but before we were aware of the

TABLE 3—Continued

Staining intensity of following isolate:														
Plasmidless Environmental							Plasmid-bearing Environmental						Control	
45	82	89	120	121	129	B4-3	42	116	D7-4 and D6-1 ^c	71	88	99	87	Knox
+	+	+	+	+	+	+	+	+	+	+	+	+	—	+
—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
—	—	—	—	—	—	+	—	—	—	—	—	—	—	+
—	—	—	—	—	—	—	+	—	—	—	—	—	—	—
—	—	—	—	—	—	—	+	+	+	+	+	+	—	+
—	—	—	—	—	—	—	+	+	+	+	+	+	—	—
—	—	—	—	—	—	—	+	+	+	+	+	+	—	—
—	—	—	—	—	—	+	+	+	+	+	+	+	—	—
—	—	—	NT	—	NT	—	+	+	+	+	+	+	—	—

^a Antibody binding was detected by indirect immunofluorescence by using fluorescein-conjugated rabbit anti-rat immunoglobulin. +, 3+ to 4+ staining; —, 0 staining to $\pm$ intensity of staining. See Table 2 for index to strains used.

^b The serum for all reactions indicated was obtained from a rat (D5-6) infected intratracheally with isolate 116 (HWT3), a plasmid-containing environmental isolate. The titer of this serum was 1:320 by IFA.

^c Isolates D7-4 and D6-1, obtained from animals infected with isolate 116, gave identical reactions, as expected.

^d NT, Not tested.

onset of this outbreak, routine environmental surveillance had been initiated at 10 sites chosen to represent a geographic cross section of the medical center. To our surprise, since some sites selected had been negative on several previous surveys, *L. pneumophila* was isolated from all sites sampled on that occasion. Table 1 shows the difference in attack rates on the wards from which plasmid-bearing or plasmidless isolates had been recovered on that survey. A total of 6 of the 15 nosocomial cases occurred on wards which had not been sampled at the time of the outbreak. Of the remaining nine cases, six occurred on wards from which only plasmidless isolates had been obtained; only one patient acquired the disease on a unit from which only a plasmid-containing isolate had been recovered. The risk of developing disease, calculated from the ward-specific attack rates, was over 10 times greater if a patient was hospitalized on a ward from which only a plasmidless isolate had been obtained rather than on a unit with a plasmid-containing isolate. When the outbreak subsided, *L. pneumophila* could no longer be recovered from five of the six sites tested from which plasmidless strains had been isolated; one site with a plasmidless isolate before the outbreak contained a plasmid-bearing isolate after the outbreak, and all four sites tested which had plasmid-containing isolates remained positive ($P < 0.05$ by the Fisher exact test).

Specific surface antigens were studied by reacting isolates with serum from rats infected with the sera from the other animals are not shown. Unabsorbed sera from all animals with three types of isolates: clinical, without

plasmid; environmental, without plasmid; and environmental, with plasmid. The strains tested are listed in Table 2. Sera were absorbed with strains as indicated. The data obtained with serum from an animal which had been infected with the plasmid-containing environmental isolate HWT-3 are shown in Table 3; the reactions stained every serogroup 1 isolate tested. When sera were selectively absorbed, however, several different reaction patterns became apparent. A reactivity that was present in the serum of the animal infected with the plasmid-bearing isolate was eliminated by absorption with other plasmid-bearing isolates, but not by absorption with plasmidless isolates. This reactivity was not present in the sera of the animals infected with clinical or environmental plasmidless isolates obtained from the Pittsburgh VA Medical Center (data not shown). Plasmidless environmental isolates had two patterns of reactivity. Six of the seven tested reacted only with the unabsorbed antiserum; the pattern of one isolate differed slightly. The clinical isolates were similar or identical to the majority of plasmidless isolates tested in their reactivity. Although further studies are needed to clarify some of the slight differences observed, the data support the utility of such refined antigenic studies in the investigation of an outbreak and suggest, as do the plasmid studies, that the plasmidless environmental strains were associated with the observed outbreak.

DISCUSSION

At one time or another *L. pneumophila* was isolated from 38 of the 55 sites studied (69%) at

the Pittsburgh VA Medical Center. The hot water circuit was positive more often and usually contained larger numbers of organisms than did the cold water system. The majority (61%) of these environmental isolates contained a single 80-Mdal plasmid. The rapid, simple alkaline sodium dodecyl sulfate extraction procedure of Kado and Liu (7) allowed a more efficient recovery of plasmid DNA than did other extraction procedures previously used for the study of plasmids in *Legionellaceae* (1, 8, 10). Oddly, in none of the isolates obtained from 23 patients with Legionnaires disease could a plasmid be detected.

Experiments with rats demonstrated that both plasmid-containing and plasmidless strains could infect animals on intratracheal inoculation and that plasmids were not lost on passage in these animals (data not shown). Experiments to accurately quantitate the relative virulence of these strains and strains cured of plasmids are in progress. However, the isolates obtained from the lungs of lethally infected animals and survivors at 8 to 14 days after infection remained either plasmid bearing or plasmidless, depending on the infecting strain used.

At the onset of an explosive outbreak of Legionnaires disease involving 15 nosocomial cases over a 5-week period in the spring of 1981, the routine sampling of 11 hospital sites was initiated coincidentally. This sampling was continued monthly thereafter. At the onset of the outbreak, all 10 sites sampled contained *L. pneumophila*, 4 with plasmid-containing isolates and 6 with plasmidless isolates. For 3 months after the outbreak (before an attempt at eradication), all sites with plasmid-containing isolates continued to harbor these organisms, whereas the organism could no longer be isolated from five of the six sites with plasmidless *L. pneumophila*. At the 1 site which remained positive, only a plasmid-bearing isolate was obtained on several subsequent samplings. This suggested that the plasmid-bearing strains were better adapted to survival in our environment; however, other explanations cannot be excluded.

The case attack rate during the 1981 outbreak was highest on the wards from which plasmidless strains of *L. pneumophila* were isolated. On the wards with plasmid-containing isolates, the attack rate (4.9 cases per 1,000 discharges) was similar to that which we previously reported (5.0 cases per 1,000 discharges) for endemic disease at the Pittsburgh VA Medical Center (4). The higher attack rate associated with the presence of plasmidless strains may be due to the enhanced virulence of those organisms, a property which may be independent of the presence or absence of the observed plasmid. Alternatively, those strains marked by the absence of a plasmid

may have been introduced into an environment from which they could be more efficiently disseminated, without there being any difference in virulence among the various isolates.

Because of the broad and repeated environmental sampling, estimates of the prevalence and distribution of plasmid-bearing and plasmidless strains should correspond to the actual distribution of these isolates; however, since we were not aware of the presence of plasmids in our strains at the time of sampling, several colonies of each isolate were not available for plasmid analysis. Although random colonies were selected for preservation, it is possible that some bias was introduced into colony selection. It would, however, be a great coincidence if it were only by happenstance that the attack rate of disease was higher on the units from which the plasmidless isolates were preferentially preserved and that these isolates were no longer culturable after the subsidence of the outbreak. In addition, with the presence of the plasmid-containing isolates in our environment, it is peculiar that such an isolate was never obtained from our patients.

By selective absorption, serum from a rat infected with an environmental plasmid-containing isolate provided a set of reagents which could be used for the serological subtyping of isolates. With these reagents, certain patterns emerged which suggested that specific surface antigens were present in plasmid-bearing strains and that the most common plasmidless environmental isolate may have been associated with the outbreak of disease.

The utility of applying these techniques to studies of the epidemiology of Legionnaires disease is clear. Our inability to distinguish one strain from another of the same serotype has seriously hampered our ability to investigate outbreaks. In addition, the exciting possibility that the plasmid present in our strains may modify virulence or environmental adaptability obviously warrants further study. As previously noted, the strain differences may, of course, be unrelated to the presence or absence of this plasmid, and obviously the properties of these strains cannot be regarded as representative of all strains until confirmed by detailed studies elsewhere. It should be pointed out that the strains Atlanta 1, 2, and 4 and the isolate OLDA are plasmid-bearing clinical isolates (1, 10), clearly indicating that not all plasmids in *L. pneumophila* possess the properties that we postulate to be associated with the plasmids in the isolates we studied.

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LITERATURE CITED

1. Aye, T., K. Wachsmuth, J. C. Feeley, R. J. Gibson, and S. R. Johnson. 1981. Plasmid profiles of *Legionella* species. *Curr. Microbiol.* 6:389-394.
2. Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2:95-113.
3. Brown, A., L. Davis, R. Yee, and B. Postic. 1978. Endemic *Serratia marcescens* at the Veterans Administration Hospital, Pittsburgh, PA, 1971-1977. *Health Lab. Sci.* 15:159-167.
4. Brown, A., V. L. Yu, E. M. Elder, M. H. Magnussen, and F. Kroboth. 1980. Nosocomial outbreak of Legionnaires' Disease at the Pittsburgh Veterans Administration Medical Center. *Trans. Assoc. Am. Physicians* 93:52-59.
5. Garrity, G. M., A. Brown, and R. M. Vickers. 1980. *Tatlockia* and *Fluoribacter*: two new genera of organisms resembling *Legionella pneumophila*. *Int. J. Syst. Bacteriol.* 30:609-614.
6. Garrity, G. M., E. M. Elder, B. Davis, R. M. Vickers, and A. Brown. 1982. Serological and genotypic diversity among serogroup 5-reacting environmental *Legionella* isolates. *J. Clin. Microbiol.* 15:646-653.
7. Kado, C. I., and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* 145:1365-1373.
8. Knudson, G. B., and P. Mikesell. 1980. A plasmid in *Legionella pneumophila*. *Infect. Immun.* 29:1092-1095.
9. McKinney, R. M., H. W. Wilkinson, H. M. Sommers, B. J. Fikes, K. R. Sasseville, M. M. Yungbluth, and J. S. Wolf. 1980. *Legionella pneumophila* serogroup six: isolation from cases of legionellosis, identification by immunofluorescence staining, and immunological response to infection. *J. Clin. Microbiol.* 12:395-401.
10. Mikesell, P., J. W. Ezzell, and G. B. Knudson. 1981. Isolation of plasmids in *Legionella pneumophila* and *Legionella*-like organisms. *Infect. Immun.* 31:1270-1272.
11. Parisi, J. T., and D. W. Hecht. 1980. Plasmid profiles in epidemiologic studies of infections by *Staphylococcus epidermidis*. *J. Infect. Dis.* 141:637-643.
12. Pasculle, A. W., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Myerowitz, C. M. Patton, G. W. Gorman, L. L. Carmack, J. W. Ezzell, and J. N. Dowling. 1980. Pittsburgh Pneumonia Agent: direct isolation from human lung tissue. *J. Infect. Dis.* 141:727-732.
13. Riess, G., H. Burkardt, and A. Pultler. 1978. Molecular characterization of R68.45, a plasmid with chromosomal donor ability. *Hoppe-Seyler's Z. Physiol. Chem.* 359:1139.
14. Smith, H. R., G. O. Humphreys, and E. S. Anderson. 1974. Genetic and molecular characterization of some non-transferring plasmids. *Mol. Gen. Genet.* 129:229-242.
15. Stout J., V. L. Yu, R. M. Vickers, J. Zuravleff, M. Best, A. Brown, R. B. Yee, and R. Wadowski. 1982. Ubiquitousness of *Legionella pneumophila* in the water supply of a hospital with endemic Legionnaires' disease. *N. Engl. J. Med.* 306:466-468.
16. Wilkinson, H. W., D. D. Cruce, B. J. Fikes, L. P. Yealy, and C. E. Farshy. 1979. Indirect immunofluorescence test for Legionnaires' disease, p. 112-116. *In* G. L. Jones and G. A. Hébert (ed.), *Legionnaires': the disease, the bacterium, and methodology*. Publ. no. 79-8375, Centers for Disease Control, Atlanta.
17. Wong, K. O., W. O. Schalla, R. J. Arko, J. C. Bollard, and J. C. Feeley. 1979. Immunochemical, serologic, and immunologic properties of major antigens isolated from the Legionnaires' disease bacterium. *Ann. Intern. Med.* 90:634-638.