Molecular Epidemiology of *Legionella* Species by Restriction Endonuclease and Alloenzyme Analysis

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Received 20 March 1987/Accepted 12 June 1987

As part of an ongoing investigation into nosocomial *Legionella* infections at Stanford University Medical Center (SUMC), we applied the technique of restriction endonuclease analysis (REA) to determine strain differences among three species, including *Legionella pneumophila*, *Legionella dumoffii*, and *Legionella micdadei*. A total of 26 human and environmental water isolates from SUMC were selected for REA and compared with control strains that were not epidemiologically linked to SUMC. REA results were compared with results of alloenzyme typing, typing by monoclonal antibodies, and plasmid fingerprinting in all but *L. micdadei* strains. REA and alloenzyme typing showed that SUMC patient isolates were derived from distinct strains of three species. *L. pneumophila* strains from SUMC patients were genotypically identical to those isolated from potable water. REA was especially useful in proving that SUMC *L. dumoffii* patient isolates were derived from a single strain and that patients may have been exposed to a common source(s). REA typing correlated well with alloenzyme typing. These methods complement serologic typing of *L. pneumophila* and provide discriminating capability between strains of other *Legionella* species such as *L. dumoffii*, for which serologic types have not been identified. In addition, REA typing is somewhat easier to perform than alloenzyme typing and can be done in clinical laboratories.

In recent years epidemiologic investigations have been substantially improved by the application of molecular techniques, including restriction endonuclease analysis (REA) of genomic DNA. This method elucidates restriction site heterogeneity between strains and has been used successfully to subtype many species of bacteria (1, 5, 12). REA fingerprinting has been used to distinguish a few strains of *Legionella pneumophila* (14), but the method has not yet been used for other *Legionella* species. While investigating nosocomial *Legionella* infections at Stanford University Medical Center (SUMC), we had the opportunity to evaluate REA fingerprints of *Legionella pneumophila*, *Legionella dumoffii*, and *Legionella micdadei* isolates recovered from more than 50 clinical and environmental samples from SUMC; of these we selected 16 patient isolates and 10 water isolates, including a small number of SUMC *L. micdadei* isolates that were analyzed by REA only. SUMC isolates were also compared with 34 control strains. This study demonstrates that REA successfully differentiated SUMC strains of all three *Legionella* species isolated from SUMC patients or environmental sites from control strains that were not linked to the outbreak and that REA was especially helpful in typing *L. dumoffii*. A comparison of the results obtained by REA with monoclonal antibody typing, alloenzyme analysis, and plasmid fingerprinting was also made.

MATERIALS AND METHODS

Strains. Selected isolates of *L. pneumophila* serogroup 1, obtained from 10 patients and 10 potable water sources at SUMC, were studied. A total of 21 control strains were obtained from the Centers for Disease Control (CDC), Atlanta, Ga., 3 were from the California State Health Laboratory, and 1 was from SUMC. A total of 12 *L. dumoffii*

patient isolates from SUMC were studied; 6 control strains were obtained from CDC and from Paul Edelstein, University of Pennsylvania. Two SUMC *L. micdadei* clinical isolates were compared with three control strains obtained from Victor Yu, Pittsburgh Veterans Administration Medical Center, and from the California State Health Laboratory. All isolates were maintained by freezing at -70°C until they were used.

Monoclonal antibody and alloenzyme typing. *L. pneumophila* serogroup 1 isolates were typed by monoclonal antibody reactivity patterns (MAPs) by methods previously described (7). Alloenzyme analysis of *L. pneumophila* and *L. dumoffii* isolates was performed at CDC by methods described by Selander et al. (11). A total of 20 different enzymes were evaluated from each *L. pneumophila* strain. Enzyme electrophoretic mobility types (ETs) of *L. pneumophila* strains are designated by numbers according to the scheme of Selander et al. ET numbers for *L. dumoffii* strains are preceded by the letter d to distinguish them from the ET assignments for *L. pneumophila*. Thus, ET d1 for *L. dumoffii* does not represent the same mobility pattern as ET 1 for *L. pneumophila*. Detailed analysis of *L. dumoffii* by using multilocus enzyme electrophoresis will be described in a separate report. *L. micdadei* isolates were only examined by using REA.

REA. *Legionella* strains were grown at 37°C for 48 h on BCYE agar (Remel, Lenexa, Kans.). Cells were scraped from two plates, and whole-cell DNA was extracted by the method of Roussel and Chabbert (10). Whole-cell purified DNA was reacted with four restriction endonucleases individually, including *EcoRI*, *HindIII*, *BglII*, and *BamHI*, under conditions specified by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), except that incubation was performed for 18 h at 37°C. Restriction fragments were separated by overnight electrophoresis in 0.7% agarose gels (13). Gels were stained with ethidium

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bromide and photographed with a Polaroid camera under UV light. REA patterns were visually inspected. Lambda DNA restriction fragments were included as molecular weight standards.

Plasmid profile analysis. Plasmid DNA was prepared by the method of Kado and Liu (4) and analyzed by gel electrophoresis on 0.7% agarose gels that were stained with ethidium bromide and examined under UV light (8). The approximate molecular weights of plasmids were determined by the rate of migration relative to molecular weight standards run in the same gel.

Surveillance studies at SUMC. Beginning in 1983, we have conducted ongoing surveillance of hospital-acquired *Legionella* infections at SUMC. Water samples have been obtained from a large number of sites within the hospital, including hot water tanks, sinks, faucets, and showers in patient care areas and from cooling towers in the vicinity of the medical center. Duplicate water samples were examined by James Barbaree, at CDC, and at SUMC. At SUMC, 0.1-ml samples of water were plated directly and after low-speed centrifugation, without acid wash treatment, onto BCYE agar and BCYE agar containing antibiotics (Remel). All *Legionella* isolates, including *L. dumoffii* and *L. micdadei* isolates recovered from clinical specimens, grew without inhibition on each lot of both types of agar media. Although *L. pneumophila* colony counts were higher by the CDC procedures, in which 1 liter of water was concentrated prior to culturing, the same strains were recovered by using both procedures. Plates were incubated in ambient atmosphere at 37°C for 1 week and subsequently screened for *L. dumoffii* colonies by using UV light. Colonies resembling *Legionella* species were identified by the absence of growth on blood agar, Gram stain morphology, reaction with fluorescein-tagged polyvalent (Zeus Laboratories, Raritan, N.J.) and monoclonal antibody typing (Genetic Systems, Seattle, Wash.) sera, and colony fluorescence under UV light. The identification of all isolates was confirmed by the California State Department of Health Services Laboratory or by CDC.

Epidemiology of *Legionella* infections at SUMC. Since 1980, more than 50 patients have acquired infection with *Legionella* species at SUMC. Most were cardiac transplant recipients and were immunosuppressed; however, several infections occurred in patients who had undergone other types of cardiovascular surgery. All but one of these patients had received steroids in the perioperative period (S. C. Redd, L. S. Tompkins, B. Davis, M. L. Cohen, and J. Barbaree, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1150, 1986). Three *Legionella* species were isolated from SUMC patients: *L. pneumophila* serogroup 1, *L. dumoffii*, and *L. micdadei*. *L. pneumophila* organisms were consistently recovered from various potable water samples during this period. However, neither *L. dumoffii* nor *L. micdadei* strains have been isolated from water samples obtained at the medical center, including cooling towers located on or near SUMC.

RESULTS

Comparison of SUMC *L. pneumophila* isolates with controls. Table 1 gives the results of REA fingerprinting, monoclonal antibody typing, and alloenzyme analysis of 20 *L. pneumophila* SUMC isolates (10 patient isolates and 10 water isolates) and 25 control isolates. The remaining 19 SUMC patient isolates and all other SUMC water isolates which were also studied by these techniques (not included in this report) gave results which were identical to those shown

FIG. 1. REA patterns of SUMC *L. pneumophila* isolates produced by digestion of whole-cell DNA with *EcoRI*. Lanes: 1, Stanford L7 (patient; type 1ap); 2, CRC 326 (water; type 1ap); 3, phase 1 (water; type 1p); 4, Stanford L2 (patient; type 1p); 5, NICU (water; type 1p); 6, Stanford L8 (patient; type 1ap); 7, Stanford L7 (patient; type 1ap). Arrow indicates the location of extra bands seen in type 1ap isolates which are not well visualized.

in Table 1. Individual digestions with four enzymes produced distinct patterns; for example, REA patterns by digestion with *HindIII* were substantially different from fingerprints produced by *EcoRI* digestion. Examples of fingerprints produced by digestion of whole-cell DNA with *EcoRI* endonuclease followed by agarose gel electrophoresis are shown in Fig. 1. All patient and environmental SUMC isolates displayed either REA fingerprint patterns 1p or 1ap after digestion with each restriction endonuclease. These two patterns were nearly identical but showed minor, reproducible differences in restriction site distribution, which were most easily observed following digestion with *EcoRI*. Restriction endonuclease digestion of the 80-megadalton (MDa) plasmid contained in the isolates having the 1ap pattern did not produce fragments corresponding to one of the additional bands seen in the 1ap strain (data not shown). The majority of isolates having the prototype REA fingerprint pattern (type 1p; Table 1) were monoclonal antibody type 1,4,5,6,7,8,9 and alloenzyme type 21 (ET 21). Three patient isolates and two environmental isolates had an REA 1ap fingerprint; these had a different monoclonal antibody type (type 1,2,3,4) but had the identical ET 21 type (Table 1).

Of the 25 controls isolated from different geographic areas, we observed 13 different major REA fingerprint patterns following individual digestions with the four restriction endonucleases, and we observed 9 different ETs; 4 isolates that had unique REA patterns were not analyzed by ET analysis (Table 1). Representative REA patterns produced by digestion with *HindIII* are shown in Fig. 2. Five control strains (Camperdown, OLDA, Tucson 1, Burlington 2, and Bloomington) had REA patterns which were identical to that of the SUMC REA 1p strain. These strains were also ET 21, and all but one had identical MAPs (Table 1). All of these strains also contained an 80-MDa plasmid that had a restriction digestion fingerprint pattern identical to that of the 80-MDa plasmid contained in the SUMC REA 1ap strain (data not shown).

All *L. pneumophila* isolates containing the same al-

TABLE 1. Phenotypic and genotypic analysis of *L. pneumophila* strains

Strain	Source	REA	ET	MAP	Plasmid profile ^a
NICU 11/83	SUMC; water	1p	21	1,4,5,6,7,8,9	0
Core 11/85	SUMC; water	1p	21	1,4,5,6,7,8,9	0
Phase 1, small	SUMC; water	1p	21	1,4,5,6,7,8,9	0
Phase 1, large	SUMC; water	1p	21	1,4,5,6,7,8,9	0
NICU 209	SUMC; water	1p	21	1,4,5,6,7,8,9	0
NICU 217	SUMC; water	1p	21	1,4,5,6,7,8,9	0
CRCA 322	SUMC; water	1p	21	1,4,5,6,7,8,9	0
CRC 326, f	SUMC; water	1p	21	1,4,5,6,7,8,9	0
Stanford L1	SUMC; patient	1p	21	1,4,5,6,7,8,9	0
Stanford L2	SUMC; patient	1p	21	1,4,5,6,7,8,9	0
Stanford L3	SUMC; patient	1p	21	1,4,5,6,7,8,9	0
Stanford L4	SUMC; patient	1p	21	1,4,5,6,7,8,9	0
Stanford L5	SUMC; patient	1p	21	1,4,5,6,7,8,9	20
Stanford L6/s	SUMC; patient	1p	21	1,4,5,6,7,8,9	0
Stanford L6/l	SUMC; patient	1p	21	1,4,5,6,7,8,9	0
OLDA	CDC	1p	21	1,4,5,6,7,8,9	80
Tucson 1	CDC	1p	21	1,4,5,6,7,8,9	80
Bloomington 1	CDC	1p	21	1,4,5,6,7,8,9	80
Burlington 2	CDC	1p	21	1,4,5,6,7,8,9	0
Camperdown	CDC	1p	21	1,4,6	80
CRC 326	SUMC; water	1ap	21	1,2,3,4	80
CRC 326, d	SUMC; water	1ap	21	1,2,3,4	80
Stanford L7	SUMC; patient	1ap	21	1,2,3,4	80
Stanford L8	SUMC; patient	1ap	21	1,2,3,4	80
Stanford L9	SUMC; patient	1ap	21	1,2,3,4	80
Philadelphia 1	CDC	2p	25	1,2,4,5	0
Miami Beach 1	CDC	2p	25	1,2,3	0
W. Palm Beach	CDC	2p	25	1,2	0
Bonn 1	CDC	2p	25	1,2,5	0
D4999	CDC	2p	25	1,2,3	0
L.p. SG1-C1	Calif. State ^b	3p	NT ^c	NT	0
Stanford L10	SUMC; patient ^d	4p	NT	NT	20, 40
Westhaven 2	CDC	5p	31	1,4,6	0
L.p. SG8	Calif. State	6p	NT	NT	0
Glochester 1	CDC	7p	6	1,2,3	15
L.p. SG1-C2	Calif. State	8p	NT	NT	0
Pontiac 1	CDC	9ap	17	1,2,4	0
Alameda 1	CDC	9bp	17	1,2	0
Berkeley 1	CDC	9cp	17	1,2,4,5	7, 10
SMH 4	CDC	9dp	17	1,2,4,5,9	0
Lyon Cedex 1	CDC	10p	29	1,2,3	85
Indianapolis 3	CDC	11p	1	1,4,6	80
Davenport 1	CDC	11p	1	1,2,3	80
Naples	CDC	12p	19	1,2,3	0
Burlington 1	CDC	13p	27	1,2,3	0

^a Approximate molecular size in megadaltons.^b Calif. State, California State Health Laboratory.^c NT, Not tested.^d Community acquired.

loenzyme type had very similar REA fingerprints, and conversely, isolates having different REA patterns also had different ETs. Thus, the results of the two methods were comparable. As was shown by a previous study of *L. pneumophila* (9), MAPs did not show a high degree of correlation with alloenzyme and REA typing (Table 1).

The SUMC REA 1p isolates contained no plasmids, except for one isolate (Stanford L5; 20-MDa plasmid), whereas SUMC REA 1ap isolates each contained an 80-MDa plasmid. Some of the control strains contained one or more plasmids, although most did not contain plasmids (Table 1).

REA and alloenzyme analysis of *L. dumoffii* strains. REA and ET assignments for 10 *L. dumoffii* strains are shown in Table 2. All SUMC patient isolates had identical REA fingerprints (type 1d). The results obtained after whole-cell DNA digestion with *EcoRI* are shown in Fig. 3. Digestion with *EcoRI* and *BglII* produced the best patterns, whereas

digestion with *HindIII* did not produce good cleavage fragments. The SUMC REA 1d pattern differed markedly from that of the control strains. The ET patterns of all SUMC isolates were identical (ET d1) and different from all those of the control strains tested. Two control isolates recovered from the same hospital in Quebec had identical REA fingerprints, distinct from all others tested, and also had identical ET d2 patterns and were therefore believed to be derived from a single strain. By alloenzyme typing, the Quebec strain was identical to the Wadsworth strain, although minor differences in restriction sites could be detected by REA. Two isolates recovered from different geographic locations (Tucson, Ariz., and New Zealand) had indistinguishable REA fingerprints but slightly different alloenzyme patterns. REA typing and alloenzyme typing each placed all isolates examined in this study into five groups. The correlation between REA and alloenzyme typing for *L. dumoffii* was

FIG. 2. REA patterns of SUMC and control *L. pneumophila* strains following digestion of whole-cell DNA with *Hind*III. Lanes: 1, Westhaven 2 (control; type 5p); 2, Stanford L2 (patient; type 1p); 3, Bonn 1 (control; type 2p); 4, Lyon Cedex 1 (control; type 10p); 5, Stanford L5 (patient; type 1p); 6, hot water tank (SUMC; type 1p); 7, Pontiac 1 (control; type 9ap); 8, Alameda 1 (control; type 9bp); 9, Berkeley 1 (control; type 9cp); 10, SMH 4 (control; type 9dp).

generally good for the limited number of strains that were available, although ET d2 encompassed REA types 2d and 3d, whereas REA type 5d encompassed ETs d4 and d5.

The SUMC isolates examined each contained an 85-MDa plasmid, whereas none of the control strains contained this plasmid (Table 2).

Analysis of *L. micdadei* by REA. Both SUMC isolates were similar by REA and differed from three control strains. Two control isolates from the Pittsburgh Veterans Administration Medical Center appeared to be genotypically identical but different from the SUMC strain; the third strain from California was also different (data not shown).

DISCUSSION

Previous work by vanKetel et al. (14) suggested that REA is useful in analyzing a limited number of *L. pneumophila* isolates, but the technique has not been used to examine other *Legionella* species. Endonuclease restriction digestion of total cellular DNA from three species of *Legionella* produced patterns that distinguished SUMC nosocomial isolates from other strains that were studied, with the exception of five control *L. pneumophila* strains isolated from areas outside California.

REA fingerprinting was found to correlate highly with alloenzyme results but less well with MAP typing. In the

TABLE 2. Phenotypic and genotypic typing of *L. pneumophila* strains

Strain	Source	REA	ET	Plasmid profile ^a
Stanford Ld1	SUMC; patient	1d	d1	85
Stanford Ld2	SUMC; patient	1d	d1	85
Stanford Ld3	SUMC; patient	1d	d1	85
Stanford Ld4	SUMC; patient	1d	d1	85
Quebec 1J	Quebec city; patient	2d	d2	0
Quebec 2J	Quebec city; patient	2d	d2	0
Wadsworth	Patient	3d	d2	0
Tex KL	Patient	4d	d3	40
84/c/118	Patient	5d	d4	0
Tucson 2	Patient	5d	d5	0

^a Approximate molecular size in megadaltons.

FIG. 3. REA fingerprints of SUMC and control *L. pneumophila* strains following whole-cell DNA digestion with *Eco*RI. Lanes: 1, lambda DNA digested with *Hind*III; 2, Stanford Ld1 (patient; type 1d); 3, Quebec 1J (control; type 2d); 4, Quebec 2J (control; type 2d); 5, Tex KL (control; type 4d); 6, Stanford Ld2 (patient; type 1d); 7, Stanford Ld3 (patient; type 1d); 8, Tucson (control; type 5d); 9, 84/c/118 (control; type 5d); 10, Wadsworth (control; type 3d); 11, Stanford Ld4 (patient; type 1d); 12, lambda DNA digested with *Hind*III.

more comprehensive alloenzyme typing study of *L. pneumophila* by Selander et al. (11), it was found that of a total of 292 isolates studied, 26 were ET 21, which was the ET of the SUMC *L. pneumophila* isolates described in this study. This ET encompassed five MAPs and 1 serologically nontypeable strain (11). Further, 2 ETs were represented by isolates of two serogroups. Statistical evidence indicated that although there is overlap of serogroups in some multilocus genotypes, the serogroups are not randomly distributed (11). This was not the case for the MAPs, however, which appeared to be randomly distributed among the various ETs (11). Although the MAPs showed no relationship to the underlying genetic structure as determined by REA and alloenzyme typing, they do provide a useful epidemiologic marker system that complements the other systems, and in many instances they provide important additional discriminating capability.

It is not surprising that a high correlation was observed between the results obtained by REA and by alloenzyme analysis, since both are genetically determined. Minor differences between REA and ET analysis might be expected, because small mutations or base rearrangements may not result in an amino acid substitution affecting the enzyme electrophoretic mobility. Although in one case we observed strains that produced different alloenzyme patterns but that had identical REA 5d patterns (strains Tucson 2 [ET d4] and 84/c/118 [ET d5]), the mobility difference was slight and was found in only one enzyme (data not shown). Although plasmid DNA was present in some of the preparations that we evaluated, the differences in restriction fragment patterns did not seem to be caused by intact plasmid bands or by plasmid restriction fragments, including the subtle difference between the *L. pneumophila* type 1p and type 1ap strains.

The REA results also indicate that the SUMC REA type 1p strain was indistinguishable from five other *L. pneumophila* strains, including OLDA, originally isolated more than 40 years ago. Of interest is the observation that the 80-MDa

plasmid carried by the SUMC *L. pneumophila* REA type 1ap strain appears to be very similar to the plasmid contained in the epidemiologically and geographically unrelated control strains, including OLDA, that had identical REA and al-enzyme patterns. Two *L. dumoffii* strains isolated in Tucson, Ariz. (Tucson 2), and New Zealand (84/c/118) also appear to be genotypically related. These data suggest clonal dissemination of some *Legionella* strains. As more sophisticated genotyping schemes have been applied to bacteria, it has become apparent that clonal dissemination of certain strains of many pathogenic bacterial species is not unusual (11, 13). One could interpret our findings to indicate that certain *Legionella* strains have been selected and survive in many geographic locations throughout the world, perhaps because they are more virulent. Although some investigators have noted an association between *Legionella* strains lacking plasmids and human infection (2, 9), there appears to be no correlation between the presence or absence of plasmids and infection or virulence in SUMC patients. However, we have not had the opportunity to assess the virulence characteristics of these strains and therefore cannot interpret the significance of the 80-MDa plasmid in the REA type 1ap strain or its absence in the type 1p strain.

In one instance, we noted that four *L. pneumophila* control strains that were obtained from different geographical locations and that were identical by REA and ET analysis had different plasmid profiles. In our view, these data indicate that plasmid carriage reflects recent genetic events and that plasmid profiles are most useful in discriminating between strains isolated during outbreaks (6). On the other hand, in agreement with the findings of Edelstein et al. (3), we found that techniques of REA and ET typing that analyze the chromosome, a genetic unit that is more stable, were more useful for examining clonal dissemination. For example, although both SUMC *L. pneumophila* strains had different plasmid profiles, the homogeneity of the restriction sites indicated that they are probably closely related and presumably arose from a common progenitor. The differences we observed between the *L. pneumophila* REA type 1p and type 1ap patterns could merely reflect modification of an endonuclease restriction site in one strain. Presumably, the progenitor strain acquired a plasmid which was subsequently lost in one line of daughter cells. Therefore, although plasmid profiles of isolates derived from the same outbreak are helpful in assigning strain designation, strains containing identical restriction sites and identical genes encoding al-enzymes may have different plasmid profiles.

The REA and ET data showing that SUMC isolates were derived from unique strains suggest that there is a common source(s) from which SUMC patients acquired infection. The isolation of *L. pneumophila* serogroup 1 from potable water in the medical center cannot necessarily be used as evidence of acquisition from a common nosocomial source, since this is the most prevalent serogroup isolated from patients throughout the country. However, phenotypic and genotypic analyses of patient and environmental isolates at SUMC constitute strong evidence that two strains of *L. pneumophila* serogroup 1 have been disseminated in the hospital. Additional data which support the concept of a common source are the identification of three SUMC patients who were infected with multiple SUMC *Legionella* species. In one case, the patient was infected simultaneously with *L. pneumophila* REA 1a, *L. dumoffii* REA 1d, and *L. micdadei* REA 1m (*L. Tompkins*, unpublished results). The reservoir of these strains may be potable water, but other reservoirs may still exist. Although we have been unable to

culture *L. dumoffii* from any environmental water samples to date, the discovery that all *L. dumoffii* SUMC isolates are derived from a single strain has now allowed us to design additional studies to identify the source and mode of transmission of this uncommon species. These studies are now in progress.

Although REA has not yet been adopted in many clinical laboratories, this technique is easier to perform than al-enzyme analysis and could be easily developed in clinical laboratories which are familiar with plasmid fingerprinting methods. Like plasmid fingerprints, REA fingerprints can be developed within a single day. Investigators should be aware, however, that whereas different REA patterns clearly indicate molecular dissimilarity if all samples have been completely digested with restriction endonucleases, REA patterns which appear to be identical by visual inspection could be derived from isolates that have a small number of genetic differences which cannot be resolved by this technique. Unless a more quantitative and specific method of assessing the number and concentration of linear fragments, such as densitometry, is utilized to assess REA patterns, genetic identity should not necessarily be assumed. For practical purposes, however, this method certainly appears to be an ideal technique to classify strains genotypically as an aid in epidemiologic studies.

In summary, the epidemiologic investigations into nosocomial *Legionella* infections at SUMC support the REA and ET analysis data, demonstrating that SUMC isolates of three *Legionella* species are derivatives of unique strains and that strains of *L. pneumophila*, *L. dumoffii*, and *L. micdadei* can be reliably distinguished by these techniques.

ACKNOWLEDGMENTS

We acknowledge the many contributions of the staff of the SUMC Clinical Microbiology Laboratory to this project.

This work was supported in part by Public Health Service grant RR5353 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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