

Isolation of *Legionella longbeachae* Serogroup 1 from Potting Mixes

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Following a statewide outbreak of legionellosis due to *Legionella longbeachae* serogroup 1 in South Australia in 1988 and 1989, studies were performed to find a source of the organism. A number of water and soil samples with and without acid decontamination were examined for *L. longbeachae* by using a selective medium containing vancomycin, aztreonam, and pimafulin. There were no isolations of *L. longbeachae* from water samples. Organisms resembling *L. longbeachae* were isolated from a number of samples of potting mixes and from soil surrounding plants in pots collected from the homes of four patients. The organisms were found to persist for 7 months in two potting mixes stored at room temperature. Legionellae were isolated with difficulty from potting mixes which were allowed to dry out. Identification of isolates as *L. longbeachae* serogroup 1 was confirmed by quantitative DNA hybridization and serological tests. Restriction-fragment-length-polymorphism studies showed minor differences between patient and environmental isolates but differentiated these readily from *L. longbeachae* serogroup 2 and other antigenically related legionellae. The isolation of *L. longbeachae* from some potting mixes and the prolonged survival of the organisms in this medium suggest that soil rather than water is the natural habitat of this species and may be the source of human infections.

Legionella longbeachae was first described as a new species in 1981 after it was isolated from a patient with pneumonia who resided in Long Beach, Calif. (8). Within a short time, a second serogroup of this organism was described (1). Despite reports over the next 8 years of sporadic infections due to these organisms, an environmental source for infections by *L. longbeachae* has not been found. In May 1987, *L. longbeachae* serogroup 1 was isolated for the first time from a patient in South Australia, and two more cases occurred in October 1987 and June 1988. Two of these cases were reported by Lim et al. (5).

In October 1988, a statewide outbreak of infection with *L. longbeachae* serogroup 1 began in South Australia and involved 23 patients in 3 months. Sporadic single cases were diagnosed in February, April, and June 1989. Between May 1987 and June 1989, the diagnosis of infection with this serogroup was made in 30 patients. The diagnosis was confirmed by isolating the organism from 11 patients, 8 of whom showed seroconversion, and by demonstrating seroconversion to *L. longbeachae* alone in another 8 patients. A presumptive diagnosis of infection was made on the basis of an elevated level of antibody (1 in 256 or more) to *L. longbeachae* in the remaining 11 patients.

Epidemiological investigations carried out by the South Australian Communicable Diseases Control Unit suggested that gardening was a major risk factor in acquiring *L. longbeachae* infection. Between October and December 1988, laboratory investigations concentrated on the examination of water samples collected from the homes of confirmed cases; particular attention was paid to water collected from polypropylene watering systems, since these were thought to be a possible source of infection. When these investigations proved negative, it was decided to extend the search to soil samples. The Communicable Diseases Control Unit staff collected soil samples from the gardens of four patients whose infection occurred in or after December 1988 and was confirmed by culture or seroconversion. All patients from whose homes soil samples were collected were active

gardeners and had used commercially available potting mixes shortly before becoming ill. A limited number of water samples from the homes of two patients were also examined for *L. longbeachae*.

This paper describes the investigation of these environmental samples, the methods used to isolate *L. longbeachae* from soils, and the characteristics of the strains of *L. longbeachae* found in these samples.

MATERIALS AND METHODS

Environment. Samples were collected from the homes of four patients (Table 1).

VAP medium. An antibiotic medium containing *Legionella* charcoal yeast extract agar base (25 g/liter; CM 655; Oxoid Ltd., Basingstoke, United Kingdom), ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid; 10 g/liter]; ferric PP₁ (0.25 g/liter), L-cysteine hydrochloride (0.4 g/liter), α -ketoglutarate (1 g/liter), vancomycin (2 mg/liter), aztreonam (8 mg/liter), and pimafulin (250 mg/liter; Gist-Brocades, Delft, Netherlands) (VAP medium) was prepared. This followed tests which showed that seven isolates of *L. longbeachae* serogroup 1 from patients and the type strain ATCC 33462 were relatively resistant to aztreonam (MIC, 16 to 32 mg/liter) and were not significantly inhibited by this medium and that colonies of these organisms could be detected after 3 days of incubation at 35°C. In quality control tests, VAP medium selectively inhibited most common gram-negative and gram-positive bacteria found in clinical bacteriological specimens, some soil bacteria, and a wide range of fungi. This medium was prepared by autoclaving charcoal yeast extract base agar containing ACES and α -ketoglutarate. The pH was adjusted to 6.9 with KOH. Ferric PP₁ and L-cysteine were filter sterilized and added separately to the molten agar. Antibiotics were added after the medium was cooled to 50°C.

Processing of samples. Water samples from the homes of patients 1 and 3 were concentrated by centrifuging 30 ml at 6,000 \times g for 15 min. The deposit was suspended in 1 ml of original water. After treatment with acid for 5 and 10 min, it was inoculated in duplicate onto VAP medium with a 5-mm nichrome wire loop. Acid-treated samples were not neutralized. Plates were incubated at 35°C for 7 days and were

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TABLE 1. Garden samples from four patients with confirmed *L. longbeachae* infection

Case no.	Sample(s) (n)
1	Water from garden and house (12) Home-made compost (1) Potting mixes A and B (2) Garden soil (3) Potted-plant soil (4)
2	Potting mix C and D (2) Homemade compost (1) Composted cow manure (1) Blood and bone fertilizer mix (1)
3	Potting mix E (1) Potted plant-soil (brand E) (1) Garden soil (2) Rain water sample from tank (1)
4	Potting mix F (1) Homemade potting mix (2)

examined daily with an Olympus plate microscope after 72 h of incubation.

Soil samples, including samples of potting mixes, were prepared for culture by making a 1-in-5 (wt/vol) dilution of soil in 70 ml of sterile tap water. The suspensions were held at room temperature for 2 to 4 h and were shaken two or three times before being filtered through gauze. A portion of unconcentrated sample was retained for testing. A concentrated sample was prepared by centrifuging 30 ml of soil suspension at $6,000 \times g$ for 15 min. Most of the supernatant was discarded, and the deposit was suspended in the remaining 1 to 2 ml of water. Concentrated and unconcentrated samples of soil suspensions were decontaminated by making a 1-in-20 (wt/vol) dilution of the sample in 0.2 M HCl-KCl buffer. A loopful (5-mm nichrome wire loop) of each sample was plated in duplicate on VAP medium after 5 and 10 min of acid treatment. Suspensions of soil samples, including potting mix samples from the homes of patients 1 and 2, were cultured with and without acid treatment and were plated to *Legionella*-selective agar (Oxoid) containing cefamandole, polymyxin, and anisomycin; to modified Wadowsky and Yee (MWY) medium (Oxoid) containing vancomycin, polymyxin, and anisomycin as selective agents; and to charcoal yeast extract agar without antibiotics. Plates were incubated at 35°C in a humidified incubator and were examined daily for 7 days with an Olympus plate microscope after 72 h of incubation.

A sample of potting mix brand A placed in a plastic flower pot (50 by 75 mm) was left outdoors for 18 weeks and watered weekly with legionella-free rainwater when the weather was warm and dry but not during autumn and winter (May through August 1989). Samples of water were collected from the bottom of this flower pot by watering the soil at 4, 6, 8, and 18 weeks and were tested for legionellae by the methods described above.

Several soil samples found to be positive for *L. longbeachae* were suspended in water. These were treated with acid for various times and heated at 50°C for 15 and 30 min. Heated samples were plated in duplicate with a wire loop directly without acid treatment and after acid treatment.

Resistance to drying. The effect of drying on two potting mixes found to contain *L. longbeachae* was examined. Two mixes were dried in uncovered petri dishes, one at 36°C for 7 days and one at 36°C for 2 h and at room temperature for

TABLE 2. Pattern of agglutination reactions shown by latex reagents coated with antibodies to *L. longbeachae* serogroup 1 and *L. cincinnatiensis*

Test organism (ATCC no.)	Latex agglutination reactions ^a	
	<i>L. longbeachae</i> serogroup 1	<i>L. cincinnatiensis</i>
<i>L. longbeachae</i> serogroup 1 (33462)	3+	—
<i>L. longbeachae</i> serogroup 2 (33484)	—	—
<i>L. cincinnatiensis</i> (43753)	2+	3+
<i>L. sainthelensi</i> (35248)	1+	1+
<i>L. santicrucis</i> (35301)	1+	1+

^a Grading of agglutination reactions: 3+, 75% to 100% of cells agglutinated; 2+, approximately 50% of cells agglutinated; 1+, approximately 25% of cells agglutinated; —, no agglutination.

5 days. Undried samples of both mixes in sealed containers were held at room temperature and 36°C to serve as controls. All samples were tested in duplicate after acid treatment.

Characterization of isolates. Colonies of legionellalike organisms appearing on VAP plates and other antibiotic-containing plates after 3 or more days of incubation were tested for agglutination with latex reagents prepared with *L. longbeachae* serogroup 1 antiserum and *L. cincinnatiensis* antiserum obtained by immunizing rabbits with suspensions of American Type Culture Collection type strains. These unabsorbed antisera were diluted by adding different amounts to 1% latex suspensions which were then tested against a panel of 26 *Legionella* species. Latex reagents showing reasonably good specificity and rapid agglutination with the lowest concentration of antiserum were used throughout this study. The pattern of reactions found with these latex reagents when tests were performed on antigenically related *Legionella* spp. is shown in Table 2. Organisms were identified as *L. longbeachae* serogroup 1 if they had a typical colonial morphology on VAP medium, were dependent on L-cysteine, did not show blue-white autofluorescence, and agglutinated rapidly (within 30 s) with the latex antiserum to *L. longbeachae* serogroup 1 but not with the antiserum to *L. cincinnatiensis*. In this study, all isolates which showed agglutination on slide tests were checked by direct fluorescent-antibody (DFA) staining with fluorescein isothiocyanate-conjugated antisera prepared against *L. longbeachae* serogroups 1 and 2 (Centers for Disease Control, Atlanta). The pattern of reactions shown by these DFA reagents is shown in Table 3.

DNA homology and RFLP studies. The identity of the isolates was confirmed by DNA homology studies using the quantitative method of Brenner et al. (2), which was modified for use with small volumes (5). The standard strain used in the homologous reaction was *L. longbeachae* serogroup 1 ATCC 33462. The antigenically related *L. longbeachae* serogroup 2 ATCC 33484, *L. cincinnatiensis* ATCC 43753, *L. santicrucis* ATCC 35301, and *L. sainthelensi* ATCC 35248 were also included. Whole chromosomal DNA for restriction-fragment-length-polymorphism (RFLP) studies was purified from each isolate by the method of Manning et al. (7). Samples of the preparations were electrophoresed on 0.8% agarose gels to confirm that minimal fragmentation of the chromosomal DNA had occurred.

The DNA was fully digested with restriction enzymes *Hind*III and *Bam*HI, and the fragments were separated by overnight electrophoresis on 0.8% agarose gels in TAE buffer (6). The fragments were then transferred by the procedure of Southern (11) to nylon filter membranes (Biotrace RP; Gelman Sciences, Inc., Ann Arbor, Mich.)

TABLE 3. Pattern of reactions shown by DFA reagents tested on serologically related American Type Culture Collection cultures of *Legionella* spp.

Species and group	DFA reaction to <i>L. longbeachae</i> serogroup:	
	1	2
<i>L. longbeachae</i> serogroup 1	4+	1-2+
serogroup 2	1+	4+
<i>L. cinцинатиensis</i>	3+	1-2+
<i>L. sainthelensi</i>	1+	2+
<i>L. santicrucis</i>	4+	1-2+
<i>L. bozemanii</i> serogroup 1	-	-
serogroup 2	-	2+
<i>L. anisa</i>	-	3+

^a Grading of reactions: 4+, brilliant yellow-green staining of bacterial cells; 3+, bright yellow-green staining; 2+, definite but dim staining; 1+, barely visible staining; -, no staining.

which were then baked at 80°C for 2 h and stored at room temperature.

Derivation of DNA probe. A genomic bank for *L. longbeachae* serogroup 1 ATCC 33462 was constructed in LambdaGEM-11 (Promega Biotec, Madison, Wis.) according to the instructions of the manufacturer.

Amplified stocks of several clones were prepared and used to infect lawns of *Escherichia coli* LE392 at concentrations which would yield confluent lysis. Bacteriophage was harvested from the plaques by the method detailed by Maniatis et al. (6). The phage suspension was then clarified at 17,500 × g for 20 min at 4°C. The pellet was discarded. The phage was pelleted from the supernatant by centrifugation at 40,000 rpm for 2 h at 4°C in an L8, 70 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was discarded, and the phage pellet was suspended in phosphate-buffered saline. The vector with the *L. longbeachae* insert was purified from the phage particles by the method of Maniatis et al. (6). The vector with insert was labeled with [α -³²P]dCTP by nick translation (6).

Hybridization. The filters were hybridized overnight at 42°C in hybridization fluid (50% formamide, 1% skim milk

powder, 7% sodium dodecyl sulfate, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA), 200 µg of salmon sperm DNA per ml, radiolabeled probe DNA [5 × 10⁵ cpm/ml]). The next day, filters were washed twice for 15 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% sodium dodecyl sulfate and then once at 68°C in 1× SSC–0.1% sodium dodecyl sulfate for 45 min and autoradiographed at –70°C.

RESULTS

Organisms resembling *L. longbeachae* were isolated from the soil of two potted plants and three potting mixes on VAP medium (Table 4) but not from any garden soil or water samples. All media, including VAP plates inoculated with samples and not treated with acid, were overgrown with soil bacteria, and legionellae were not detected on these plates. In addition, charcoal yeast extract and both Oxoid selective media inoculated with samples treated with acid were overgrown with soil bacteria and soil fungi after 3 days of incubation and had to be discarded.

L. longbeachae could still be isolated from soil suspensions treated with acid for 5 to 20 min and from those heated at 50°C for 30 min, although acid treatment of the heated samples was necessary. Acid treatment of soil suspensions for 10 min proved to be the most effective way of reducing the numbers of soil organisms present in these samples. When present in moderate to large numbers (>10⁴ CFU/g of soil), legionellae were generally isolated from both acid-treated samples. When lower numbers were present, they were more easily detected in samples treated for 10 min, because of better suppression of other soil bacteria. The numbers found in the potted plant soil and the potting mix samples were calculated to range from 10² to 10⁵ CFU/g of soil. Potting mix brands A and D remained positive on testing for 7 months, and *L. longbeachae* was isolated from a concentrated sample of leachate after 18 weeks of exposure of the flower pot to natural climatic conditions. Legionellae were not isolated from known positive soil samples which were dried for 7 days, but they remained detectable in undried samples held at room temperature and at 36°C.

The *L. longbeachae* strains isolated from environmental samples and patients showed similar colonial morphologies on VAP medium. Incubation of plates for longer than 4 days resulted in the development of colonies with concentric rings, which gave them a characteristic target appearance. All three human and five environmental *Legionella* isolates required L-cysteine for growth. All isolates reacted strongly and rapidly with the *L. longbeachae* serogroup 1 latex reagent and showed bright brilliant staining with the DFA reagent for this serogroup. They did not react with the *L. cinцинатиensis* latex reagent but did react weakly with the *L. longbeachae* serogroup 2 DFA reagent.

The identity of the environmental isolates was confirmed by DNA homology studies with *L. longbeachae* ATCC 33462. The DNA relative binding ratios of the three human and five environmental isolates were above 80% at the stringent temperature of 75°C. *L. cinцинатиensis* ATCC 43753, *L. santicrucis* ATCC 35301, and *L. sainthelensi* ATCC 35248 gave relative binding ratios of 13, 13, and 11%, respectively.

Figure 1 shows the RFLP patterns obtained by Southern hybridization of isolates digested with *Hind*III and *Bam*HI using a probe made from a cloned fragment of the *L. longbeachae* genome. The type strain of *L. longbeachae*

TABLE 4. Five garden samples found to contain legionellae resembling *L. longbeachae*

Patient ^a	Date of illness	Sample		Laboratory designation of isolates
		Type	Date of purchase	
1	February 1989	Potting mix A	January 1989	E1
		Soil from one potted plant	Unknown	E2
2	December 1988	Potting mix D ^b	February 1989	E3
3	April 1989	Soil from potted plant	April 1989	E4
4	June 1989	Potting mix F	April 1989	E5

^a *L. longbeachae* serogroup 1 strains isolated from patients were designated H1, H2, and H3. Legionellae were not isolated from patient 4, who showed seroconversion to *L. longbeachae*.

^b Brand normally used by the patient but purchased after his illness. Mix used before illness was unavailable for testing.

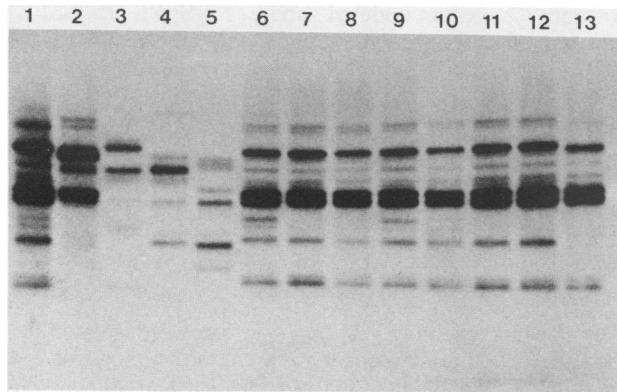


FIG. 1. Southern hybridization of *Hind*III and *Bam*HI digests of genomic DNA. Lanes: 1, *L. longbeachae* serogroup 1 ATCC 33462; 2, *L. longbeachae* serogroup 2 ATCC 33484; 3, *L. cincinnatiensis* ATCC 43753; 4, *L. santicrucis* ATCC 35301; 5, *L. sainthelensi* ATCC 35248; 6 to 13, isolates H1, E1, E2, H2, E3, H3, E4, and E5, respectively.

serogroup 1 differed from the American Type Culture Collection strains of *L. longbeachae* serogroup 2, *L. cincinnatiensis*, *L. santicrucis*, and *L. sainthelensi*. The human and environmental isolates closely resembled each other and *L. longbeachae* ATCC 33462. Among the *L. longbeachae* serogroup 1 strains tested, only minor differences in profiles were observed. Two human isolates, H1 and H2, had an extra band below the heavily labeled double band not seen in the third human strain or in the environmental strains, while E5 lacked a smaller band found in all other strains. The American Type Culture Collection strain of *L. longbeachae* serogroup 1 had a single band of high molecular weight, whereas Australian strains all had a fainter double band in that region.

DISCUSSION

Organisms identified as *L. longbeachae* serogroup 1 were isolated from three potting mixes and from the soil of two potted plants obtained from the homes of four patients who had had pneumonia due to this species. The organisms were shown to persist in some of these samples for 7 months. They were not isolated from any water or natural soil samples examined in this study.

Several reports that *L. longbeachae* has been found in aquatic environments have been published (3, 9, 10). In these studies, identification relied mainly on serological tests. Definitive DNA homology tests were not performed to confirm the identity of the *L. longbeachae* serogroup 2 isolates in Europe (3) or of the isolate of *L. longbeachae* (serogroup not stated) from a hotel in the Caribbean (10). *L. longbeachae* was reported to be present in seawater in Puerto Rico when DFA tests were used, but isolation of these organisms was not reported (9). Serological cross-reactions between *L. longbeachae* serogroup 2 and two species which show blue-white autofluorescence, *L. anisa* and *L. bozemanii* serogroup 2 (13), are known to occur. Cross-reactions also occur between *L. longbeachae* serogroup 1, *L. cincinnatiensis*, *L. santicrucis*, and *L. sainthelensi* (12, 13) and were demonstrated in this study with DFA tests. Therefore, DFA cannot be used to differentiate among these species reliably. All these *Legionella* spp. have common ubiquinone profiles and belong to group B of Lambert and Moss (4). The slide agglutinating latex reagents used in

this investigation distinguished *L. longbeachae* serogroup 1 from the other serologically related nonautofluorescing species. Our soil isolates reacted in the same way as the type strain of *L. longbeachae* serogroup 1 when tested with DFA and slide agglutination. The identification of these environmental isolates as *L. longbeachae* was confirmed by DNA homology studies.

RFLP analysis provided a more precise comparison of *L. longbeachae* isolates from patients and the environment. Minor differences in profiles were noted between human isolates H1 and H2 and isolate H3 and between environmental isolates E1 through E4 and isolate E5. RFLP analysis clearly differentiated *L. longbeachae* serogroup 1 from the serogroup 2 strain and from other legionellae which have very similar ubiquinone patterns and show some DNA and serological relatedness. This result is compatible with the view that these strains are closely related and that *L. longbeachae* in soil could be the source of some human infections. The potting mixes and potted plant soils from which the environmental isolates E1, E3, and E4 of *L. longbeachae* were grown were made by one manufacturer but distributed under separate brand names. Isolate E5 was obtained from a potting mix (brand F) made by a different company.

It is generally accepted that pneumonia results from inhalation of aerosols containing *Legionella* spp. It is not known if this is the route for *L. longbeachae* infections. Our investigations have shown that legionellae are leached from the soil during watering of potted plants. It is possible that aerosols containing *L. longbeachae* are formed by vigorous watering of plants in pots or when water falls onto hard surfaces from potted plants placed above ground level. Case-controlled studies are needed to determine if potting mixes are responsible for infection and how such infections are acquired. Now that an environmental source of *L. longbeachae* has been found, it should be possible to investigate the environmental, host, and organism factors which lead to infection.

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