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Distribution of *Legionella longbeachae* Serogroup 1 and Other Legionellae in Potting Soils in Australia

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***Legionella longbeachae* serogroup 1 and other *Legionella* spp. were isolated from 73% of 45 potting soils made in Australia by 13 manufacturers but were not detected in 19 potting soils made in Greece, Switzerland, and the United Kingdom examined between March 1989 and May 1990. Several *Legionella* species were isolated from a small number of samples of uncomposted pine sawdusts, but it is not known whether sawdust was the source of some of the legionellae found in potting soils. *Legionella* spp. persisted for periods ranging from 3 to 10 months in a potting soil held at temperatures between −20 and 35°C. Isolates of *L. longbeachae* serogroup 1 from soil did not grow at 43°C, a temperature which was also lethal for this species in soil. Most *Legionella* spp. isolated from potting and natural soils belonged to one distinct group according to analysis of ubiquinones and were serologically related to several known species in this group. A small number of potting soils contained *L. pneumophila* and *L. micdadei*.**

The natural habitat of less commonly encountered pathogenic legionellae is unknown, and their ecology is poorly understood. Although it was suggested in 1978 (9) that soil was implicated in an outbreak of Legionnaires disease, there have been few reports of the isolation of legionellae from soils since their discovery in 1976. During an investigation of an outbreak of Legionnaires disease associated with the Indiana Memorial Union, several serogroups of *L. pneumophila* were isolated from mud and soil obtained from a creek nearby but legionellae were not detected in soils obtained from grounds near the Union or from some distance away (5). These *L. pneumophila* strains (from the Indiana Memorial Union Soils) and *L. gormanii* and *L. jamestowniensis* (from different sites) were isolated from soils by first inoculating these soils into guinea pigs, passaging guinea pig tissues through embryonated eggs, and then culturing the infected eggs on charcoal-yeast extract agar. More recently, *L. bozemanii* was isolated from 2 of 14 samples of wet or semifrozen soil without animal inoculation by using buffered charcoal-yeast extract medium containing antibiotics during the investigation of an outbreak of nosocomial pneumonia in immunocompromised patients (7).

In 1989, *L. longbeachae* serogroup 1 was isolated from potting soils and from a number of patients with pneumonia in South Australia (8). No other source of *L. longbeachae* was found, and potting soils were considered to be a possible source of these infections. In Australia, potting soils are made from composted waste products such as sawdust and hammermilled bark by methods which were originally developed in the United States where these materials were abundant. In Europe, peat rather than composted sawdust and bark is the major component used in potting soils.

Between March 1989 and May 1990, potting soils made in Australia and in Europe as well as a number of natural soils were investigated to determine the prevalence of potentially pathogenic legionellae in the environment. As *L. longbeachae* serogroup 1 was known to cause infection in Australia, the study was primarily directed toward detecting this species and defining the conditions which permitted its growth and survival in soils. It was hoped that these studies would provide information about the presence of *Legionella* spp. in potting soils at various times of the year and that this knowledge would assist in controlling their spread to humans.

MATERIALS AND METHODS

Potting soils. Sixty-four potting soils were tested for legionellae. The origins of these soils are shown in Table 1. Fifty-three of these were ready-to-use products in 4- or 20-liter bags purchased from stores in Australia, England, Greece, and Switzerland. Samples from five potting soils made in states of Australia other than South Australia were obtained from Kevin Handreck of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), and samples from six potting soils were obtained from home owners concerned about the risks of acquiring legionellosis. Further bags of potting soil made by each manufacturer in South Australia and by two interstate manufacturers whose products were readily available in this state were purchased and tested at 3- to 6-month intervals throughout the study.

Components of potting soils. Twenty-one components used by two manufacturers to make potting soils in South Australia were tested (Table 2). Generally, three to five samples (1 to 4 liters) of the most commonly used components were examined.

Natural soils. Thirty-four natural soil samples were tested. Twenty-four were obtained from gardens near Adelaide, four were from two industrial sites in South Australia where potting soils were made, and six were from gardens and parks in England.

Preparation of leachates. Polystyrene centrifuge tubes (15 by 115 mm) were used to prepare the samples of potting soils and components for leaching. Holes were made in the conical base of the tube with a red-hot needle, a small plug of glass wool was inserted, and the tube was filled with potting soil moistened with sterile water. Sterile tap water was slowly added to the tube with a Pasteur pipette until approximately 1 ml of effluent was collected in a test tube for testing. If legionellae were not detected in the leachate and

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TABLE 1. Source and prevalence of legionellae in potting soils made in Australia and Europe

Source	No. of manufacturers	No. of soils tested	No. (%) with:	
			L. longbeachae serogroup 1	*Legionella* spp.[a]
Australia				
South Australia	6	34	16 (47)	20 (59)
Western Australia	1	2	2 (100)	2 (100)
New South Wales	2	3	2 (67)	2 (67)
Victoria	4	6	6 (100)	3 (50)
Europe				
United Kingdom	6	14	0	0
Greece	2	4	0	0
Switzerland	1	1	0	0

[a] The serological reactions of these species are shown in Table 3.

the sample contained small numbers of other soil microorganisms, either a second subsample was prepared for leaching or a suspension of a fresh subsample of the potting soil was prepared and tested within 10 days of the first test.

Preparation of soil suspensions. Suspensions of natural soil samples and potting soils were prepared by making a 1-in-4 (wt/vol) dilution by mixing an approximately 5-g sample with 15 ml of sterile tap water in a screw-cap polystyrene universal container (28 by 78 mm). The suspension was shaken well and allowed to stand at room temperature (RT) for 5 min.

Test procedure. All soils were tested for legionellae within 10 days of receipt or purchase, with the majority being examined within 24 h. Potting soils and porous materials such as components were tested by the protocol shown in Fig. 1. This procedure was not suitable for testing natural soils. All suspensions and leachates were treated with acid to reduce unwanted soil microorganisms by preparing a 1-in-20 dilution of the suspension or leachate in 0.2 M HCl-KCl acid buffer (pH 2.2). The acid-treated samples were mixed intermittently for 10 min and were then streaked in duplicate on selective media with a calibrated 5-mm nichrome wire loop by a standard streaking procedure (8). Australian samples were streaked on buffered charcoal-yeast extract medium containing vancomycin (2 mg/liter); polymyxin B (80,000 IU/liter), and Pimafucin (250 mg/liter) with aztreonam (4 mg/liter) (VAPP) (8) or without aztreonam (VPP). These media were supplemented with 1% bovine serum albumin. Samples from Europe were streaked on VPP medium only.

TABLE 2. Twenty-one components used by two manufacturers to make potting soils

Component	State	No. of batches tested	Proportion of samples with *Legionella* spp.
Hammermilled pine bark	Fresh	2	0/4
	Composted	2	3/4
Sawdust			
Pine	Fresh	3	3/10
	Composted	2	1/2
Eucalypt	Fresh	3	0/3
	Composted	3	4/4
Paunch contents	Composted	1	0/1
Peat and peat moss		2	0/2
Sands		2	0/5
Mineral fertilizer		1	0/1

Plates were incubated at 35 ± 1°C. They were examined after overnight incubation for rapidly growing bacteria and daily from days 3 to 7 for legionellae with a stereoscopic dissecting microscope (Olympus T-Xr, magnification ×6.3).

Centrifugation of samples. Bacteria from 67 samples obtained in Australia were concentrated by centrifuging 15 ml of a 1-in-4 (wt/vol) suspension of each sample in sterile tap water at 3,500 × *g* for 15 min. The suspension was filtered through gauze before centrifugation. The supernatant was discarded, and the sediment was resuspended in 1 ml of sterile tap water. This suspension was diluted 1 in 10 with 0.2 M acid buffer and was mixed for 10 min before being streaked in duplicate onto VAPP and VPP media. The sedimented material from a small number of samples was examined for fluorescing organisms with *L. longbeachae* serogroup 1 direct fluorescent antibody (DFA) reagents obtained from the Centers for Disease Control (Atlanta, Ga.).

Dilution of samples. When cultures of leachates or suspensions contained more than 10^4 CFU of other soil bacteria per g or when swarming organisms obscured the growth of legionellae, a subsample was retested by diluting it 1 in 200 (Fig. 1).

Identification of legionellae. The methods used to identify legionellae have been described previously (8, 10). Culture plates were examined for autofluorescent colonies with a UV lamp emitting light at 366 nm. Legionella-like colonies were tested by slide agglutination with latex reagents prepared with antisera to appropriate *Legionella* strains obtained from the American Type Culture Collection (Rockville, Md.) or by DFA testing with reagents supplied by the Centers for Disease Control, SciMedx (Denville, N.J.), and Zeus Technologies, Inc. (Raritan, N.J.). Legionellae which showed atypical or negative serological reactions were examined for ubiquinone content by high-performance liquid chromatography (10). The majority of strains of soil *Legionella* species were analyzed for restriction fragment length polymorphisms (RFLP) by methods previously described (4).

RESULTS

Legionellae were isolated from 33 (73%) of 45 potting soils made in Australia but were not detected in any potting or natural soils originating in Europe (Table 1). Potting soils made by two manufacturers were found to contain legionellae only during autumn and winter months, whereas the remainder were positive throughout the year.

***L. longbeachae* serogroup 1 in soils.** Of the 45 potting soils made in Australia, 26 contained *L. longbeachae* serogroup 1. This species was also isolated from one of a number of samples of fresh pine sawdust tested (Table 2) and from one natural soil sample collected from the ground near a stockpile of potting soil found to contain this species. Sand and humus were detected in the positive sawdust sample on microscopic examination, indicating contamination with finished potting soil. Strains of *L. longbeachae* serogroup 1 isolated from various potting soils had RFLP patterns similar to those previously reported (4).

Other *Legionella* species in soils. A number of *Legionella* species other than *L. longbeachae* serogroup 1 were isolated from 27 potting soils, 20 of which also contained the latter species. They were the only legionellae found in two potting soils made by one South Australian manufacturer. *Legionella* spp. were also isolated from two samples of fresh pine sawdust not obviously contaminated with potting soil (Table 2) and from one garden soil. They were found to coexist with

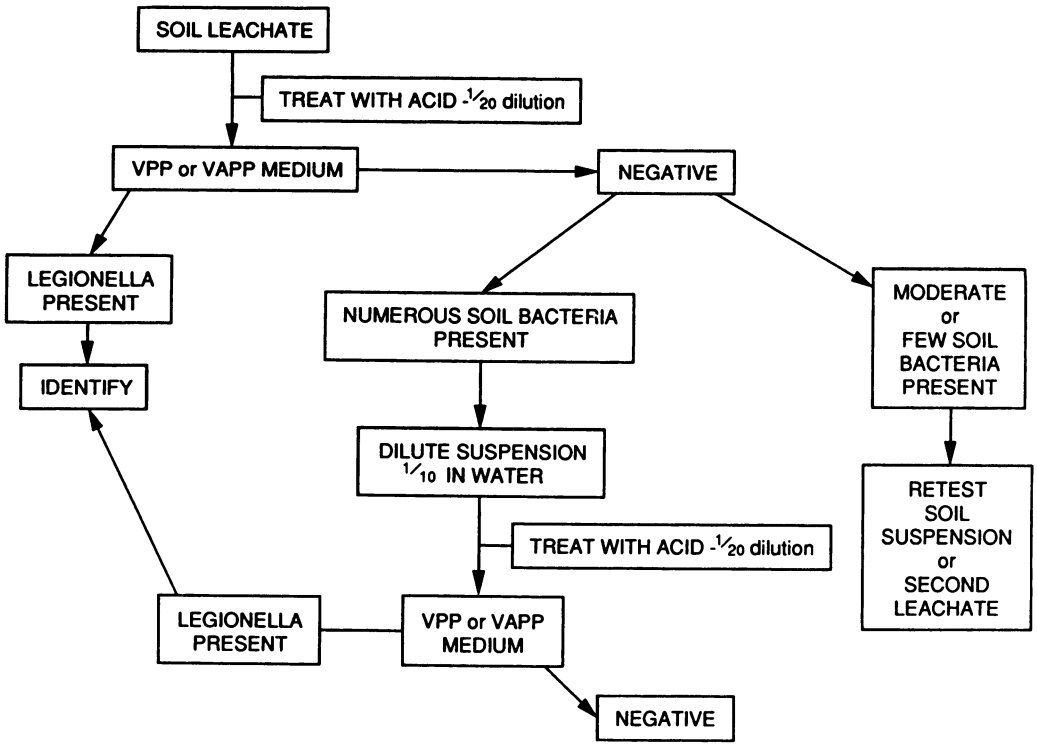

FIG. 1. Test protocol for potting soil samples.

L. longbeachae in components being composted and in unfinished potting soils. The serological reactions of these species are shown in Table 3, but definitive DNA studies by quantitative hybridization have not been done to confirm the identities of these isolates. Thirteen additional *Legionella* isolates, which remain unidentified, were excluded from Table 3 because they reacted slowly or weakly with latex agglutinating reagents to *L. longbeachae* serogroup 1 and *L. cincinnatiensis*. They did not show blue-white autofluorescence and belonged to group B (3) as determined by analysis of ubiquinones. Two had RFLP patterns similar to those obtained with the ATCC type strains of *L. cincinnatiensis* and *L. santicrucis*, but the remaining 11 had patterns which distinguished them from these and the other serologically related species in this ubiquinone group, *L. longbeachae* and *L. sainthelensi* (data not shown). The *Legionella* spp. which reacted strongly with the *L. anisa* latex reagent did not show blue-white autofluorescence. They reacted strongly with a polyvalent DFA reagent for *L. longbeachae* serogroups 1 and 2 and had RFLP patterns which were indistinguishable from those shown by *L. longbeachae* serogroup 2 ATCC 33484. The RFLP patterns of three of these isolates assigned the numbers 276/E7, 349/G7, and 350/F5 have been previously reported (4). These four strains were considered to be *L. longbeachae* serogroup 2.

TABLE 3. Number of *Legionella* isolates from individual potting soils, components, and natural soils reacting strongly in serological tests

Serologic reagent	No. and source of isolates reacting strongly with serological reagents		
	Potting soils	Components of potting soils	Natural soils
Latex for:			
L. longbeachae serogroup 1	26	1	1
L. cincinnatiensis	0	0	0
L. anisa	4	0	0
L. bozemanii serogroup 1	8	3	0
L. micdadei	3	0	0
L. pneumophila serogroups 1–14	6	0	0
DFA for:			
L. bozemanii	10	3	0
L. gormanii	1	1	0
L. micdadei	3	0	0
L. dumoffii	1	0	0
L. longbeachae serogroups 1 and 2	31	1	1

With the exception of *L. pneumophila* and *L. micdadei*, the legionellae isolated from potting soils, components of potting soils, and natural soils belonged to group B as determined by analysis of ubiquinones. Legionellae which showed blue-white autofluorescence were particularly common in potting soils. Most were serologically related to *L. bozemanii*, but other legionellae identified serologically as *L. gormanii* and *L. dumoffii* were detected in a small number of potting soils.

Concentration and dilution of samples. Centrifugation of 67 samples detected only one sample which did not contain legionellae when tested by the protocol (Table 4). Centrifugation detected 13 (48%) of 27 positive samples, whereas the procedure with unconcentrated samples detected 26 (96%). Five potting soils containing numerous other soil microorganisms were found to contain *L. longbeachae* or other legionellae when a more diluted sample was tested. Examination of centrifuged deposits by DFA was unsatisfactory for the detection of *L. longbeachae* because of considerable

TABLE 4. Effect of centrifugation on detection of legionellae in 67 soil samples

Sample (no.)	Positive samples			Negative samples (all tests)
	Both[a]	Uncentrifuged only	Centrifuged only	
Potting soils (28)	10	11	0	7
Components[b] (21)	1	2	1	17
Natural soils (18)	1	1	0	16

[a] Legionellae were detected in both unconcentrated and centrifuged samples.
[b] Described in Table 2.

background fluorescence and the presence of moderate numbers of fluorescent organisms which did not resemble legionellae morphologically. Small numbers of DFA-positive organisms morphologically resembling legionellae were seen within or attached to small soil particles in one or two samples.

Selectivity of VPP and VAPP media. Aztreonam inhibited some soil bacteria including several *Legionella* species but permitted the detection of *L. longbeachae*, *L. dumoffii*, and *L. pneumophila* in potting soils. *L. longbeachae* was isolated with equal frequency on both selective media. *L. bozemanii* and *L. micdadei* as well as several other *Legionella* species found in soils failed to grow on VAPP medium.

Effect of temperature on survival of legionellae in soil. One western Australian soil which contained 4×10^3 to 8×10^3 CFU of legionellae per g of soil when repeatedly tested by the standardized loop and streaking method was used to study the effect of temperature on the long-term survival of strains of *L. longbeachae* serogroup 1 and *L. dumoffii* present in this soil. The soil was moistened with sterile tap water to contain 30% total water as measured by drying at 160°C for 6 h. Six subsamples (30 to 40 g) of moistened soil were placed in polystyrene screw-cap containers (46 by 57 mm) and were held at temperatures ranging from −20 to +50°C for periods ranging from 2 weeks to 10 months. The moisture content of each sample was kept relatively constant by replacing water lost by evaporation in excess of 0.5 g during the interval between tests. Water was not replaced in the sample frozen at −20°C. Each incubated sample was tested at 3, 5, 7, and 14 days and then at monthly intervals up to 10 months by removing a 1-to-2-g sample and preparing a 1-in-10 (wt/vol) dilution in sterile tap water. This suspension was further diluted 1 in 10 in 0.2 M acid buffer, treated for 10 min, and streaked in duplicate on VPP medium. The survival of legionellae in these samples is shown in Table 5. Legionellae survived in soils held at −20°C for several months and at temperatures between 4 and 35°C for 6 to 10 months. The population of legionellae declined rapidly in soils incubated at 43 and 50°C but appeared to show a 5- to 10-fold increase in samples held at RT and 35°C in month 1 of the study. Additional studies with six strains of *L. longbeachae* serogroup 1 from six different potting soils showed that they grew slowly at 21 and 41°C but did not grow at 43°C on buffered charcoal-yeast extract agar incubated for 14 days.

Multiplication of legionellae in soil suspensions. To study whether multiplication of legionellae occurred in soils, we reexamined samples from 13 separate potting soils for legionellae using more quantitative methods. Each soil sample (1.0 to 1.5 g) was suspended in an equal volume of sterile water. Samples were held at RT for 29 days and then incubated at 35°C for 7 days. These suspensions were examined 24 h after preparation and then at weekly intervals by making a 1-in-10 dilution in 0.2 M acid buffer, leaving it for 10 min, and spreading 25 μl (Oxford Sampler P7000 sampling pipette) in duplicate on VPP medium. Plates were incubated at 35°C for 7 days, and legionellae were enumerated and identified. Of the 13 samples examined, 6 did not yield any legionellae. In five samples, including a new subsample of the western Australian soil previously tested, the population of legionellae remained constant in the range of 10^2 to 10^3 CFU/ml of suspension. In the remaining two suspensions of soils which had been acquired 1 month previously, the population of legionellae appeared to increase (Table 6).

TABLE 5. Survival of legionellae in a western Australian soil sample incubated at various temperatures for up to 10 months

Legionella species	Length of survival at a temp (°C) of:					
	−20	4	RT (19–23)	35	43	50
L. longbeachae	3 mo	7 mo	9 mo	6 mo	5 d[a]	3 d
L. dumoffii	4 mo	10 mo	10 mo	6 mo	5 d	3 d

[a] d, Days.

DISCUSSION

Difficulties in isolating legionellae directly from soil and the widely held view (2) that *L. pneumophila* and other legionellae are principally aquatic organisms may account for the paucity of reported investigations of soil for these organisms. Although legionellae could be readily isolated from potting soils made by many manufacturers in Australia, they were only rarely isolated from fresh components or from natural soils. Manufacturers of potting soils obtained

TABLE 6. Numbers of legionellae in two South Australian potting soils held at RT for 29 days and then at 35°C for 7 days

Soil and species	No. of legionellae (CFU/ml) on the following day:					
	1	8	15	22	29	36
Soil 407						
L. longbeachae	ND[a]	ND	ND	9.2×10^3	4.8×10^4	1.6×10^4
Legionella spp.[b]	8×10^2	ND	4×10^2	4.4×10^4	4.8×10^4	4×10^4
Soil 409						
L. pneumophila	6.4×10^3	3.2×10^3	9.2×10^3	2.4×10^3	8×10^2	ND
L. bozemanii	1.1×10^4	6×10^3	9.6×10^3	2×10^3	8×10^2	4×10^2
L. longbeachae	ND	ND	8.4×10^3	2.8×10^3	ND	ND

[a] ND, Not detected on VPP medium (estimated CFU less than 400/ml).
[b] This *Legionella* species has not been identified.

their components from many independent suppliers and used separate water sources. Differences in the prevalence of legionellae in potting soils made by different manufacturers remain unexplained but may be due to the nature of the components used by individual manufacturers and the methods of composting these materials.

The methods used in this study, while they were capable of detecting 10^3 CFU of legionellae per g of soil, did not reliably detect smaller populations. It is possible that small numbers of legionellae in some source material could multiply to form populations of detectable size during the composting process. On the other hand, contamination of fresh components with composted material or unfinished and even finished potting soils containing legionellae was commonly found on the industrial sites studied. Because of the limitations of the methods and the finding of contamination of fresh components, the source of legionellae in potting soils could not be determined.

Laboratory investigations to demonstrate multiplication of legionellae in potting soils gave equivocal results. These experiments did not re-create the conditions which occurred during the composting process. In particular, the range of nutrients released by microbial degradation of organic matter was absent in laboratory tests. Survival of legionellae in the environment for prolonged periods as observed in the field and demonstrated in laboratory tests would facilitate their spread on industrial sites where potting soils are made.

While pasteurization of potting soils would use temperatures capable of killing soil legionellae, its value in preventing outbreaks of legionellosis remains to be defined. Although many potting soils containing legionellae were used by persons in the community during the time this study was performed, no outbreak of legionellosis due to *L. longbeachae* serogroup 1 was detected in the state of South Australia. Serological evidence of infection as demonstrated by a greater than fourfold rise in antibody to *L. longbeachae* was detected in five patients, with one case occurring each month in 5 of the 6 months from September 1989 to February 1990. None of these infections was confirmed by culture. Infections due to *L. bozemanii* and the other *Legionella* species commonly associated with potting soils have not been documented in this state. Further studies should be done to determine the relationship between legionellae in potting soils and legionellosis in Australia.

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