**Legionella longbeachae** serogroup 1 infections linked to potting compost

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Four cases of legionellosis caused by **Legionella longbeachae** serogroup (sg) 1 were identified in Scotland from 2008 to 2010. All case patients had exposure to commercially manufactured growing media or potting soils, commonly known as multipurpose compost (MPC), in greenhouse conditions, prior to disease onset. Two patients had been using the same brand of MPC but the clinical isolates were distinct genotypically by amplified fragment length polymorphism (AFLP) analysis. However, an indistinguishable AFLP profile was also found in an environmental isolate from the supply of MPC used by each patient. The third patient was diagnosed by immunofluorescent antibody serology only; however, the MPC to which this patient was exposed contained **L. longbeachae** sg 1 in large quantities (80 000 c.f.u. g⁻¹). The fourth patient was **L. longbeachae** sg 1 culture-positive, but **L. longbeachae** was not identified from 10 samples of garden composting material. As compost is commonly used, but **L. longbeachae** infection seemingly rare, further work is required to ascertain (i) the prevalence and predictors of **L. longbeachae** in compost and (ii) the conditions which facilitate transmission and generate an aerosol of the bacteria. As most cases of legionellosis are diagnosed by urinary antigen that is **Legionella pneumophila**-specific and does not detect infection with **L. longbeachae**, patients in cases of community-acquired pneumonia with a history of compost exposure should have serum and respiratory samples sent to a specialist **Legionella** reference laboratory for analysis.

**INTRODUCTION**

**Legionella longbeachae** serogroup (sg) 1 was first identified in 1981 in Long Beach, California (McKinney et al., 1981). Since then it has become a common cause of Legionnaires’ disease (LD) in Australia, where a third of cases are caused by **L. longbeachae**, and infection associated with exposure to potting mixes, compost and gardening (Yu et al., 2002; O’Connor et al., 2007). In the UK, only 11 cases of **L. longbeachae** have been reported since 1984 (Health Protection Agency, personal communication), of which seven were identified in Scotland. Across Europe, there has been a steady increase in notifications of **L. longbeachae** (Whiley & Bentham, 2011). This may be an underestimation as current urinary antigen enzyme immunoassays (EIAs) would not detect **L. longbeachae** infection. However, the reason for the apparent increase in Scotland in recent years is not known. Four cases of legionellosis caused by **L. longbeachae** sg 1 were identified in Scotland from 2008 to 2010. All case patients had exposure to growing media or potting soils, commonly known as multipurpose compost (MPC), in greenhouse conditions, prior to disease onset. Two patients had been using the same brand of MPC. This study describes (i) the microbiological investigative methods which allowed the epidemiological association with a particular brand of MPC to be confirmed, (ii) the benefit of serology to identify the cases and (iii) two further cases as a result of the public health attention. In addition, we hypothesize as to possible reasons for the apparent increase in **L. longbeachae** and make recommendations as to the further investigations required to elucidate the

**Abbreviations:** AFLP, amplified fragment length polymorphism; EIA, enzyme immunoassay; IFA, immunofluorescent antibody; LD, Legionnaires’ disease; MPC, multipurpose compost; sg, serogroup.
relationship between legionellae in compost and human cases of LD.

METHODS

Human samples: diagnostic tests. Urinary antigen EIA testing was performed as per the manufacturers’ instructions (Biotest and Binax) and immunofluorescent antibody (IFA) testing for all Legionella species that cause human disease was performed as previously described (Wilkinson et al., 1981). Patient respiratory samples were cultured both untreated and after heating to 50 °C for 30 min then plated onto buffer charcoal yeast extract (BCYE) and BMPA media (Oxoid) and incubated at 37 °C for up to 10 days.

Respiratory PCR. DNA was extracted from the respiratory samples using a Nucleospin C & T kit (Macherey-Nagel) and was amplified with 16S rRNA gene Legionella-specific primers 16S rDNA 1 (5'-TAC GTA CCC TTA CGA TAC AGT G-3') and 16S rDNA 2 (5'-CTT CCT CCG GTT TGT CAC-3') and digoxigenin-labelled dNTPs at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 1 min and 70 °C for 30 s, then one cycle of 72 °C for 5 min. PCR products (~200 bp) were visualized on a 1.5 % agarose gel. A PCR ELISA was specific probe (5'-ATG TGA TGG TGG GGA CTC-3') using a Nucleospin C & T kit (Macherey-Nagel) and was amplified by electrophoresis in 1.5 % agarose (Molecular Biology Certified beads (Amersham Biosciences). Amplified products were separated with 16S rRNA gene using a Nucleospin C & T kit (Macherey-Nagel) and was amplified described (Wilkinson et al., 2002; Lindsay et al., 2006).

Strain identification. The cultures of L. longbeachae sg 1 were identified by polyclonal IFA serology (Wilkinson et al., 1981) and mip gene speciation (Ratcliff et al., 1998) and then genotyped by amplified fragment length polymorphism (AFLP) using the standard European Working Group for Legionella Infection method (Fry et al., 2000).

AFLP. Briefly, restriction–ligation reactions were performed at 37 °C for 3 h. Each mix comprised approximately 1.5 μg genomic DNA, 200 ng each adaptor oligonucleotide (AFLP-LG1, 5’-CTCGTAG-3’; AFLP-LG2, 5’-TCTACGCTACGCT-3’), 20 U PstI (Boehringer), 1 U T4 DNA ligase (Roche) and 1 x ligation buffer (10 x ligation buffer is 660 mM Tris pH 7.5, 50 mM magnesium chloride, 10 mM dithiothreitol, 10 mM ATP (Roche). PCR was performed in a reaction mixture of 25 μl comprising approximately 1 ng template and 400 nM selective primer (AFLP-PstG-G, 5’-GACTGGTACATGCTACG-3’)) using Ready-To-Go beads (Amersham Biosciences). Amplified products were separated by electrophoresis in 1.5 % agarose (Molecular Biology Certified Agarose; Bio-Rad) gels in 0.5 x TBE for 4 h at 100 V. The 100 bp GeneRuler ladder (Fermentas) was used as the molecular size marker. Gels were visualized under UV light and photographed digitally using a GelDoc XR (Bio-Rad). Images were analysed with BioNumerics v6.01 (Applied Maths) and AFLP patterns were compared using the Pearson correlation similarity coefficient with optimization set at 2 % and curve smoothing to 1.5 %.

Environmental samples: compost. For each compost sample, 5 g compost was added to 50 ml sterile distilled water in a Falcon tube and rotated for 1 h at 20 g at room temperature (RT). The compost samples were left for 30 min at RT then centrifuged for 1 min at 1000 g. Two hundred microlitres of compost supernatant was added to 200 μl of a 0.2 M HCl/KCl acid solution for 10 min at RT. The sample was then immediately diluted 1:10 and 1:100 in water and 200 μl was added to glycine (1.5 g l⁻¹), polymyxin B sulfate (40 000 IU l⁻¹), vancomycin hydrochloride (0.5 mg l⁻¹) and cycloheximide (40 mg l⁻¹) (GVPC medium) and modified Wadowsky-Yee (MWY) (Oxoid) plates. The plates were allowed to dry, placed at 37 °C in a moistened chamber, left for 3–4 days and examined daily until day 10. Any suspect colonies were inoculated onto blood agar and BCYE. In the case of blue-white Legionella strains, these were viewed visually under long-wave UV (366 nm) for autofluorescence. Legionella species were identified by polyclonal IFA (Wilkinson et al., 1981) and mip speciation (Ratcliff et al., 1998).

RESULTS

The laboratory results from all patients are detailed in Table 1. The patient in case 1 was urinary antigen-negative by the Biotest urinary antigen ELISA but produced a single high titre of 256 to L. longbeachae sg 1 that remained raised for several months. The patient had been using MPC in a glass conservatory in early spring when the infection was identified. The MPC contained a variety of Legionella species, including Legionella anisa, Legionella londiniensis, Legionella sainthelenensis and two different strains of L. longbeachae sg 1. The patients in cases 1, 2 and 4 were all culture-positive and L. longbeachae sg 1 was identified by both mip speciation and serological examination. In case 2, as well as a respiratory isolate, we identified an environmental isolate from bedding plant mixture that had been handled in a greenhouse environment prior to disease onset. This patient had cultivated plants from seed using the same brand of MPC from the same commercial outlet.

Table 1. Patient laboratory findings showing urinary antigen, IFA serology, culture and PCR results

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>Urinary antigen EIA</th>
<th>IFA antibody titre to L. longbeachae sg 1</th>
<th>Culture</th>
<th>16S rDNA PCR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59/M</td>
<td>Negative†</td>
<td>Single high titre of 256</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>65/M</td>
<td>Negative‡</td>
<td>Negative</td>
<td>Positive</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>66/F</td>
<td>NA</td>
<td>&gt;Fourfold rise in titre</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>68/M</td>
<td>NA</td>
<td>&gt;Fourfold rise in titre</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*Positive after probe hybridization with Legionella species-specific probe. †Biotest EIA. ‡Binax EIA.
and manufacturer as in case 1. In cases 1 and 2, strains of \textit{L. longbeachae} sg 1 indistinguishable from those of the patients' infections were isolated from their respective supplies of this MPC. The bag that the patient in case 1 used contained the distinct AFLP genotype (1a) that the patient in case 1 was infected with and the bag that the patient in case 2 used contained the AFLP genotype (1b) of \textit{L. longbeachae} sg 1 (Fig. 1) that the patient in case 2 was infected with. The patient in case 1 was infected with AFLP genotype (1a) and the patient in case 2 was infected with the other AFLP genotype (1b), which was the only genotype isolated from the MPC brand at the time of case patient 2 illness. In case 1, both AFLP genotypes were isolated from the MPC.

Case 3 was serology-positive and gave a fourfold rise in titre to \textit{L. longbeachae} sg 1 that eventually produced a high titre of 8182. The implicated MPC contained 80 000 c.f.u. \textit{L. longbeachae} sg 1 g\(^{-1}\) that gave an identical genotype (1b) by AFLP as case 2, although the patient in case 3 had used an MPC from a different manufacturer to that used by the patients in cases 1 and 2. The patient in case 3 did not produce any respiratory samples to test in either the PCR or by culture.

In case 4, \textit{L. longbeachae} sg 1 was only isolated from the patient and not from ten MPC samples taken from the home. The AFLP genotype (2) was distinct from any other type isolated from the previous three cases. In conclusion, three distinct genotypes of \textit{L. longbeachae} sg 1 were identified by AFLP from the four cases and the implicated MPC.

**DISCUSSION**

Previous studies have shown a link between \textit{L. longbeachae} infection and compost exposure (den Boer et al., 2007; Kümper et al., 2008); however, to our knowledge, this is the first time that the patients in two geographically linked (but genotypically dissimilar) cases of \textit{L. longbeachae} sg 1 infection have been shown to have had exposure to the same compost brand. Two further cases were identified as a result of raised awareness. In all of the cases, domestic water supplies were excluded as potential sources. No other obvious source or travel exposure was identified.

MPC consists of a mixture of coconut husk, wood fines, green waste and sometimes peat, with a variety of growth-enhancing additives depending on the manufacturer. A number of studies have investigated the diversity of \textit{Legionella} species in compost (Steele et al., 1992; Hughes & Steele, 1994; O’Connor et al., 2007; Lindsay et al., 2009; Casati et al., 2009; Pravinkumar et al., 2010) and found a variety of species in high numbers, ranging from \(10^3\) to \(10^6\) c.f.u. g\(^{-1}\) (Casati et al., 2009). In an international collaborative paper from 2002, involving the USA, New Zealand, Switzerland and Italy, the most commonly isolated non-\textit{pneumophila} \textit{Legionella} species were \textit{L. longbeachae} (3.2 %), \textit{Legionella bozemanae} (2.4 %), \textit{Legionella micdadei}, \textit{Legionella dumoffii} and \textit{Legionella feeleii} (2.2 % combined) (Yu et al., 2002). In contrast, a recent European study showed a different mix of organisms isolated from compost: \textit{L. bozemanii} (26.1 %), \textit{L. pneumophila} 2–15 (serogroups 3, 6 and 10) (19.6 %), \textit{L. sainthelensi} (13.0 %), \textit{L. micdadei} (8.7 %), \textit{L. pneumophila} 1 (mAbs subgroup Philadelphia, France/Allentown, Benidorm) (6.5 %) and \textit{L. longbeachae} from only two samples (4.3 %) (Casati et al., 2009). These studies demonstrate the diversity of \textit{Legionella} species found in composts and potting soils. Most recently, Velonakis et al. (2010) recovered a total of 21 \textit{Legionella} isolates from six out of 22 samples of potting soil. It is interesting that MPC contains a large number of potentially pathogenic \textit{L. pneumophila} and \textit{Legionella} species but \textit{L. longbeachae} infection is the most commonly associated with compost exposure.

MPC production is a large industry and there has been a move in recent years to replace peat with green waste for ecological reasons. It is possible that this change goes some way to explain the recent rise in cases of \textit{L. longbeachae} in Scotland.

**Fig. 1.** AFLP analysis of \textit{L. longbeachae} sg 1 isolates carried out using BioNumerics v6.01 (Applied Maths). Normalized patterns were compared using the Pearson correlation similarity coefficient with optimization set at 2 % and curve smoothing to 1.5 %. The dendrogram was generated using UPGMA clustering. The percentage similarity at the nodes is indicated.
A recent study by Casati et al. (2010) showed that green waste that was stored for long periods of time contained greater numbers of Legionella species than green waste that was stored for shorter timescales. In a preliminary study in this laboratory, there was an increase in Legionella numbers in samples stored under greenhouse conditions (39%) compared to under ambient conditions (19%) (personal communication). This hypothesis warrants further investigation via an adequately powered study to identify whether the difference is real or coincidental.

The significance of Legionella species in MPC and the interaction with amoebae also merits further exploration. Conza et al. (2009) investigated free-living amoebae and Legionella from composting facilities in southern Switzerland and confirmed that viable amoebae and L. pneumophila and L. bozemanii were present in composts and bio-aerosols developed during the composting process. They also found L. longbeachae in the compost but never isolated it in the aerosol experiments, which may suggest an alternative route of infection.

MPC in the UK is subjected to the PAS 100 standard (BSI, 2005) which involves a sanitization phase. In the above investigative work, the implicated composts were manufactured in the UK and would have been subjected to this sanitization phase. They were also tested as a whole so we were unable to demonstrate the particular component of the compost which was contaminated. Although the PAS 100 standard uses indicator species such as Escherichia coli and Salmonella, it is uncertain that the processes used to achieve the required standard would be adequate to completely eliminate legionellae, particularly as they can survive high temperatures inside encysted amoebae.

While compost is the likely source of L. longbeachae infection, the exact mode of transmission is not clear; it may be as a result of aerosols being created during gardening or watering of the compost mixture, usually in an enclosed space, but other modes, such as ingestion via contaminated hands, have also been postulated (Steele et al., 1990).

In Europe, there is a growing tendency to rely on urinary antigen tests to identify cases of LD; however, as these assays are specific for L. pneumophila infection they cannot detect Legionella infection caused by other Legionella species including L. longbeachae.

In summary, the microbiological investigation of MPC, in conjunction with that of human cases of L. longbeachae, can establish the source of infection for public health purposes; however, the distribution, characteristics and associated potential modes of transmission of Legionella species in MPC require to be described, and viewed in the context of the human cases (taking into account likely under-ascertainment given the limitations of current diagnostic techniques). Based on practice in other countries, labelling of compost bags to highlight the risk of L. longbeachae associated with compost use should be considered. Perhaps prior to this given the still low, albeit increased, number of cases, there may be value in examining the various points of the manufacturing processes, and the components used, to identify the source or medium that is most likely to promote the growth of Legionella, allowing measures to reduce the risk of legionella infection to be taken. LD associated with MPC is an emergent disease in Europe (Whiley & Bentham, 2011) but because of diagnostic bias the number of cases identified is low. Therefore, greater vigilance is required in identifying sources and reducing exposures.

In instances where an individual presents with a community-acquired pneumonia (CAP) and has been gardening prior to infection, legionellosis should be considered. As the aetiology of CAP remains undetermined in between 30 and 60% of cases (Yu et al., 2002), it is important that serum and respiratory samples are sent to a national reference centre that has the capability to identify Legionella species.

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REFERENCES


