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Transparency Declaration

The authors declare that they have no conflict of interest.

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Enhanced isolation of *Legionella* species from composted material

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Abstract

Legionella pneumophila and *Legionella* species were isolated from composted material when freshly prepared buffered charcoal yeast extract (BCYE) was supplemented with glycine (1.5 g/L), polymyxin B sulfate (40 000 IU/L), vancomycin hydrochloride (0.5 mg/L) and cycloheximide (40 mg/L) (GVPC medium) and Modified Wadowsky–Yee (MWY) (Oxoid, Cambridge, UK) plates were used for cultivation, but not with commercially sourced pre-poured GVPC and MWY plates (Oxoid). *Legionella cincinnatiensis* and pathogenic *L. pneumophila* serogroup (Sg) 1 Benidorm and France/Allentown were identified, as well as a non-typeable (NT) strain of *L. pneumophila*. As most laboratories no longer produce their own media, this may contribute to the lack of positive cultures from composted material. The antigenicity of the NT strain is discussed.

Keywords: Compost, EIA testing, growth media, IMS, *Legionella* isolation

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Legionellosis is most commonly associated with inhalation or aspiration of contaminated water droplets, but some cases are associated with manipulation and exposure to composted material or growing media. *Legionella longbeachae* is the species most commonly associated with infection from growing media, and was first identified in 1981 in Long Beach, California [1]. Recently, a number of studies have looked at the diversity of *Legionella* species in composted material [2–8], and found a variety of species, ranging from 10^3 to 10^5 CFU/g. In this blind study, six samples of composted material were analysed for the presence of *Legionella* species by direct plating and immunomagnetic separation (IMS) techniques on pre-poured and in-house-prepared glycine (1.5 g/L), polymyxin B sulfate (40 000 IU/L), vancomycin hydrochloride (0.5 mg/L) and cycloheximide (40 mg/L) (GVPC) and Modified Wadowsky–Yee (MWY) plates (Oxoid, Cambridge, UK).

Briefly, 5 g of compost material was added to 50 mL of sterile distilled water. The samples were rotated at 10 ***g*** for 1 h, and then briefly spun down at 1400 ***g*** for 1 min. For direct isolation, 500 μL of the supernatant was added to 500 μL of 0.1 M HCl/KCl (pH 2.2) for 10 min at room temperature Two hundred microlitres of neat, 10^{-1} and 10^{-2} samples were inoculated onto pre-poured GVPC and MWY plates and plates freshly prepared in-house. For IMS, 10 mL of supernatant was added to 35 mL of 18-MΩ water in a falcon tube. Five millilitres of 10× buffer (IMS kit, Invitrogen, Paisley, UK) and 150 μL of IMS beads were added. Tubes were rotated for 1 h at 10 ***g***, and then placed in the magnet holder for 10 min. The supernatant was vacuum extracted, and the magnetic beads were washed in 1× wash buffer. The sample was finally placed in the magnet holder for a further 5 min, and the liquid was vacuum-extracted. The IMS beads were resuspended in 1 mL of 1× wash buffer. Two hundred microlitres was added to pre-poured and in-house-prepared GVPC and MWY plates, and spread over the plates. The IMS samples were also subjected to acid treatment for 10 min and diluted, and 200 μL was plated onto pre-poured and in-house-prepared GVPC and MWY plates. The plates were allowed to absorb the liquid, and then incubated in a moistened chamber at 37°C for a maximum of 10 days. 16S RNA PCR was performed on all samples; all gave negative results, but inhibitors were present that could not be reduced by polyvinylpyrrolidone, Chelex or column extraction. The presence of *Legionella* sp. DNA in the samples cannot therefore be excluded. An antigen preparation for enzyme immunoassay (EIA) testing (*Legionella* urinary antigen EIA; Bartels, County Wicklow, Ireland) was prepared from the *L. pneumophila* serogroup (Sg) 1 Benidorm strain and the *L. pneumophila* non-typeable (NT) strain, as previously described [9], with a McFarland standard starting concentration of 4, which equates to a concentration of 8×10^8 CFU/mL. Fig. 1 shows the EIA titration curve of the two antigen preparations.

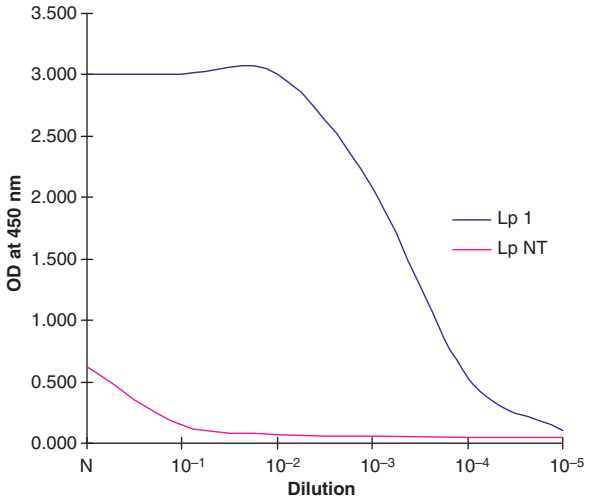

FIG. 1. Antigen titration of *Legionellapneumophila* serogroup (Sg) 1 Benidorm and *L. pneumophila* NT in enzyme immunoassay. Lp 1, *L. pneumophila* Sg 1 Benidorm; Lp NT, *L. pneumophila* non-typeable strain.

Discussion

This research note reports a preliminary study that warrants further testing with a larger sample size to ascertain the differences between media formulations in relation to the isolation of *Legionella* species from compost material. Other

TABLE 1. Isolation of *Legionella* sp. using two types of Oxoid Modified Wadowsky–Yee and glycine, polymyxin B sulfate, vancomycin hydrochloride and cycloheximide plates

Compost material	Identification on pre-poured plates (Oxoid)	Identification on in-house-prepared plates (Oxoid)	Monoclonal subtype	Sequence type	CFU/g
1	ND	ND	–	–	–
2	ND	ND	–	–	–
3	LD	*L. cincinnatiensis*	NA	NA	1000
4	ND	*L. pneumophila*	Non-typeable	New	1000
5	ND	ND	–	–	–
6a	ND	*L. pneumophila* Sg 1	Benidorm	84	2000
6b (IMS)	ND	*L. pneumophila* Sg 1	France/Allentown	84	1000

IMS, immunomagnetic separation; NA, not applicable; ND, not detected; Sg, serogroup; ST, sequence type.
ST 84: 12, 9, 2, 5, 3, 17, 15 (allele numbers).
New ST: 2, 9, 26, 10, 3, 17, 0 (allele numbers).

methods of isolation were reviewed, but the method used is novel and was developed in this laboratory. The fact that this is a preliminary study means that other factors may influence the ability to isolate *Legionella* from composted material that have not been covered in detail. This includes determining how adding other growth inhibitors to the media, e.g. natamycin [10] or propiconazol [2], and how the pH of culture media could affect the growth and isolation of *Legionella*.

All *Legionella* isolates were detected by use of the direct method, with the exception of sample 6, where *Legionella pneumophila* Sg 1 France/Allentown was identified with the IMS technique (Table 1). It is also interesting that *Legionella* was never isolated when pre-poured MWY and GVPC plates were used, but only with freshly poured MWY and GVPC plates (Table 1). This may be a result of the fungicidal properties of the pre-poured plates diminishing on storage, as the major contaminant that caused overgrowth on the plates was fungus. The MWY plates were far better for isolating *Legionella* than the GVPC plates. MWY contains glycine, polymyxin B, anisomycin, vancomycin, bromothymol blue and bromocresol purple. GVPC contains glycine, vancomycin, polymyxin B and cycloheximide. MWY contains more antifungal agents, and GVPC contains more antibacterial agents.

Half of the samples (three) were positive for *Legionella* species, with one sample containing two different *L. pneumophila* Sg 1 monoclonal subtypes (Benidorm and France/Allentown). These were both sequency type (ST) 84 with allele number 12, 9, 2, 5, 3, 17, 15. This is not unusual, as the same ST is often seen in different monoclonal antibody profiles. *L. pneumophila* Sg 1 is the most commonly found *Legionella* strain associated with human disease, and has caused major outbreaks in the past [11]. There has been one publication showing an association between *L. pneumophila* in soil and infection in humans [12].

This was a blind study, and on completion it was revealed that samples 1, 2, 4, 5 and 6 were from green waste, with some containing food waste, and sample 3 was from wood fines. Sample 3 contained *Legionella cincinnatiensis*, which is closely related to *L. longbeachae* serologically, and they may have some common ancestry. When antigen preparations of the *L. pneumophila* Sg 1 Benidorm and *L. pneumophila* NT strains were titrated in the urinary antigen EIA (Fig. 1), the results suggested that infection caused by *L. pneumophila* NT would not be detected in the urinary antigen EIA, owing to its low reactivity when compared with antigen prepared from the Benidorm strain.

In conclusion, the use of pre-poured plates was the single most important factor resulting in the inability to isolate *Legionella* from compost. As most laboratories no longer produce their own media, this may contribute to the lack of positive cultures in laboratory settings. Two compost samples contained potentially pathogenic *Legionella* species, including one strain that would not be detected by the currently preferred method of diagnosis (urinary antigen EIA).

Transparency Declaration

There is no conflict of interest.

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Clonal dissemination of *Klebsiella pneumoniae* ST258 harbouring KPC-2 in Argentina

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Abstract

The present work describes the abrupt emergence of *Klebsiella pneumoniae* carbapenemase (KPC) and characterizes the first 79 KPC-producing enterobacteria from Argentina (isolated from 2006 to 2010). The emergence of bla_{KPC-2} was characterized by two patterns of dispersion: the first was the sporadic occurrence in diverse enterobacteria from distant geographical regions, harbouring plasmids of different incompatibility groups and bla_{KPC-2} in an unusual genetic environment flanked by IS*Kpn8*-Δbla_{TEM-1} and IS*Kpn6-like*. bla_{KPC-2} was associated with IncL/M transferable plasmids; the second was the abrupt clonal spread of *K. pneumoniae* ST258 harbouring bla_{KPC-2} in Tn*4401a*.

Keywords: bla_{KPC-2}, *Enterobacteriaceae*, ST11, ST258, Tn*4401a*

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The emergence and rapid dissemination of *Klebsiella pneumoniae* carbapenemase (KPC)-producing Gram-negative bacilli has become an important therapeutic and infection control problem in the healthcare setting [1]. The worldwide spread of KPC-producing *K. pneumoniae* strains has revealed the successful dissemination of a major clone defined as sequence type (ST)258 [2–4]. bla_{KPC} has been commonly associated with Tn*4401*, which is possibly responsible for its acquisition [5,6]. A different bla_{KPC} environment was described in plasmids from Chinese enterobacteria, where bla_{KPC-2} was associated with a Tn*801*-like transposon [7]. Moreover, ST11 was demonstrated to be the dominant clone of KPC-producing *K. pneumoniae* in China, where ST258 was not detected [8].

We previously reported the emergence of bla_{KPC} in *Enterobacteriaceae* from Argentina (2006), and designed an algorithm to detect class A carbapenemases and metallo-β-lactamases at the level of the clinical microbiology laboratory [9,10]. Since 2008, as the National Reference Laboratory in Antimicrobial Resistance (NRLAR), we have used the algorithm to implement active surveillance of such carbapenemases among 432 laboratories across Argentina that participate in the National Quality Control Programme in Bacteriology (Argentinean Ministry of Health). All enterobacteria were screened through the algorithm: isolates that showed (i) imipenem inhibition zones ≤21 mm, (ii) resistance or intermediate resistance to expanded-spectrum cephalo-