Journal of Applied & Environmental Microbiology, 2021, Vol. 9, No. 1, 1-4
Available online at http://pubs.sciepub.com/jaem/9/1/1
Published by Science and Education Publishing
DOI:10.12691/jaem-9-1-1

Presence of *Legionella Longbeachae* in Pine but not Composted Green Waste Used in Manufacture of Potting Soils in New Zealand

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Received April 17, 2021; Revised May 22, 2021; Accepted May 31, 2021

Abstract In this research we compared the proportion of samples taken at different stages in the manufacturing process of potting soils that were qPCR positive for *Legionella longbeachae*. This included samples of composted feed stock, mixing sites and bagged products. Feedstock, manufacturing sites, and final retail products at three locations across the North and South Islands of New Zealand were tested for *L. longbeachae* DNA by qPCR. Positive tests were found on samples of composted pine bark 14/360 (3.9%), pine sawdust 5/185 (2.7%) and pre-bagged or bagged potting soils 9/200 (4.5%). All samples from peat (n = 20), green waste (n = 100), and mixing areas (n = 73) were negative. Samples that contained pine bark or pine sawdust were more likely to be qPCR positive (28/818, 3.4%) than other samples (0/355, 0%; P<0.001). *L. longbeachae* probably reaches the manufacturing sites from fresh pine bark and persists through the composting and manufacturing process, leading to a wide distribution following the purchase.

Keywords: *Legionella longbeachae, compost, pine, Legionnaires' disease, LD, potting soil*

Cite This Article: Ali Mohammadi, Stephen T. Chambers, Sandy Slow, Trevor Anderson, Jonathan Williman, Pippa Scott, David Murdoch, and Amy Scott-Thomas, "Presence of *Legionella Longbeachae* in Pine but not Composted Green Waste Used in Manufacture of Potting Soils in New Zealand." *Journal of Applied & Environmental Microbiology*, vol. 9, no. 1 (2021): 1-4. doi: 10.12691/jaem-9-1-1.

1. Introduction

Legionella longbeachae was first isolated from three patients with pneumonia in California and one from Georgia in 1980 [1], and subsequently it has been found in patients in Australia, New Zealand, Japan, Europe, United States and Asia. [2-8] A recent nationwide study of Legionnaires' disease (LD) demonstrated that *L. longbeachae* caused 150 (63%) of 238 cases of LD in New Zealand and was widely distributed across the country. [9] There was a marked seasonal variation in LD incidence with most cases occurring in spring and early summer (October to January) confirming previous observations. [10] Case control studies in New Zealand and Australia have linked the recent use, tipping, or trowelling of commercial potting soil, and poor hygiene practices with *L. longbeachae* infection [11,12,13], as well as exposure to aerosolised commercial potting soil with Pontiac fever. [14] Infection with *L. longbeachae*, has also been associated with contact with compost and potting soil in Asia, Europe and North America. [7,15-21]

Steele *et al*. isolated living *L. longbeachae* in Australian made potting soils that contained composted pine, eucalyptus and pine sawdust. [22,23] Subsequent environmental studies in Australia, Europe, Japan and North America, have demonstrated that *L. longbeachae* has been identified in compost, soil and contaminated water bodies. [24,25,26,27] In Europe *L. longbeachae* has never been identified in peat, and studies on green waste reported variable results where *L. longbeachae* was identified in composted but not fresh green waste. [27,28,29] It is possible that green waste becomes contaminated from external sources such as rain and wind-blown material during the composting process. [28]

We have recently demonstrated that *L. longbeachae* DNA is present on the bark of most living pine species including *Pinus radiata*, but was seldom found on bark from non-pine species. [30] This suggests that living pine species provide a reservoir for these organisms. Bark and sawdust from *P. radiata* are a major component of feed stocks used in the manufacture of potting soils in New Zealand. Because of this we thought that *L. longbeachae* would be present in pine bark, at the potting soil manufacturing site and persist in pine containing

manufactured products. Our aim was to determine if *L. longbeachae* could be found in the feedstock at these manufacturing plants. If so, *P. radiata* products may be the major source of *L. longbeachae* in commercial potting soils and could provide a control point for reducing *L. longbeachae* content in such products, thereby reducing the incidence of human disease.

2. Materials and Methods

A point prevalence study conducted at three commercial potting soil and compost production sites widely dispersed across the North and South Islands of New Zealand. Sampling of feed stock was unrestricted but was conducted within the manufacturing site's health and safety protocols.

2.1. Potting Mix Manufacturing Sites

Production site 1 was located in the lower South Island. Samples were taken from large windrows of graded pine bark or piles of pine sawdust outside the plant, the indoor mixing area, and locally produced peat that had been stored inside the plant. Samples were taken at approximately 3 m intervals across the surface and edges of the feedstock at depths between 5 and 50 cm, from a grid at 1 m intervals over the floor of the mixing area, and from bagged finished product stored at the site. There was no composted green waste on site. Samples (N=348) were taken on 12 April 2017.

Production site 2 was in the upper South Island providing access to large piles of pine bark, partially composted sawdust, some imported peat, the mixing area and finished product but no green waste. All feedstock, mixed composted bagged material and not yet bagged product was located outside. The mixing area was indoors. Samples (N=255) were taken on 17 April 2017.

Production site 3 was in the upper North Island of New Zealand. Most of the feedstock was located offsite and could not be sampled at that location. Feedstock that could be sampled was brought inside to a temporary storage area and included locally produced bark products and composted green-waste. Peat was imported from Europe. Mixing and bagging of the products was done inside, and the bagged product was stored outside with bags on large plastic covered pallets awaiting shipping. Samples (N=570) were taken on 31 May 2017.

2.2. Quantitative PCR (qPCR)

The samples were pulverised and 5 g were mixed with 50 mL of ultrapure distilled water (Invitrogen, Thermo Fisher Scientific, MA, USA) and shaken for 5 minutes. Two hundred microliters of the supernatant were used for DNA extraction using the GenElute Bacterial DNA extraction kit (Sigma, MO, USA) as per the manufacturer's instructions. The presence of *L. longbeachae* DNA was detected using the qPCR parameters and the primer and probes designed and validated as specific for *L. longbeachae* as previously described [10,30]. Positive and negative controls were included in each run and negative qPCR results were validated by using internal controls.

The crossing threshold (CT) cut off of ≤44 cycles for the lower limit of detection and quantification was determined by spiking samples of sterilised potting mix with dilutions of a pure culture of *L. longbeachae* sg1 ATCC33462 from $x10^1$-$x10^7$ cfu/mL (R^2 = 0.9964 y = -1.7ln(x) + 48.811). The lower limit of quantification was 10 CFU/mL.

2.3. Statistical Analysis

The prevalence rates of samples meeting qPCR CT values for *L. longbeachae* were summarised by site and type as counts and percentages with 95% binomial (Wilson) confidence intervals. Differences in rates were compared across subgroups using Fisher's exact tests. All statistical testing was conducted using R software (version 4.0.3) [31] and P values < 0.05 were considered significant.

3. Results

Fourteen (4%) pine bark samples were qPCR positive, with all but one coming from production site 1 (Table 1). There were significant differences in the percentages of qPCR positive samples between the manufacturing sites (18/348 from site 1, 3/255 from site 2 and 7/570 from site 3; Fisher's exact p<0.001).

None of the samples from the mixing area, which is the beginning of the blending and bagging cycle, were qPCR positive but 9 of 200 (4.5%) samples taken from pre-bagged or bagged compost or potting mix products were qPCR positive. These were predominantly from samples taken at production site 3.

Table 1. qPCR results of samples collected from three widely separated potting mix production sites across both the North and South Islands of New Zealand in Autumn 2017

	Production Site 1		Production Site 2		Production Site 3		Total	
Sample type	Sample number	PCR positive N (%, 95% CI)	Sample number	PCR positive N (%;95% CI)	Sample number	PCR positive N (%;95% CI)	Sample number	Number positive N (%;95% CI)
Bark	100	13 (13;7.8-21.0)	170	1 (0.6;0.1-3.3)	90	0 (0%)	360	14 (3.9; 2.3-6.4)
Sawdust	95	5 (5.3;2.2-11.7)	40	0 (0;0-8.8)	50	0 (0%)	185	5 (2.7;1.2-6.2)
Peat	100	0 (0;3.7-4.6)	5	0 (0;0-43)	100	0 (0%)	205	0 (0;0-1.8)
Green waste	0	na	0	na	130	0 (0%)	130	0 (0;0-2.9)
Pumice	0	na	0	na	20	0 (0%)	20	0 (0;0-16)
Mixing area	53	0 (0;0-6.8)	20	0 (0;0-16.1)	0	na	73	0 (0;0-5)
Pre-bagged or bagged product	0	na	20	2 (10;2.8-30.0)	180	7 (3.9;1.9-7.8)	200	9 (4.5;2.4-8)
Total	348	18 (5.2, 3.3-8.0)	255	3 (1.2, 0.4-3.4)	570	7 (1.2, -.6-2.5)	1173	28 (2%)

Overall of the 1153 samples 28 (2.4 %) were qPCR positive (Table 1). There was a significant difference in the proportion of qPCR positive samples from bark and bark containing products (pine bark, pine sawdust, mixing area and bagged product, 28/818, 3.4%) compared with non-bark containing samples (peat, green waste and pumice 0/355, 0%; Fisher exact p<0.001).

4. Discussion

In this study we have found that *L. longbeachae* DNA could be detected in pine bark and pine sawdust feedstock, or pine bark containing potting soil products from three widely separated production sites across New Zealand. In contrast, *L. longbeachae* DNA was not detected from peat and green waste feedstock. As pine bark is a major constituent of potting soils, it is likely to be the major source of *L. longbeachae* in these products. In addition, pine products may facilitate the persistence of *L. longbeachae* in the manufactured potting soil products.

The presence *L. longbeachae* DNA in pine bark and pine sawdust is consistent with the finding that this organism was present on bark from living *P. radiata* and with studies from Steele *et al*. who found living *Legionella* spp. in fresh pine sawdust and composted bark. [22,30] The lack of evidence for *L. longbeachae* in composted green waste is compatible with other studies that have reported variable results. In Switzerland, Casati *et al* found multiple pathogenic *Legionella* species, (e.g. *Legionella pneumophila* sg 1-15, *Legionella bozemanii*, *Legionella cincinnatiensis*, *Legionella jamestowniensis*, *Legionella micdadei* and *Legionella oakridgensis*) but not *L. longbeachae* in samples from stored composted green waste. In contrast they found only a single legionella isolate (*L. cincinnatiensis*) in samples taken from fresh green waste at three separate sites. [28] They suggested that green waste becomes contaminated from other environmental sources such as wind and rain. In the Netherlands, Huss et al., found that many legionella species were present in 97 (68%) of both fresh and composted green waste but there was only one isolate of *L. longbeachae* identified (0.7%). [29] In Scotland Currie et al., did not find any *Legionella* species in the single green waste product they had sampled, but found multiple isolates of many species including *L. longbeachae* in peat containing composts. [27] As *Legionella* species have never been isolated from peat the organisms are likely to have come from another source.

L. longbeachae was found on a relatively low number of samples of pine bark and pine containing products and there were differences in the proportion of positive samples between the sites. This may have been related to multiple factors. Firstly, the study was done in winter when there is both a low rate of clinical LD caused by *L. longbeachae* and where we have previously shown that only a low proportion of bark samples from living trees had detectable levels of DNA present. [9,10,30] *L. longbeachae* favours growth over a temperature range of 4°C – 25°C rather than higher temperatures, but will persist at temperatures down to -20 °C in potting mix for months. [22,32] Secondly, the time of sampling may be important as the load of *L. longbeachae* may vary with local variations in temperate and humidity. This has previously been reported for *L. pneumophila* and it is likely, these factors will also influence *L. longbeachae* in similar manner. [33,34] Thirdly, variation in accessibility of samples may also have contributed to differences found between production sites. The feedstock at production sites 1 and 2 were outside, so that samples could only be taken close to the surface of the windrows. In contrast, at production site 3 samples could be taken from the indoor low capacity depots that were refilled by a high capacity digger that mixed variable portions from across the windrow types. Similarly, *L. longbeachae* may have been inhibited in inner portions where composting produces high temperatures, while outer portions are cooler with temperatures that are supportive of *L. longbeachae* replication, either in the environment or within protozoa or nematodes. [33,34,35]

Other limitations to this study include the use of qPCR as the primary endpoint. Our findings do not exclude the presence of *L. longbeachae* in numbers below the level of detection. The samples have not been cultured and positive cultures would have added more certainty to our findings but it is less sensitive than qPCR. [36] Secondly, the green waste tested was produce by a well-controlled commercial process that may have eliminated *L. longbeachae* so that these results may not apply to green waste compost produced by other processes.

5. Conclusion

Pine bark provides an important niche for *L. longbeachae* and the use of pine bark in the manufacture of potting soils is likely to be a major source, but not necessarily the only source of this organism. Failure to find *L. longbeachae* in peat and composted green waste suggests that control measures on *P. radiata* feedstock at the site of manufacture may offer a way to reduce the rates of human disease. In addition, *P. radiata* is planted in extremely large commercial plantations in several countries and there are also large industries based on other pine species around the world that could pose a hazard to human health. The use of pine based product as a substitute for peat, as this resource becomes scarce, needs careful consideration.

Acknowledgements

We sincerely thank all of the manufacturing site staff for access to sites and samples. We have no actual or potential competing interests regarding this study. This work was funded by an unrestricted grant from the Canterbury Medical Research Foundation, a University of Otago Scholarship grant and an unrestricted grant from Tui Products Ltd, Tauranga, New Zealand.

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