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Pine Species Provide a Niche for *Legionella Longbeachae*

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Abstract *Legionella longbeachae* is the commonest cause of Legionnaires' disease (LD) nationwide in New Zealand (NZ). Most cases occur in spring and summer (October - January) and are associated with the use of commercial potting mix, which usually contains pine bark. *L. longbeachae* is an environmental organism but its niche has not yet been defined. Bark samples were taken at chest height from trees in three stands of *Pinus radiata* (Monterey pine) located in the central South Island of NZ. *L. longbeachae* DNA was detected by qPCR in 28/400 (7%) samples and from 22/50 (44%) different trees. There was a significant difference in the proportion of positive tests by season: summer 0/50 (0%); autumn 0/50 (0%); winter1/50 (2%); spring 22/50 (44%); ($p<0.001$). Bark samples from non-*P. radiata* pine species and adjacent mixed species were then tested. More samples from pine species 22/28 (79%) than non-pine species 6/37 (16%) tested positive for *L. longbeachae* ($p<0.001$). Pine species appear to be an important ecological niche for *L. longbeachae.* To our knowledge this is the second human pathogen to have an arboreal niche. The use of bark from *P. radiata* in commercial potting mix may contribute to the incidence of LD in New Zealand.

Keywords: *legionella longbeachae, pine trees, season, reservoir*

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1. Introduction

Legionella longbeachae was first isolated from patients with pneumonia in California and Georgia in 1980. [1] Those from California were from Long Beach, Los Angeles and Concord, which is close to Monterey near the San Francisco metropolis. It has subsequently been found in patients in Australia, New Zealand, Japan, Europe, and Asia. [2,3,4,5,6,7,8] A recent nationwide study of Legionnaires' disease (LD) in New Zealand demonstrated that *L. longbeachae* was the commonest cause of LD and 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]

A case control study in New Zealand found that using commercially produced potting soils, but not homemade compost, was strongly associated with LD (OR 6.2, 95% CI 2.2-17.3). LD with *L. longbeachae* infection was also linked to tipping, or troweling of commercial potting soils, and poor hygiene practices. [11] These results were similar to a case control study conducted in Australia. [12] Exposure to aerosolised commercial potting mix may also cause Pontiac fever. [13] Reports from Asia, Europe and North America have also found contact with compost and potting soils to be associated with *L. longbeachae* infection. [3,14,15,16,17]

The seminal environmental studies by Steele and co-workers in Australia demonstrated the presence of *L. longbeachae* in a large proportion of Australian potting soils. [18,19] It was also isolated from a very small sample of fresh and composted pine sawdust, composted but not fresh hammer milled pine bark (unspecified species), and composted but not fresh eucalyptus (unspecified species) used to manufacture potting soils. [19] They did not find evidence of *L. longbeachae* in imported potting soils but it has since been identified in the potting soils of several other countries. [14,20,21,22,23,24] It is possible that composting may contribute to the content of *L. longbeachae* in these samples but the results do not exclude the possibility that *L. longbeachae* is present on bark on living trees prior to composting. This is of particular interest in New Zealand as hammer milled *Pinus radiata* bark is a major component of potting mixes.

The objectives of this study were to determine firstly, whether *L. longbeachae* DNA was present on bark of living pine trees, and whether there was a seasonal variation in proportion of trees on which it could be detected, and secondly, whether *L. longbeachae* DNA was present on other tree species.

2. Methods

The primary endpoint of the study was a positive qPCR test for *L. longbeachae* DNA. A cohort of *P. radiata* trees at three locations in the central South Island of New Zealand was sampled over four seasons. A survey of pine and other mixed trees that could be identified to the species level, in the central South Island of New Zealand was then conducted during late spring time/early summer. Approximately 10 g of superficial bark, avoiding the cambium, was taken at chest height. Samples were placed into separate, labelled plastic containers. Trees were identified by a member of the research group (JC). Permission for sampling was obtained from the owners of the trees.

Pinus radiata. Stands of *P. radiata* trees at three sites separated from each other by 10-15 kilometres, around Christchurch city were identified. All 22 *P. radiata* trees in a pinetum were sampled. Plantations 2 and 3 contained hundreds of trees which were sampled by starting from the northwest corner and taking samples from sequential trees on a 45-degree diagonal leading toward the heart of the stand. Individual trees were identified by GPS, aerial photographs and local photographs to ensure the same trees were sampled on each occasion during January (summer), April (autumn), July (winter), and October (spring) 2018.

We estimated that if *L. longbeachae* was present on 10% (+/-9%) of pine trees sampling 50 trees would give us 95% chance of detecting it on at least one tree.

Other pine species and non pine tree species. Bark from all pine trees other than *P. radiata* growing in the pinetum (40 trees of 28 species) and a similar number of non-pine well identified species (41 from 38 species) growing at the same location in Christchurch were collected. The non-pine species were selected by convenience from approximately 100 mixed tree species located within 400 metres of the pine trees. All trees were numbered sequentially and results for the first tree sampled of any species was included in the analysis of tree species. Results from analysis of bark samples from subsequent trees of the same species were included in results as duplicates.

The samples of non-*P. radiata* pine species were taken on 15 October 2018 and other mixed trees on 21 November 2018.

2.1. 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) 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] Positive and negative controls were included in each run and negative qPCR results were validated by using internal controls.

A positive result was defined as a crossing threshold (CT) of $\leq$44 cycles. This lower limit of quantification was determined with dilutions of a pure culture of *L. longbeachae* sg1 ATCC33462 from $x10^1$-$x10^7$ cfu/mL ($R^2 = 0.9964$ $y = -1.7\ln(x) + 48.811$).

2.2. Statistical Analysis

For each study the rates for *L. longbeachae* were summarised as counts and percentages of samples meeting the qPCR CT values of $\leq$44 cycles. Differences in rates were compared across subgroups using Chi-square or Fisher's exact tests. McNemar tests were used for comparison of paired data.

3. Results

3.1. Seasonal Sampling from Stands of Living *Pinus radiata* Trees

Four hundred bark samples were collected from 50 trees (two samples per tree; one north and one south aspect) over four seasons (100 samples per season). The qPCR result was positive in 29 bark samples over all season but 28 of these were from samples taken in spring (Table 1). Twenty-two (44%) trees were qPCR positive and six of these had two positive samples at the same time. One tree was positive on two occasions (winter and spring). Across sites, the proportion of qPCR positive trees was broadly similar (pinetum = 7/22, 32%; plantation 2 = 8/12, 67%; plantation 3 = 7/16, 44%; p = 0.12).

Table 1. Results of testing bark from tree species during spring

	Pinus radiata	**non-*P. radiata* species**	**Non-pine species**
Trees tested, number	50	40	41
Bark samples, number	100	80	82
Bark qPCR positive, number (%)	28 (56%)	47(59%)	8(10%)
Trees tested positive, number (%)	22(44%)	31(78%)	6(15%)
Species tested number (%)	1	28	38
Species qPCR positive first tree of duplicates number (%)	1(100%)	22(79%)*	6 (16%)*
Duplicate species tested.	49	12	2 trees of a single species
Duplicates trees qPCR positive	21	9 of 5 species	0

When more than one tree of the same species was tested only results from the first one was included for that species. The others were entered as duplicates. *p<0.001.

There was strong evidence that the percentage of samples that were qPCR positive differed by sampling times (Chi squared p <0.001). Twenty-two (44%) trees were qPCR positive in spring, one in winter (2%) but none in either summer or autumn. *L. longbeachae* was more often detected in samples from the south side of the trees (less sun exposed), with 19/200 (9.5%) compared to 9/200 (4.5%) on the north side (more sun exposed) (McNemar's test, p = 0.013).

3.2. Survey of non *P. radiata* Pine Species in Spring

Forty-seven (59%) bark samples taken from 40 non-*P radiata* trees (2 from each tree; n=80 samples) were qPCR positive (Table 1), with 31 (78%) of the 40 individual trees having at least one qPCR positive sample. Of the 28 species tested 22 (78%) had at least one positive bark sample (Table 2). Samples taken from the south side of the tree were positive in 25 and 22 from the north side.

3.3. Non-pine Tree Species in Spring

Eight (10%) bark samples taken from the 41 individual trees (2 from each tree; n=82 samples) were qPCR positive (Table 1), with 6 (16%) of the 38 species tested having at least one positive sample (Table 3). Four qPCR positive species were endemic to New Zealand (*Agathis australis*, *Lophozonia menziesii*, *Dacrycarpus dacrydioides*, *Prumnopitys ferruginea*) and two to Australia (*Eucalyptus delegatensis, E. fastigata*).

Samples taken from the south side of the tree were positive in 5 and 3 from the north side.

The proportion of qPCR positive pine trees was significantly greater than the other mixed trees (p<0.001).

Table 2. qPCR results of samples collected from 40 trees of 28 non-*P. radiata* pine tree species tested on a single occasion in spring

Species	Continent / Region	No of trees tested	qPCR CT positive
	Asia		
Pinus densiflora	Northeast Asia including Japan	**1**	**1**
Pinus gerardiana	Central Asia	**1**	**1**
Pinus thunbergii	Northeast Asia including Japan	**1**	**1**
Pinus wallichiana	Central Asia from Afghanistan to China	**3**	**2**
Pinus yunnanensis	Southwest China	**2**	**2**
	Europe/Eurasia		
Pinus brutia	Eastern Mediterranean	**1**	**1**
Pinus canariensis	Canary Islands	**1**	**0**
Pinus mugo	Southwestern to southeast Europe	**1**	**0**
Pinus nigra	Europe and north Africa	**3**	**2**
Pinus pinea	Mediterranean	**7**	**6**
Pinus sylvestris	Eurasia from western Europe to Siberia	**1**	**0**
	Central America and Mexico		
Pinus durangensis	Northwest Mexico	**1**	**1**
Pinus hartwegii	Mexico and Central America	**1**	**1**
Pinus montezumae	Mexico and Central America	**1**	**2**
Pinus patula	Mexico	**1**	**1**
Pinus pseudostrobus	Mexico	**1**	**0**
	North America		
Pinus banksiana	Eastern USA	**1**	**1**
Pinus glabra	Southeast North America	**1**	**1**
Pinus ponderosa	Western USA	**1**	**1**
Pinus pungens	Appalachian Mountains, USA	**1**	**1**
Pinus rigida	Eastern USA	**1**	**1**
Pinus sabiniana	California, USA	**1**	**1**
Pinus serotina	Atlantic Plain USA	**1**	**1**
Pinus strobus	Eastern North America	**1**	**1**
Pinus torreyana	Coastal California, USA	**1**	**1**
Pinus virginiana	Appalachian Mountains, USA	**1**	**1**
Pinus edulis	Southern USA	**2**	**0**
Pinus muricata	Coastal California, USA	**1**	**0**
Total		**40**	**31**

Table 3. qPCR results of samples collected from 41trees from 38 non-pine tree species tested on a single occasion in spring

Family	Genus species	Common name	Continent / Region (other than New Zealand)	Trees tested	qPCR positive
			Africa		
Podocarpaceae	*Podocarpus latifolius*		Southern Africa	1	0
			Asia		
Pinaceae	*Abies fabri*	fir	China	2	0
Aceraceae	*Acer cappadocicum*	maple	Caucuses to Himalayas	1	0
Aceraceae	*Acer griseum*	maple	China	1	0
Cupressaceae	*Cunninghamia lanceolata*		China	1	0
Taxodiaceae	*Metasequoia glyptostroboides*	dawn redwood	China	3	0
Podocarpaceae	*Podocarpus macrophyllus*		China and Japan	1	0
Rosaceae	*Prunus serrula*	cherry		1	0
			Australia		
Fabaceae	*Acacia melanoxylon*	wattle	Eastern region	1	0
Myrtaceae	*Eucalyptus delegatensis*	eucalypt	Southeastern Australia	1	1
Myrtaceae	*Eucalyptus fastigata*	eucalypt	Southeastern Australia	1	1
Myrtaceae	*Eucalyptus macarthurii*	eucalypt	New South Wales	1	0
Myrtaceae	*Eucalyptus viminalis* subsp. *cygnetensis*	eucalypt	Southeastern Australia	1	0
			Central America		
Pinaceae	*Abies religiosa*	fir	Mexico/Guatemala	1	0
			Europe		
Hippocastanaceae	*Aesculus hippocastanum*	horse chestnut	Balkans	1	0
Betulaceae	*Alnus glutinosa*	alder	Eurasia	1	0
Ericaceae	*Arbutus canariensis*		Canary Islands	1	0
Betulaceae	*Betula pendula*	birch	Eurasia	1	0
Oleaceae	*Fraxinus excelsior*	ash	Europe/Russia	1	0
Tiliaceae	*Tilia platyphyllos*	lime	Europe	1	0
			New Zealand		
Araucariaceae	*Agathis australis*	kauri		1	1
Taxaceae	*Dacrydium cupressinum*	rimu		1	0
Dicksoniaceae	*Dicksonia fibrosa*	tree fern			
Fagaceae	*Fuscospora fusca*	red beech		1	0
Fagaceae	*Lophozonia menziesii*	silver beech		1	1
Fagaceae	*Fuscaspora solandri*	black beech		1	0
Myrtaceae	*Metrosideros umbellata*	southern rata		1	0
Scrophulariaceae	*Myoporum laetum*	ngaio		1	0
Oleaceae	*Nestegis cunninghamii*	black maire		1	0
Podocarpaceae	*Dacrycarpus dacrydioides*	kahikatea		1	1
Podocarpaceae	*Podocarpus hallii*	Hall's totara		1	0
Podocarpaceae	*Prumnopitys ferruginea*	miro		1	1
Podocarpaceae	*Prumnopitys taxifolia*	matai		1	0
Podocarpaceae	*Podocarpus totara*	totara		1	0
			North America		
Cupressaceae	*Cupressus macrocarpa*	macrocarpa	California	1	0
Juglandaceae	*Juglans nigra*	walnut	Eastern USA	1	0
Cupressaceae	*Juniperus flaccida*	juniper	South West USA/Mexico	1	0
Taxodiaceae	*Taxodium distichum* var. *imbricarium*	pond cypress	Southeast USA	1	0
Other					
Betulaceae	*Betula* x *fetisowii*	birch	Garden origin	1	0

4. Discussion

The primary aim of this study was to determine whether *L. longbeachae* DNA was detectable on the bark of living pine trees. We found qPCR positive bark samples taken during spring from *P. radiata* trees (22/50) and from most of the other living pine species tested (31/40) but seldom from *P. radiata* at other times of year. This result is consistent with the isolation of *L. longbeachae* from fresh pine sawdust although it is possible these samples had been contaminated at the production site. [18] In contrast, *L. longbeachae* DNA was found on the bark of a small minority of other mixed tree species (6/37), including from two of four *Eucalptus* species. These results suggest that of the many living trees, pine species may be a natural reservoir of this organism. If so, this would be the second human pathogen to have an arboreal reservoir following *Cryptococcus gattii*. [25]

There was some evidence that *L. longbeachae* favoured moist conditions in the *P. radiata* plantations, as samples taken from the south-facing, moister side of the tree were more often qPCR positive than the drier north-facing side (in the Southern Hemisphere). The conditions needed to promote growth and survival of *L. longbeachae* in the environment have not been well defined but our results suggest that moist conditions with rising temperatures found during spring may be important. *L. longbeachae* favours a temperature range between 4 °C - 25 °C rather than 25°C and 42°C for *L. pneumophila*. [26] Adaptation to this temperature range may contribute to the abundance of *L. longbeachae* in spring. We are unaware of any studies linking the time that cases of *L. longbeachae* LD occur with environmental conditions, such as water flows and humidity, although this has been documented in LD cases caused by *L. pneumophila*. [27,28]

The finding that *L. longbeachae* was more common on living pine species than on living non-pine species ($p<0.001$) tested suggests that pine species offer a more favourable niche. The nature of this niche is unknown but may include an acidic environment as *L. longbeachae* tolerates pH as low as 4.0 and water extracts of pine barks have a pH at that level. [29,30] Bark may also provide nutrients as the genome of *L. longbeachae* encodes genes for proteins that can degrade cellulose, hemicellulose, pectin and other plant material. [31] The niche may also overlap with the range of protozoa that can host *L. longbeachae.* [32]

Pine species form the basis of major industries across the globe. *Pinus radiata* plantations are managed commercially in Australia, Spain, South Africa and Chile as well as in New Zealand. There are also industries in Europe, Asia and the Americas associated with other pine species. [33] It is unlikely that these industries will cause human disease unless they facilitate close contact between humans and pine products, and conditions allow the organism to amplify to an infectious dose. There may be some risk if pine products are substituted for peat in commercial potting mix products in Europe as peat supplies may not be sustainable as it is a non-renewable resource. [34]

There are several limitations to these studies. Firstly, we used qPCR as the primary endpoint and have not cultured qPCR positive samples. This would have added to certainty about the result but culture is less sensitive than qPCR. [35] The qPCR we used had a relatively low efficiency due to the nature of the binding of the primers. In contrast, the binding of the probes is highly specific and the assay has been used diagnostically with success. [9,10] A negative qPCR does not preclude the presence of *L. longbeachae* at numbers below the detection limit of the assay. Finally, we have not tested our samples for evidence of other legionella species, or potential hosts for *L. longbeachae*, both of which would add to our understanding of the possible niche of *L. longbeachae*.

5. Conclusions

Living pine bark appears to provide an important niche for *L. longbeachae* with a major increase in qPCR positive samples found during spring. *L longbeachae* was named for Long Beach in California where the first human case was described (McKinney *et al*., 1981). [1] *P radiata* is also known as Monterey pine, which is endemic to that locality. It would not be surprising that many other pine species across Western USA, Mexico and further afield have co-evolved with *L. longbeachae* to maintain a niche for this organism.

The observed seasonal increase in percentage of *L. longbeachae* DNA positive trees may contribute to the seasonal increase in LD found in New Zealand as large quantities are used in commercial potting mixes.

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. Infection with *L. longbeachae* needs to be considered if there are outbreaks of unexplained respiratory illness in people working in these industries.

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Competing interests

The authors have no competing interests

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