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RESEARCH ARTICLE



Molecular typing of *Leptospira* spp. in farmed and wild mammals reveals new host-serovar associations in New Zealand

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ABSTRACT

Aims: To apply molecular typing to DNA isolated from historical samples to determine *Leptospira* spp. infecting farmed and wild mammals in New Zealand.

Materials and methods: DNA samples used in this study were extracted from urine, serum or kidney samples (or *Leptospira* spp. cultures isolated from them) collected between 2007 and 2017 from a range of domestic and wildlife mammalian species as part of different research projects at Massey University. Samples were included in the study if they met one of three criteria: samples that tested positive with a *lipL32* PCR for pathogenic *Leptospira*; samples that tested negative by *lipL32* PCR but were recorded as positive to PCR for pathogenic *Leptospira* in the previous studies; or samples that were PCR-negative in all studies but were from animals with positive agglutination titres against serogroup Tarassovi. DNA samples were typed using PCR that targeted either the *glmU* or *gyrB* genetic loci. The resulting amplicons were sequenced and typed relative to reference sequences.

Results: We identified several associations between mammalian hosts and *Leptospira* strains/serovars that had not been previously reported in New Zealand. *Leptospira borgpetersenii* strain Pacifica was found in farmed red deer (*Cervus elaphus*) samples, *L. borgpetersenii* serovars Balcanica and Ballum were found in wild red deer samples, *Leptospira interrogans* serovar Copenhageni was found in stoats (*Mustela erminea*) and brushtail possums (*Trichosurus vulpecula*), and *L. borgpetersenii* was found in a ferret (*Mustela putorius furo*). Furthermore, we reconfirmed previously described associations including dairy cattle with *L. interrogans* serovars Copenhageni and Pomona and *L. borgpetersenii* serovars Ballum, Hardjo type bovis and strain Pacifica, sheep with *L. interrogans* serovar Pomona and *L. borgpetersenii* serovar Hardjo type bovis, brushtail possum with *L. borgpetersenii* serovar Balcanica, farmed deer with *L. borgpetersenii* serovar Hardjo type bovis and hedgehogs (*Erinaceus europaeus*) with *L. borgpetersenii* serovar Ballum.

Conclusions: This study provides an updated summary of host–*Leptospira* associations in New Zealand and highlights the importance of molecular typing. Furthermore, strain Pacifica, which was first identified as Tarassovi using serological methods in dairy cattle in 2016, has circulated in animal communities since at least 2007 but remained undetected as serology is unable to distinguish the different genotypes.

Clinical relevance: To date, leptospirosis in New Zealand has been diagnosed with serological typing, which is deficient in typing all strains in circulation. Molecular methods are necessary to accurately type strains of *Leptospira* spp. infecting mammals in New Zealand.

Abbreviations: Cq: Quantification cycle; MAT: Microscopic agglutination test; qPCR: Quantitative polymerase chain reaction

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Introduction

Leptospirosis is an emerging zoonosis of global importance (Dunay *et al.* 2016). It is caused by the bacteria *Leptospira*, a genus with 71 species (Korba *et al.* 2021) and over 300 serovars (Picardeau 2017) worldwide. This genus is divided into two major clades:

pathogenic (P) and saprophytic (S) and four subclades (P1, P2, S1 and S2). P1 is the virulent clade and P2 is the intermediate low-virulence clade, both with 21 species in each clade, while S1 and S2 are the saprophytic clades with 24 and 5 species in each clade, respectively (Vincent *et al.* 2019; Korba *et al.* 2021). The pathogenic


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species primarily infect mammals and can colonise a broad range of domestic and wild species (Picardeau 2017). Disease transmission to humans occurs via contact with infected animal urine or the contaminated environment. Historically in New Zealand, only two species from the P1 clade and six serovars were known to be endemic in animals (Marshall and Manktelow 2002). These included *Leptospira borgpetersenii* serovars Ballum, Tarassovi, Balcanica and Hardjo type bovis, and *Leptospira interrogans* serovars Pomona and Copenhageni. *L. interrogans* serovars Canicola and Australis have also been isolated but from human cases only (Marshall and Manktelow 2002), are observed occasionally in low numbers in humans, and are considered exotic (Nisa *et al.* 2020).

Surveillance of *Leptospira* strains circulating in New Zealand, both in humans and animals, relies on serology using the microscopic agglutination test (MAT) (Musso and La Scola 2013). However, typing strains with serology is challenging as this assay cannot distinguish between species, requires accurate knowledge of locally circulating strains, the interpretation of results can be subjective, and there is cross-reactivity within and between serogroups (Musso and La Scola 2013). For example, in New Zealand, cattle are often infected with serovar Hardjo type bovis which belongs to the species *L. borgpetersenii*. This strain serologically reacts like serovar Hardjo type prajitno which belongs to the species *L. interrogans* (Ramadass *et al.* 1990; Chideroli *et al.* 2017). Furthermore, both Hardjo type bovis and Hardjo type prajitno cross-react with serovar Balcanica as they are all in one serogroup, Sejroe. Serovars Hardjo type bovis and Balcanica are both endemic in New Zealand, but current typing methods using serology cannot distinguish between them. However, we recently developed a molecular typing method where we identified and demonstrated that genomic data from the *glmU* loci can be used to achieve serovar distinction for all serovars circulating in New Zealand, and it can discriminate all the pathogenic species in the P1 clade (Wilkinson *et al.* 2021).

In New Zealand, dairy farmers, meat workers and pig farmers were identified as high-risk occupations in the 1970s (Marshall and Manktelow 2002). Cattle were the recognised maintenance host for serovar Hardjo and pigs for serovars Pomona and Tarassovi (Marshall and Manktelow 2002). In the early 1980s, dairy cattle vaccines against serovars Hardjo and Pomona and pig vaccines against serovars Pomona and Tarassovi were implemented as an intervention to reduce the risk of disease in humans (Marshall and Cheresky 1996; Fairly 1997). Analysis of serological data of notified human cases from 1999 to 2017 showed a decline in leptospirosis cases with serovars Hardjo (0.81–0.44 per 100,000) and Pomona (0.43–0.24 per 100,000) but an increase in cases with serovars Ballum (0.23–0.38 per 100,000) and Tarassovi (0.11–0.15 per 100,000) (Nisa *et al.* 2020). Tarassovi cases were associated with dairy farmers, while

Ballum cases were associated with occupations other than farming and meat processing (Benschop *et al.* 2021). Serovar Ballum was first identified in the late 1970s in wildlife including the house mouse (*Mus musculus*), ship rat (*Rattus rattus*), brown rat (*R. norvegicus*), and hedgehog (*Erinaceus europaeus*) (Hathaway *et al.* 1978) and these host–serovar associations remain today (Moinet 2020). A study from 2016 to 2018 showed Ballum prevalence in mice varied seasonally from 83 (95% CI = 61–95)% to 86 (95% CI = 65–97)% in spring, to 31 (95% CI = 21–43)% to 37 (95% CI = 26–50)%, in autumn while prevalence in ship rats and hedgehogs was 44 (95% CI = 26–62)% and 27 (95% CI = 11–50)%, respectively (Moinet 2020). It is unclear whether the increase in human cases with serovar Ballum is linked to exposure to an increasing number of infected wild hosts, a more contaminated environment, or to a change in serovar-associations within livestock.

Serovar Tarassovi has historically been associated with pigs (Marshall and Manktelow 2002). However, a survey of 4,000 dairy cattle in 2016 identified 2.4% of cattle shedding *Leptospira* spp. and over 50% of the shedders were seropositive to Tarassovi. Since cattle have historically been associated with serovars Hardjo and Pomona, Tarassovi is now considered an emerging strain in dairy cattle posing public health risk (Yupiana *et al.* 2019a), especially for dairy farmers (Nisa *et al.* 2020). Application of molecular typing methods identified the strain shed by dairy cattle at the species level as *L. borgpetersenii*; however, the sequence was unlike serovar Tarassovi, thus this strain is called Pacifica (Bingham 2021; Wilkinson *et al.* 2021). Pacifica is currently included in serogroup Tarassovi since most animals shedding Pacifica have high titres against serovar Tarassovi, but no isolate has been obtained to date for confirmation by cross-agglutination absorption test (CAAT) (Babudieri 1971). It is unclear whether the increase in human cases of serovar Tarassovi is due to Tarassovi or Pacifica, as all human data to date has been acquired through serology and these two strains cannot be differentiated serologically.

Given the multi-host, multi-pathogen complexity of leptospirosis, the establishment of routine screening and typing as part of surveillance work in both wild and domesticated animals is necessary to fully understand its epidemiology. In this study, we used our recently developed molecular assay that is able to type all pathogenic species of *Leptospira* identified to date in the P1 clade and the serovars known to be endemic in New Zealand (Wilkinson *et al.* 2021) to identify *Leptospira* spp. in circulation in New Zealand mammals.

Materials and methods

Sample source

All DNA samples used in this study were collected as part of other research projects between 1 May 2007

Table 1. Sample types from different mammals sourced between 2007 and 2017 in New Zealand, tested with *lipL32*, *glmU* and *gyrB* PCR to identify pathogenic *Leptospira* spp.

Animal species	Sample types				N	Samples positive to <i>lipL32</i> screening PCR (N)	Samples positive to screening and speciating PCR (N)		Samples positive to ≥ 1 PCR test (N)
	Culture	Kidney	Serum	Urine			<i>glmU</i>	<i>gyrB</i>	
Dairy cattle	0	0	0	741	741	74	26	25	85
Beef cattle	6	27	0	0	33	33	13	15	33
Farmed deer (<i>Cervus elaphus</i>)	0	43	0	84	127	55	62	ND	59
Sheep	16	4	5	0	25	24	20	4	24
Wild red deer (<i>Cervus elaphus</i>)	0	24	0	0	24	12	16	ND	18
Brushtail possum (<i>Trichosurus vulpecula</i>)	0	28	0	2	30	7	7	5	10
Hedgehog (<i>Erinaceus europaeus</i>)	0	5	0	2	7	2	2	3	4
Ship rat (<i>Rattus rattus</i>)	0	3	0	0	3	2	1	0	2
Stoat (<i>Mustela erminea</i>)	0	5	0	0	5	2	0	2	2
Mouse (<i>Mus musculus</i>)	0	1	0	0	1	0	ND	ND	0
Ferret (<i>Mustela putorius furo</i>)	0	1	0	0	1	ND	1	0	1
Total	22	141	5	829	997	211	148	54	238

and 18 November 2017 at the Molecular Epidemiology and Public Health Laboratory (mEpiLab; Massey University, Palmerston North, NZ) (Supplementary Table 1). Sample types included DNA extracted from serum, urine, and kidney, or cultures from these samples of domestic and wild mammals. A total of 997 DNA samples were tested in this study including samples from beef and dairy cattle ($n = 774$), farmed red deer (*Cervus elaphus*; $n = 127$), sheep ($n = 25$), wild red deer (*Cervus elaphus*; $n = 24$), brushtail possum (*Trichosurus vulpecula*; $n = 30$), European hedgehog ($n = 7$), stoat (*Mustela erminea*; $n = 5$), ship rat ($n = 3$), house mouse ($n = 1$) and ferret (*Mustela putorius furo*; $n = 1$) (Table 1). Domestic animal samples were collected from farms and abattoirs located in the lower North Island of New Zealand. Wildlife samples were collected via opportunistic sampling in and around Palmerston North. All DNA had been extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and stored at -20°C during the previous studies.

PCR

If PCR data were available from the previous studies, only samples that were previously positive for pathogenic *Leptospira* were tested in this study unless they met selection criteria for typing 3 (Figure 1). This study used three different PCR assays as described previously (Wilkinson *et al.* 2021): a *lipL32* quantitative PCR (qPCR) to screen for pathogenic *Leptospira* (Stoddard *et al.* 2009) which was used to identify *Leptospira*-positive samples, and a *glmU* (Wilkinson *et al.* 2021) or *gyrB* (Slack *et al.* 2006) speciating PCR which generated amplicons for sequencing to allow differentiation between different *Leptospira* taxa and their associated serovars. Samples were preferentially analysed with the *glmU* PCR as it can differentiate all seven endemic genotypes of *Leptospira* in New Zealand, while the *gyrB* locus has identical sequence for Balcanica and Tarassovi. If the *glmU* amplicons were insufficient in providing quality sequence data, then the samples were subjected to the *gyrB* PCR. Samples were selected for typing with the *glmU* or

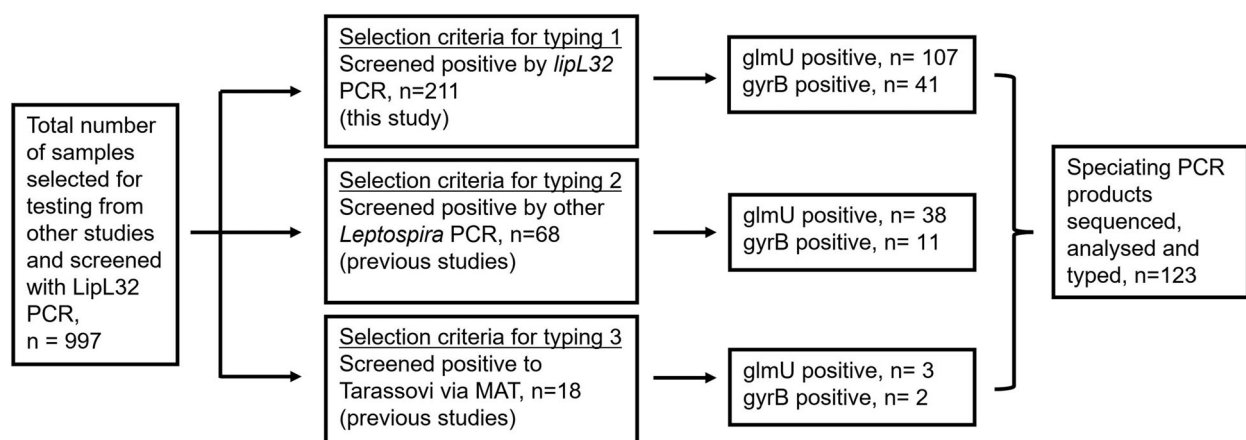


Figure 1. Flowchart showing selection of DNA samples typed as part of a study of *Leptospira* spp. infection in mammals in New Zealand. Selection criteria for inclusion in the study were: criteria 1 – samples that were positive to *lipL32* PCR; criteria 2 – samples negative to *lipL32* PCR but positive to PCR for *Leptospira* spp. in previous studies; and criteria 3 – samples negative to *lipL32* PCR but that came from animals seropositive to Tarassovi in previous studies. Samples were subjected to typing with the speciating PCR (*glmU* and/or *gyrB*) if they were positive to any one of these selection criteria.

gyrB speciating PCR based on three selection criteria: (1) samples that tested positive with the *lipL32* screening qPCR; (2) samples that tested negative to the *lipL32* screening qPCR but were positive for PCR used to identify pathogenic *Leptospira* in previous studies; or (3) samples that were negative to PCR tests in all studies but were from animals with positive agglutination titres against serogroup Tarassovi in previous studies (Figure 1). A cut-off of 96 was used to signify positive titres against Tarassovi, all of which were from dairy cattle.

A total of 45 amplification cycles were used for the *lipL32* qPCR and a sample was considered positive if a quantification cycle (Cq) value was generated. The *lipL32* qPCR assays were performed in sterile DNase- and RNase-free, 100-well rotor discs (Qiagen) in a Rotor-Gene 6000 PCR cyclers (Corbett Research, Mortlake, Australia). Speciating PCR assays were performed using FIREPol master mix (Solis BioDyne, Tartu, Estonia) in sterile DNase and RNase-free 0.2-mL SnapStrip II PCR tubes (SSiBio, Lodi, CA, USA) in the Labcycler (SensoQuest, Göttingen, Germany) and visualised in an agarose gel. All primers used in this study are listed in Supplementary Table 2. All PCR assays included positive controls consisting of purified *Leptospira* DNA from laboratory reference strains of *L. borgpetersenii* serovar Hardjo type bovis and *L. interrogans* serovar Pomona. UltraPure distilled water (Invitrogen, Waltham, MA, USA) was used for no-template controls.

PCR purification and amplicon sequencing

The amplicons from the *glmU* and *gyrB* PCR were analysed by gel electrophoresis in 1% agarose gels stained with RedSafe (iNtRON Biotechnology, Seongnam, South Korea) and analysed under a Gel Doc XR UV transilluminator (Bio-Rad, Hercules, CA, USA). The gel bands of the expected size (567 bp for *glmU* and 504 bp for *gyrB*) were cut out and incubated with 50 µL of elution buffer (QIAamp DNA mini kit) overnight at 4°C. Extracted DNA was submitted for Sanger sequencing on an ABI 3730 DNA Analyzer (ThermoFisher Scientific) at Massey Genome Services (Palmerston North, NZ).

Molecular typing

Forward and reverse sequences were aligned to generate a consensus sequence in Geneious software, version 10.2.6 (Biomatters Inc., Auckland, NZ). Consensus sequences were aligned with reference sequences to assign species and serovars (Supplementary Table 3). Reference sequences included the *glmU* and *gyrB* regions of eight strains associated with two species, i.e. *L. interrogans* serovar Pomona str68, herein referred to as Pomona; *L. interrogans* serovar Copenhageni

strain M20, herein referred to as Copenhageni; *L. borgpetersenii* serovar Hardjo type bovis strain I89, herein referred to as Hardjo type bovis; *L. borgpetersenii* serovar Balcanica strain Bal_possum, herein referred to as Balcanica_NZ; *L. borgpetersenii* serovar Balcanica strain Bal_Burgas, herein referred to as Balcanica_Burgas; *L. borgpetersenii* serovar Ballum strain Bal_mus127, herein referred to as Ballum; *L. borgpetersenii* serovar Tarassovi strain Perepelitsin, herein referred to as Tarassovi; and *L. borgpetersenii* strain Pacifica, herein referred to as Pacifica.

Ethics

This study was approved by the Royal (Dick) School of Veterinary Studies' Veterinary Ethical Review Committee (reference number 15.19). According to Massey University's animal ethics committee guidelines, no additional ethical approval was required beyond that obtained for the original sampling as part of the previous projects.

Results

Molecular typing of *Leptospira*

A total of 997 samples were screened with the *lipL32* qPCR, of which 211 tested positive and were subjected to the speciating PCR (Figure 1). An additional 86 *lipL32*-negative samples were also tested with the speciating PCR because they had either tested positive to pathogenic *Leptospira* PCR in previous studies ($n = 68$) or were samples from animals with high titres against serovar Tarassovi in previous studies ($n = 18$). In total, 297 samples were tested with the speciating PCR, 148 samples were successfully amplified with the *glmU* PCR and 54 with the *gyrB* PCR (Table 1). A total of 202 amplicons were sent for sequencing, however 28 samples were amplified by both the *glmU* and *gyrB* PCR, thus 174 different samples were typed. Of the 174 amplicons, 123 were successfully typed following sequencing; 100 samples with the *glmU* PCR and 41 samples with the *gyrB* PCR, with 18 samples being typed with both PCR. Typing identified six strains/serovars belonging to two species: *L. borgpetersenii* strain Pacifica and serovars Balcanica_NZ, Ballum and Hardjo type bovis, and *L. interrogans* serovars Copenhageni and Pomona (Figure 2). All generated sequences were identical to the reference strains and no new types were detected in any of the positive samples using the speciating PCR. All Balcanica sequences were determined by the *glmU* locus and were identical to the reference strain Bal_NZ, which has only been identified in New Zealand. Bal_possum has a single nucleotide polymorphism within the *glmU* region compared to the Balcanica_Burgas strain that was isolated from a human in Bulgaria (Wilkinson *et al.* 2021).

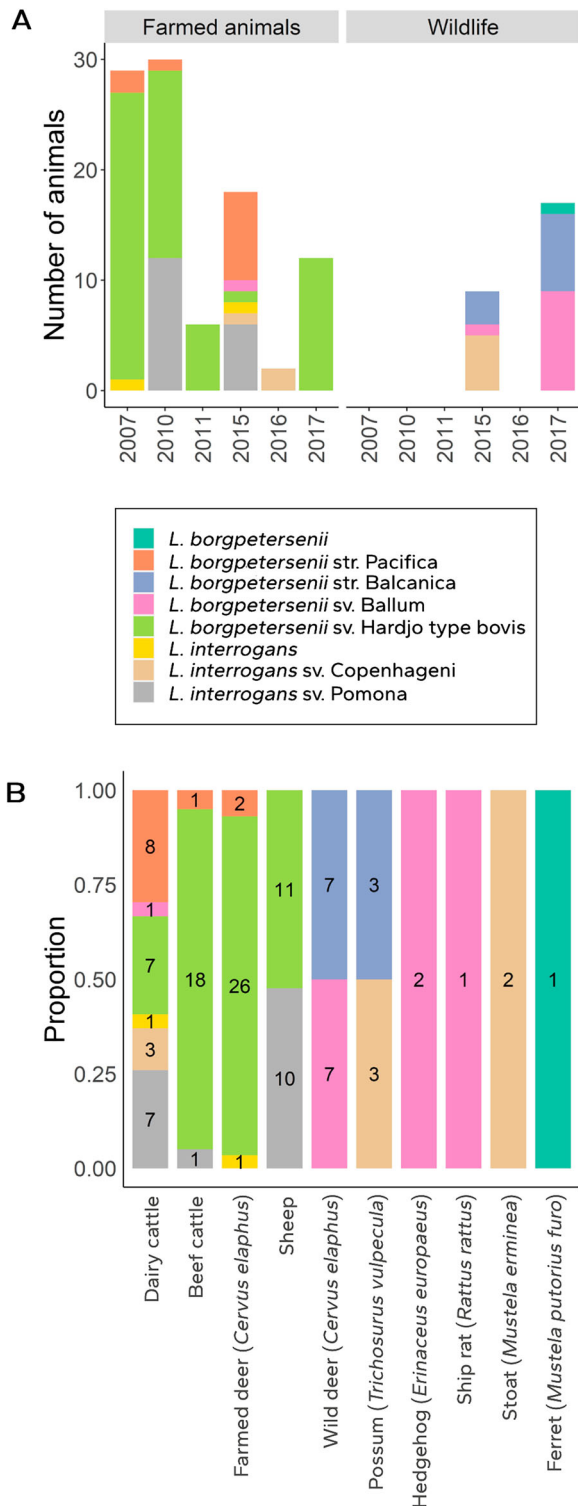


Figure 2. *Leptospira* species and serovars identified in DNA samples from New Zealand mammals stratified (A) by the year of sample acquisition and whether they were found in farmed animals or wildlife; and (B) by the mammalian hosts in which they were identified (numbers within each bar show the number of samples from each host in which each *Leptospira* strain/serovar was identified).

Host–*Leptospira* associations

Host–*Leptospira* associations were stratified by whether the host was farmed or wildlife over the study period (Figure 2A). Farmed animals constituted

dairy cattle, beef cattle, farmed deer, and sheep, while wildlife constituted wild red deer, brushtail possum, hedgehog, ship rat, stoat, mouse, and ferret. Farmed animals were positive for Hardjo type bovis (62/97; 64%), Pomona (18/97; 19%), Pacifica (11/97; 11%), Copenhageni (3/97; 3%) and Ballum (1/97; 1%). Wildlife was positive for Balcanica_NZ (10/26; 38%), Ballum (10/26; 38%) and Copenhageni (5/26; 19%).

Samples that were typed ($n = 123$), were also stratified by mammalian host and infecting *Leptospira* spp. (Figure 2B). This stratification shows that each host type was positive to a range of different *Leptospira* spp. with dairy cattle being infected with five of the seven types detected in this study. One dairy cow had a mixed infection that limited typing to species level as *L. interrogans*, one deer sample could only be identified at species level as *L. interrogans*, and the ferret sample had poor-quality sequence data, limiting typing to species level as *L. borgpetersenii* (Figure 2B).

Of the 11 samples that were positive for Pacifica in this study, six came from dairy cattle for which MAT data generated in previous studies were available. Four animals had titres to Tarassovi (1:96, 1:96, 1:768, and 1:1536); one animal had combined titres to Tarassovi (1:768), Copenhageni (1:48), Hardjo (1:48), and Ballum (1:24); and one animal had combined titres to Pomona (1:96), Copenhageni (1:96) and Hardjo (1:48). Samples without a positive titre were either not tested by MAT (one beef animal and two farmed deer) or only tested for Hardjo and Pomona (two dairy cattle).

Discussion

New Zealand has a high burden of leptospirosis in humans, which is atypical for a high-income nation with a temperate climate. A cornerstone for preventing disease in humans is the vaccination of pigs and dairy cattle (Marshall and Manktelow 2002). The combined cost of leptospirosis to the New Zealand economy including human disease (treatment and absence from work), loss of production in animals (cattle, sheep, and deer) and disease control measures (animal vaccinations) was estimated at \$NZD24M annually (Sanhueza *et al.* 2020). Surveillance is an important tool for monitoring the *Leptospira* spp. in circulation and can have implications for disease control and vaccine development. In this study, we applied our recently developed molecular assay to historical samples, which allowed the correct identification of *Leptospira* species and serovars infecting farmed and wild mammals in New Zealand.

The molecular typing results revealed previously unidentified host–*Leptospira* associations in New Zealand, i.e. farmed deer infected with Pacifica (Figure 2B). Farmed deer in New Zealand have largely been tested serologically for Hardjo type bovis, Pomona, Copenhageni, Ballum and Tarassovi

(Subharat *et al.* 2012; Wilson *et al.* 2021). Although Tarassovi seropositivity is common in deer, molecular typing to identify the specific strain being shed was not available at the time the study was carried out (Wilson *et al.* 2021). As dairy cattle serologically positive to Tarassovi (Yupiana *et al.* 2019a) shed Pacifica (Wilkinson *et al.* 2021), previous serological classifications of Tarassovi in deer could likely be Pacifica. Pacifica has been considered an emerging public health risk for dairy farmers, as it was identified in dairy cattle samples collected in 2016 (Yupiana *et al.* 2019a) and environmental samples from a dairy farm in 2017 (Wilkinson *et al.* 2021). However, results from this study suggests that deer farmers could also be at risk from Pacifica. Thus, the Pacifica lineage may be more widespread than initially thought, as it has now been identified in two farming systems (Figure 2B). Furthermore, Pacifica may not be an emerging strain, as it has been in circulation since at least 2007, as identified from the deer samples tested in this study (Supplementary Figure 1). It is difficult to ascertain how long Pacifica has been circulating in livestock as no samples from before 2007 were available for molecular typing. Since Pacifica has exclusively been found in farmed animals to date, it is likely maintained in livestock; however, only 7.1% (71/997) of the study's samples came from wildlife. A comprehensive survey of wildlife needs to be conducted to test this hypothesis.

We also found some new host–*Leptospira* associations in wildlife in New Zealand, including stoats and brushtail possums infected with Copenhageni, and a ferret infected with *L. borgpetersenii* (Figure 2B). This finding is not surprising given the limited number of wild animals tested in New Zealand to date and the wide host range of leptospires in general. Globally, mustelids have been known to harbour *Leptospira* from serogroups Icterohaemorrhagiae (which includes serovar Copenhageni), as well as serogroups Australis, Autumnalis, Grippotyphosa and Sejroe (Ayrat *et al.* 2016). Mustelids were first released in New Zealand in an attempt to control the rodent population (King 2017). Earlier studies show ship and brown rats harbouring Copenhageni in New Zealand (Brockie 1977; Carter and Cordes 1980) as well as other countries (Maas *et al.* 2018; Boey *et al.* 2019). The distribution of *Leptospira* infection in rats varies widely and has mainly been studied in urban environments (Boey *et al.* 2019). The infected brushtail possums and stoats in this study came from urban habitats that are shared with both ship and brown rats. Whether Copenhageni is maintained in both rats spp. and mustelids in New Zealand or whether there is inter-species transmission is difficult to say with the limited sample size in this study.

This study also reported a wide range of serovars associated with dairy cattle (Figure 2B). These findings reinforce recent observations that serovar

diversity in cattle is not just limited to serovars Hardjo type bovis and Pomona included in currently available vaccines (Yupiana *et al.* 2017, 2019b; Moinet 2020). While other livestock were infected with Hardjo type bovis, Pomona and Pacifica, dairy cattle were additionally infected with Ballum and Copenhageni, the two serovars that have been associated with wildlife in New Zealand (Moinet *et al.* 2021). In New Zealand, 99.5% of dairy farms vaccinate against serovars Hardjo type bovis and Pomona (Yupiana *et al.* 2017) and this has been shown to reduce shedding of the vaccinated strains (Yupiana *et al.* 2019b). However, this likely leaves an empty niche for new serovars. While historically dairy cattle were the recognised maintenance host for Hardjo type bovis and Pomona, these data indicate that Pacifica may also be maintained by dairy cattle. Human leptospirosis notification data (classified serologically) show an increase in Tarassovi cases in dairy farmers (Nisa *et al.* 2020). Due to lack of molecular typing, it is unclear if the genetic strain causing disease in dairy farmers is Tarassovi or Pacifica; however, molecular typing of dairy cattle in this study and the previous study (Yupiana *et al.* 2019a) only show evidence of Pacifica in dairy cattle with no genetic evidence of Tarassovi infection. The results from this study as well as the previous survey of dairy cattle (Yupiana *et al.* 2019a) suggest a relationship between Tarassovi seropositivity and Pacifica infection where both likely belong to serogroup Tarassovi. However, this can only be determined upon isolation and characterisation of strain Pacifica and all attempts to isolate this strain to date have been unsuccessful. Serological testing with Tarassovi before the availability of molecular typing tools explains why Pacifica remained undescribed prior to 2019 (Yupiana *et al.* 2019a).

In previous studies, mice, rats, and hedgehogs were found to harbour serovar Ballum, where mice were considered the primary maintenance host and rats and hedgehogs as secondary maintenance hosts (Brockie 1977; Moinet 2020; Moinet *et al.* 2021). In this study, hedgehogs and rats were infected with Ballum, but so were wild red deer (Figure 2B). This is the first report of Ballum infection in wild red deer, but it is unclear if wild red deer are a maintenance host or spill-over host from being in the same environment with mice, rats and hedgehogs. Similarly, while brushtail possums have been known to be the maintenance host for Balcanica_NZ in New Zealand (Hathaway *et al.* 1978), this is the first report of Balcanica_NZ in wild red deer. A comprehensive survey needs to be conducted to determine if wild red deer can maintain Ballum and Balcanica_NZ, as sample numbers in this study were low. Overall, the wild mammals in New Zealand appear to maintain Balcanica_NZ, Ballum and Copenhageni while farmed animals appear to be the maintenance

host for Hardjo type bovis, Pomona and Pacifica, except for dairy cattle, which are also infected with wildlife-associated serovars Ballum and Copenhageni. The results from this study also verified that the Hardjo type bovis in farmed animals that were previously typed serologically, and are serologically indistinct from Balcanica, were indeed Hardjo type bovis and not Balcanica. Lastly, the results of this study also reconfirmed previously described host–*Leptospira* associations including dairy cattle infected with Copenhageni, Pomona, Ballum, Hardjo type bovis and strain Pacifica; sheep infected with Pomona and Hardjo type bovis; brushtail possum infected with Balcanica_NZ; farmed deer infected with Hardjo type bovis; and hedgehogs infected with Ballum (Figure 2B).

While this study revealed some new host–*Leptospira* associations, there are several limitations. Firstly, not all samples from the previous studies were tested in this study, i.e. samples that were previously determined to be negative for pathogenic *Leptospira* using PCR were excluded from this study unless they met selection criteria for typing 3 (Figure 1). This was to conserve resources on samples known to be negative. Thus, this

convenience sampling and a low number of available samples for some species prevented an accurate estimation of prevalence of the different serovars in their respective mammalian species. Further research with appropriate sampling methods, e.g. investigating the difference in serovars from vaccinated and unvaccinated dairy cattle, or wildlife from rural and urban regions, within a defined period and with larger sample sizes is necessary to estimate prevalence. Secondly, typing in this study was often limited by the bacterial load in the samples as determined by the Cq values of the qPCR, where a higher Cq value denoted lower bacterial load. Of the 211 samples positive with the *lipL32* qPCR, 103 were typed, of which 32% (33/103) had Cq values of ≥ 30 while of the 108 samples that were not typed, 90% (97/108) had Cq values of ≥ 30 . Thus, while samples with lower bacterial load were positive, the quantity of DNA was insufficient to provide quality sequence for typing, therefore decreasing the number of positive samples that could be typed.

In conclusion, this study has updated understanding of host–*Leptospira* associations in New Zealand (Table 2). Many studies in the last decade have

Table 2. An updated summary of *Leptospira* spp. detected by culture or molecular typing in New Zealand mammals.

Species	Serogroup	Serovar	Host animals	Reference
<i>Leptospira interrogans</i>	Icterohaemorrhagiae	Copenhageni	Dairy cattle	This study; Ris <i>et al.</i> 1973; Yupiana <i>et al.</i> 2019a Brockie 1977 This study This study; Brockie 1977
			Brown rat (<i>Rattus norvegicus</i>)	
			Stoat (<i>Mustela erminea</i>)	
			Brushtail possum (<i>Trichosurus vulpecula</i>)	
	Pomona	Pomona	Dairy cattle	This study; Yupiana <i>et al.</i> 2019a; Moinet 2020 This study; Fang <i>et al.</i> 2014 This study This study; Dorjee <i>et al.</i> 2008; Fang <i>et al.</i> 2014 Moinet 2020 Mackintosh <i>et al.</i> 1980a Robinson <i>et al.</i> 1982; Bolt 1990
			Beef cattle	
			Farmed deer (<i>Cervus elaphus</i>)	
			Sheep	
			Hedgehog (<i>Erinaceus europaeus</i>)	
			Dog	
<i>Leptospira borgpetersenii</i>	Ballum	Ballum	Dairy cattle	This study; Ris <i>et al.</i> 1973; Yupiana <i>et al.</i> 2019a; Moinet 2020 This study This study; Brockie 1977; Moinet 2020 This study; Brockie 1977; Hathaway <i>et al.</i> 1981; Moinet 2020 Brockie 1977; Hathaway <i>et al.</i> 1981 Brockie 1977; Hathaway <i>et al.</i> 1981; Moinet 2020 This study; Fang <i>et al.</i> 2014; Yupiana <i>et al.</i> 2019a; Moinet 2020 This study; Fang <i>et al.</i> 2014 This study; Subharat 2010 This study; Bahaman <i>et al.</i> 1980; Fang <i>et al.</i> 2014 Schollum and Blackmore 1981 Mackintosh <i>et al.</i> 1980b This study This study; Hathaway <i>et al.</i> 1978; Moinet 2020 Moinet 2020 Schollum and Blackmore 1981 Ryan and Marshall 1976 Mackintosh <i>et al.</i> 1980a This study; Yupiana <i>et al.</i> 2019a This study This study This study
			Wild red deer (<i>Cervus elaphus</i>)	
			Hedgehog	
			Ship rat (<i>Rattus rattus</i>)	
	Sejroe	Hardjo type bovis	Brown rat	
			Mouse (<i>Mus musculus</i>)	
			Dairy cattle	
		Balcanica	Beef cattle	
			Farmed deer	
			Sheep	
Tarassovi	Tarassovi	Feral goat		
		Dairy cattle		
		Wild red deer		
	Pacifica ^b	Brushtail possum		
		Hedgehog		
		Feral goat		
Not determined	Not determined	Pig	This study; Yupiana <i>et al.</i> 2019a This study This study This study	
		Dog ^a		
Not determined	Not determined	Dairy cattle	This study; Yupiana <i>et al.</i> 2019a This study This study This study	
		Beef cattle		
Not determined	Not determined	Farmed deer	This study This study	
		Ferret (<i>Mustela putorius furo</i>)		

^aReported as *L. interrogans* serovar Tarassovi, however, it was only characterised serologically. Molecular typing method has identified the strain found in the dog as *L. borgpetersenii* serovar Tarassovi.

^bPacifici is not confirmed as part of the Tarassovi serogroup as no isolate has been cultured to date for a cross agglutination absorption test, however, since most dairy cattle shedding Pacifica have high titres against serovar Tarassovi by microscopic agglutination test (Yupiana *et al.* 2019a), Pacifica was included in serogroup Tarassovi in this table.

focused solely on Hardjo type bovis and Pomona with reliance on serology (Dorjee *et al.* 2008; Vallée *et al.* 2015; Dreyfus *et al.* 2018) and very few studies have investigated Ballum, Copenhageni and Tarassovi (Yupiana *et al.* 2019a; Moinet 2020; Wilson *et al.* 2021). While serology remains a useful tool, it is not uncommon for seronegative animals to shed leptospire (Brockie 1977; Hathaway *et al.* 1981) and there can be cross-reactivity and inaccurate classification. Future surveillance and research will need to incorporate molecular typing and will need to focus on a wider diversity of serovars to better understand their distributions, prevalence, and overall impacts.

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No potential conflict of interest was reported by the authors.

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