Research Article

iMedPub Journals
www.imedpub.com

Annals of Clinical and Laboratory Research
ISSN 2386-5180

2018
Vol.6 No.3:244

DOI: 10.21767/2386-5180.100244

Epidemiological Investigation of *Leptospira* spp. in a Dairy Farming Enterprise after the Occurrence of Three Human Leptospirosis Cases

Wilson P[1], Heuer C[1*], Vallée E[1], Weston J[1], Yupiana Y[1,2], Collins-Emerson J[3], Benschop J[3] and Scotland T[4]

1 School of Veterinary Science, Massey University, Palmerston North, New Zealand
2 Ministry of Agriculture, Jakarta, Indonesia
3 Molecular Epidemiology and Veterinary Public Health Laboratory (mEpilab) and Infectious Disease Research Centre (IDReC), Hopkirk Research Institute, Massey University, Palmerston North, New Zealand
4 Southern Rangitikei Veterinary Services, Bulls, New Zealand

***Corresponding author:**
Heuer C

C.Heuer@massey.ac.nz

School of Veterinary Science, Massey University, Palmerston North 4441, New Zealand.

Tel: +64 - (0)6-9519037

Citation: Wilson P, Heuer C, Vallée E, Weston J, Yupiana Y, et al. (2018) Epidemiological Investigation of *Leptospira* spp. in a Dairy Farming Enterprise after the Occurrence of Three Human Leptospirosis Cases. Ann Clin Lab Res Vol.6 No.3: 244

Abstract

An epidemiological investigation was conducted in an unvaccinated dairy farming enterprise in which three workers on one of the milking herds (Herd 1) were diagnosed with leptospirosis due to serovars Hardjo (H) (n=2) and Pomona (P) (n=1) between January and March 2015. Blood and urine samples were collected from milking cows in Herd 1 (N=230) and Herd 2 (N=400), rising-one- (R1, N=125) and rising-two-year-old (R2, N=130) replacement heifers, and four pigs associated with Herd 1, in March 2015. Sera were tested using the MAT for serovars H, P, Copenhageni (C), Ballum (B) and Tarassovi (T), and urine samples were tested by qPCR. Seventy five percent of 109 cows in Herd 1 and 36% of 121 in Herd 2 were seropositive (≥ 48), predominantly to H and P, and 23% of 74 cows in Herd 1 and 1% of 90 cows in Herd 2 were qPCR positive. Fifty five percent of 42 R2 heifers were seropositive to T. No R1 and 17% of 42 R2 heifers were qPCR positive. Subsequently, all cattle were vaccinated for H and P, and Herds 1 and 2 were given amoxicillin. After the booster vaccination, 7% of 91 in Herd 1, 2% of 82 in Herd 2 and 1% of 38 R1 heifers (sampled as R2) were PCR positive. After the amoxicillin treatment, no cows in Herd 1 and 5% of 62 cows in Herd 2 were urine PCR positive. Calves and pigs were seropositive to H, P, C and B. Vaccination, followed by antibiotic treatment, appeared effective in reducing the risk of exposure of workers to vaccine serovars. However, serological and PCR evidence suggested a dynamic infection with non-vaccine serovars resulting in urine shedding in Herd 2 and heifer replacements, indicating that workers likely remain at risk of exposure to Leptospira.

Keywords: *Leptospira*; Epidemiology; Case investigation; Human leptospirosis; Dairy cattle

Abbreviations: *B- Leptospira borgpetersenii* sv. Ballum; C- *Leptospira interrogans* sv. Copenhageni; H- *Leptospira borgpetersenii* sv. Hardjo; H1 and H2- Herds 1 and 2; R1 and R2- Rising one-year-old and rising two-year-old, respectively; MAT- Microscopic Agglutination Test; P- *Leptospira interrogans* sv. Pomona; qpcr- Quantitative Polymerase Chain Reaction; T- *Leptospira borgpetersenii* sv. Tarassovi

Received: August 13, 2018; **Accepted:** August 21, 2018; **Published:** August 31, 2018

Introduction

Leptospirosis is a zoonotic disease caused by pathogenic bacteria of the genus *Leptospira*. Transmission to humans usually occurs via contact with urine from infected animals, directly or indirectly via contamination of the environment, entering the body through cuts or across mucosal membranes [1,2]. In New Zealand, six serovars belonging to two pathogenic species are known to be endemic in animals, namely *Leptospira borgpetersenii* serovars Hardjo (H), Ballum (B), Balcanica and Tarassovi (T) and *Leptospira interrogans* serovars Pomona (P) and Copenhageni (C) [3-15]. Cattle are considered to be maintenance hosts for serovar H and pigs for serovars P and T [7]. Other serovars are maintained by wildlife, namely serovar Balcanica by possums (*Trichosurus*

vulpecula) and wild deer (*Cervus elaphus*), serovar B by house mice (*Mus musculus*), ship rats (*Rattus rattus*) and hedgehogs (*Erinaceus europaeus*) and serovar C by Norway rats (*Rattus norvegicus*) [13]. Accidental infection of humans from livestock commonly occurs in New Zealand (ESR reports 2012-16) but human-to-human infections are rarely reported globally [1]. All serovars endemic in animals have been reported in human leptospirosis cases in New Zealand, with serovars H, P, B [14-27] and T (ESR 2012-16) reported most frequently. In the early 1980s, leptospirosis vaccination was initiated in dairy cattle and pigs in New Zealand due to high *Leptospira* transmission from these livestock to humans. Vaccination was associated with a significant decrease in the number of human cases [15].

Currently, approximately 95% of dairy herds in New Zealand use either a bivalent vaccine with serovars H and P or a trivalent vaccine with serovars H, P and C [4,28,29]. In New Zealand, farmers have a legal requirement to protect workers from health and safety risks including zoonotic diseases. For leptospirosis protection, animal vaccination has been recommended as a long term strategy [28]. However, leptospirosis cases are still reported in dairy farm workers [4,16]. From 2012-2016, there were 376 reported cases of human leptospirosis in New Zealand among which 297 cases were in people working in high-risk occupations including farmers, meat workers, cattle exporters, hunters, and trappers. Of those, 63% were farmers (ESR 2012-16). Most reported being in contact with unvaccinated or poorly vaccinated herds [5,14]. There have been no recent published reports of epidemiological investigations of *Leptospira* infection on farms where workers have been affected, or of the effectiveness of livestock vaccination programmes per se in minimizing shedding and risk to workers.

This case study describes an epidemiological investigation of *Leptospira* infection in two unvaccinated dairy herds in a farming enterprise that had three cases of leptospirosis in workers within three months [4]. The investigation was undertaken to establish whether livestock were a possible or probable source of exposure and if so, to quantify the risk, and to determine the apparent effectiveness of animal vaccination and antibiotic treatment in reducing the risk of exposure to workers.

Materials and Methods

This was an opportunistic case study arising from clinical leptospirosis in three workers on a seasonal-supply dairy farming enterprise located in the lower North Island of New Zealand, diagnosed between January 25 and March 14, 2015 [4]. Two cases were confirmed as H and one as P.

Farming enterprise and animals

The farming enterprise consisted of Herd 1 (H1) comprising adult (3-years and older) cows only and Herd 2 (H2) comprising adult cows and first lactation heifers, grazed separately without direct contact on adjacent areas (Farms 1 and 2, respectively) **(Figure 1)**. There were 230 milking cows in H1 grazing 130 hectares and milked in a rotary shed, and 400 milking cows in H2 grazing 190 hectares and milked in a herringbone shed. Rising 1-year-old (R1) and pregnant R2 replacement heifers were managed on a third area (Farm 3) **(Figure 1)**, a short distance from the milking herd farms. Breeding bulls and pigs were present on Farm 1. There was no clinical evidence of leptospirosis in either cattle or pigs. Before the outbreak of leptospirosis in farm workers, *Leptospira* vaccination had not been undertaken for at least twenty years, and there was no rodent control programme in place. The three affected workers had been working solely with the cattle in H1.

Study design

On March 6, within a week of the second human confirmed case, an initial screening investigation was undertaken with blood and urine samples collected from adult milking cows in H1 and H2 to establish their *Leptospira* serological status. Positive MAT results reinforced the need for further sampling in herds, as well as rising 1-year-old (R1) and pregnant rising 2-year-old (R2) heifer replacements on Farm 3, and calves and pigs on Farm 1, between March 2015 and January 2016 according to the schedule presented in **Table 1**.

For the initial screening, sample sizes were calculated to detect *Leptospira* urinary shedding, given an expected prevalence of 10%, at p=0.05, with 80% power, using PCR with sensitivity (Se) of 0.53 and specificity (Sp) of 0.96. Forty cows needed to be sampled from H1 and 45 cows from H2. Actual numbers sampled are presented in **Table 1**. Based on positive serological and PCR findings from the initial screening, further power analyses were undertaken for each herd for testing the effectiveness of vaccination and antibiotic intervention on the reduction of shedding. To detect a reduction in *Leptospira* shedding from 30% before to 6% after intervention, with 80% power and 95% confidence, 60 animals sampled three times were required from H1. To detect a reduction in *Leptospira* shedding from 20% before to 4% after vaccination and antibiotic treatment, with 80% power and 95% confidence, 80 animals sampled three times were required from H2. Additional sampling was therefore undertaken on March 18 and 19 to achieve the required power prior to intervention. Based on the assumption that the prevalence would be similar in R1 and R2 heifers, to detect a decrease in prevalence of shedding from 40% before to 4% after vaccination with 80% power and 95% confidence, 40 animals were required in each age category. Sampling four of the six pigs was sufficient to determine exposure rate, and sampling of 60 calves born July-August during the 2015 calving period was sufficient to investigate maternal antibody and/or early post-natal infection.

Thus, the first stage of the initial investigation involved collection of blood and urine samples from adult cows in H1 and H2 on March 6 with additional sampling 12-13 days later. As there was no significant difference (Pearson's Chi-squared, >0.05) in seroprevalence for H and P between those sampling days **(Table 1)** the data were combined and designated as the initial investigation. Subsequent sampling episodes for H1 and H2 are referred to as "post-vaccination" (May 20/27 2015) and "post-vaccination/antibiotic" (Jan 19/20, 2016). Sampling of R1 and R2 heifers in March 2015 is referred to as "pre-vaccination". Sampling in November 2015 of those R1 heifers, which became R2 heifers in July/August is referred to as "post-vaccination". Animal ethics approval was granted by the Massey University Animal Ethics Committee, protocol 15/27.

Blood and urine collection

Sampling of adult cows, H1 and H2, was conducted during milking. Where possible paired blood and urine samples were collected for the initial and post-vaccination/antibiotic samplings **(Table 1)**. At the post-vaccination sampling only urine samples were collected, targeting previously sampled cows where possible in both herds.

Blood and urine samples were collected from R1 and R2 (6-8 and 18-20 month old, respectively) heifer replacements in March 2015, before vaccination, and the R1 heifers were re-sampled in November 2015 as R2s after sensitizer and booster vaccination. Additionally, heifer calves born in July/August from cows having undergone vaccination and antibiotic treatment were blood sampled at 2-3 months of age in October 2015. Pigs were blood sampled in March 2015.

Blood samples were collected by venipuncture from the coccygeal vein in adult cattle, the jugular vein in calves, and by anterior vena cava puncture in pigs, into a 10 ml plain (red top) evacuated plastic tube without anticoagulant. Urine samples were collected into a 50 ml clean plastic container either from spontaneous urination or urination induced by stimulating the ventral vulva. Blood and urine samples were packed separately in plastic bags and taken in an insulated container on ice to the mEpilab, Massey University where they were processed within 24 hours of collection.

Vaccination and antibiotic treatment

Intervention involved both vaccination and treatment with antibiotic in H1 and H2, and vaccination alone in R1 and R2 heifers according to the schedules described in **Table 1**.

Vaccination was undertaken by subcutaneous injection using a bivalent *Leptospira* vaccine (Leptoshield®, Pfizer Animal Health, West Ryde, NSW, Australia) that contained antigens from serovars H and P. Antibiotic treatment consisted of a single dose of long-acting amoxicillin (15 mg/kg, IM, Betamox LA, Noorbook, VIC Australia) administered subcutaneously. Antibiotic administration was delayed until the end of lactation to avoid withholding of milk and difficulty in disposing of this.

MAT

The MAT was performed at the mEpilab, Massey University. Blood samples were centrifuged at 1,300 g for 10 minutes and sera collected as supernatant. Thirty µL of each serum was mixed with 150 µL sterile standard saline into 96 well plates as a masterplate to make 1/6 dilution for testing. Master plates were then stored at -20°C. The remaining sera were stored at -80°C. Serum samples were tested against serovars H, P, C, B and T. The MAT was performed as described by Fang et al. [6], based on the method described by Faine. Eight serial, two-fold dilutions were prepared in standard saline and ranged from 1: 24 to 1: 3072 (final dilution inclusive of antigen). A positive control using standard antisera against each serovar and a negative control using standard saline were prepared in a similar way. The dilutions were incubated with live cultures for 2 hours at 20-30°C. A reciprocal titre of >1:48 test was considered positive. The end-point titre was the lowest dilution where approximately 50% or more of the Leptospires were agglutinated or lysed.

Quantitative real-time PCR

Ten mL of each urine sample was centrifuged at 1,300 g for 10 minutes after which approximately 8 mL of supernatant was discarded using a transfer pipette (Raylab, Auckland, New Zealand). A 1.2 mL aliquot of the remaining urine and pellet was transferred into a 1.5 mL microfuge tube and centrifuged at 10,625 g for 20 minutes and then re-suspended in 200 µL PBS after discarding the supernatant. DNA extraction was performed using the QIAamp DNA mini kit (Qiagen) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µL of elution buffer and stored at -20°C for Polymerase Chain Reaction (PCR) testing.

The qPCR assay was based on the method developed in the mEpiLab by Subharat et al. [21] and refined by Fang et al. [6]. Green-fluorescent nucleic acid stain SYTO9 was used as the intercalating dye. Primers 2For (5'-TGAGCCAAGAAGAAACAAGCTACA-3') and 504Rev (5'-MATGGTTCCRCTTTCCGAAGA-3') were used to amplify the gyrB gene. The 25 µL reaction included 2.5 µM SYTO9, 1 × PCR buffer, 1.5 mM magnesium chloride ($MgCl_2$), 200 µM deoxyribonucleotide tri-phosphates, 5 pmol of 2For and 504Rev, 1 unit of Taq DNA polymerase, 2 µL of DNA extract, and double-distilled water (ddH_2O). Thermal cycling comprised initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 63°C for 20 sec, and extension at 72°C for 20 sec. Fluorescence readings were taken at the end of each extension cycle in the F1 (SYBR Green) channel. Melting curve analysis was performed by heating the PCR product from 78°C to 90°C and monitoring the fluorescence change every 0.2°C. The positive control was serovar Pomona (mEpilab laboratory strain), and distilled water was used as the negative control. Samples were considered positive, if a similar melting temperature (± 0.5°C) and a similar melting curve to the positive controls were produced.

Statistical analysis

All statistical analyses were performed using R version 3.3.2 (2016-10-31). Geometric Mean Titre (GMT) was calculated for positive samples i.e. titres ≥ 48. Student's T-test was used to compare the GMT between herds and between sampling times within herds, and Pearson's Chi-square with Yates' continuity correction was used to compare the proportion of positive to PCR and MAT between herds and sampling times. The 95% confidence intervals for proportions were calculated using Wilson's method [19].

Result

Herds 1 and 2

Initial investigation: Seroprevalence and GMT data from the initial investigation are presented in **Table 2**, with MAT titre distributions presented in **Figures 2 and 3**. Eighty-two cows (75%, 95% CI: 66-82%) in H1 and 43 (36%, 95% CI: 28-44%) cows in H2 were seropositive to at least one serovar. Cattle in H1 were positive against serovars H, P, C, B and T and cattle in H2 were positive against H, P, and B. The highest seroprevalence was for H and P in both herds. Urine qPCR prior to intervention **(Table 3)** was positive in 17 (23%, 95% CI: 15-34) cows in H1 and one (1%, 95% CI: 0-6) cows in H2.

Rising one- and two-year-old heifers

Pre-vaccination: Seroprevalence and GMT data are presented in **Table 4** and the proportion at each titre is presented in **Figure 4**. While few R1 heifers were seropositive at the pre-vaccination

Table 1 Timeline for blood (B) and urine (U) sampling (number of samples in brackets), vaccination and antibiotic treatment for adult cows in Herds 1 (H1) and 2 (H2) and rising-1-year-old (R1) and rising 2-year-old (R2) heifers, calves (C; born August & September 2015), and pigs (P), in 2015 and 2016. *These animals have transitioned from R1 in July/August 2015.

Animal group	2015														2016	
	Mar,6	Mar,17	Mar,18	Mar,19	Mar,20	Mar,27	Mar,27	Apr,16	Apr,24	May,20	May,27	May,27	Oct,7	Nov,3	Jan,19	Jan,20
H1	B(41)	Sensitiser Vaccination		B(68)				Booster Vaccination				antibiotic at drying off				B(85)
	U(41)			U(33)							U(91)					U(60)
H2	B(39)		B(82)												B(81)	
	U(22)		U(68)							U(89)					U(62)	
R1						B(41)	Sensitiser Vaccination		Booster Vaccination							
						U(41)										
R2						B(42)								B(38)*		
						U(42)								U(38)*		
C													B(61)			
P					B(4)											

Table 2 Number of cows tested in herds 1 (H1) and 2 (H2) and % MAT positive (titre ≥ 48) (95% CI) to five serovars, and overall, and geometric mean titre (GMT) (95% CI) of positive samples, at the initial investigation in March 2015 (Initial) and January 2016, 8-10 months after vaccination and antibiotic treatment (post V/Ab).

Herd	Sampling occasion	No.	Seropositivity	Serovar					
				Hardjo	Pomona	Copenhageni	Ballum	Tarassovi	Overall
H1	Initial	109	Prev (%) (95%CI)	41 (33-51)	46 (37-55)	19 (13-28)	8 (4-15)	2 (0-6)	75 (66-82)
			GMT (95%CI)	186 (136-255)	435 (293-646)	117 (76-179)	56 (44-71)	272 *(48-768)	--
	Post-V/Ab	85	Prev (%) (95%CI)	17 (10-26)	51 (40-61)	11 (6-19)	2 (1-8)	4 (1-10)	61 (51-71)
			GMT (95%CI)	68 (55-84)	192 (134-272)	76 (48-121)	68 *(48-96)	76 (28-206)	--
H2	Initial	121	Prev (%) (95%CI)	31 (24-40)	16 (10-23)	0 (0-3)	1 (0-5)	0 (0-3)	36 (28-44)
			GMT (95%CI)	226 (163-314)	107 (71-160)	0 NA	0 NA	0 NA	--
	Post-V/Ab	81	Prev (%) (95%CI)	22 (15-32)	15 (9-24)	1 (0-7)	4 (1-10)	0 (0-5)	32 (23-43)
			GMT (95%CI)	100 (72-138)	102 (68-152)	96 *(96)	60 (22-163)	0 NA	--

Note: January 2016 titres for Hardjo and Pomona are post-vaccination. * Range of MAT titre instead of CI.

Table 3 Number of urine samples qPCR tested and proportion positive in March 2015 (initial), May 2015 (post-vaccination) and January 2016 (post-vaccination and antibiotic) in herds H1 and H2.

Herd	Sampling occasion	No. samples	% positive (95% CI)
H1	Initial	74	23 (15-34)
	Post vaccination	91	7 (3-14)
	Post vaccination/antibiotic	60	0 (0-6)
H2	Initial	90	1 (0-6)
	Post vaccination	89	2 (0-8)
	Post vaccination/antibiotic	62	5 (2-13).

screening, all were positive to at least one serovar post-intervention in November. None were positive for C or T at that sampling. The post-vaccination GMT in November was higher for H, P and B ($p<0.001$) than in March. Pre-vaccination screening

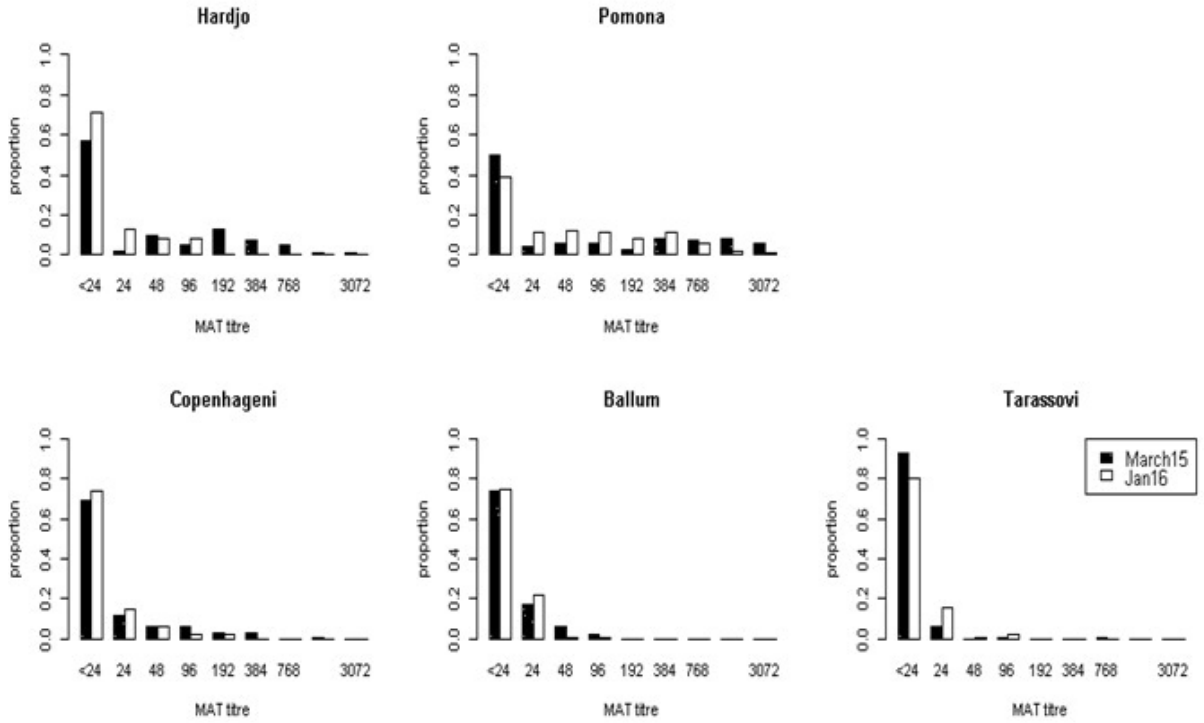

Figure 1 Proportion of cows in Herd 1 at each MAT titer for each serovar, at the initial sampling in March 2015 (n=109) and at the post-vaccination/antibiotic sampling in January 2016 (n=85). Note: January 2016 titres for Hardjo and Pomona are post-vaccination.

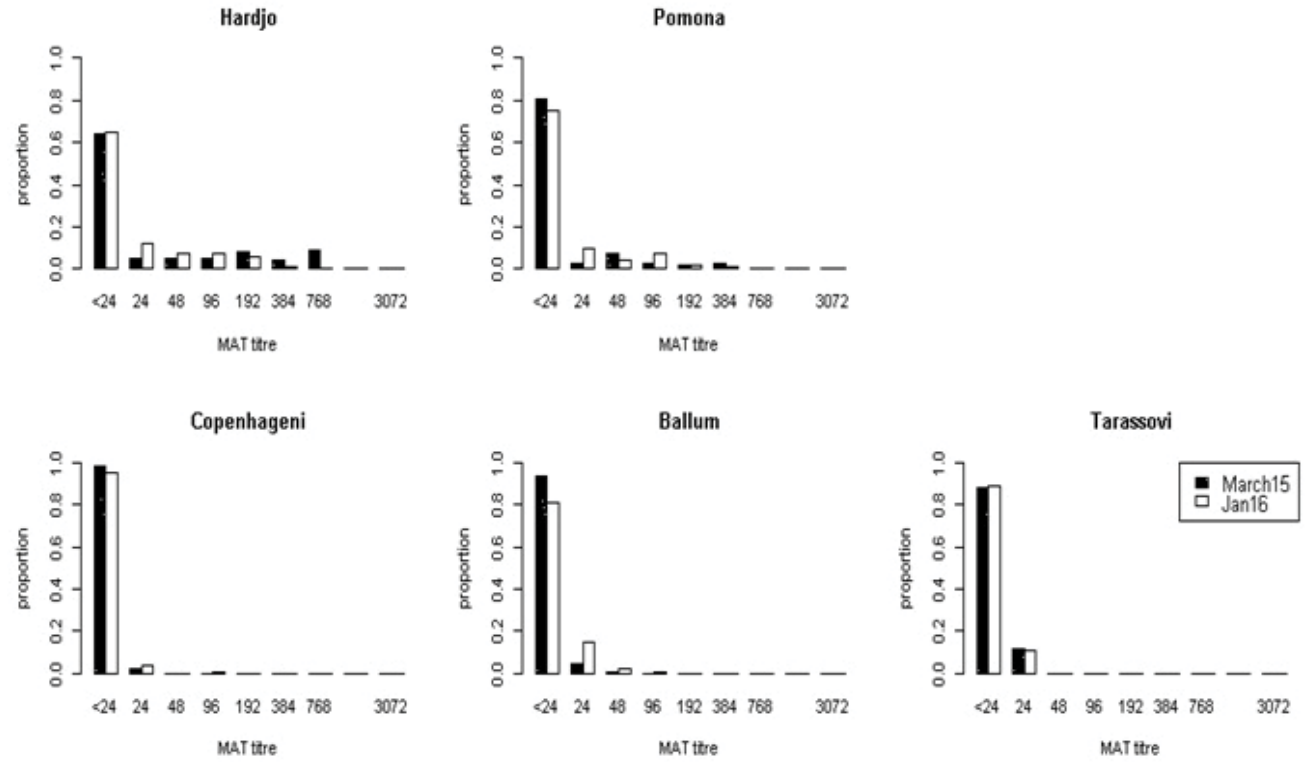

Figure 2 Proportion of cows in Herd 2 at each MAT titre for each serovar at the initial sampling in March 2015 (n=121) and at the post-vaccination/antibiotic sampling in January 2016 (n=81). Note: January 2016 titres for Hardjo and Pomona are post-vaccination.

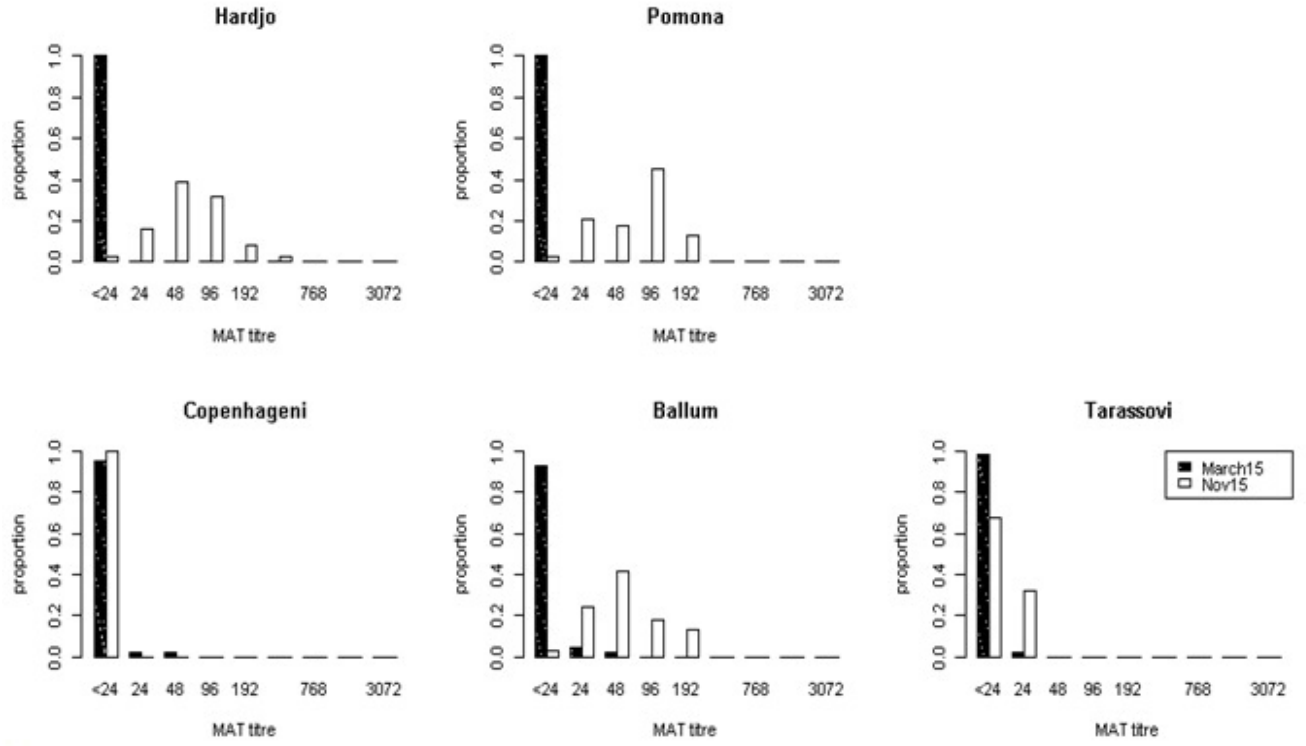

Figure 3 Proportion of rising one-year-old heifers at each MAT titre for each serovar in March 2015, pre-vaccination (Hardjo/Pomona) (n=41) and the same animals as R2 in November 2015, post-vaccination (n=38).

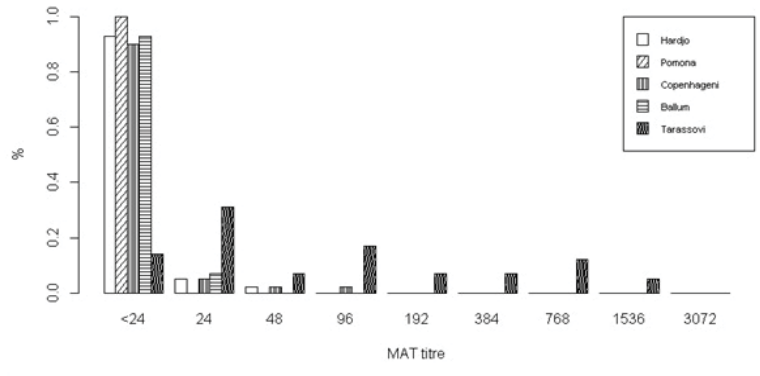

Figure 4 Proportion of R2 heifers at each MAT titer each serovar pre-vaccination in March 2015 (n=41).

showed that the majority of R2 heifers were seropositive to at least one serovar with 55% seropositive to T. The highest GMT was for T.

Calves

Data are presented in **Table 5**.

Pigs

Data are presented in **Table 6**. Titres suggest recent exposure to P, C and B.

Discussion

This was an opportunistic epidemiological investigation of *Leptospira* spp. in a dairy farming enterprise after notification of three human leptospirosis cases amongst workers in the enterprise to the authors. It was designed to identify the sources of exposure to the workers, and to evaluate the effectiveness of vaccine and antibiotic interventions.

Since almost all dairy herds in New Zealand are vaccinated for serovars H and P [29] this was a rare opportunity to reinforce the

Table 4 Number of Rising 1- (R1) and Rising two-year old (R2) heifers tested and % MAT positive (titre ≥ 48) (95% CI) to five serovars, and overall, and geometric mean titer (GMT) (95% CI) of positives, pre-vaccination, in March (pre-vaccination) and November 2015 (post-vaccination).

Age	Sampling occasion	No.		Serovar					
				Hardjo	Pomona	Copenhageni	Ballum	Tarassovi	Overall
R1	Pre-vaccination	41	Prev (%) (95%CI)	0 (0-9)	0 (0-9)	2 (0-13)	2 (0-13)	0 (0-9)	5 (1-16)
			GMT	0	0	48 (0)	48 (0)	0	
	Post-vaccination*	38	Prev (%) (95%CI)	97 (87-100)	76 (61-87)	0 (0-9)	73 (58-85)	0 (0-9)	100 (91-100)
			GMT	127 (102-158)	92 (77-109)	0	73 (59-90)	0	
R2	Pre-vaccination	42	Prev (%) (95%CI)	2 (0-12)	0 (0-8)	5 (1-16)	0 (0-8)	55 (40-69)	57 (42-71)
			GMT	48 (NA)	0	68 (1-5537)	0	230 (142-376)	

Note: November titres for Hardjo and Pomona are post-vaccination. *These are categorized as R2 animals from July/August 2015

Table 5 Seroprevalence (95% CI) and MAT titre for each serovar in calves (n=61) born from Hardjo/Pomona vaccinated dams in July/August and sampled in October 2015.

Serovar tested	Seroprevalence (%) (95% CI) (%)	GMT (95% CI)	MAT Titre				
			48	96	192	384	768
Hardjo	36 (25-49)	75 (60-93)	11	8	3	0	0
Pomona	24 (16-37)	101 (69-146)	5	5	4	1	0
Copenhageni	2 (0.2-9)	48 (0)	1	0	0	0	0
Ballum	7 (3-16)	323 (113-926)	0	0	2	1	1
Tarassovi	0	0	0	0	0	0	0

Note: These samples were prior to vaccination as calves.

Table 6 MAT titres for 5 serovars from four pigs sampled in March 2015.

Pig	Hardjo	Pomona	Copenhageni	Ballum	Tarassovi
1	0	1536	384	48	0
2	0	3072	192	24	0
3	48	1536	768	24	0
4	48	1536	1536	96	0

link between failure to vaccinate and leptospirosis cases in workers. The study demonstrated a high *Leptospira* seroprevalence in cattle and pigs, and significant urinary shedding in cattle. Concurrence of serovars H and P between worker cases [4] and lactating cattle strongly supports that transmission was from that source either directly or indirectly. This study demonstrated that vaccination, alone or in combination with antibiotic, was effective in reducing and possibly eliminating urinary shedding of vaccine serovars. However, evidence of shedding of serovars C, B and T, which are not in the vaccine used, demonstrated that workers remained at risk of exposure of *Leptospira* per se, and therefore, that other protective measures should be routinely adopted.

In New Zealand, H and P have historically been the predominant serovars found in leptospirosis cases among farm workers [27]. This study confirms that the risk remains high in unvaccinated herds. However, in addition to infection with H and P, recent reports (ESR 2012-16) show an increasing proportion of cases associated with Ballum and Tarassovi, both of which were identified in this study, particularly in replacement heifers.

That all the worker cases were from H1 using the rotary milking shed, could suggest that this system may have inherently greater risk for transmission than the herringbone system used for H2. However, at the initial investigation, implemented immediately after notification of the disease among workers, the proportion of cows shedding *Leptospira* was 23 times higher in H1 than H2 despite that H1 had 42% fewer cows milked. Extrapolation

suggests that approximately 53 cows in H1 were shedding at the initial investigation compared with four in H2. This suggests that workers were infected as a result of the high challenge associated with the urinary shedding rate per se rather than inherent risk of rotary milking systems. Higher seroprevalence, and higher urinary shedding rate in H1 at the time of three worker cases within a short period, suggests that there was active epidemic infection in H1 likely related to recent exposure, whereas the serology and PCR results for H2 suggest endemic infection. Alternatively, exposure may have been by indirect contact with effluent since at least one worker reported gross contamination during effluent management. Other environmental exposure cannot be discounted.

During the initial investigation, serovars C and T were detected serologically in H1 but not H2. Serovar B was detected in both herds, at low prevalence. Exposure to wildlife might explain the differences as about 50% of Farm 1 was bordered by forest, while less than 10% of Farm 2 was bordered by forest. Various wildlife species are reservoir hosts for serovars C, B and T in New Zealand [8]. Antibodies to those serovars were also variably observed in R1 and R2 heifers, with seroprevalence up to 55% for T, while seroprevalence was 2% for H in R2 and zero for P in both R1 and R2. These age-groups also had exposure to wildlife.

At the post vaccine/antibiotic sampling in January, it was notable that none of the cows sampled in H2 were positive to T despite the heifers, which were initially sampled as R2 and which were combined with H2 prior to calving, had a seroprevalence of 55% at the initial sampling. This suggests that this serovar had not been transmitted from the introduced heifers to the older cows in the herd, since only the latter were sampled after amalgamation. Identification of higher than previously reported seroprevalence of T in dairy cattle has occurred only recently [29], so little is understood about its epidemiology, indicating that further study is required.

Despite a relatively high proportion of cattle being seropositive and some having high antibody titres, no signs of clinical leptospirosis were detected in any age group. Similarly, no signs were detected in pigs despite high titres. One possible reason for this is that most cows were infected with serovar H, a cattle-adapted serovar for which infection is usually subclinical [11]. However, serovar P, a non-adapted serovar in cattle, was also found in both herds, as were serovars C and B, and additionally T in H1. This suggests that herd immunity was sufficient to prevent clinical disease, but not shedding, or that these serovars were not particularly virulent in this herd. *Leptospira* infection in pigs possibly occurred through transmission from cattle as both pigs and cattle on Farm 1 had serovars P and C. However, transmission from pigs to cattle, or concurrent exposure from an external source, particularly rodents in the case of C, cannot be discounted. Pomona is an adapted serovar in pigs [3]. Copenhageni has also been detected in pigs in New Zealand [9]. However, there is a possibility of cross-reaction between strains of P and C that could have contributed to these results from the pigs cited in [9] though given the observed distribution of titres, this appears unlikely.

High seroprevalence of T was found in R2 heifers at the initial investigation but seroprevalence in other groups was low. Additionally, C and B were present in heifers and PCR data suggest that some or all of these serovars were being shed in urine. These serovars could therefore pose a risk to workers directly, or subsequently, via amplification in older cows once those heifers were merged with the adult milking cows prior to calving. A recent study of 200 dairy herds in New Zealand has shown evidence of *Leptospira* shedding in 26.5% of herds and 2.4% of cows in vaccinated herds, with serological evidence for Tarassovi, and DNA evidence of a Tarassovi-like strain [29]. Serological and PCR evidence from this herd is therefore not unlike that of many herds throughout New Zealand in which evidence is emerging for infection with this non-vaccination serovar. This is supported by recent evidence of this serovar in human cases (ESR 2012-6). Workers were therefore advised to practise protective measures such as wearing protective clothing during milking, covering wounds, avoiding direct contact with effluent, and protecting their face from urine splash [14] rather than rely on vaccination and antibiotic treatment alone.

The PCR used in this study identified pathogenic *Leptospira* and did not differentiate between serovars. However, in New Zealand, since there are few serovars, with limited serological cross-reactivity between them, it has been proposed that parallel consideration of serology and urine PCR results allows reasonable specificity of diagnosis of serovar [21]. Hence, it appears reasonable to suggest that the serovars shed in urine at the initial investigation were likely to be H and P, and that as the study progressed, C, B and T were also variably shed in urine, particularly in heifers.

Serological data for H and P from calves may represent maternal antibody, but exposure cannot be excluded as titres of 192-384 are unlikely to represent maternal antibody 2-3 months after birth, and are potentially predictive of active infection in dairy cattle [29]. Serological evidence suggests environmental exposure to B, and when combined with results from other age groups, suggests that this organism may be prevalent in mice, its reservoir host species. The presence of antibodies to C and P suggest these serovars may also be circulating in wildlife endemic to the farm. A recent survey of wildlife in the proximity of this farm confirmed a high prevalence of C in mice [17].

Leptospira vaccination per se is efficacious in preventing renal colonization and urinary shedding [13], particularly if vaccination occurs prior to exposure. Long-term vaccination programmes, which are implemented in more than 95% of dairy herds with bivalent (H and P) or trivalent (H, P and C) vaccines in New Zealand, are effective in preventing shedding in adult cows [29]. In H1, reduction in shedding was observed after bivalent vaccination alone, and elimination of shedding was observed after vaccination and antibiotic. However, in H2 there was an increase in prevalence of shedding after each intervention. In this herd, serological evidence suggests that the shedding was likely due to non-vaccine serovars, particularly B but possibly also C, since there was an increase in seroprevalence from the initial sampling for these serovars. Serological observation of C in cattle and pigs on Farm 1, would have justified the use of a trivalent vaccine containing that serovar rather than the bivalent vaccine chosen by the farmer.

Some studies have suggested that treatment with antibiotics in addition to vaccination is preferred to reduce *Leptospira* infection in cattle herds [12,18]. If used simultaneously, antibiotics should reduce or eliminate renal infection and therefore shedding, before animals have sufficient vaccine-induced immunity, as vaccines do not eliminate shedding in all animals in the short term [18]. Immunity due to vaccination should prevent infection of subsequently exposed animals. Combinations of penicillin and streptomycin or streptomycin alone have been used widely, but ampicillin, amoxicillin and the third generation cephalosporins have also been used [10]. In this study, a long-acting preparation of amoxicillin was chosen since Smith et al., [20] demonstrated that this drug was effective in eliminating Leptospires from the kidney following two and possibly one injection in cattle experimentally infected with serovar H. Treatment was given only to the milking cows because the greatest risk to workers was from this group. There was little evidence of vaccine serovars in replacement heifers, hence they were vaccinated prior to infection so immunity should have been protective. Antibiotic treatment was delayed until the end of lactation to avoid milk wastage and disposal problems.

Conclusion

In conclusion, the occurrence of leptospirosis in workers in this farming enterprise confirms that the risk of *Leptospira* infection with vaccine serovars in unvaccinated dairy cattle and exposure to dairy farm workers from cattle in New Zealand persists. This study also demonstrated that a combination of whole herd vaccination and antibiotic treatment in adult cows was effective in decreasing and possibly eliminating urine shedding of vaccine serovars. It also confirmed, consistent with the study of Yupiana et al., that serovars B and T which are not present in available vaccines may be shed in vaccinated herds, supporting that personal protective measures should continue to be adopted regardless of vaccination status of herds. This study also supports that investigation of the epidemiology and production impact of serovars not currently contained in vaccines is warranted.

Acknowledgments

Authors express their appreciation to MPI, AGMARDT, Zoetis, MSD, Virbac, NZAID and others for funding, Neville Haack for organization, support and guidance in laboratory testing, and Massey students for sample collection.

References

1 Adler B (2015) Leptospira and Leptospirosis. Springer-Verlag Berlin Heidelberg p: 387.

2 Adler B, De La Peña Moctezuma A (2010) Leptospira and leptospirosis. Veterinary Microbiology 40(3): 287-296.

3 Adler B, Levett PN, Cameron CE, Picardeau M, Levett, et al. (2015) Leptospira and leptospirosis Verlag Berlin Heidelberg. Springer p: 387.

4 Benschop J, Collins-Emerson J, Maskill A, O'Connor P, Tunbridge M, et al. (2017) Leptospirosis in three workers on a dairy farm with unvaccinated cattle. The New Zealand medical journal 130(1462): 102.

5 Christmas B, Tennent R, Philip N, Lindsay P (1974) Dairy farm fever in New Zealand: A local outbreak of human leptospirosis. New Zealand Medical Journal 79(514): 901-904.

6 Fang F, Collins-Emerson JM, Heuer C, Hill FI, Tisdall DJ, et al. (2014) Interlaboratory and between-specimen comparisons of diagnostic tests for leptospirosis in sheep and cattle. Journal of Veterinary Diagnostic Investigation 26(6): 734-747.

7 Hathaway SC (1981) Leptospirosis in New Zealand: An ecological view. NZ Vet J 29(7): 109-112.

8 Hathaway SC, Blackmore DK (1981) Ecological aspects of the epidemiology of infection with *Leptospires* of the Ballum serogroup in the black rat (*Rattus rattus*) and the brown rat (*Rattus norvegicus*) in New Zealand. J Hyg (Lond) 87(3): 427-436.

9 Hellstrom JS (1978) Bovine Leptospirosis in New Zealand. (Doctor of Philosophy), Massey University, Massey University.

10 Liegeon G, Delory T, Picardeau M (2018) Antibiotic susceptibilities of livestock isolates of leptospira. International Journal of Antimicrobial Agents 51(5): 693-699.

11 Lilenbaum W, Martins G (2014) Leptospirosis in cattle: A challenging scenario for the understanding of the epidemiology. Transboundary & Emerging Diseases 61: 63-68.

12 Little TWA, Hathaway SC, Boughton ES, Seawright D (1992) Development of a control strategy for Leptospira-hardjo infection in a closed beef herd. Veterinary Record 131(17): 383-386.

13 Mackintosh CG, Marshall RB, Broughton E (1980) The use of a hardjo-pomona vaccine to prevent leptospiruria in cattle exposed to natural challenge with *Leptospira interrogans* serovar hardjo. New Zealand Veterinary Journal 28(9): 174-177.

14 Mackintosh CG, Schollum LM, Harris RE, Blackmore DK, Willis AF, et al. (1980) Epidemiology of leptospirosis in dairy farm-workers in the Manawatu: A cross-sectional serological survey and associated occupational factors. New Zealand Veterinary Journal 28(12): 245-250.

15 Marshall RB, Manktelow BW (2002) Fifty years of leptospirosis research in New Zealand: A perspective. New Zealand Veterinary Journal 50(3): 61-63.

16 McLean M (2014) A cluster of three cases of leptospirosis in dairy farm workers in New Zealand. New Zealand medical journal 127(1388): 13-20.

17 Moinet M, Nisa S, Haack N, Wilkinson DA, Oosterhof H, et al. (2017) Is wildlife a source of Leptospira infection in livestock in New Zealand? Paper presented at the 2017 Conference Proceedings of the Large Animal Veterinarian Technicians Group of the NZVA Wellington, New Zealand.

18 Mughini-Gras L, Bonfanti L, Natale A, Comin A, Ferronato A, et al. (2014) Application of an integrated outbreak management plan for the control of leptospirosis in dairy cattle herds. Epidemiology And Infection 142(6): 1172-1181.

19 Newcombe RG (1998) Two-sided confidence intervals for the single proportion: Comparison of seven methods. Statistics in medicine 17(8): 857-872.

20 Smith CR, Corney BG, McGowan MR, McClintock CS, Ward W, et al. (1997) Amoxycillin as an alternative to dihydrostreptomycin sulphate for treating cattle infected with *Leptospira borgpetersenii* serovar hardjo. Australian Veterinary Journal 75(11): 818-821.

21 Subharat S, Wilson PR, Heuer C, Collins-Emerson JM (2011) Evaluation of a SYTO9 real-time polymerase chain reaction assay to detect and identify pathogenic *Leptospira* species in kidney tissue and urine of New Zealand farmed deer. Journal of Veterinary Diagnostic Investigation 23(4): 743-752.

22 The Institute of Environmental Science and Research Ltd (2012) Notifiable diseases in New Zealand: Annual report 2012. Porirua, New Zealand.

23 The Institute of Environmental Science and Research Ltd (2013). Notifiable diseases in New Zealand: Annual report 2013. Porirua, New Zealand.

24 The Institute of Environmental Science and Research Ltd (2014) Notifiable diseases in New Zealand: Annual report 2014. Porirua, New Zealand.

25 The Institute of Environmental Science and Research Ltd (2015) Notifiable diseases in New Zealand: Annual report 2015. Porirua, New Zealand.

26 The Institute of Environmental Science and Research Ltd (2016) Notifiable diseases in New Zealand: Annual report 2016. Porirua, New Zealand.

27 Thornley CN, Baker MG, Weinstein P, Maas EW (2002) Changing epidemiology of human leptospirosis in New Zealand. Epidemiology and Infection 128(01): 29-36.

28 WorkSafe New Zealand (2015) Prevention and control of leptospirosis. Retrieved from http://saferfarms.org.nz/assets/guides/WSNZ-1368-Prevention-and-Control-of-Leptospirosis-GPG-v5-0-FA1-LR.pdf

29 Yupiana Y, Collins JC, Benschop J, Weston J, Wilson P, et al. (2017) Nationwide survey of leptospiral antibodies and shedding in New Zealand dairy herds. Paper presented at the Proceedings of the Society of Dairy Cattle Veterinarians of the NZVA Annual Conference, 2017 Conference, Wellington.