Leptospirosis diagnostics and exposure at the human and animal interface in New Zealand

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Abstract

The studies presented in this thesis investigate key questions about leptospirosis diagnostics in animals and humans in New Zealand (NZ): how do different diagnostic tests perform on various specimens collected at different stages of infection; how well do tests from a commercial and a research laboratory agree; how do serological test results and urine/kidney quantitative real-time PCR (qPCR) results compare; and what is the utility of PCRs on blood from acute human cases? Additional studies investigate occupational risk at the human-animal interface.

In trials where the animals were challenged with *Leptospira borgpetersenii* serovar Hardjobovis (Hardjobovis) and/or *Leptospira interrogans* serovar Pomona (Pomona), sequential samples were taken from sheep and cattle to evaluate diagnostic tests at various known times post-infection. Although no statistically significant differences were found, results suggested that during the early stage of a Pomona infection in sheep, qPCR on serum had the highest sensitivity for detecting leptospires in blood, followed by blood culture and qPCR on whole blood. In sheep infected under carefully controlled experimental conditions, culture tended to have higher sensitivity for detecting leptospires (either Hardjobovis or Pomona) in urine than qPCR; whereas in cattle with Hardjobovis infection, higher sensitivity was apparent using qPCR. Sensitivity was similar in culture and qPCR for detecting leptospires in kidney from sheep with either Hardjobovis or Pomona infections. There was low sensitivity and specificity of dark field microscopy for both urine and kidney samples, thus questioning the usefulness of this technique in veterinary settings.

A cross-sectional study was carried out at a NZ sheep and cattle abattoir to investigate the seroprevalence (by microscopic agglutination test (MAT)), shedding rate (by urine qPCR), and renal colonisation rate (by kidney qPCR) of slaughtered animals. Urine, kidney and blood samples were collected from carcasses of 399 sheep and 146 cattle. The animal-level seroprevalence found in sheep (57%, predominately lambs) and cattle (73%, predominately ≤18 months old) was substantially higher than in previous studies; these and the recorded shedding rate (27%) and renal colonisation rate (27%) raised occupational health concerns that meat workers from this abattoir may be at risk of exposure to leptospires during their daily work routine.

Samples from this abattoir study were used to investigate the inter-laboratory test agreements between a research (HLRL) and a commercial veterinary diagnostic laboratory (GV), and test agreements (HLRL) between specimens for leptospirosis diagnosis. Urine qPCR results on from the two laboratories had almost perfect agreement (kappa = 0.93). The MAT agreement between these two laboratories was higher for Hardjobovis (kappa = 0.94) than Pomona (kappa = 0.53). This serovar-dependent difference suggested that the different MAT results may be more likely due to the different source of antigen cultures (especially serovar Pomona) used in two laboratories than observer variation. These inter-laboratory comparisons can assist researchers and diagnosticians in understanding the sometimes discrepant test results received. Within HLRL, almost perfect agreement (kappa = 0.84) between qPCR results on urine and kidney suggested that the qPCR on these two specimens can be used interchangeably. The comparisons between MAT and qPCR on both kidney and urine, suggested that except from Hardjobovis-seropositivity in sheep, Pomona-seropositivity in sheep and seropositivity of both Hardjobovis and Pomona in cattle was not considered to be predictive for indicating shedding/renal colonisation at individual animal level.

A pilot panel of isolates from 18 sheep and five cattle kidney cultures demonstrated the utility of a multi-locus sequence typing scheme for genotyping *Leptospira* spp. field isolates from sheep and cattle in NZ. The sequence results provided sufficient genetic variability to assign the isolates to two distinct species, those being *L. borgpetersenii* and *L. interrogans*. Two dominant serovars (Hardjobovis and Kenniwicki) were identified. Identical sequences found in Hardjobovis isolates from sheep and cattle provided evidence for inter-species transmission of *Leptospira* spp.

Aiming to establish the best diagnostic test or combination of tests for the early diagnosis of human leptospirosis, suspect leptospirosis patients were recruited via rural general practitioners (GP), hospital doctors and phlebotomists within the Waikato District Health Board area. For each recruited patient (n = 14), blood culture, MAT (on acute and convalescent serum), and whole blood/serum PCRs (by three laboratories) were performed. Although it is difficult to make conclusions based on findings from 14 patients recruited from one region, this is the first attempt to compare different diagnostic tests for acute leptospirosis cases in NZ. The information of clinical

symptoms, demographics, and exposure to risk factors can contribute to the GPs' suspicion of future leptospirosis cases.

A cross-sectional study was conducted to determine the seroprevalence and quantify putative risk factors for both intra- and extra-curricular exposure to leptospirosis among undergraduate veterinary students at Massey University, NZ. All participating students (n = 302) were MAT negative for each serovar (Hardjobovis, Pomona, and Ballum), using a cut-point of ≥ 48. This study demonstrated that these veterinary students were at low risk of contracting leptospirosis, despite frequent exposure to potential sources of infection (e.g. animal urine within and outside veterinary curriculum, home slaughtering, hunting, and outdoor activities involving fresh water). The similar frequency of exposure to the non-work putative risky activities (hunting and home slaughtering) reported in veterinary students as previously reported in meat workers, added strength to the finding that non-work activities are less important risk factors compared to within-work activities.

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List of Publications

Fang F, Benschop J, Wilson PR, Collins-Emerson JM, Heuer C and Prattley D. Seroprevalence and exposure to risk factors for leptospirosis among veterinary students at Massey University. *New Zealand Veterinary Journal* 62, 130-5, 2014.

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Fang F, Collins-Emerson JM, Wilson PR, Heuer C, Tisdall D and Benschop J. Leptospirosis infection in sheep and cattle in a New Zealand Abattoir: sero-prevalence, shedding and diagnostic test comparison. *Poster session presented at the meeting of 13th International Symposium on Veterinary Epidemiology and Economics (ISVEE)*. Maastricht, Belgium/Netherlands, 2012.

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Chapter 1 General Introduction

This general introduction covers a brief overview of leptospirosis in both humans and animals, and the epidemiology and diagnostics of leptospirosis in New Zealand. The aim and structure of this thesis are explained.

1 Leptospirosis Overview

1.1 Leptospira (the organism)

Leptospirosis is a globally important zoonotic disease, which is caused by the pathogenic spirochetes of the genus *Leptospira* (Bharti *et al.* 2003; Adler and de la Peña Moctezuma 2010). Leptospires are usually about 6-25 μm long and 0.1 to 0.2 μm in diameter, and have a typical double membrane structure as found in other spirochetes (Levett 2001; Bharti *et al.* 2003). Leptospires are highly motile, obligate aerobes, with an optimum growth at a temperature of 28 to 30 °C and at pH range 6.8 to 7.4 (Bharti *et al.* 2003; Adler and de la Peña Moctezuma 2010). Leptospires can survive in a moist environment (e.g. soils, mud, swamps, streams and rivers), in organs and tissues of live or dead animals, or in diluted milk (Faine *et al.* 1999; Hirsh *et al.* 2004).

Based on DNA relatedness, 20 species of *Leptospira* spp. have been identified (Nalam *et al.* 2010). The traditional serological classification that is based on agglutinating lipopolysaccharide antigens, classifies *Leptospira* spp. into 20 serogroups and over 300 serovars (Picardeau 2013). The seven main pathogenic *Leptospira* species are considered to be *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. kirschneri* and *L. alexanderi* (Ahmed *et al.* 2006). Approximately half of the pathogenic serovars belong to *L. interrogans* and *L. borgpetersenii* (Victoriano *et al.* 2009).

1.2 Epidemiology of leptospirosis

Numerous mammalian species are natural carriers (maintenance hosts) of pathogenic leptospires, including feral, farm and pet animals (Faine *et al.* 1999; Levett 2001). Infected animals may have leptospires persistently colonising the proximal renal tubules and excrete the organism intermittently for months or years, or even for lifetime (Faine

et al. 1999). Humans are incidental hosts for *Leptospira* spp. (Ko *et al.* 2009). Although excretion of leptospires in human urine for weeks, or rarely, months has been reported, humans are not regarded as a source of transmission (Bharti *et al.* 2003). Animals or humans are infected through direct contact with animals, animal tissues, body fluids (especially urine), or through indirect contact with contaminated environment (e.g. soil or water contaminated with urine of carrier animals) (Bharti *et al.* 2003; Sharma and Yadav 2008) (Figure 1.1). Leptospirosis is an occupational disease for farmers, abattoir workers, butchers, veterinarians, pet traders, hunters, rodent control workers, and other occupations requiring frequent contact with animals (Hartskeerl *et al.* 2011; Musso and La Scola 2013). Due to the fact that leptospires survive longer in warm and humid environments (Levett 2001), such as in tropical climates, occupations that involve indirect contact with contaminated wet soil or water (e.g. rice and taro farming, sewer workers) are at highest risk (Musso and La Scola 2013). In addition to occupationally exposed groups, urban slum dwellers in areas with poor sanitation are communities at particularly high risk (Abela-Ridder *et al.* 2010). Infection associated with outdoor recreational exposure, international travel, especially to endemic areas in the tropics, and flooding is increasing (Lau *et al.* 2010; Lau *et al.* 2010b).

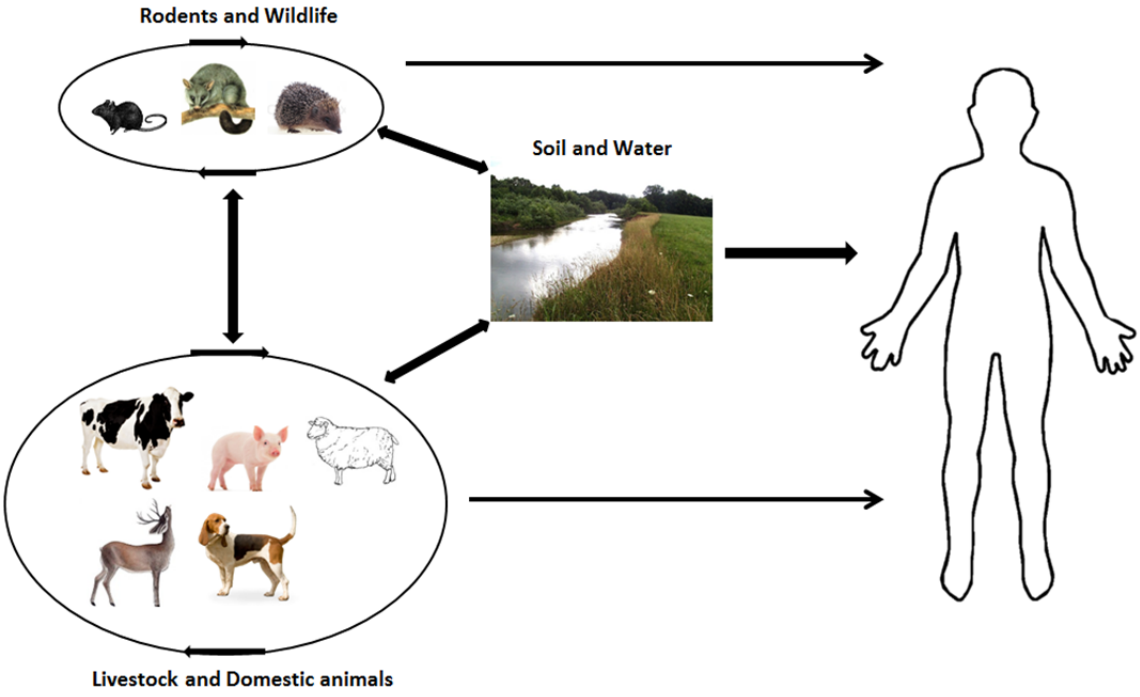

Figure 1.1 Flow diagram of epidemiology of leptospirosis in animals and humans

1.3 Clinical features

The manifestation of leptospirosis depends on the virulence characteristics of the infecting serovar, infectious dose, and the age, health and immunological competence of the host (Ko *et al.* 2009; Adler and de la Peña Moctezuma 2010). In animals, clinical signs are usually mild or inapparent when infected by host-adapted serovars. However, for infections with non-host-adapted serovars, clinical symptoms may range from mild to severe, with severe cases observed more commonly in young than in older animals (Ellis 1994). Signs of acute disease are similar in all animals. The most common signs are elevated temperature, listlessness, anorexia, irritability, ruffled fur, red eyes, and sometimes diarrhoea. There may be signs of haemorrhages, haemoglobinuria and jaundice. Animals that recover may have weight loss and renal failure (Faine *et al.* 1999). Chronic leptospirosis usually involves infection by host-adapted serovars, which can lead to chronic interstitial nephritis (Faine *et al.* 1999; Radostitis *et al.* 2007), poor reproductive performance (Dhaliwal *et al.* 1996), abortion, stillbirth, weak offspring (Heath and Johnson 1994), reduced milk production (Dhaliwal *et al.* 1996), and poor growth (Subharat *et al.* 2012).

The clinical presentation of leptospirosis in humans ranges from asymptomatic to lethal, with a broad spectrum of clinical signs and symptoms (Levett 2001). The common onset symptoms recorded include fever, headache, myalgia, conjunctival suffusion, nausea, and a transient skin and mucosal rash (Faine *et al.* 1999; Vinetz 2000). Severe complications such as renal failure, liver failure, pulmonary haemorrhage, meningitis, and myocarditis have also been reported (Bharti *et al.* 2003). The majority of cases may have subclinical disease or show very mild symptoms, and do not require medical treatment (Plank and Dean 2000). The mild symptoms of leptospirosis in both animals and humans are not disease-specific. For example, clinical symptoms in animals mimic other infectious abortifacient diseases (e.g. brucellosis, neosporosis bovine virus diarrhoea, porcine circa virus) (Hartskeerl *et al.* 2011), while clinical symptoms in humans mimic many other illnesses with febrile syndromes (e.g. dengue fever, influenza, hepatic disease, Hantavirus infections) (Plank and Dean 2000; Levett 2001). Therefore, the disease is often misdiagnosed, which contributes to the underestimation of the occurrence of leptospirosis (Yang 2007; Victoriano *et al.* 2009; Hartskeerl *et al.* 2011).

1.4 Burden of human and animal leptospirosis

The World Health Organization (WHO) has estimated that the annual incidence of leptospirosis is 0.1–1 case/100,000 people in temperate non-endemic areas, and 10–100 cases/100,000 people in humid, tropical, endemic areas (Abgueguen *et al.* 2008; Sethi *et al.* 2010). The number of severe cases were reported to be approximately 300,000 to 500,000 each year worldwide, with case fatality rates of up to 30% (Tulsiani *et al.* 2010). The annually death rate due to leptospirosis worldwide is estimated to be 58,900 (Hagan *et al.* 2013).

Leptospirosis has been recognized as an (re-)emerging global public health problem due to the increased incidence in both developing and developed countries (Vijayachari *et al.* 2008). Global warming that leads to extreme weather events such as cyclones and floods, increased rainfall, and increased world population and urbanization are considered as the factors associated with the upsurge in the incidence of leptospirosis, as well as the magnitude of outbreaks (Lau *et al.* 2010b; Hartskeerl *et al.* 2011). Numerous outbreaks have occurred worldwide during the past decade. The most recently published examples that were due to either heavy rainfall or flooding are in Nicaragua in 2007 and 2010 (Schneider *et al.* 2012), in Sri Lanka in 2008 (Agampodi *et al.* 2011), in New Caledonia in 2008 (Goarant *et al.* 2009), in Philippines in 2009 (McCurry 2009), and in Martinique in 2011 (Hochedez *et al.* 2013). Since leptospirosis is a generally neglected disease and often under- or mis-diagnosed, there are no reliable global disease burden estimates for human leptospirosis. Therefore, WHO has established the Leptospirosis Burden Epidemiology Reference Group (LERG) in 2010 to coordinate the assessment, aiming to quantify and compare the health of populations across the globe (Abela-Ridder *et al.* 2010). The burden estimates by LERG will guide public health policy on leptospirosis disease control and prevention, with the aim of reducing the impact on human health.

Besides the significant impact on public health, leptospirosis is also an animal health problem that causes economic losses in the livestock industries, due to reproductive failure, decreased milk and meat production (Pearson *et al.* 1980; Ellis 1994; Langoni *et al.* 1999), reduced growth (e.g. in non-vaccinated deer (Subharat *et al.* 2012)), and clinical illness (e.g. sudden death, haemoglobinuria (Cordes *et al.* 1982; Ayanegui-

Alcerreca *et al.* 2007)). In addition, infected animals can develop chronic renal infection and excrete the organisms in urine, thereby disseminating leptospires to other animals and constituting a potential zoonotic threat to those engaged in animal production and related industries (Higgins *et al.* 1980; Cousins *et al.* 1989; Gerritsen *et al.* 1994; Magajevski *et al.* 2005). Despite its negative impact, animal leptospirosis is usually not routinely monitored. Case findings and reporting in veterinary medicine have been limited (Cachay and Vinetz 2005).

1.5 Diagnosis

Other than the lack of clinical suspicion, imperfect diagnostics for acute human leptospirosis is an important reason that contributes to the under-reporting of this disease. Currently, no diagnostic technique is completely satisfactory (details of strengths and weakness of most of the diagnostic tests will be discussed in Chapter 2). Standard tests, such as culture (isolation of the organism from clinical specimens) and the microscopic agglutination test (MAT) (serological test on paired serum collected during acute and convalescent phases of the disease to establish sero-conversion or rise in titre), are time-consuming, laborious, and require well-equipped laboratories, great expertise and patient follow-up (for MAT) (Vijayachari *et al.* 2008; Hartskeerl *et al.* 2011). Amplification of specific fragments of leptospiral DNA using polymerase chain reaction (PCR) has also been used for the diagnosis of leptospirosis (Musso and La Scola 2013). However, this technique also has its own drawbacks such as inability to identify the infecting serovars in most PCR assays, requirement of special equipment and the relatively high cost (Faine *et al.* 1999; Picardeau 2013). Several rapid tests are currently available (e.g. IgM-ELISA, Lateral flow test, lepto dipstick); however, they are recommended for providing presumptive evidence of infection, due to the lack of sensitivity or specificity. Robust and easy to use diagnostic tests are still lacking for diagnosis in both humans and animals. Improving the diagnostics is important for both public health and assessment of health economics, since it will contribute to improved case detection and consequently increased awareness and control of leptospirosis (Hartskeerl *et al.* 2011).

2 Leptospirosis in New Zealand

An unusual native fauna, lack of native terrestrial mammals (only two bat species), and recent incursions of exotic fauna characterize the unique infectious disease epidemiology in New Zealand, and unusually high rates of some endemic infectious diseases in this country (Crump *et al.* 2001). Compared with other temperate developed countries, leptospirosis occurs at a higher frequency in New Zealand (Thornley *et al.* 2002). In a study that investigated the annual country-specific incidence of human leptospirosis from 1996 onwards, among countries that have data available, New Zealand ranked 9th in the 28 countries with the highest incidence recorded (Pappas *et al.* 2008). In the Asia- Pacific region, the incidence risk of 2.5 per 100,000 placed New Zealand in the moderate incidence category (Victoriano *et al.* 2009).

Understanding of the eco-epidemiological and cultural characteristics of a community that faces the problem of leptospirosis is an important prerequisite for setting up effective prevention and control strategies for this disease (Vijayachari *et al.* 2008). In New Zealand, the epidemiology of leptospirosis follows the pattern that occurs in temperate regions (Levett 2001), as it involves relatively few serovars and is dominated by exposure to domestic livestock. Ruminant livestock species, including cattle, sheep, deer, and pigs are the key maintenance hosts of leptospires in New Zealand (Marshall and Manktelow 2002; Ayanegui-Alcerreca *et al.* 2007; Dorjee *et al.* 2008). Previous Massey University surveys of farms and abattoirs have shown that up to 69% of beef cattle herds, 44% of sheep slaughter lines, and 81% of deer herds were seropositive for leptospirosis (Heuer *et al.* 2007; Dorjee *et al.* 2008; Ayanegui-Alcérreca *et al.* 2010).

Only eight serovars from two *Leptospira* species have been isolated in New Zealand. Of these, the six seovars *L. interrogans* serovars Copenhageni and Pomona, and *L. borgpetersenii* serovars Balcanica, Hardjobovis, Tarassovi and Ballum have been isolated and are known to be endemic in animals. Serovars *L. interrogans* Australis and Canicola have rarely been isolated from humans and cannot be ascribed an endemic status in New Zealand (Marshall and Manktelow 2002). The most commonly identified serovars in human leptospirosis infections in the past ten years are Hardjobovis, Pomona, and Ballum (Figure 1.2). Serovar Ballum has emerged as an important cause of human disease in New Zealand, based on a review of leptospirosis notification data from 1990 to 1998 (Thornley *et al.* 2002). This trend continued, and in 2011, Ballum

was the most frequently notified serovar in human cases (The Institute of Environmental Science and Research Ltd. 2003-2013). Serological evidence of leptospirosis in domestic livestock (e.g. cattle, sheep and deer) is most commonly found with serovars Hardjobovis and Pomona in New Zealand (Dreyfus *et al.* 2011). Considering the increased trend of human infections with serovar Ballum, investigation of seroprevalence of Ballum in livestock is in progress by our research group, with regard to potential inclusion of this serovar in animal vaccines.

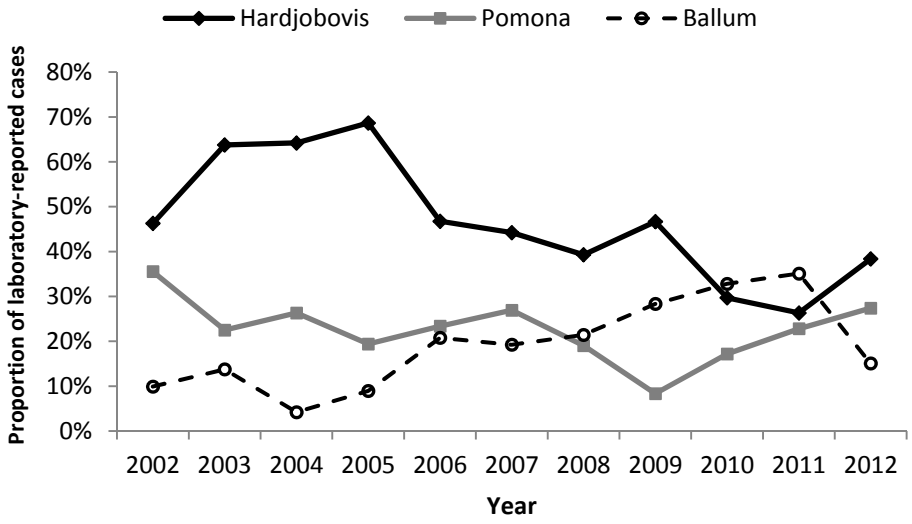

Figure 1.2 Proportion of laboratory-reported cases infected with serovar Hardjobovis, Pomona, and Ballum by year 2002-2012 (The Institute of Environmental Science and Research Ltd. 2003-2013).

The number of notified human leptospirosis cases peaked at 875 in 1974 (Thornley *et al.* 2002). Since the widespread uptake of vaccination of dairy cattle in 1979, notifications of human infection dropped from 677 in 1979, to 325 in 1981 (Marshall and Chereshsky 1996). The reduction of human cases continued and has remained at approximately 100 cases over the period from 1997 to 2000 (Thornley *et al.* 2002) and to present day (Figure 1.3). Leptospirosis continues to be a severe disease for rural New Zealanders, with 50% of notified cases hospitalized (The Institute of Environmental Science and Research Ltd. 2003-2013). Leptospirosis is the most important occupationally-acquired zoonotic disease in New Zealand, mainly associated with agricultural and meat workers (Thornley *et al.* 2002). On average, farmers and meat workers together accounted for 82% of the leptospirosis cases in the past ten years (Figure 1.3). New Zealand's Department of Labour recognized leptospirosis as an

important occupational disease, with "Guidelines for the Control of Occupationally Acquired Leptospirosis" published in 2001 to provide practical suggestions for the management of the health hazard presented by the bacteria (Keenan 2007). Until now, leptospirosis remains the most important illness in meat workers (Annonymous 2009). Massey University sero-surveys of leptospirosis in meat workers from eight abattoirs (sheep, beef and deer) reported an average seroprevalence of leptospirosis as 11% (range from 5 to 31%) (Benschop *et al.* 2009; Dreyfus 2013), and suggested the ongoing public health burden of leptospirosis in this occupational group in New Zealand. Little information is yet available about seroprevalence of leptospirosis in farmers.

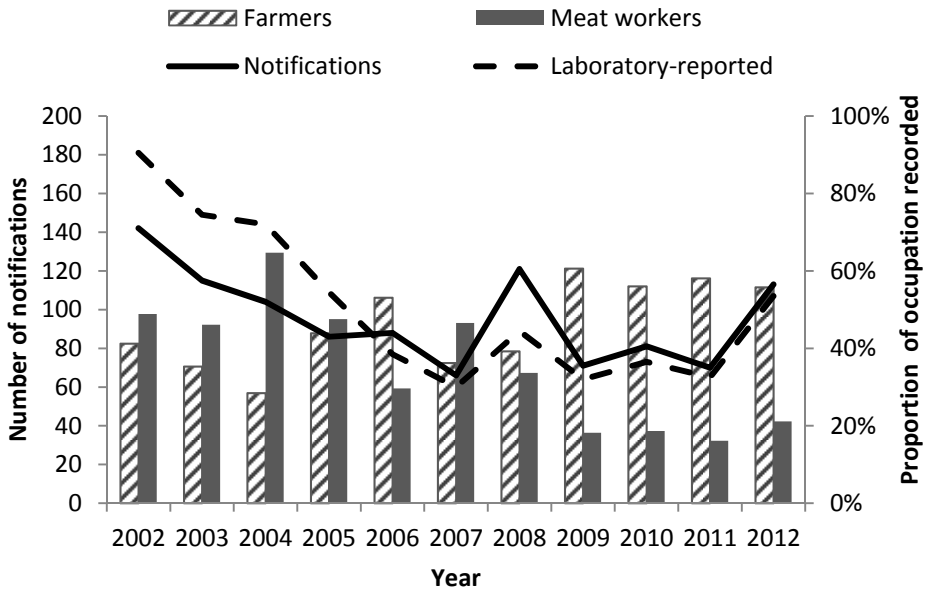

Figure 1.3 Leptospirosis notifications and laboratory reported cases by year 2002-2012 (lines); proportion of notified cases with occupations recorded as farmers and meat workers (bars) (The Institute of Environmental Science and Research Ltd. 2003-2013).

As is the case worldwide, there is considerable under-reporting of leptospirosis in New Zealand (Keenan 2007; Heuer *et al.* 2008). The underreporting may be due to the lack of awareness of the disease, non-specific symptoms, and the currently accepted diagnostic methods to confirm leptospirosis, especially in the acute setting (Levett *et al.* 2001; Bharti *et al.* 2003; Vickery *et al.* 2006). In New Zealand, the (laboratory-confirmed) case definition of human leptospirosis used to base on isolation of *Leptospira*, or a four-fold or greater rise in MAT titre or a single high titre ≥ 400. From

May 2012, detection of leptospiral DNA by PCR from a clinical specimen was added into the list of laboratory techniques for the confirmation for leptospirosis cases (Annonymous 2012). Previous studies have shown that PCR assays are able to detect leptospires in clinical specimen at an early stage of infection, before antibodies become detectable by the MAT (Merien *et al.* 1995; Ooteman *et al.* 2006). Therefore the change of case definition is likely to contribute to the reduction of the under-reporting of leptospirosis cases in New Zealand.

Diagnostic tests are important tools that contribute to the prevention of leptospirosis (Faine *et al.* 1999), which is essential for livestock industries in New Zealand, since considerable economic impact due to the poor reproductive performance, reduced milk production, poor growth, and clinical illness in livestock may result from this disease. Moreover, since livestock are the main reservoir hosts for this disease in New Zealand, identifying the carriage status in these animals is important for preventing the spread of leptospirosis infection from the carriers to other animal species and humans. Compared to the commonly used serological tests (MAT) that detect antibodies in serum rather than the presence of leptospires directly, the direct detection of leptospiral DNA by PCR can differentiate current infection from past exposure, and also avoid false-positive serological results due to vaccination induced antibodies (Faine *et al.* 1999; Adler and de la Peña Moctezuma 2010). The PCR also provides information as to the carrier status of the host (Çetinkaya *et al.* 2000), which may fail to be detected by serological tests (Cole *et al.* 1973; Thiermann 1984). Therefore, quantitative real-time PCRs (qPCRs) have been introduced to veterinary diagnostic laboratories in New Zealand (e.g. in Gribbles Veterinary (GV) Palmerston North and New Zealand Veterinary Pathology, Palmerston North) as screening tests, and to Hopkirk Leptospirosis Research Laboratory (HLRL) from Massey University for research purposes (Subharat *et al.* 2011).

3 Thesis aim and structure

The aim of this thesis is to answer some key questions about leptospirosis diagnostics in both animals (sheep and cattle) and humans in New Zealand, and also investigate the occupational risks at the human-animal interface. Leptospiral diagnostic tests, including the standard tests (culture and MAT) and molecular techniques, were evaluated in both animals (sheep and cattle) and humans. Multi-locus sequence typing (MLST) was first

applied to animal field isolates to test the utility of this technique to identify leptospiral strains in New Zealand. The shedding/renal colonisation rates and seroprevalence of *Leptospira* spp. were examined in slaughtered animals to raise awareness of the potential risk of contracting leptospirosis in meat workers. The seroprevalence of leptospirosis among veterinary students at Massey University and exposure to putative risk factors both within the veterinary curriculum and personal life were examined to contribute to the understanding of risk factors for leptospirosis infection in other occupational groups in New Zealand. Three chapters (Chapter 4, 5, and 8) are presented in the format of manuscripts for peer-reviewed publication. As a consequence of this style of thesis presentation, the reference styles of these chapters are different, as required by the journals. Also, there are some similarities in Chapters 4 and 5, because they are companion papers. For example, some background description and information about correlation between MAT and qPCR on urine and kidney samples were repeated in these two chapters. In Chapter 8, the process of publication required a substantial amount of distilling of material. This chapter includes some additional tables and text that do not appear in the published version.

The second chapter of the thesis is a literature review of laboratory diagnostic tests for leptospirosis, focussed primarily on the standard diagnostic tests (culture and MAT), ELISA, and PCR assays that have been increasingly used, with other available tests briefly described.

Chapter 3 evaluates leptospiral diagnostic tests at various known times post-infection, by taking sequential samples from *Leptospira* challenged sheep and cattle, and testing them by MAT, culture, qPCR and dark-field microscopy.

Chapter 4 describes a cross-sectional study of 399 sheep and 146 cattle slaughtered at a New Zealand abattoir from September to November 2010, aiming to investigate the supplier-specific shedding rate, renal colonisation rate, and seroprevalence of leptospires.

Chapter 5 used blood, urine and kidney samples from sheep and beef cattle described in Chapter 4 to investigate the inter-laboratory (between a research (HLRL) and a commercial veterinary diagnostic laboratory (GV)) and between-specimen (HLRL) test agreements on leptospirosis diagnosis.

In Chapter 6, *Leptospira* isolates from 18 sheep and five cattle kidney cultures described in Chapter 5 were used as a pilot panel to test the utility of a MLST scheme for genotyping *Leptospira spp.* field isolates in New Zealand.

In Chapter 7, suspect leptospirosis patients were recruited from August 2011 to December 2012 within the Waikato District Health Board area, with blood culture, MAT, and whole blood/serum PCRs (by three laboratories) performed to establish the best diagnostic test or combination of tests for the early diagnosis of human leptospirosis.

In Chapter 8, a cross-sectional study was conducted from September 2010 to November 2011 to determine the seroprevalence and quantify putative risk factors for both intra- and extra-curricular exposure to leptospirosis among undergraduate veterinary students at Massey University, New Zealand.

The thesis concludes with a general discussion (Chapter 9) of the study findings in context, a reflective critique of study design and methodology, and suggestions for future work.

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Chapter 2 Literature Review: Laboratory Diagnosis of Leptospirosis

Reviews about various aspects about leptospirosis, including biology, epidemiology, pathogenesis, clinical features, and prevention and treatment have been generally covered in published papers (Levett 2001; Bharti *et al.* 2003; Adler and de la Peña Moctezuma 2010), and also in recent PhD thesis about leptospirosis at Massey University (Ayanegui-Alcerreca 2006; Subharat 2010; Dreyfus 2013). However, literature reviews focusing on laboratory diagnosis of leptospirosis are limited, except a brief review by O'Keefe (2002). New techniques have been introduced in the last decade, and the use of polymerase chain reaction (PCR) has increased substantially for leptospirosis diagnosis since the review by (O'Keefe 2002). An updated review of laboratory diagnosis has recently been published (Musso and La Scola 2013), but again, that review covers each category of diagnostic test only briefly.

Considering this thesis is predominantly about a range of aspects of laboratory diagnosis of leptospirosis, both in animals and humans, this literature review is confined to this area. In general, reviews of each category of diagnostic test are presented to fit the circumstances of this thesis for both humans and animals, with particular aspects differentiated.

1 Introduction

As a result of the variety of clinical and often "flu like" symptoms, human leptospirosis is often undiagnosed or misdiagnosed as other illnesses with febrile syndromes (e.g. aseptic meningitis, influenza, hepatic disease, Hantavirus infections) (Plank and Dean 2000; Levett 2001). This is also the case in domestic animals, where most cases are difficult to diagnose clinically, due to non-specific clinical presentation or inapparent clinical signs with host-adapted serovars (Adler and de la Peña Moctezuma 2010; Hartskeerl *et al.* 2011). Therefore, diagnosis of leptospirosis in both humans and animals cannot be made with confidence without laboratory confirmation (Faine 1982; O'Keefe 2002).

Leptospirosis is characteristically a biphasic disease, with the acute phase lasting about four to seven days as described in Figure 2.1 (Faine *et al.* 1999; Levett 2001). During

this stage, leptospiraemia occurs, and leptospires can be present in high numbers in multiple body fluids including blood and cerebrospinal fluid (CSF) (Levett 2001; Segura *et al.* 2005). The immune (convalescent) phase usually occurs during the second week after onset of symptoms, and is characterized by excretion of leptospires in the urine and appearance of antibodies in the serum (Faine *et al.* 1999; Levett 2001). Antibodies generally reach maximum levels within two to three weeks then gradually recede (Faine *et al.* 1999; Levett 2001), but may remain detectable for two to ten years in humans, and for similar periods or for lifetime at low levels (particularly in reservoir hosts) in animals (Blackmore *et al.* 1984; Everard and Bennett 1990; Faine *et al.* 1999). The duration of human leptospiruria varies, but may last days or several weeks (Faine *et al.* 1999; Levett 2001). In animals, the urinary excretion period may be prolonged (months, up to a year or more), especially for renal carriers (Faine 1982). For example, heifers naturally infected with serovar Hardjo were reported to shed the organism from six to nine months (Leonard *et al.* 1993); while in a longitudinal study of leptospira in New Zealand deer that were naturally infected, shedding of serovar Hardjo was reported to last at least eight months (Ayanegui-Alcerreca 2006).

The classifications of laboratory diagnosis for leptospirosis will be covered in this review: direct examination of clinical specimens for organisms; culture of leptospires from clinical specimens; detection of leptospiral antibodies; and detecting the presence of leptospiral DNA (Faine *et al.* 1999; Bharti *et al.* 2003). The tests available have different capacity for diagnosis and this is depending on the type of specimen that is available, the course of the illness, and the purpose for testing (O'Keefe 2002; Jouglard *et al.* 2006). The questions “What is the aim of diagnosis?; what kind of specimen?; what test?; and when to test?” were regarded as important for consideration before selecting a particular test for diagnosis (Faine *et al.* 1999; Picardeau 2013), and these will be addressed in the literature synthesis for each category of diagnostic tests.

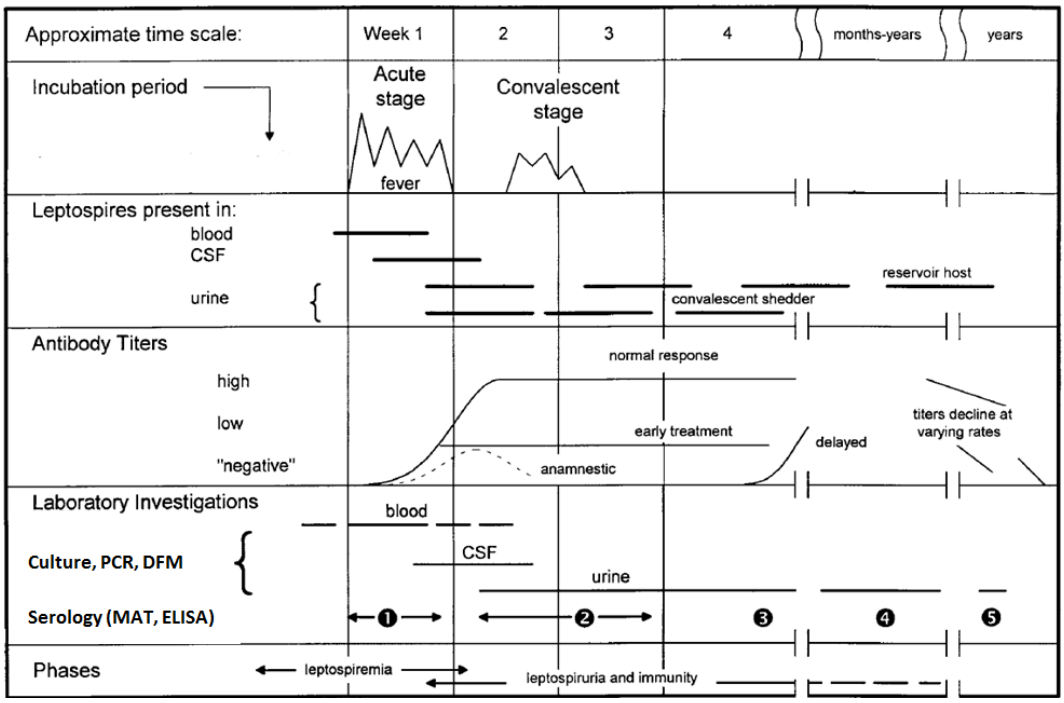

Figure 2.1 Schematic representation of the biphasic nature of leptospirosis and relevant diagnostic investigations at different stages of disease. For serology specimens 1 and 2 are acute-phase specimens, 3 is a convalescent-phase specimen which may facilitate detection of a delayed immune response, 4 and 5 are follow-up specimens which can provide epidemiological information, such as the presumptive infecting serogroup. This figure was adapted with permission from (Levett 2001).

2 Direct examination for leptospires and antigen detection

2.1 Dark field microscopic examination

Dark field microscopic examination (DFM) of body fluids such as blood, urine, CSF, and dialysate fluid can be used to rapidly detect the presence of leptospires (Levett 2001; O'Keefe 2002). This technique can also be applied on tissues removed for surgical or experimental reasons from animals, or necropsy specimens, tissues from carcasses or abortion products (Faine *et al.* 1999). Although DFM is useful in situations where laboratory resources are limited (O'Keefe 2002), this technique has low sensitivity and specificity (Levett 2001). Approximately 10^4 leptospires/ml is the detection threshold for one cell per field to be visible (Turner 1970). The risk of false positives due to misinterpretation of fibrin or protein threads, cell debris and other artefacts can be high, even for experts (Faine *et al.* 1999; Vijayachari *et al.* 2001). DFM also suffers from other disadvantages, such as technical difficulties with obtaining suitable specimens (specimens should be taken aseptically and sent to a laboratory

without delay), the requirement of a high level of operator skill, and this method provides no information of infecting serovars (Faine *et al.* 1999; Bharti *et al.* 2003).

A variety of staining methods have been used to increase the sensitivity of direct microscopic examination of leptospires in veterinary specimens, including immunofluorescence staining of bovine urine (Hodges and Ekdahl 1973; Bolin *et al.* 1989b) and bovine and porcine kidney (Skilbeck 1986), immunoperoxidase staining of bovine blood and urine (Terpstra *et al.* 1983) and bovine kidney (Yener and Keles 2001), silver staining of possum and bovine kidneys (Skilbeck and Chappel 1987), and Warthin-Starry staining of porcine kidney (Elliott 1988).

2.2 Detection of leptospiral antigen

Monoclonal antibody (mAb) based methods have been developed to detect leptospiral antigen in clinical specimens from animals and humans, and although not in general use, they have potential for rapid diagnosis of leptospirosis at a low cost. An antigen-capture detection system based on the use of mAb coated on magnetic beads was developed for detecting leptospiral antigen in bovine urine (Yan *et al.* 1998). The system was reported to be rapid and to detect as few as 10^2 to 10^3 leptospires/ml of urine from Hardjobovis-positive cattle. Saengjaruk *et al.* produced a mAb (clone LD5) which reacted to the 35 kDa components of all serogroups of the pathogenic species of *Leptospira* and was used in the development of a dot blot–enzyme-linked immunosorbent assay (dot-ELISA) for detecting *Leptospira* antigen in urine samples (Saengjaruk *et al.* 2002). The assay was tested on urine samples collected from 36 clinically diagnosed leptospirosis patients serially on days 2, 3, 7, and 14, and reported cumulative positivity as 88.9%, 97.2%, 97.2%, and 100%, respectively (Saengjaruk *et al.* 2002). The assay developed by Saengjaruk *et al.* (2002) was evaluated later with urine samples from cattle which were predominantly infected with *Leptospira* serovars Hardjobovis, Ranarum, and Sejroe, and reported the detection limit as 10^3 leptospires/ml of bovine urine (Suwimonteerabutr *et al.* 2005). More recently, using sandwich dot-ELISA with mAbs that reacted to four *Leptospira* serovars Autumnalis, Australis, Grippotyphosa and Icterohaemorrhagiae, leptospiral antigens were detected in plasma from experimentally infected mice and guinea pigs (Sharma *et al.* 2008).

3 Culture of leptospires from clinical specimens

Detection of leptospires by culture is considered as the definitive diagnosis of leptospirosis (Adler and de la Peña Moctezuma 2010). During the first week of illness, leptospires can be isolated from blood, CSF and dialysate fluid (Ahmad *et al.* 2005). At this early stage of infection, urine is unlikely to contain many leptospires (Faine *et al.* 1999); it can be more successfully cultured from the beginning of the second week of symptoms. The highest success rate of culturing from urine is 14-28 days after infection when significant leptospiruria is seen (Faine *et al.* 1999; Bharti *et al.* 2003). Given the optimum growth pH for leptospires is 6.8-7.4, the organisms die or lyse rapidly in acid urine (Faine *et al.* 1999; Bharti *et al.* 2003). Therefore, voided human urine and acidic animal urine should be processed immediately by centrifugation and the sediment resuspended with phosphate-buffered saline afterwards to neutralize the pH (Levett 2001). In addition, urine can be made neutral by drinking alkaline water before sampling (Faine 1982). For fatal cases in humans and animals, leptospires can be cultured from tissue specimens at post-mortem. The most commonly used tissues are kidneys, liver and brain (Faine 1982) and the specimens should not be frozen before culture (Bharti *et al.* 2003). Leptospires may also be cultured from aborted or stillborn animal foetuses, placentas and abortion products (Faine *et al.* 1999).

A commonly used medium for culture is Ellinghausen- McCullough-Johnson-Harris (EMJH) medium, which contains 1% bovine serum albumin and polysorbate 80 (source of long-chain fatty acids) (Ellinghausen and McCullough 1965). Antibiotics 5-fluorouracil, gentamicin, nalidixic acid or rifampicin need to be added for culture of clinical specimens to inhibit contamination (Faine *et al.* 1999; Levett 2001). The preparation of the culture medium is expensive and technically demanding, thus the medium is stocked in few laboratories (Bharti *et al.* 2003). Due to the requirement of prolonged incubation and special media, culture is not often performed as a routine diagnostic method (Levett and Branch 2002; Villumsen *et al.* 2012). However, it is widely used in research (Faine *et al.* 1999; Bharti *et al.* 2003). Cultures need to be incubated at 28 to 30°C and examined weekly under dark-field microscopy. Even if optimal conditions are provided, the organisms may grow slowly and the cultures need to be examined for up to 13 weeks before being regarded as negative for growth (Levett 2001; Bharti *et al.* 2003). Therefore, this technique provides retrospective diagnostic confirmation and does not contribute to immediate disease management (Faine 1982).

Culture of leptospires from human and animal specimens may be influenced by many factors including the sample type and the condition of sample taken (Faine *et al.* 1999), survival of the organism in the collected primary tissue or body fluid sample, host immune system responses (Smythe *et al.* 2002), exposure of the host to administered antibiotics (antibiotic administration can eliminate leptospires from blood rapidly and stop urinary shedding) (Gerritsen *et al.* 1993; Picardeau 2013), and the fastidious nature of some leptospires (e.g. *L. borgpetersenii* serovar Hardjobovis is relatively difficult to culture) (Cousins *et al.* 1985). Although it is considered that during the first few days (especially during fever), cultures taken in appropriate conditions can frequently be positive (Faine 1982), the reported sensitivity of blood culture from humans varied from 14% to 90% (McClain *et al.* 1984; Sasaki *et al.* 1993; Truccolo *et al.* 2001). Further, the sensitivity of culture from aqueous humour and CSF was as low as 3% in one study (Brown *et al.* 2003). Culture of leptospires from urine may be poorly sensitive due to intermittent shedding of bacteria in both human and animal cases, problems with contamination, and the acidic urine environment (Plank and Dean 2000; Magajevski *et al.* 2005; Fearnley *et al.* 2008). In a French study, among eight human cases confirmed by sero-conversion, three (38%) had leptospires isolated from urine (Merien *et al.* 1995). Similarly, a Barbados study that tested urine samples from 20 patients admitted to the hospital with history and clinical manifestations of leptospirosis and diagnosed serologically, seven (35%) were culture positive (Brown *et al.* 1995). In a study that compared different tests for detecting *L. borgpetersenii* serovar Hardjobovis in bovine urine, 13 urine samples (17%) tested positive by culture from 75 samples collected from 18 cows (sampling schedule was not detailed) that were conjunctively challenged (Bolin *et al.* 1989b). As leptospires usually persist and multiply in the kidney of maintenance and accidental hosts (Guerra 2009), culture of *Leptospira* organisms from kidney can provide definitive diagnosis of carrier status of the hosts. However, low success rate of culturing leptospires from kidneys has been reported in animals, for example, sheep and pigs (Fearnley *et al.* 2008; Fornazari *et al.* 2012). It is considered that the post-mortem changes, which can reduce the number of viable bacteria in organs, may have an impact on the sensitivity of this assay (World Health Organization 2003).

Although culture is labour intensive and may be poorly sensitive in clinical settings, leptospiral isolates obtained by culture can be identified to serovar level and contribute

to the study of outbreaks and global epidemiology (Wuthiekanun *et al.* 2007). Traditional methods of isolate identification relied on the cumbersome and time-consuming cross-agglutination absorption test, which is performed in a very small number of laboratories (Cerqueira and Picardeau 2009). The characterization of leptospiral isolates has evolved from serological methods to the more reliable and robust genotypic methods, including restriction endonucleases analysis (Thiermann *et al.* 1986), restriction fragment length polylmorphism (Zuerner *et al.* 1993), pulsed field gel electrophoresis (Herrmann *et al.* 1992), variable number of tandem repeats analysis (Majed *et al.* 2005), fluorescent amplified fragment length polymorphism (Vijayachari *et al.* 2004), and more recently, multi-locus sequence typing (Ahmed *et al.* 2006; Boonsilp *et al.* 2013).

4 Serological tests

Serology is the most frequently used diagnostic approach for leptospirosis (Toyokawa *et al.* 2011). The serological tests available are mainly based on detection of immunoglobulin (Ig) M and IgG class antibodies. The response patterns of IgM and IgG class antibodies found in human and animals cases are similar (Adler *et al.* 1980). IgM antibodies appear first (as early as second day of onset of symptom) during the infection, followed by IgG class antibodies (Figure 2.2) (Chernukha *et al.* 1976; Adler *et al.* 1982; Silva *et al.* 1995). Both IgM and IgG antibodies commonly persist after infection, but the persistence of IgM antibodies is generally shorter than IgG antibodies (Cousins *et al.* 1985; Terpstra *et al.* 1985; da Silva *et al.* 1997; Cumberland *et al.* 2001). Therefore, tests for detection of IgM antibodies can identify infections at earlier stage than those for detection of IgG antibodies. Tests for detection of IgG antibodies are more appropriate for detecting residual antibodies from past infections.

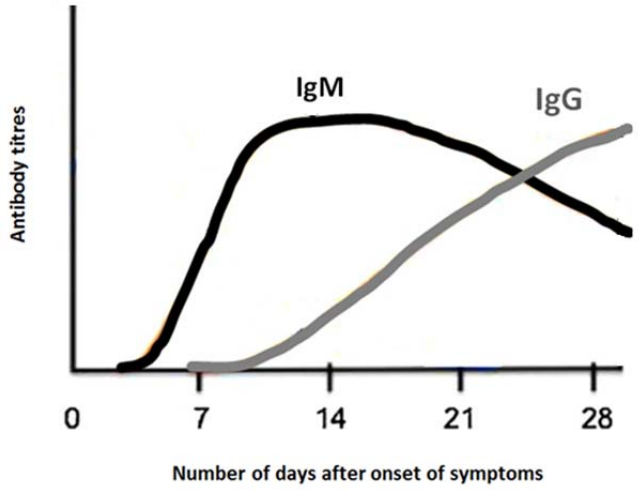

Figure 2.2 Response patterns of anti-leptospiral IgM and IgG class antibodies in leptospirosis infection. This figure was adapted with permission from (Picardeau 2013).

4.1 The microscopic agglutination test (MAT)

The MAT remains the reference method for serological diagnosis of leptospirosis for both humans and animals today (Subharat *et al.* 2011; Picardeau 2013). During this procedure, sera are titrated with live antigen suspensions of leptospiral serovars and inspected for agglutination with dark-field microscopy (Faine 1982). Approximately 7 to 10 days after the onset of symptoms, antibodies can be detected by the MAT (Dutta and Christopher 2005).

For both human and animal cases, the standard criterion for confirming clinical leptospirosis cases by the MAT is sero-conversion or a fourfold or more increase in antibody titre between paired sera (Faine *et al.* 1999; Levett 2001; Bharti *et al.* 2003). The required interval between paired sera to detect rising titres depends on the delay between onset of symptoms and presentation of the individual. If the first serum is collected while the symptoms of overt leptospirosis are present, an interval of three to five days may be adequate to detect rising titres (Levett 2001). However, an interval of two to three weeks is used more commonly, especially when the date of onset is not known precisely (Faine *et al.* 1999; Levett 2001). As the collection of a second serum sample is often difficult and not guaranteed, a single high MAT titre can be considered diagnostic for humans with acute febrile illness and an appropriate history of animal contacts (Faine *et al.* 1999; Shivakumar and Krishnakumar 2006). The optimal determination of cut-off for this presumptive diagnosis depends on the prevalence of the

disease in the population of interest (Levett 2001). Contemporary global criterion is the use of a single titre ≥ 400 for laboratory confirmation of a current *Leptospira* infection (World Health Organization 2011). For a single sample collected from a clinically normal animal, a titre of ≥ 100 is often considered indicative of infection (O'Keefe 2002). A lower cut-off may need to be used for detecting infections of serovars in maintenance hosts. For example, it was reported in New Zealand and the Netherlands that cattle that are actively infected may have anti-Hardjo titres <100 (Mackintosh *et al.* 1980; Ellis *et al.* 1986). Lower or higher titre cut-points are necessary to define probable cases in non-endemic and endemic areas, respectively (Faine *et al.* 1999).

The sensitivity and specificity of the MAT can be high, reported as 100% and 100%, respectively, for detecting antibodies in convalescent sera (collected 23.3 ± 7.4 days after the onset of illness) from patients that were identified as leptospirosis cases by MAT on paired sera and culture isolation in Brazil (McBride *et al.* 2007). However, in the same study, the sensitivity of MAT on acute-phase sera (collected less than seven days after the onset of illness) was 46% (McBride *et al.* 2007). A similar low sensitivity of MAT on sera collected at the early stage of infections has been reported in other studies. In another Brazilian study, with leptospirosis cases confirmed by a fourfold or greater increase in MAT titres for paired sera, 34% (23/68) were detected positive by MAT on the sera collected within six days after onset of symptoms (Brandão *et al.* 1998). A study including 276 sera from 133 leptospirosis cases (from United States, Thailand, and Palau) submitted to the Centers for Disease Control and Prevention (CDC) between 1992 and 1998 reported sensitivity of MAT as 48.7% for sera collected less than 14 days (median = 6 days) after the onset of symptoms (Bajani *et al.* 2003). A recent study in Thailand estimated that the MAT had a sensitivity of 49.8% for 1652 patients that had suspected leptospirosis and were admitted to hospital within four days of illness between 2000 and 2010 (Limmathurotsakul *et al.* 2012). In both Bajani *et al.* (2003) and Limmathurotsakul *et al.* (2012), the sensitivity of the MAT was estimated by Bayesian latent class analysis, a useful tool for evaluating diagnostic tests where a "gold standard" does not exist (Boelaert *et al.* 1999). The low sensitivity of the MAT on acute-phase sera makes it inappropriate for early diagnosis and management of disease. Paired (acute-phase and convalescent-phase) sera are required to increase the sensitivity of this test and confirm the diagnosis (Cumberland *et al.* 1999; Levett 2001).

As the MAT detects both IgM and IgG antibodies, antibodies can be detected during a prolonged period after recovery (Cumberland *et al.* 1999). Examples can be found in both human and animal studies. However, it needs to be noted that the reported duration of MAT titres may be over-estimated due to re-infection, which is difficult to be controlled in study designs. In a Brazilian study among 70 patients who had convalescent titres detected during different observation periods (from three to 13 months) after leptospirosis infection, two had titres of 800 (against serogroups Pyrogenes and Australis) 13 months after infection (Romero *et al.* 1998). In a study of 69 meat inspectors that had positive MAT titres to leptospiral antigens (predominantly with serovar Pomona, followed by Hardjobovis, Tarassovi, Copenhageni and Ballum) and were re-bled/re-examined over a period of 53 months in New Zealand, some individuals maintained titres of 384 or 192 for at least 30 months, and those with initial low titres (48 and 24) maintained these for 53 months (Blackmore *et al.* 1984). No apparent differences in magnitude and duration of titres were found between different serovars (Blackmore *et al.* 1984). In a cohort study of meat workers from eight abattoirs (Dreyfus 2013), sera were collected twice from 592 participants between 50 and 61 weeks apart, and tested by MAT for Pomona and Hardjobovis. Based on the analysis of the relationship between mean seroprevalence of leptospirosis at first sampling and mean study period incidence, the estimated duration of MAT titres over 48 after infection was 10 months for Pomona and 29 months for Hardjobovis (Dreyfus 2013). In natural infection of animals, the MAT titre declines gradually after reaching maximum, and persists for variable periods (range from months to two years) (Hodges 1979; Mackintosh *et al.* 1980; Worthington 1982). Therefore, the MAT based on a single serum sample is not a reliable technique to differentiate current infection from previous infection in both humans and animals. Paired sera are required to confirm the stage of infection (Faine *et al.* 1999). If the titre remains the same, it suggests previous infection; but if the titre increases, it suggests recent infection.

The interpretation of MAT results can be confounded by the vaccination history of both animals and human (Chapman *et al.* 1990; Grooms and Bolin 2005). This may cause particular problems in animals, for example, in screening animal disease status for import or export purposes (Adler and de la Peña Moctezuma 2010). The influence of vaccination on the persistence of antibodies has been investigated in cattle and dogs. In general, cattle develop relatively low titres after vaccination against serovars

Hardjobovis and/or Pomona (Grooms and Bolin 2005). However, variable MAT titres from undetectable to a maximum of about 3000 had been reported in experimental trials that examined serological responses in cattle after vaccination (Marshall *et al.* 1979; Hodges and Day 1987). The vaccine induced antibodies in cattle usually decrease rapidly and persist no longer than six months after vaccination (Marshall *et al.* 1979; Allen *et al.* 1982; Hodges and Day 1987). The number of doses of vaccine given and the age of cattle are possible reasons for the variance in the magnitude and persistence of MAT titres after vaccination (Hodges and Day 1987; Smith *et al.* 1994). Vaccination of dogs against leptospirosis infection (mainly containing serovars Icterohaemorrhagiae or Canicola and Copenhageni) is widely practised (Langston and Heuter 2003). In general, dogs develop low antibody titres (100 to 400) in response to vaccination. However, titres up to 3200 have been observed in the early post-vaccination period in dogs vaccinated against these serovars (van de Maele *et al.* 2008). The vaccination-induced titres typically persist for less than three months, but may last for six months or more (Bolin 1996). Agglutinating antibody titres detected after vaccination are usually lower and persist for a shorter time than those detected after natural infections (Marshall *et al.* 1979; Kingscote and Proulx 1986; Hodges and Day 1987; Miller *et al.* 2011). Therefore, the magnitude of the titre and information on the interval since vaccination can, in some instances assist differentiating MAT titres due to natural infection from those due to vaccination (Smith *et al.* 1994).

For animals, diagnostic testing for leptospirosis is not only about detecting infection in animals with clinical disease, but also identification of non-clinical *Leptospira* spp. carriers. Identifying animals that develop chronic renal infection, excrete leptospires in the urine intermittently, and disseminate the organism to other animal species and humans is important for setting up management strategies to prevent the spread of leptospirosis infection (Faine *et al.* 1999; Magajevski *et al.* 2005). Although the MAT is commonly used for leptospirosis diagnosis in animals, this test detects antibodies in serum, rather than the presence of leptospires directly in urine and kidney, and therefore does not reflect the carrier status of the host (Cole *et al.* 1973; Thiermann 1984). The correlation between serological status of an animal and its carrier status is reported to be poor (Ellis *et al.* 1981). Leptospires have been recovered from seronegative carriers by urine or kidney culture (Hanson 1977; Ellis *et al.* 1981; Thiermann 1984). In a sheep abattoir study in New Zealand, leptospires were isolated from kidneys of 1% (5/499)

Hardjobovis and Pomona-negative sheep (Dorjee *et al.* 2008). In a deer abattoir study in New Zealand, among 19 deer that had leptospiral DNA detected in kidney samples by PCR, 15% were seronegative against serovar Hardjobovis and Pomona (Subharat *et al.* 2011). On the other hand, the detection rate of leptospires in urine or kidney from seropositive animals was also reported to be low. A Brazilian study reported that among 40 sheep with anti-*Leptospira* titres (predominately represented by serovar Hardjo), six (15%) tested positive by urine PCR (Lilenbaum *et al.* 2009). In the sheep abattoir study of Dorjee *et al.* 2008, leptospires were isolated from kidneys of 21% Hardjobovis or Pomona-seropositive sheep (Dorjee *et al.* 2008).

The MAT can provide a general impression of which serogroups/serovars are circulating within a population, and is considered as the most appropriate test to employ in epidemiological serosurveys (Levett 2001). The titre cut-off for determining exposure to leptospires is different from that determining clinical disease. It is suggested that a titre cut-off of 50 should be used to indicate exposure to *Leptospira spp.* for both human and animals (Blackmore *et al.* 1982; Faine *et al.* 1999; Shivakumar and Krishnakumar 2006). When applied to early infections, the high degree of cross-reaction that occurs between serovars from the same or different serogroups prevents MAT from being a reliable test to predict the infecting serogroup/serovar (Levett 2003; Grooms and Bolin 2005; Miller *et al.* 2011). Whether the MAT can provide an accurate guide to the infecting serogroup/serovars of *Leptospira* also depends on the adequate coverage of the locally circulating serovars in the battery of test antigens (Katz *et al.* 2003). Diagnostic laboratories commonly use a minimum of six and up to 15 serovars that are representative of the locally enzootic and endemic serogroups (Faine *et al.* 1999). However, a larger battery of serovars may need to be tested to ensure that the infecting serovar is included, and the battery may need to be reviewed regularly for possible emergence of new serovars. For example, in 2006, five serum samples from each of 70 deer farms were forwarded to the World Health Organisation/Food and Agriculture Organisation/World Organisation for Animal Health reference laboratory in Brisbane, Australia and tested by MAT for 23 reference panel serovars (Subharat 2010). Serological evidence of serovar Arborea, a serovar novel to New Zealand, was found in 14 samples from 11 farms (Subharat 2010). However, an attempt to confirm the serovar by culture was unsuccessful. Another example can be taken from a cross-sectional seroprevalence study of leptospirosis in American Samoa, which selected a MAT panel

of 23 pathogenic serovars, based on the known epidemiology of leptospiral serovars in the Pacific region (Lau *et al.* 2012a). Results from this study suggested emergence of three predominant serovars (possibly as result of ecological change) that were not identified in a previous CDC study in 2004, and were not previously known to occur in American Samoa (Lau *et al.* 2012b). In areas of high endemicity, the possibility of co-infection with multiple serovars is an additional confounding factor for interpreting the MAT results (Turner 1968). In a prospective study that identified 78 patients from Thailand with culture-proven leptospirosis and with diagnostic MAT titres, the MAT correctly determined the infecting serovar in 26 cases (33%) (Smythe *et al.* 2009). In another study that included 151 culture-proven leptospirosis cases in Barbados, the sensitivity and specificity of MAT for prediction of infecting serovar was 44.4% and 64.8%, respectively (Levett 2003). These indicated that MAT may be a poor predictor of infecting serovar in individual cases, especially in areas which have high endemicity of a large number of circulating serovars.

In addition to the issues mentioned above which need to be considered when using MAT for diagnosis, there are other drawbacks of MAT. The performance of MAT requires maintaining a collection of living strains (O'Keefe 2002). The repeated subculture of a large number of strains presents hazards for laboratory staff (Ahmad *et al.* 2005). Other potential problems associated with the maintenance of antigen cultures are strain contamination and mislabelling or switching of strains. However, these problems are more likely to occur in laboratories for which the capacity for quality control and long-term storage of culture collections is limited (Cerqueira *et al.* 2010b). In addition, inter-laboratory variation of MAT results is commonly found, due to the subjective effect of observer variation (Levett 2001; Chappel *et al.* 2004). Finally, early antibiotic treatment given in the first days after onset of symptoms may eliminate leptospires rapidly and decrease the level of antibody response in the host and hence that detected by the MAT later (Faine *et al.* 1999).

4.2 Enzyme-linked immunosorbent assay (ELISA)

The complexity of performing the MAT has led to the development of tests that provide rapid results without the need for culture or MAT facilities (Bharti *et al.* 2003). Enzyme-linked immunosorbent assay (ELISA) is often used as an alternative to MAT

for screening for leptospiral infection in both human and animals (Winslow *et al.* 1997; Aslantaş 2005). As well as being easier to perform, ELISA is inexpensive, safer (as it uses killed antigen and therefore reduces the risk of infection for laboratory personnel), and gives a less subjective result than MAT (Brandão *et al.* 1998; Sakhaee *et al.* 2007).

Another advantage of ELISA over MAT is the serological response of IgM and IgG can be detected separately. The earliest time post-infection that antibody maybe detected by ELISA is affected by which class of antibody the ELISA is testing (O'Keefe 2002). In a study of specific IgM and IgG class antibody response in human leptospirosis, IgM antibodies were detected from the second day of symptoms, and in all patients, versus IgG antibodies which were first detected on the seventh day of symptoms and in 87.5% of patients (Silva *et al.* 1995). In another study that tested serum collected from patients at the acute phase infection (mean was 7 days after the onset of symptoms), IgM antibodies were detected in 98% of the patients, while IgG were detected in 70% of them (da Silva *et al.* 1997). Cousins *et al.* reported that in 18 experimentally-infected cattle, IgM antibody levels increased from the first week after infection and became negative within three to five weeks. IgG antibody was detected about the same time as IgM, but persisted for much longer (Cousins *et al.* 1985). As IgM antibodies were detected earlier in the acute phase of infection and persisted for shorter periods than IgG, the IgM ELISA is deemed as a more suitable method for detecting acute infection in humans and animals and is more commonly used (Bharti *et al.* 2003).

Using human sera collected during the acute phase of infection, IgM ELISA was reported to be more sensitive than the MAT by several studies (Cumberland *et al.* 1999; Bajani *et al.* 2003; Limmathurotsakul *et al.* 2012). In a study in Australia, serial serum samples (n=117) were collected after the onset of symptoms from 41 cases that were diagnosed with MAT and/or blood culture. For 29% of these cases, a positive ELISA ratio was detected in early stage of infection (range from 4 to 9, or not specified days after the onset of symptoms), before MAT titres reached 50 (Winslow *et al.* 1997). The sensitivity of the ELISA assay for acute-phase sera is important for patient management, because early antibiotic treatment has been shown to be efficient for preventing leptospiruria and shortening the duration of clinical illness (McClain *et al.* 1984; Edwards *et al.* 1988; Guidugli *et al.* 2000; Katz *et al.* 2001).

The reported sensitivity and specificity estimates for ELISA are quite variable, as demonstrated by the performance of a commercial IgM ELISA (Panbio's IgM ELISA). This ELISA has been recommended by the World Health Organization as a diagnostic test for leptospirosis where healthcare resources are limited (World Health Organization 2003). The evaluation of this test in different countries found variable sensitivity and specificity as 100% and 93% in Barbados (Winslow *et al.* 1997), 90% and 94% in UK (Zochowski *et al.* 2001), 87% and 97% in USA (Bajani *et al.* 2003), 76% and 82% in Thailand (Desakorn *et al.* 2012), and 36% and 98% in Hawaii (Effler *et al.* 2002). This variation in performance across geographical regions indicates that ELISA assays need to be validated for distinct epidemiological situations including differences in the studied population, the prevalence of various different infecting serogroups, and also the definition of confirmed cases (predominately defined by MAT and/or isolation of *Leptospira* spp.) (Effler *et al.* 2002; Picardeau 2013).

The IgM ELISA is less specific than MAT test, hindering its use as a single test of diagnosis of leptospirosis (de Abreu Fonseca *et al.* 2006). A limitation to the use of single serum samples for IgM ELISA test is the persistence of IgM antibodies. IgM was reported to be detectable for several months or even years after infection in human cases (da Silva *et al.* 1997; Cumberland *et al.* 2001). However, the range of IgM levels were reported to decline after 2 months, and to be lower for persistent cases than recently infected cases (Winslow *et al.* 1997; Brandão *et al.* 1998). Compared with the MAT, false positive reactions detected in animals due to vaccination by IgM ELISA may happen with higher frequency. It is reported that significant levels of antibody can be detected by ELISA after a single dose of vaccine in cattle, compared with low MAT titres to serovar Hardjo detected after two doses of vaccine (Tripathy *et al.* 1976; Bolin *et al.* 1989a). In dogs following vaccination with serovars Canicola and Icterohaemorrhagiae, a rise in IgM was detected using the ELISA, and persisted for up to one year, compared with negative or transiently positive titres detected by the MAT (Hartman *et al.* 1984).

Reactivity to the ELISA in various non-leptospiral infections has been documented (Cinco *et al.* 1992; Winslow *et al.* 1997; Bajani *et al.* 2003). However, levels of IgM were lower in patients with non-leptospiral infection than those with leptospiral infection (Winslow *et al.* 1997). Due to the lower specificity of ELISA compared with the MAT, a positive result from a single sample can only be considered as presumptive

evidence of infection. Subsequent confirmation of a positive test is required by testing a convalescent sample with an alternative method, preferably MAT (Winslow *et al.* 1997; Cumberland *et al.* 1999).

For human cases, since the most important goal is to confirm clinical cases and initiate patient management, broadly reactive assays to a wide range of leptospiral serovars are generally desirable. Therefore, few serovar-specific assays have been developed (Milner *et al.* 1985). However, identifying the infecting serovar in diagnosis of human cases is also very important, as it can inform control measures related to the animal reservoirs. Detection of serovar-specific IgM antibodies has been developed for the veterinary field, aiming to understand the serovar-host relationship, and in turn contributed to the vaccination strategies. ELISA methods for detection of serovar Pomona (Surujballi and Elmgren 2000) and Hardjo (Adler *et al.* 1982; Surujballi *et al.* 1997; Yan *et al.* 1999) infection in cattle and Hardjo in sheep (Adler *et al.* 1981) have been developed.

Most of the ELISA assays use whole-cell lysates, usually the saprophytic strain *L.biflexa* serovar Patoc as the antigen, which shares several surface antigens with pathogenic strains (Picardeau 2013). This resulted in low specificity as reported for whole-cell *Leptospira* assays in some studies (Vijayachari *et al.* 2002; Blacksell *et al.* 2006). Recently, recombinant lipoprotein-based ELISA tests have been accessed, with improved specificity and reproducibility (Adler and de la Peña Moctezuma 2010). Recombinant cell-surface lipoprotein antigen lipL32 has proved to be a useful antigen for the ELISA test in humans, cattle and dogs (Flannery *et al.* 2001; Dey *et al.* 2004; Bomfim *et al.* 2005). In a study that evaluated the use of *Leptospira* immunoglobulin (Ig)-like (Lig) proteins as serodiagnostic markers for acute-phase leptospirosis, anti-LigB antibodies were found in sera from 57% of the patients who did not have detectable anti-whole-*Leptospira* response as detected by IgM ELISA. Specificity of single samples has also been improved, and reported as 93% to 100% (Croda *et al.* 2007). Researchers also attempted to use the conserved regions of the Lig proteins A and B as serodiagnostic markers to differentiate between vaccinated and naturally infected animals (Palaniappan *et al.* 2004).

4.3 Other serological methods

Several other serological tests have been used as screening tests for antibodies, including macro-agglutination (Arimitsu *et al.* 1994), complement fixation reaction

(Terzin 1956), indirect immunofluorescence (Appassakij *et al.* 1995), indirect hemagglutination assay (Sulzer *et al.* 1975), and latex agglutination (Ramadass *et al.* 1999). Nevertheless, these tests are rarely used due to their lack of sensitivity or specificity (Picardeau 2013).

A lepto dipstick test has been developed as a rapid field test for leptospirosis that does not require special laboratory equipment and trained personnel (Smits *et al.* 1999). The presence of *Leptospira*-specific IgM antibodies in the serum sample was tested by reading the staining of the antigen band. The strength of the staining is determined by comparing to the reference strip, scored from 0 to 4 (Plank and Dean 2000). The assay was evaluated for detecting *Leptospira*-specific IgM antibodies in human sera from a large number of countries. The sensitivity reported ranged from 35% to 86%, while specificity was from 88% to 99% on acute phase sera (Gussenhoven *et al.* 1997; Sehgal *et al.* 1999; Smits *et al.* 1999). Given the ease of use and good negative predictive value (Smits *et al.* 1999), this assay can be used as a quick screening for leptospirosis, especially where laboratory resources are limited. However, considering the variability of this assay's sensitivity, a second serum sample should be tested to rule out the suspicion of leptospirosis, when the first sample is negative.

The lateral flow assay is another "bedside" test that has been developed for leptospirosis and is commercially available. The assay uses stabilized components and is simply performed by adding serum and sample fluid to the sample well of the assay device. The assay is read after 10 minutes and a positive result is obtained when staining of the test line is observed. Evaluation of the test was performed in different studies and revealed sensitivity from 52.9% to 85.8% and specificity from 89.4% to 93.6%; while good correlation with ELISA was obtained (Smits *et al.* 2001; Eapen *et al.* 2002; Sehgal *et al.* 2003). It is recommended that the assay results are interpreted in conjunction with clinical findings, and are followed up with a second serum sample tested at later stage of infection.

5 Molecular methods

5.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) assay has been used since the 1990s for detecting leptospiral DNA (Adler and de la Peña Moctezuma 2010). The PCR-based assays use

specific primers to amplify a target DNA fragment of pathogenic leptospiral strains. There are two sets of primers that have been subjected to extensive clinical evaluation and gained widespread use for diagnosis. The primer from the *L. interrogans* serovar Canicola *rrs* (16S) was developed by Merien *et al.* (1992) and claimed to detect as few as 10 leptopires/ml in urine, CSF and blood when combined with the DNA hybridization technique (Mérien *et al.* 1992). However, this assay amplified DNA from both pathogenic and non-pathogenic serovars. The second mixed primer sets G1/G2 (*L. interrogans* serovar Icterohaemorrhagiae-derived) and B64-I/ B64-II (*L. kirschneri* serovar Bim-derived) are used in the approach described by Gravekamp *et al.* (1993) to detect all seven pathogenic species and differentiate them from non-pathogenic species (Gravekamp *et al.* 1993).

A number of PCR assays based on these two sets of primers have been applied to detect *Leptospira* spp. from both human and animals in different clinical specimens including serum (Merien *et al.* 1995), urine (Bal *et al.* 1994; Brown *et al.* 1995), and aqueous humour from humans (Merien *et al.* 1993; Chu *et al.* 1998); bovine urine (Wagenaar *et al.* 1994; Magajevski *et al.* 2005; Bomfim and Koury 2006), and urine/vaginal fluids/semen from goats and sheep (Lilenbaum *et al.* 2008; Lilenbaum *et al.* 2009). Besides these two widely used primers, assays with primers targeted at other genes were also developed, including *flaB* (Kawabata *et al.* 2001), *ompL1* (Heinemann *et al.* 1999; Reitstetter 2006), *LipL32* (Jouglard *et al.* 2006; Bomfim *et al.* 2008), and 23s rDNA (Harkin *et al.* 2003).

Compared with culture, PCR assays are reported to be more sensitive for detecting leptospires in clinical materials from both humans and animals (Mérien *et al.* 1992; Brown *et al.* 1995; Heinemann *et al.* 2000; Wagenaar *et al.* 2000). The high sensitivity of PCR assays may be due to the fact that these assays detect both viable and dead bacteria, while culture requires sufficient numbers of viable bacteria for sample preparation (which is difficult to ensure due to the limited survival time of leptospires), and may be affected by poor sample quality (O'Keefe 2002; Brown *et al.* 2003). The PCR assays also provide a considerable time advantage, compared with the long incubation time for culture and the long reporting times for MAT (involves diagnosis with paired sera) (Bal *et al.* 1994; Ooteman *et al.* 2006).

The use of PCR for early leptospirosis diagnosis, that is before antibodies appear in the blood, can provide a major improvement in disease management, since treatment should be started as soon as possible after disease onset. The PCR assay developed by Merien *et al.* (1995) was capable of detecting leptospires in serum as early as two days after infection in one study, and was recommended as an efficient tool for leptospirosis diagnosis during the first 10 days of disease (Merien *et al.* 1995). A study which compared PCR, MAT and IgM ELISA results on serum samples from clinically suspected patients showed that PCR verified 29% of cases unconfirmed by MAT, while ELISA verified 7% (Ooteman *et al.* 2006). Leptospires were also detected in urine by Merien *et al.* (1995) during the first week of disease. Bal *et al.* (1994) sampled urine from 29 patients with clinical leptospirosis, seven of whom had an onset of disease eight days prior to sampling. All urine samples from the seven patients were tested positive by PCR, and indicated the utility of urine PCR for early diagnosis (Bal *et al.* 1994). Compared with the serological tests (MAT, ELISA) that detect antibodies in serum rather than the presence of leptospires directly, the direct detection of leptospiral DNA by PCR can differentiate current infection from past exposure (Faine *et al.* 1999). For animals, the PCR has additional advantages over serological tests, such as avoiding false-positive serological results due to vaccination induced antibodies, and PCR also informs the carrier status of the host (Çetinkaya *et al.* 2000; Bomfim *et al.* 2008; Lilenbaum *et al.* 2009).

In the last decade, quantitative real-time PCRs (qPCRs) were introduced for diagnosing leptospirosis. This further improved the conventional PCRs (described above) by taking less time and reducing false positive results caused by carry-over contaminations (contamination with products from previous reactions) (Ahmed *et al.* 2009; Picardeau 2013). The two main fluorescence detection formats used in the qPCRs for detection of leptospiral DNA are fluorescent dye SYBR Green I that binds non-specifically to double-stranded DNA (Levett *et al.* 2005; Merien *et al.* 2005; Slack *et al.* 2006) or the fluorescent labelled TaqMan probe that allows detection of a specific sequence of the 16S rRNA or *lipL32* gene of *Leptospira* spp. (Smythe *et al.* 2002; Slack *et al.* 2007; Villumsen *et al.* 2012). A double-stranded DNA intercalating dye from the SYTO family (SYTO9) was also introduced as an alternative to the conventional SYBR Green I, and considered to be far less inhibitory to PCR, easier to use than SYBR Green I and does not appear to selectively detect particular amplicons (Monis *et al.* 2005).

A number of qPCR assays have been developed for detection of leptospires. These assays can be divided into two categories based on the detection of genes that are universally present in bacteria, such as *gyrB* (Slack *et al.* 2006; Subharat *et al.* 2011), *rrs* (16S rRNA gene) (Smythe *et al.* 2002; Slack *et al.* 2007) and *secY* (Ahmed *et al.* 2009); or detection of genes that are restricted to pathogenic *Leptospira* spp. , such as *lipL32* (Levett *et al.* 2005; Stoddard *et al.* 2009; Rojas *et al.* 2010; Villumsen *et al.* 2012), *ligA*, *B* (Palaniappan *et al.* 2005), and the conserved hypothetical protein coding locus LA0322 in *L. interrogans* serovar Lai (Merien *et al.* 2005).

The detection threshold reported from the developed qPCR assays on human specimens ranged from two to 30 genome copies per reaction in blood/serum (Smythe *et al.* 2002; Levett *et al.* 2005; Palaniappan *et al.* 2005; Slack *et al.* 2007; Ahmed *et al.* 2009; Stoddard *et al.* 2009), and approximately 10 genome copies per reaction in urine (Smythe *et al.* 2002; Levett *et al.* 2005). The detection limit was two to 10 genome copies per reaction in deer urine for the SYTO9 qPCR assay (Subharat *et al.* 2011).

Although most of the assays were developed with *Leptospira* reference strains and were not tested on clinical specimens, some assays have been validated for diagnostic use. These include validation on human blood/serum samples (Slack *et al.* 2007; Ahmed *et al.* 2009; Thaipadungpanit *et al.* 2011) and urine samples (Villumsen *et al.* 2012). In animals, qPCR assays have been validated for deer kidney and urine samples (Subharat *et al.* 2011) and canine urine samples (Rojas *et al.* 2010). The diagnostic sensitivity for human serum/blood ranged from 43.0% to 99.5%, specificity ranged from 90% to 100%, using culture and/or MAT results as the gold standard. Villumsen *et al.* (2012) reported the sensitivity was 100% for both *lipL32* and 16S qPCR for detecting leptospires in urine from patients diagnosed by MAT, while the specificity was 100% for *lipL32* qPCR and 91.5% for 16S qPCR (Villumsen *et al.* 2012). The SYTO9 qPCR developed by Subharat *et al.* (2011) had a sensitivity and specificity of 85% and 99.2%, respectively, for deer kidneys, comparing with kidney culture; while the sensitivity and specificity was reported as 96.7% and 100%, respectively, for deer urine, using known inoculated samples (Subharat *et al.* 2011).

Polymerase chain reaction assays can also quantify the bacterial load in biological samples with valuable clinical and research applications. This can facilitate the monitoring of antibiotic treatment, pathogenesis studies, and standardization of assays

between laboratories (Truccolo *et al.* 2001). If a panel of standard DNA concentrations (standard curve) is included in the PCRs, the bacterial load can be obtained (Picardeau 2013). A quantitative microplate PCR assay was developed by Truccolo *et al.* (2001) with 27 biological samples (blood, urine) collected during the acute phase of infection (average from Day 1 to Day 4 after onset of symptoms) from 12 confirmed leptospirosis cases. The clinical evolution of these cases indicated that a density of 10^4 leptospires/ml of blood can be considered as a critical threshold for the vital prognosis of the patients (Truccolo *et al.* 2001). The same protocol was applied to evaluate the efficacy of selected antibiotics during the course of *L. interrogans* serovar Icterohaemorrhagiae infection, using a Syrian hamster model. The potential value of doxycycline for the treatment of leptospirosis cases was demonstrated (Truccolo *et al.* 2002). To establish the level of exposure for seven leptospirosis patients who demonstrated pulmonary symptoms and were from an area in Peru with high endemicity, a quantitative qPCR (based on Smythe *et al.* (2002)) was set up. High levels of leptospiraemia ($\geq 10^4$ leptospires/ml) were found in most fatal cases; one patient, from whom tissue specimens were obtained at autopsy, had $\geq 10^5$ leptospires/g of lung, kidney, and muscle tissue (Segura *et al.* 2005). Despite the cost, this assay has advantages over the microplate assay approach described by Truccolo *et al.* (2001) as it is rapid, sensitive, specific, and also minimises cross-contamination.

A new quantitative qPCR that uses TaqMan primers and an optimized temperature step-down protocol was used to analyse blood/serum samples from 105 patients during a 2008 outbreak of leptospirosis in Sri Lanka (Agampodi *et al.* 2012). The median leptospiral load in serum/blood for clinical manifestations (included renal failure, myocarditis and multi-organ failure) ranged from 1.1×10^4 to 3.6×10^4 leptospires/ml among 58 qPCR positive cases. Although the median leptospiral load was higher in complicated patients than in uncomplicated patients (0.8×10^4 leptospires/ml), the difference was not statistically significant (Agampodi *et al.* 2012).

The most recognised limitation for the PCR-based diagnosis of leptospirosis is the inability of most assays to identify the infecting serovar (Picardeau 2013). Although this is not crucial for individual patient/animal management, the identity of the infecting serovar has important epidemiological and public health value (Levett 2001). Strategies designed to overcome this drawback include restriction endonuclease digestion of PCR products (Savio *et al.* 1994; Brown and Levett 1997), analysing the amplification

products by melting curves (Merien *et al.* 2005), and direct sequencing of amplicons (Slack *et al.* 2006; Perez and Goarant 2010; Cerqueira *et al.* 2010a). Most of these techniques allow identification of the speices. However, a qPCR based on Slack *et al.* (2006) and evaluated with kidney and urine samples from New Zealand farmed deer, was reported to distinguish serovars Hardjobovis and Pomona by sequencing amplicons of *gyrB* gene and matched fully with MAT results (Subharat *et al.* 2011). This indicated that in regions with few pathogenic *Leptospira* species and serovars prevalent, PCR may be appropriate to identify *Leptospira* at serovar level.

Other drawbacks of PCR include the need for special equipment and the relatively high cost of the reagents, that may hinder the use of this technique for large sets of samples and in the areas where laboratory resources are limited (Faine *et al.* 1999). Moreover, DNA extracted from clinical specimens may contain inhibitors to PCR and lead to false-negative results. In blood samples, substances such as urea, creatinine, and haemoglobin derivatives are likely to inhibit DNA amplification (de Abreu Fonseca *et al.* 2006). The chemical components in the blood collection systems may also interfere with PCR. A study comparing the results from a number of standard blood collection systems demonstrated that the collection tubes containing lithium heparin interfered with the PCR (Smythe *et al.* 2002). The use of commercially available DNA extraction kits can provide high DNA yields and reduce inhibition. A study which evaluated two commercial extraction kits (QIAamp DNA Blood Mini Kit and Maxwell® 16 Blood DNA Purification Kit) on spiked whole blood, plasma and serum suggested little inhibition (Bourhy *et al.* 2011). Similarly, in urine samples, components such as haemoglobin, bile salts, and acidic polysaccharides are likely to be inhibitors of *Taq* DNA polymerase, and thus affect the reproducibility and sensitivity of urine PCR. The extreme pH variations, the chelated free magnesium ions produced during DNA extraction, and the phenol and chloroform that are often used for DNA extraction and purification are also considered to be inhibitors (Lucchesi *et al.* 2004). An extra washing step in the extraction procedure for urine samples was proposed to reduce inhibition. Although this approach has the disadvantage of losing or diluting target DNA, in one study on human urine samples it is reported to lead to markedly higher success rates (Ahmed *et al.* 2009). Bovine serum albumin (BSA) is a protein that can absorb residual quantities of PCR inhibitors and increase the stability in solution of *Taq* DNA polymerase. Therefore, adding 0.1% BSA in the PCR reaction mix can be utilised

as a cheap and more time-saving option to reduce inhibition, compared to extra purification steps (Lucchesi *et al.* 2004).

5.2 Isothermal methods

Since the loop mediated isothermal amplification (LAMP) was originally reported by Notomi *et al.* in 2000 (Notomi *et al.* 2000), the technique has been established in recent years as a nucleic acid amplification method that offers simple, rapid, and cost-effective diagnosis for a variety of pathogenic bacteria and viruses (e.g. *Salmonella*, *Escherichia coli,* influenza subtype 1, 3, H5N1) (Hara-Kudo *et al.* 2005; Imai *et al.* 2006; Ito *et al.* 2006; Yano *et al.* 2007). The current focus on LAMP development is the use of this technique in resource-limited laboratories in developing countries, where many fatal tropical diseases are endemic (Mori and Notomi 2009).

In contrast to the conventional and qPCRs that require a thermal cycler, this method simply requires a conventional water bath or heating block to incubate the mixture of samples, primers and DNA polymerase at a constant temperature of 60 to 65 °C (Notomi *et al.* 2000). The reaction results in the accumulation of much higher number of copies of the target (around 10^9) compared to PCR and in less than an hour. Moreover, it has been shown that LAMP exhibits less sensitivity to inhibitory substances present in biological samples than PCR (Kaneko *et al.* 2007). The LAMP requires a set of six primers comprising two outer, two internal and two loop primers that recognise eight distinct regions on the target sequence (Parida *et al.* 2008). Therefore, high specificity can be achieved with good primer designs. The amplified DNA from this method can be detected by simple eye-observation either in the form of visual turbidity (detecting white precipitate) or visual fluorescence (with calcein added before the amplification, or SYBR Green I added in the amplified products) (Parida *et al.* 2008).

Loop mediated isothermal amplification methods targeting genes *lipL41* or *rrs2* have recently been developed for the rapid detection of pathogenic leptospires (Lin *et al.* 2009; Sonthayanon *et al.* 2011; Koizumi *et al.* 2012). The detection limit for these assays ranged from two to 100 genome equivalents per reaction mixture. All of the assays reported high analytical sensitivity and specificity. Koizumi *et al.* (2012) reported that the LAMP assay was more sensitive for testing clinical urine samples

(field rats, farmed pigs and buffaloes) than *flaB* nested PCR (Koizumi *et al.* 2012). Lin *et al.* (2009) reported equal sensitivity and specificity of LAMP to qPCR by testing human leptospiral strains and other clinically encountered bacterial species (e.g. *Escherichia coli*) (Lin *et al.* 2009). However, in a case-control study conducted in Thailand to evaluate the diagnostic accuracy of two LAMP assays with 133 laboratory-confirmed (by isolation of *Leptospira* spp. from blood and/or a positive MAT result) leptospirosis cases and 133 patients who had other febrile illnesses, the diagnostic sensitivity and specificity was reported as 43.6% and 83.5%, respectively, for the *rrs* LAMP, and 37.6% and 90.2%, respectively, for the *lipL41* LAMP on blood samples (Sonthayanon *et al.* 2011). This indicated that further clinical evaluations were recommended to determine the utility of LAMP in routine clinical practice, especially in endemic areas.

6 Conclusion

There are a number of diagnostic tests currently available for leptospirosis, but none are completely satisfactory for all purposes. Therefore, tests used should be chosen carefully with consideration to the purpose of the investigation, the specimens available, and, if applicable, the course of the illness (in the case of tests used for patient management).

For diagnosis at the acute stage of leptospirosis infection, PCR assays on blood or urine are the most appropriate to provide rapid diagnosis with high sensitivity and specificity. In addition, the detection of bacterial load by including a standard curve in the PCR assays may provide an early indication of the prognosis. IgM-ELISA is also able to provide rapid diagnosis at the early stage of infection (as early as three days). However, due to its relatively low specificity, the diagnosis should be confirmed by testing a convalescent serum with an alternative serological method (preferably MAT). DFM and culture are not recommended, either due to the poor sensitivity and specificity, or the requirement of long incubation time and therefore does not allow rapid diagnosis.

For detection of the immune response following leptospirosis infection, the MAT is still the most widely used reference test. The MAT titres can be used to confirm the diagnosis, indicate previous exposure in humans and animals, and also identify infecting serogroups. However, the interpretation of MAT titres needs to include consideration of factors such as the stage of infection, host species, infecting serovars; and particularly in

animals, the vaccination history. Compared with the MAT, the sensitivity and specificity of other serological tests (e.g. IgM-ELISA, lepto dipstick test, and lateral flow assay) are lower. Given the latter methods are easier to perform, they can be used as 'screening' or rapid diagnostic tests; however, they need to be followed by confirmation with MAT.

For diagnosis of leptospirosis in animals, an important point is to identify carriers, which shed leptospires into the environment through their urine, and may cause the spread of infection to other susceptible animals and to human populations at risk. Amplification of leptospiral DNA by PCRs is the test of choice here. Although culture of the organism from kidney has been used as the definitive method for identifying carrier status in animals and is considered as the gold standard to evaluate diagnosis tests on kidneys, it is time-consuming, requires a large number of viable leptospires in samples, and only available in dead animals. Culture from urine can occur in the live animal; however, it has the same disadvantages as kidney culture and the added disadvantages of higher level of contamination and poor survival of the bacteria in urine. The commonly found poor correlation between serological and carrier status of animals hampers the ability of MAT to identify carriers.

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Chapter 3 Appraisal of diagnostic assays following challenge with *Leptospira borgpetersenii* serovar Hardjobovis and *Leptospira interrogans* serovar Pomona in sheep and cattle.

1 Introduction

Leptospirosis is a zoonotic disease of worldwide importance and significantly impacts on livestock production (Faine *et al.* 1999; Levett 2001). Infection with pathogenic leptospires causes economic losses in the cattle and sheep industries due to reproductive failure (Ellis *et al.* 1983; Ellis 1994; Langoni *et al.* 1999), decreased milk and meat production (Pearson *et al.* 1980), and clinical illness (e.g. sudden death, haemoglobinuria (Cordes *et al.* 1982)). In addition, infected animals can develop chronic renal infection and excrete the organisms in the urine, disseminating leptospires to other animals and constituting a potential zoonotic threat to those engaged in animal production and related industries (Higgins *et al.* 1980; Cousins *et al.* 1989; Gerritsen *et al.* 1994; Magajevski *et al.* 2005).

In New Zealand, cattle and sheep are important animal reservoirs of *Leptospira* (Vickery *et al.* 2006). Surveys on farms and in abattoirs have shown that 69% of beef cattle herds and 44% of sheep slaughter lines have been exposed to leptospires (Heuer *et al.* 2007; Dorjee *et al.* 2008). Among the six serovars isolated from animals in New Zealand (NZ) from two species (*L. borgpetersenii* and *L. interrogans*) (Marshall and Manktelow 2002), *Leptospira borgpetersenii* serovar Hardjobovis (Hardjobovis) and *Leptospira interrogans* serovar Pomona (Pomona) are most commonly found in domestic livestock (Keenan 2007). This is consistent with findings worldwide (Faine *et al.* 1999). These two serovars were also reported among the three most commonly identified serovars for human cases in NZ from 2009 to 2011 (The Institute of Environmental Science and Research Ltd. 2003-2013). A better understanding of the responses to infection with these two serovars in cattle and sheep, and the application of appropriate tests to detect infection, will benefit both production animal industries and public health issues associated with leptospirosis.

Laboratory diagnosis of leptospirosis is based on direct examination of clinical specimens for organisms, culture of leptospires from clinical specimens, showing the

presence of leptospiral DNA, or detection of leptospiral antibodies (Faine *et al.* 1999; Bharti *et al.* 2003). Leptospires can be visualised by dark field microscopy (DFM) in clinical material, such as urine or blood (Levett 2001), but in general, this is not the preferred method due to the low sensitivity and specificity and the requirement for expert skills in identification (Bharti *et al.* 2003). Culture has been well established as a definitive diagnosis through isolation of the bacteria from a variety of samples (Smith *et al.* 1994; Faine *et al.* 1999). However, culture is laborious and time-consuming, the result is often influenced by the overgrowth of other bacterial and fungal contaminants, and some serovars, e.g. Hardjobovis, are difficult to culture (Cousins *et al.* 1985; Çetinkaya *et al.* 2000; Fearnley *et al.* 2008). The microscopic agglutination test (MAT) is the most commonly used standard test for detecting leptospiral antibodies (Faine *et al.* 1999; Lilenbaum *et al.* 2009). However, the assay requires significant expertise to perform and interpret (Levett 2001). The MAT detects antibodies that are based on the infected host's response, rather than the direct detection of leptospires *per se* (Magajevski *et al.* 2005). Hence, it does not indicate if the infection is active, and is not able to differentiate titres of natural infection from titres caused by vaccination (Bomfim *et al.* 2008). Nor is it able to reliably identify carrier status (Thiermann 1984). Polymerase chain reaction (PCR) has been reported as a reliable method for the rapid diagnosis of leptospirosis by detecting leptospiral DNA in various specimens (Wagenaar *et al.* 2000; Fearnley *et al.* 2008; Hernández-Rodríguez *et al.* 2011), and may be especially useful when the immune response of the maintenance host to the infecting serovar is poor (e.g. the MAT has low sensitivity for detecting Hardjo infection in cattle)(O'Keefe 2002).

The systemic response to *Leptospira* in cattle and sheep has been reported by other studies (Morse *et al.* 1957; Hodges 1974; Mackintosh *et al.* 1981; Cousins *et al.* 1985; Dhaliwal *et al.* 1996). However, for each study, the responses were examined by serological tests, culture or DFM on limited types of specimens while PCR assays were not applied. The limited information available for the performance of each test at various stages post-infection in animals limits diagnostic test selection and interpretation.

This study aimed to evaluate leptospiral diagnostic tests at various known times post-infection, and to help refine test usage and interpretation for future studies in animals and, potentially in humans. For this study, the opportunity was taken to obtain

sequential samples from *Leptospira* challenged animals from challenge trials conducted in an allied research facility. Immune and bacteriological responses from the challenged animals were recorded using MAT, culture, quantitative real-time PCR (qPCR) and DFM on various specimens (serum, whole blood, urine, and kidneys (sheep only)).

2 Materials and methods

2.1 Experimental Design

A commercial research organisation, Estendart Ltd., was contracted by a vaccine company to run challenge trials on sheep and cattle with serovars Hardjobovis and Pomona. The data collected will contribute to the study design of vaccination efficacy trials in the future. The designs of the challenge trials were determined by the Estendart Ltd. in conjunction with their client to meet their needs, including the number of animals and sample collection schedule. As the Hopkirk Leptospirosis Research Laboratory (HLRL) involvement was opportunistic in nature, it had no input in the protocol designs.

All animals were clinically healthy, and screened by MAT six or two weeks before challenge to ensure that they were free of pre-existing MAT-detectable antibodies (at titre cut-off 25) against Hardjobovis and Pomona (Appendix 3a). All animals were grazed on pasture (Jennersmead Farm) with other sheep or cattle before challenge. Sheep were transferred to the Animal Physiology Unit, Massey University (APU, MAF PC2 Transitional Facility) one or two days prior to challenge for acclimatisation.
In total, four trials were performed:

- A: challenge four sheep (#1-4) with Hardjobovis and four sheep (#5-8) with Pomona, Animal Ethics Committee (AEC) approval number 019/09;
- B: challenge eight sheep with Hardjobovis, AEC approval number 019/09;
- C: challenge 16 sheep with Pomona, AEC approval number 019/09; and
- D: challenge three cattle (#1-3) with Hardjobovis and three cattle (#4-6) with Pomona, AEC approval number 025/09.

Ethical approvals for all of the challenges were received from the Kaiawhina Animal Ethics Committee (Palmerston North, New Zealand). The average age of animals was 18-months for Trial A, 4.8-months for Trial B, 6.8-months for Trial C and 19-months for Trial D.

2.2 Challenge

The information regarding cultures used for each trial is presented in Table 3.1. All cultures were grown at HLRL for five to seven days, with density checked by McFarland scale of turbidity before challenge.

Table 3.1 The providers and the source of challenge cultures used for each trial

Trial	Provider	Serovar inoculated	Source of culture
A	HLRL	Hardjobovis	MU K4-12– NZ field isolate cultured from deer kidney
		Pomona	A025ii– NZ field isolate cultured from sheep kidney
	GV	Hardjobovis	Laboratory strain
		Pomona	Laboratory strain
B	HLRL	Hardjobovis	MU K4-12, passaged through sheep A2 from trial A
C	ESR	Pomona[a]	NZ Human isolate (LPC04/4)
			NZ Human isolate (LPC04/8)
D	HLRL	Hardjobovis	MU K4-12– NZ field isolate cultured from deer kidney
		Pomona	A025ii– NZ field isolate cultured from sheep kidney

HLRL= Hopkirk Leptospirosis Research Laboratory
GV= Gribbles Veterinary Pathology, Palmerston North
ESR= Environmental Science and Research group
[a] both human isolates were collected from meat workers

The concentration of leptospires in prepared culture was estimated on each day of challenge, using a Petroff-Hausser Chamber (Hausser Scientific, Horsham, UK) as per manufacturer's instructions at HLRL (except for cattle trial D, the estimation was performed by New Zealand Veterinary Pathology, Palmerston North (NZVP)). For each animal, around 2 ml of Hardjobovis or Pomona culture (containing approximately 10^7 to 10^9 organisms) was inoculated by syringe; 1 ml was administered into the nasal cavity (approximately 0.5 ml to each nostril), and 1 ml was administered through conjunctival instillation (approximately 0.5 ml into ventral conjunctival sac of each eye). Animals were observed for post-challenge adverse events for approximately one hour after administration. The inoculations were delivered on three successive days (Appendix 3b).

2.3 Sample collection

Blood and urine samples were collected from sheep and cattle, and sheep kidneys were harvested at post-mortem according to the schedules in Appendix 3a. The sample collection schedule was different for each trial. For example, besides collecting blood samples to test pre-existing MAT-detectable antibodies, urine samples were also collected to test pre-existing shedding before challenge on Day 0 for sheep Trial B and C; while for sheep Trial A and cattle Trial D, urine samples were not collected. As Trials A and D were performed as pilot trials, samples were collected from fewer sampling days compared to the other two trials.

Blood samples were collected by venipuncture using 10 ml BD Vacutainer® SST^{TM} II Advance Tubes or 10 ml BD Vacutainer® EDTA tubes (BD, Franklin Lakes, NJ.). For urine collection from sheep, approximately 1.5 mg/kg of the diuretic Salix (Intervet Ltd, Auckland, New Zealand) was administered intravenously on each occasion. Approximately 10 minutes later, the vulva of each animal was cleaned with 70% ethanol wipes to reduce contamination, and no less than 10 ml of midstream urine was collected into a sterile 120 ml container (Bio-lab, Auckland, New Zealand). No diuretic was administered to cattle. For sheep trials, the right kidney was collected directly after sacrificing sheep. The kidney capsule was removed, and then the surface was wiped with 70% Ethanol. The kidney was placed in a new plastic zip lock bag for transport fresh to the laboratory. All samples were refrigerated at 4°C and sent to HLRL for processing on the date of collection.

2.4 Husbandry of animals

For sheep trials, animals were housed in the APU. Sheep were grouped by the serovar of culture they had been inoculated with (Hardjobovis or Pomona), and each group housed in separate pens indoor with ample separation of the groups to prevent cross-infection. Feed and water was supplied *ad-libitum*. For the cattle trial, animals were grouped by the serovar of culture they had been inoculated with and grazed separately on pasture *ad-libitum* (Jennersmead Farm). All animals were examined daily for clinical symptoms post-challenge. Rectal temperatures were measured for cattle in Trial

D, from Day 0 to Day 9. For Trial B, all sheep were weighed on Day 0 (before challenge) and on the day of sacrifice (Day 42).

2.5 PCR detection of bacterial DNA

2.5.1 DNA extraction

2.5.1.1 Urine samples

For Trials A and D, 10 ml of each urine sample was added into a BD Vacutainer® Serum Tube (BD, Franklin Lakes, NJ, USA), and then centrifuged at 1512 *g* for 10 minutes. Supernatant was discarded by a transfer pipette (Raylab, Auckland, New Zealand) and 200 µl of phosphate buffered saline (PBS) was used to re-suspend the pellet. This mixture was used to extract DNA using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl.

For Trials B and C, 1.2 ml of urine was added into a 1.5 ml Eppendorf tube (Raylab, Auckland, New Zealand), and then centrifuged at 10625 *g* for 20 minutes. Supernatant was discarded by a transfer pipette (Raylab, Auckland, New Zealand) and 200 µl of PBS was used to re-suspend the pellet. This mixture was used to extract DNA using the QIAamp DNA Mini Kit (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl.

2.5.1.2 Whole blood samples

Two hundred microlitres of whole blood were used to extract DNA, using the QIAamp DNA Mini Kit (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl.

2.5.1.3 Serum samples

Blood samples were first centrifuged at 1300 *g* for 10 minutes. Two hundred microlitres of the serum was used to extract DNA, using the QIAamp DNA Mini Kit (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand) as per manufacturer's instructions. DNA was eluted in a final volume of 200µl. The rest of the supernatant was stored in a 2 ml cryovial (RayLab, Auckland, New Zealand) at -20° C and held as a reserve aliquot.

2.5.1.4 Kidney samples

Kidney surfaces were sterilised by swabbing with 70% ethyl alcohol. Approximately a 10g section of kidney, extending from the renal cortex to the medulla, was removed aseptically. This section was doused with 70% ethyl alcohol (using funnel and flask), followed by flaming with a Bunsen burner. It was then homogenised in 50 ml PBS, using the Colworth Stomacher 400 (AJ Seward Ltd, London, UK). A 160 µl aliquot of this suspension was used to extract DNA, using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl.

2.5.2 PCR amplification

The qPCR assay was a modification of the assay set up and validated by Subharat *et al.* (2011), using deer urine and kidney samples (Subharat *et al.* 2011). Green-Fluorescent Nucleic Acid Stain SYTO9 (Invitrogen Corp., Carlsbad, CA, USA) was used as the intercalating dye. The assay was performed in a Rotor-Gene Q machine (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand). Primers 2For (5'-TGAGCCAAGAAGAAACAAGCTACA-3') and 504Rev (5'-MATGGTTCCRCTTTCCGAAGA-3') were used to amplify *gyr*B gene. The 25 µl reaction mix comprised 2.5 µM SYTO9 (Invitrogen Corp., Carlsbad, CA.), 1×PCR buffer, 1.5 mM Magnesium Chloride (MgCl2), 200 µM dNTPs, 5 pmol of 2For and 504Rev, one unit of *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA), 2 µl of DNA extract and double distilled water (ddH2O). Thermal cycling was as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 63°C for 20 s and extension at 72°C for 20 s. 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. Positive controls were serovar Hardjobovis and Pomona (HLRL laboratory strains), and ddH2O was used as negative control. Samples were considered as positive, if a similar melting temperature to the positive controls were received.

2.6 Culture

The medium used for culture was Ellinghausen-McCullough-Johnson-Harris (EMJH) medium prepared at the HLRL, and contained 5'-fluorouracil to help prevent contamination, as described by Ellinghausen and McCullough (1965), Johnson and Harris (1967) and Faine *et al.* (1999). Cultures were performed and read by either HLRL or NZVP. One hundred microlitres of urine, whole blood or an aliquot of kidney suspension prepared for DNA extraction (as described above) was inoculated into 5 ml of EMJH medium. From this inoculated medium, a serial dilution was performed by transferring a 100 µl aliquot to another 5 ml of medium, followed by further transfer of 100 µl to another 5 ml of medium. All three inoculated medium bottles were then incubated aerobically at 28–30°C, and examined every week for the presence of leptospires, using dark-field microscopy. Cultures that showed no detectable growth after 12 weeks were declared negative.

2.7 MAT

Thirty microlitres of each serum sample was mixed with 150 µl standard saline to make a 1:6 dilution for testing. MAT was performed at HLRL, and tested against serovars Hardjobovis and Pomona (HLRL laboratory strains) based on the standard procedure described by Faine (1982). The antigens used for MAT were 4–14-day-old cultures of Hardjobovis and Pomona containing approximately $1–2\times10^8$ organisms/ml. Eight, two-fold serial dilutions of serum in standard saline covering the range from 1:24 to 1:3072 were prepared in 96-well, flat-bottom polystrene plate (Greiner Bio-one, Frickenhausen, Germany). A positive control using standard antisera against the serovar tested and a negative control using standard saline instead of diluted serum, were prepared in a similar manner. The plates were then placed in re-sealable plastic bags and incubated at room temperature (20–30°C) for 1.5-4 hours, after which the degree of agglutination lysis was determined by examination under an Olympus BH2 dark-field microscope (Olympus, Tokyo, Japan). The end-point titre was the lowest dilution at which approximately 50% of the organisms were agglutinated or lysed.

For Trial A, MATs on samples collected from Day 19 were performed by Gribbles Veterinary Pathology, Palmerston North (Gribbles). For Trial C, MATs on samples collected from Days 14, 28 and 42 were performed by NZVP, following the same

procedure as HLRL (used HLRL laboratory strains as antigen), but with eight two-fold serial dilutions covering the range from 1:25 to 1:3200 serum-antigen mixtures.

2.8 Dark field micoscopy (DFM) on urine and kidney cultures

DFM was performed by either HLRL or NZVP.

One and a half millilitres of urine sample was added into a 1.5 ml Eppendorf tube (Raylab, Auckland, New Zealand), and then centrifuged at 6000 *g* for 3 minutes. A transfer pipette was used to draw-off and discard the supernatant carefully, and leave approximately 100 µl of urine at the bottom of the Eppendorf tube (Raylab, Auckland, New Zealand). More urine was added into the same tube to re-suspend to 1.5 ml. The mixture was again centrifuged at 6000 *g* for 3 minutes again. The supernatant was discarded leaving approximately 100 µl of urine. One drop (or 10-20 µl) of this solution was placed onto a microscope slide (covered by a cover slip), and observed at a magnification of 20x under an Olympus BH2 dark-field microscope (Olympus, Tokyo, Japan) with a dark field condenser.

Ten to twenty microlitres of the suspensions prepared for kidney DNA extraction (described above) were used, and followed the same procedure as urine sample.

3 Results

3.1 Sheep trials A, B and C

3.1.1 Clinical observations

Challenged sheep appeared clinically normal throughout the observation period, except for sheep 4 from Trial B that died on Day 15. Post-mortem of the carcass was conducted at Massey University on the same day. The kidneys were slightly swollen, but no visible lesions (spots) were found on the cortical surface. The rumen and abomasum were moderately distended. No other abnormalities were noted on gross post-mortem.

For Trial B, eight sheep were weighed on Day 0 (before challenge) and seven survivors on Day 42 when sacrificed. Weight loss averaging 5.2 kg (range from 1.5 to 8 kg) was observed in five animals during the trial (B1, 3, 5, 6 and 8) while two animals (B2 and 7) gained 2.5 kg.

3.1.2 Bacteriological findings

3.1.2.1 Leptospires in sheep urine

For Trial A, urine samples were not collected on Day 0 for testing. In total, leptospires were detected by urine culture from two sheep while positive urine qPCR results were detected from one (Table 3.2). Leptospires were detected in urine as early as Day 19 and retained until Day 30. Two of eight sheep tested positive by urine DFM on Day 27.

Table 3.2 Detection of leptospires in urine on each sampling day after challenge, and kidney on Day 42, by culture (C), qPCR (P) and Dark Field Microscopy (D) and antibody titres against serovars Hardjobovis and Pomona by MAT for sheep Trial A.

Sheep ID	Serovar inoculated	Urine*					Kidney	MAT titre					
		Day					Day	Day					
		19	22	27	30	33	42	0	1	2	3	19 †	42
A1	Hardjobovis [a]	–	–	D	–	–	–	–	–	–	–	–	–
A2	Hardjobovis [b]	P	P,C	C	P	–	P,C,D	–	–	–	–	100	192
A3	Hardjobovis [a]	–	–	–	–	–	–	–	–	–	–	–	–
A4	Hardjobovis [b]	C	C	C	C	–	P,C	–	–	–	–	1600	768
A5	Pomona [b]	–	–	–	–	–	P,D	–	24	–	–	–	24
A6	Pomona [a]	–	–	–	–	–	–	–	–	–	–	–	–
A7	Pomona [a]	–	–	D	–	–	–	–	–	–	–	–	–
A8	Pomona [b]	–	–	–	–	–	–	–	–	–	–	–	–

[a] inoculum was provided by GV
[b] inoculum was provided by HLRL
- = negative
* urine samples were not collected on Day 0 for testing
† MAT was performed by Gribbles on Day 19

For Trial B, positive urine cultures were detected from one animal on all sampling days but Day 28. Urine qPCR was positive for that animal on all sampling days (Table 3.3). Urine DFM was not performed on Day 0, but was positive for that animal on three sampling days. For four other animals, positive DFM results were detected on one sampling day (Day 21 or 37).

Table 3.3 Detection of leptospires in urine on each sampling day after challenge, and kidney on Day 42, by culture (C), qPCR (P) and Dark Field Microscopy (D) and antibody titres against serovar Hardjobovis by MAT for sheep Trial B.

Sheep ID	Urine								Kidney	MAT titre against serovar Hardjobovis							
	Day								Day	Day							
	0[b]	5	8	12	14	21	28	37	42	0	5	8	12	14	21	28	37
B1	–	–	–	–	–	–	–	–	P	–	–	–	384	192	384	48	96
B2	–	–	–	–	–	–	–	–	D	–	–	96	1536	768	384	192	48
B3	–	–	–	–	–	–	–	D	D	–	–	48	1536	768	96	48	24
B4[a]	–	–	–	–	–				–	–	–	96	768	768			
B5	–	–	–	–	–	–	–	D	D	–	–	48	3072	1536	384	192	192
B6	P,C	P,C	P,C	P,C	P,C,D	P,C,D	P	P,C,D	P,D	24	48	192	96	–	192	96	24
B7	–	–	–	–	–	D	–	–	D	–	–	384	768	1536	384	384	96
B8	–	–	–	–	–	–	–	D	D	–	–	768	1536	768	192	96	96

- = negative
[a] died on Day 15 at which time the kidney was sampled
[b] urine DFM was not performed on Day 0

For Trial C, positive urine cultures were detected on Day 0 from 13 of 16 animals, while no positive urine qPCR result was detected from any animal. Urine DFM was not performed on Day 0 (Table 3.4). Leptospires were first detected by qPCR and DFM on Day 7, and by culture on Day 14. Leptospires were observed in urine from a maximum number of sheep on Day 28 (by culture) or Day 35 (by qPCR and DFM) (Figure 3.1). Among eight sheep inoculated with LPC 04/08, leptospires were detected in seven by urine culture, eight by urine qPCR, and four by urine DFM. Among eight sheep inoculated with LPC 04/04, leptospires were detected in urine by culture, qPCR and DFM from eight, seven, and five sheep, respectively.

Table 3.4 Detection of leptospires in blood, serum, urine and kidney by culture (C), qPCR (P) and Dark Field Microscopy (D) and antibody titres against serovar Pomona by MAT from each sampling day after challenge for sheep Trial C.

Inoculated strains	Sheep ID	Blood					Serum					Urine							Kidney	MAT[a] titre against serovar Pomona					
		Day					Day					Day							Day	Day					
		0	3	5	7	14[b]	0	3	5	7	14	0*	7	14	21	28	35	42	42	0	5	7	14	28	42
LPC 04/08	C1	–	–	–	–	–	–	P	P	–	–	C	–	P	P,C	C	P	–	–	–	96	3072	800	50	–
	C2	–	–	P	P	–	–	P	P	–	–	–	–	–	–	–	P	–	–	–	96	192	50	25	–
	C3	–	–	–	–	–	–	–	–	–	–	C	D	P	C	–	P,D	D	–	–	24	768	100	50	–
	C4	–	C	–	–	–	–	P	P	–	–	C	–	P,C	P,C	P,C,D	P,C,D	P,C	P,C,D	–	48	3072	800	25	–
	C5	–	C	P	–	–	–	P	P	–	–	C	–	P,C	P,C,D	P,C	P,C,D	P,C	P,C	–	24	1536	800	50	–
	C6	–	C	C	–	–	–	P	P	–	–	C	P	C	C	P,C	P,C	–	P,C	–	–	1536	800	25	–
	C7	–	P,C	P	–	–	–	P	–	–	–	C	D	P,C	P,C	P,C,D	P,C,D	P,C	P,C	–	48	3072	800	100	–
	C8	–	C	P	–	–	–	P	P	–	–	–	–	C	P,C	P,C	P,C	P,C	P,C	–	48	3072	200	100	–
LPC 04/04	C9	–	P,C	–	–	–	–	P	P	P	–	C	–	C	C	P,C	P,C	P,C	P,C	–	24	384	400	50	–
	C10	–	C	C	P	–	–	P	P	–	–	C	–	P,C	P,C	P,C,D	P,C	P,C	P,C	–	–	384	400	25	–
	C11	–	C	P	–	–	–	P	P	–	–	C	–	–	P,C,D	P,C	P,C	P,C	P,C	–	48	1536	400	25	–
	C12	–	P,C	–	–	–	–	P	–	–	–	C	–	C	P,C	P,C,D	P,C	P,C	P,C	–	24	768	800	100	50
	C13	–	P,C	P	–	–	–	P	P	–	–	–	–	P,C	P,C	P,C	P,C,D	P,C	P	–	48	1536	400	100	–
	C14	–	–	–	–	–	–	P	–	–	–	C	P	D	C	P,C	P,C,D	C	C	–	48	768	1600	100	100
	C15	–	C	–	–	–	–	P	P	–	–	C	–	C	P,C	P,C	P,C	P,C	P,C,D	–	–	384	800	50	–
	C16	–	–	–	–	–	–	–	–	–	–	C	–	–	–	C	C	–	C	–	48	768	400	25	–

- = negative

[a] MAT was performed by HLRL on Day 0 to 7, and by NZVP from Day 14 to 42

[b] blood culture was not performed on Day 14

* urine DFM was not performed on Day 0

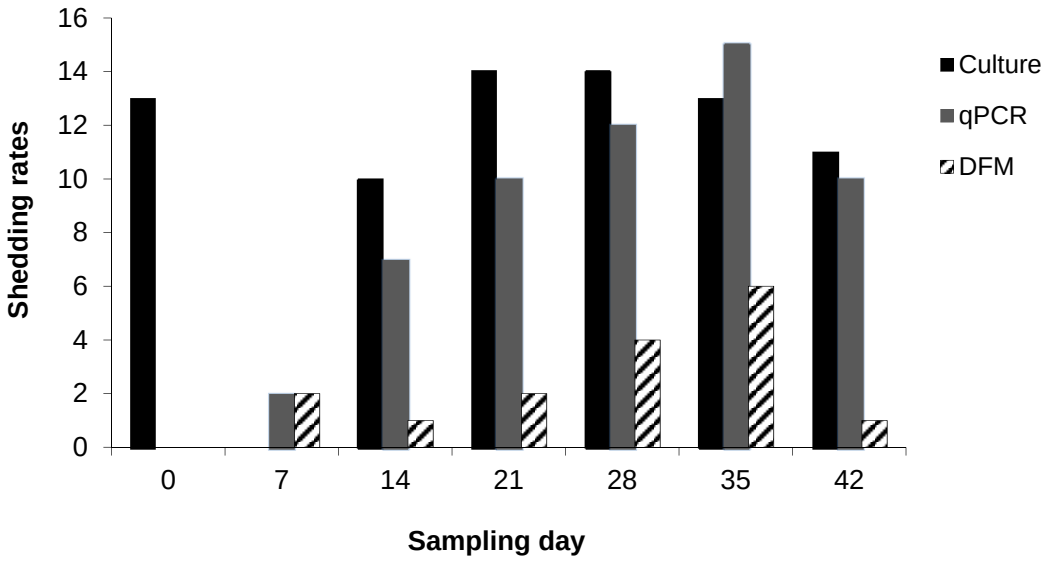

Figure 3.1 Number of sheep tested positive by culture, qPCR and DFM on urine on all sampling days (Day 0 to 42) after challenge among 16 sheep for Trial C.

3.1.2.2 Leptospires in kidneys

For Trial A, two animals tested positive for kidney culture and DFM. Positive kidney q-PCR results were obtained from three animals (Table 3.2). For Trial B, no animal was culture positive. Two animals tested positive for kidney qPCR. Positive DFM results were detected from six animals (Table 3.3). In Trial C, among eight sheep inoculated with LPC 04/08, leptospires were detected in kidney from five by culture and qPCR, and one by DFM. Among eight sheep inoculated with LPC 04/04, leptospires were detected in kidney by culture, qPCR and DFM from seven, six and one sheep, respectively (Table 3.4).

3.1.2.3 Leptospires in whole blood and serum

For Trial A, all eight sheep tested negative by serum qPCR on all five sampling days. No positive blood qPCR results were obtained on Day 22 and 42. For Trial B, on eight sampling days, negative results were obtained from blood culture, blood qPCR and serum qPCR on almost all sheep; except serum qPCR tested positive from B6 on Day 21. For Trial C, leptopsires were detected by blood tests from Day 3 to Day 7. Among eight sheep inoculated with LPC04/08, five tested positive by blood culture, seven by serum qPCR, and four by blood qPCR. Among sheep inoculated with LPC04/04, leptospires were detected in blood by blood culture, serum qPCR, and blood qPCR from six, seven and five sheep, respectively (Table 3.4).

3.1.3 Immune response

For Trial A, antibodies against leptospires were not detected from any of the animals on Day 0. Antibodies against serovar Hardjobovis were detected from two animals on Day 19 and 42. A low titre of 24 was detected in animal A5 (inoculated with serovar Pomona) against serovar Pomona on Day 1 and 42 (Table 3.2).

For Trial B, a MAT titre against serovar Hardjobovis (24) was detected in B6 on Day 0; no titres were obtained from other animals. Titre was detected in B6 on all sampling days except Day 14 (Table 3.3). This animal was most likely infected before challenge (will be elaborated on in Discussion), and the serological data of B6 was not included in the geometric mean titres and seroprevalence shown in Figure 3.2. Serological response was detected as early as Day 8 or 12 in the other seven animals, with MAT titres attained maximum on Day 12 or 14, and declined after that. The maximum titres ranged

for individual animals from 384 to 3072. Titres were still detectable in all surviving animals on Day 37 (Table 3.3).

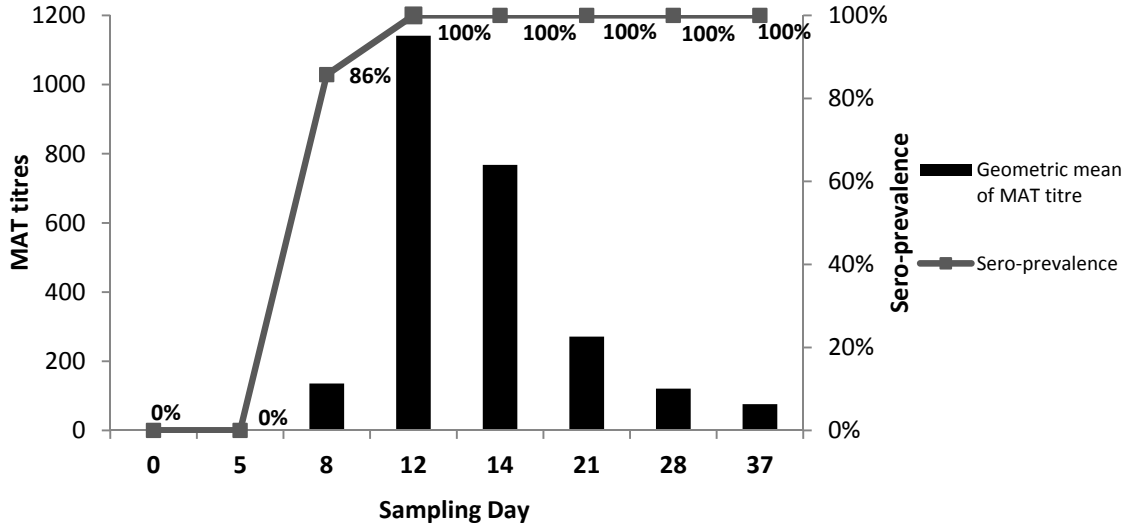

Figure 3.2 Geometric mean of MAT titres against serovar Hardjobovis, and seroprevalence on eight sampling days (Day 0 to 37) after challenge for seven sheep (B6 were excluded from this analysis) from Trial B. Data from Day 21 onwards does not include that from sheep B4 as it died on Day 15.

For Trial C, all animals developed a serological response to serovar Pomona (Table 3.4). The geometric mean of titres and seroprevalence are shown in Figure 3.3. Antibodies against leptospires were not detected from any animal on Day 0. Antibodies were first detectable in Day 5 or 7, attained a maximum titre on Day 7 or 14, and declined after that. The maximum titres for eight sheep inoculated with isolate LPC04/08 ranged from 192 to 3072, while the maximum titres for eight sheep inoculated with isolate LPC04/04 ranged from 384/400 to 1536/1600. Titres were not detected in any sheep that was inoculated with isolate LPC04/08, whereas in two sheep that inoculated with isolate LPC04/04 on Day 42 (Table 3.4).

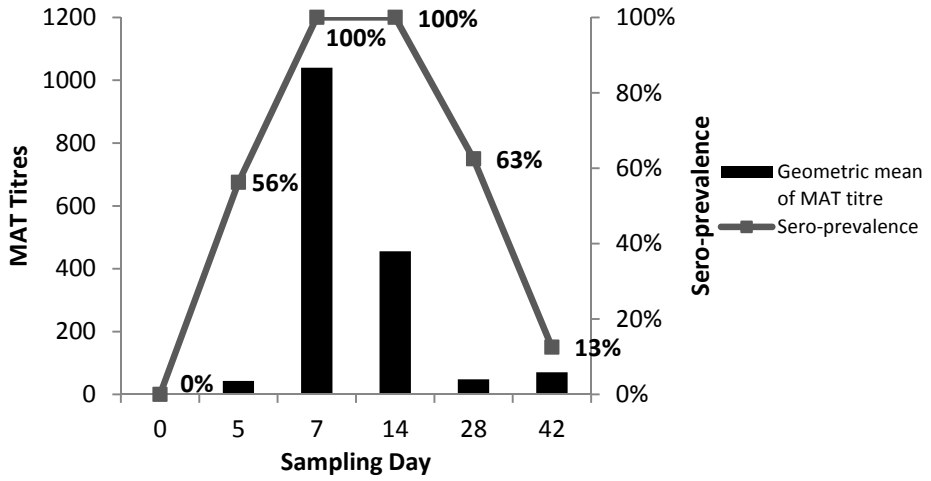

Figure 3.3 Geometric mean of MAT titres against serovar Pomona, and seroprevalence on six sampling days (Day 0 to 42) after challenge among 16 sheep for Trial C.

3.2 Cattle trial D

3.2.1 Clinical observations

All six cattle appeared clinically normal throughout the observation period. The rectal temperature dropped for all cattle from Day 0 to 7, but remained within the normal range two weeks after inoculation. Elevated temperatures were reported on Day 9 from all three cattle inoculated with serovar Hardjobovis, while the temperature remained similar for three cattle inoculated with serovar Pomona.

3.2.2 Bacteriological findings

3.2.2.1 Leptospires in urine

Urine samples were not collected on Day 0 for testing. Positive urine culture and qPCR results were detected only from D2 and D3, inoculated with serovar Hardjobovis (Table 3.5). Leptospires were first detected by culture on Day 28 and by urine qPCR on Day 23. One of the cattle, D3, tested positive by urine DFM (Table 3.5).

Table 3.5 Detection of leptospires in urine on each sampling day after challenge by culture (C), qPCR (P) and Dark Field Microscopy (D) and antibody titres against serovars Hardjobovis and Pomona by MAT for cattle Trial D.

Sample ID	Serovar Inoculated	Urine [a]						MAT titres						
		Day						Day						
		20	23	26	28	35	42	0	7	9	14	20	23	42
D1	Hardjobovis	–	–	–	–	–	–	–	–	–	192	3072	384	–
D2	Hardjobovis	–	–	–	P	P,C	P	–	–	384	192	1536	192	–
D3	Hardjobovis	–	P	P	C,D	–	–	–	–	384	384	384	96	–
D4	Pomona	–	–	–	–	–	–	–	–	–	–	–	–	–
D5	Pomona	–	–	–	–	–	–	–	–	–	–	–	–	–
D6	Pomona	–	–	–	–	–	–	–	–	–	–	–	–	–

– = not detected

[a] urine samples were not collected on Day 0 for testing

3.2.2.2 Leptospires in whole bloods and serum

Blood cultures were performed on Day 9 and 14, while qPCRs on whole blood and serum were performed on Day 0, 7, 9, 14, 20, 23 and 42; all were negative.

3.2.3 Immune response in cattle

Throughout the observation period, MAT titres were detected only from cattle challenged with Hardjobovis (Table 3.5). Antibodies were detectable as early as Day 9, and attained maximum on Day 20. MAT titres remained detectable until Day 23. Titres were not detected on Day 42.

4 Discussion

In this study, MAT, culture, qPCR and DFM were applied to test various specimens collected at different stages of leptospiral infection. Although there were no statistically significant differences, results suggested that during the early stage of Pomona infection in sheep, serum qPCR had the highest sensitivity for detecting leptospires in blood, followed by blood culture and whole blood qPCR. Under carefully controlled experimental conditions, urine culture was shown to have the highest sensitivity of detecting shedding in sheep with Hardjobovis and Pomona infection, followed by qPCR; while in cattle with Hardjobovis infection, highest sensitivity was achieved using qPCR. Comparable sensitivity was found for culture and qPCR for detecting renal colonisation of leptospires in sheep with Hardjobovis and Pomona infection. Findings in this study showed low sensitivity and specificity of DFM for both urine and kidney

samples, and questioned the usefulness of this technique in veterinary settings. Test results also provided information about the trends of leptospiraemia, immune response, and urinary shedding after infection, which can be useful for future studies in animals and, potentially in humans. However, it needs to be noted that the findings from this study were based on artificial challenges with a limited number of animals and sampling days. Further challenges with a larger number of animals, more sampling days, and a longer observation period (for identifying duration of immune responses and shedding) should be considered. Additional work on the limits of detection and diagnostic sensitivity and specificity (as previously carried out on deer samples (Subharat *et al.* 2011)) is also required to completely evaluate the utility of the test on sheep and cattle specimens.

For all four trials, results from animals that had immune responses (indicating successful infection) detected during the observation periods are included in the discussion, with two exceptions. In Trial B, positive urine culture and qPCR results and low MAT titre (24) were obtained on Day 0 (before challenge) from B6 (Table 3.3). These indicated that this animal had been exposed to *Leptospira* spp. before the challenge. Due to the fact that the animals were screened prior to the challenges and did not show detectable titres, it is possible that B6 had already been exposed to leptospires and developed kidney colonisation/shedding status but did not show detectable MAT titres prior to the screening test (MAT-negative animals are not infrequently proved bacteriologically to be carriers of leptospires (Faine *et al.* 1999; Dorjee *et al.* 2008)). It is also possible that the exposure happened during the period between MAT screening days and the relocation days (Appendix 3a), while B6 was grazed on pasture with other sheep with unknown leptospirosis status. After being challenged, the MAT titres detected in B6 increased from Day 5 (MAT titres not detected only in Day 14 may be due to detection error). However, it is not able to be determined whether that was due to re-exposure to leptospires because of the inoculation or an anamnestic reaction from previous exposure. Also, it is not able to be determined whether the leptospires detected in the urine and kidney was due to past infection or to challenge or both. Therefore, the results from B6 should be excluded from the discussion. In Trial C, 13 of the 16 animals tested positive by urine culture on Day 0, before the challenge; however, no positive urine qPCR or DFM results were obtained on the same day. Moreover, on Day 7, none of the animals tested positive by urine culture. Therefore, the positive urine cultures

obtained on Day 0 were more likely to be due to the contamination of the medium with *Leptospira* spp. from the laboratory environment. The test results from Trial C were therefore included for analysis, except the urine culture results on Day 0.

For Trial B, leptospires were not able to be detected in urine by culture or qPCR for all sero-converted sheep (except B6, which is excluded from this discussion). Compared with sheep inoculated with serovar Hardjobovis in Trial A, the sheep in Trial B were younger (average age of 18 months from Trial A and 4.8 months from Trial B). However, shedding of leptospires is considered more likely to occur in young animals (Heath and Johnson 1994). Therefore, the age differences may not be the reason for shedding/kidney colonisation not being detected in Trial B. Compared to Trial A, a higher concentration of inoculum was used in Trial B (Appendix 3b). It is suggested that a high concentration of inoculation may reduce the length of the kidney colonisation and urinary shedding (Mackintosh *et al.* 1981). However, a similar concentration of inoculum as Trial B was used in Trial C, in which shedding and renal colonisation was detected. Therefore, the most plausible reason for the shedding/kidney colonisation not being detected in Trial B is the virulence of the isolate used for challenge.

Although no statistically significant differences were found, based on the proportion of animals that tested positive and the number of sampling days that detected positive results from each urine test, among sero-converted sheep in A and C (data from sheep inoculated with strains LPC 04/08 and LPC 04/04 combined), culture was shown to have higher sensitivity of detecting urinary shedding in sheep with Hardjobovis and Pomona infection, compared to qPCR (p = 1.0 for Trial A and p = 1.0 for Trial C) and DFM (p = 0.333 for Trial A and p = 0.083 for Trial C) (Table 3.6). In Trial D, although the same number of cattle was tested positive by urine culture and qPCR, positive results were obtained in more sampling days by qPCR. Culture of *Leptospira* spp. from urine is considered to be difficult, due to the overgrowth of other bacterial and fungal contaminants (Fearnley *et al.* 2008). Leptospires remain viable in urine samples for only a few hours (Quinn *et al.* 1994). Therefore, compared with PCR assays that can detect both viable and dead bacteria, the sensitivity of culture may be lower due to the requirement of sufficient numbers of viable bacteria (O'Keefe 2002). The detection limit of leptospires in deer urine by PCRs is reported to be 10 times lower than that by culture (Subharat *et al.* 2011).

No comparison between urine culture and PCR assays was available in literature to compare with the findings in this study. However, in a Brazil study that tested urine samples from 40 seropositive (predominately serovar Hardjobovis) sheep in the field, higher sensitivity was reported for PCR than culture (PCR was positive on six samples, while no positive culture result was obtained) (Lilenbaum *et al.* 2009). The sheep urine samples were collected in Trial A and C under carefully controlled experimental conditions indoors. The APU is close to the laboratory, and the urine samples were able to be received at the laboratory within one hour after collection. Therefore, compared with field conditions in which samples are often collected and handled under less than optimal conditions and delays in submission to laboratories, the factor of contamination and limited number of viable leptospires may not have the same impact on the sensitivity of culture in this study. Findings in Trial D is consistent with those of two previous trials, which reported higher sensitivity of urine PCR than culture in cattle with Hardjobovis infection, being 100% versus 89%, respectively, by Wagennar *et al.* (2000), and 80% versus 17%, respectively, by Bolin *et al.* (1989) (Bolin *et al.* 1989; Wagenaar *et al.* 2000).

Table 3.6 Summary of number of sheep tested positive by culture, qPCR, and DFM on each sampling day and proportion of animals tested positive during the observation period for 16 sero-converted (Pomona) sheep in Trial C, two sero-converted (Hardjobovis) sheep in Trial A, and three sero-converted (Hardjobovis) cattle in Trial D.

Urine Tests	**No. tested positive**							**Proportion tested positive**
Trial C	**Sampling day**							
	0	**7**	**14**	**21**	**28**	**35**	**42**	
Culture	13	0	10	14	14	13	11	15/16 [a]
qPCR	0	2	7	10	12	15	10	15/16
DFM	/	2	1	2	4	6	1	10/16
Trial A	**Sampling day**							
		19	**22**	**27**	**30**	**33**		
Culture		1	2	2	1	0		2/2
qPCR		1	1	0	1	0		1/2
DFM		0	0	0	0	0		0/2
Trial D	**Sampling day**							
		20	**23**	**26**	**28**	**35**	**42**	
Culture		0	0	0	1	1	0	2/3
qPCR		0	1	1	1	1	1	2/3
DFM		0	0	0	1	0	0	1/3

[a] positive urine culture results obtained on Day 0 in Trial C were not included, due to the possible contamination of the medium with *Leptospira* from the laboratory environment.

Among sheep from Trial C that had sero-conversion detected, culture had the highest sensitivity for detecting the renal carrier state, followed by qPCR and DFM (Table 3.4). The rate of positive kidney culture, qPCR, and DFM in Trial C was 75% (12/16), 69% (11/16), and 13% (2/16), respectively. However, in Trial A, renal colonisation was detected by qPCR in the highest proportion (3/3) of sero-converted sheep, followed by culture (2/3) and DFM (2/3) (Table 3.2). In Trial B, a positive kidney qPCR result was obtained from B1 that did not test positive by kidney culture or DFM (Table 3.3). It is possible that the leptospires persisted in small numbers (lower than the detection limit of culture) in the kidney, but detectable by the qPCR. It can also due to the fact that qPCR can detect dead leptospires while culture is only able to detect viable leptospires. The intermittent character of shedding may explain why B1 didn't have shedding detected throughout the observation period, but had renal colonisation detected by kidney qPCR. Considering culture is laborious and the sensitivity of qPCR was comparable to the culture of kidney samples, the qPCR can be used as an alternative to provide rapid diagnosis for renal carrier state in sheep.

Dark field microscopic examination (DFM) of body fluids (e.g. urine) or tissues (e.g. kidney) can be used to rapidly detect the presence of leptospires, and is especially useful in situations where laboratory resources are limited (Faine *et al.* 1999; Levett 2001; O'Keefe 2002). However, this technique requires high level of operator skill, and the sensitivity and specificity is considered to be low (Faine *et al.* 1999; Bharti *et al.* 2003). A minimum of approximately 10^4 live organisms/ml must be present for one cell per field to be visible (Turner 1970), which leads to the low sensitivity of this test. The sensitivity of DFM was shown in the present study to be lower than culture and qPCR for detecting leptospires in urine (Table 3.6) and kidney (data presented above) in this study. The risk of false positives obtained by DFM, due to misinterpretation of fibrin or protein threads, cell debris and other artefacts, is high even for experts (Faine *et al.* 1999; Vijayachari *et al.* 2001). Test results from this study also support the low specificity of DFM. In Trial A, positive urine DFM results were obtained from two sheep that did not sero-convert (Table 3.2). In Trial B, positive urine DFM results were obtained from four sheep, while positive kidney DFM results were obtained from five sheep that did not test positive by culture or qPCR (Table 3.3). Therefore, considering the low sensitivity and specificity of DFM in this study, this technique is not recommended for detecting urinary shedding and renal carrier state of leptospires in sheep and cattle.

This is the first report of a qPCR assay being used to detect leptospires in blood from sheep and cattle at the early stages of infection. Although no statistically significant differences were found, based on the proportion of animals that tested positive and the number of sampling days that detected positive results from each blood test, test results from Trial C indicated that among sheep at early stage of Pomona infection, qPCR on serum samples may have higher sensitivity for detecting leptospires in blood, compared with blood culture ($p = 0.197$) and qPCR on blood ($p = 0.057$) (Table 3.7). Although it is considered that the sensitivity of blood culture may not be as poor as those on other specimens (e.g. urine), due to the fact that blood may have components that aid in the growth of leptospires (Turner 1970), the long incubation period is still an important concern for using this technique to diagnose acute phase infection. Rapid diagnosis is important during this period to start therapy as soon as possible. In a study that tested spiked human whole blood with serial 10-fold dilutions of leptospires stock (from 1×10^6 to 1×10^0 leptospires/ml), the spiked whole blood with highest concentration ($1 \times$

10^6 leptospires/ml) was not culture positive until three weeks after the medium was inoculated; while the spiked whole blood with lowest concentration (1×10^0 leptospires/ml) was not culture positive until week 7 (Stoddard *et al.* 2009). Therefore, considering the sensitivity and the time taken, qPCR may be a better technique than culture for diagnosis at early stage of infection.

To the author's best knowledge, no study has been conducted to compare PCRs on animal serum with whole blood. A recent human study showed that sensitivity for quantitative PCR of serum was higher than whole blood (51.0% versus 18.4%) (Agampodi *et al.* 2012), which is in contrast to other human studies that reported that serum from clotted and centrifuged blood was not the best specimen for detection of leptospires by PCR, compared to other fractions of blood (Kositanont *et al.* 2007; Stoddard *et al.* 2009). Although not quantified, *Leptospira* DNA was detected in the clot from the SST and serum tubes, which may affect the positive rate of qPCR on serum samples (Stoddard *et al.* 2009; Bourhy *et al.* 2011). Future studies involving larger numbers of clinical samples need to be conducted to evaluate the qPCR used in this study on serum versus whole blood samples. None of the blood samples collected at early stages of infection in sheep Trial B and cattle Trial D with serovar Hardjobovis tested positive by culture or qPCR. Therefore, further research needs to be undertaken to investigate the usage of these tests for detecting the early stage of infection in sheep inoculated with serovar Hardjobovis and cattle inoculated with both serovars.

Table 3.7 Summary of number of sheep tested positive by culture, serum qPCR, and whole blood qPCR on each sampling day and proportion of sheep tested positive during the observation period for 16 sero-converted (Pomona) sheep in Trial C.

Blood tests	No. tested positive					Proportion tested positive
	Sampling day					
	0	**3**	**5**	**7**	**14**	
Blood culture	0	11	2	0	/	11/16
Serum qPCR	0	14	11	1	0	14/16
Blood qPCR	0	4	6	2	0	9/16

The urine test results obtained in this study from Trial A, C, and D provided information about the trend of shedding after leptospirosis infection among sheep and cattle, during the observation period. The findings in Trial C suggested that *Leptospira* Pomona can be detected in lamb urine from Day 7 to the day of trial termination (Day 42) after

exposure, with the maximum level of leptospiruria found on Day 28 or 35 (Figure 3.1). For two hoggets that had sero-conversion detected against serovar Hardjobovis in Trial A, shedding can be detected from Days 19 to 30, but not on Day 33 (Table 3.2). For Trial D, two cattle that developed MAT titres had shedding detected from Days 23 to 28 and from Days 28 to 42, respectively. Although schedules of sampling urine in these three trials were different due to study design, results suggested that leptospiruria may occur earlier and maintained longer in animals exposed to serovar Pomona than those exposed to serovar Hardjobovis. However, it should be noted that this conclusion was made based on results from a limited number of samples collected from a small number of animals (especially in Trial A and D). Also, the age of sheep in Trial C (6.8-months-old) was different from that of sheep in Trial A (18-months-old) and cattle in Trial D (19-months-old), which may also impact on the different shedding patterns found.

Test results from this study showed that leptospires can be detected in sheep kidney 42 days (the day of sacrifice) after the exposure to *Leptospira* serovars Hardjobovis and Pomona. Compared with another sheep trial with Pomona, in which leptospires were detected in kidney no later than 37 days (Morse *et al.* 1957), renal carrier state was detected at a later stage post-infection in Trial C. No data of kidney colonisation on comparable sampling days were found in previous sheep trials with Hardjobovis. The determination of duration of renal carrier state requires kidney samples collected in various stages past infection, and thus cannot be concluded based on the findings from this study.

The blood test results obtained from Trial C provided information of leptospiraemia among sheep infected with serovar Pomona. Test results indicated that Pomona can be detected in bloodstream from Day 3 to 7 after exposure. Compared with another trial on sheep with same serovar (Pomona), in which leptospires were isolated from blood from Day 5 to 8 (Morse *et al.* 1957), leptospires were first detected in blood in Trial C earlier. Sampling of blood between Day 7 and 14 would have provided more information about the duration of leptospiraemia among the challenged sheep, however, this was not conducted due to the limitation of study design.

The immune responses observed in this study provided information about the time taken for sero-conversion and antibody to reach maximum levels in sheep and cattle, after the exposure to Hardjobovis and/or Pomona. Duration of MAT titres need to be

investigated with more sampling days, beyond those involved in this study; however, based on the study findings, detectable MAT titres can be obtained at least one month after infection. Based on the immune responses reported in Trial B, antibodies can be detected as early as Day 8 in lambs exposed to Hardjobovis, with maximum titres obtained on Day 12 (Figure 3.2). This was similar to another experimental trial on lambs with serovar Hardjobovis, which reported detectable antibodies as early as Day 7, with maximum titres obtained on Day 14 (Hathaway and Marshall 1979). Antibodies were first detected on Day 5 in Trial C, similar to Day 4 to 7 reported in a previous lamb experimental trial with serovar Pomona (Hodges 1974). However, maximum titre was recorded on Day 7 in Trial C, earlier than Day 10 reported by Hodges (1974). According to the results from this study and previous trials (Hodges 1974; Hathaway and Marshall 1979), in general, lambs challenged with serovar Pomona develop immune response and have maximum MAT titres attained more quickly than those infected with serovar Hardjobovis. The different sampling schedule for Trial B and C made the direct comparison of last day of detectable antibodies between sheep exposed to serovars Hardjobovis and sheep exposed to Pomona not possible. However, antibody titres were still detectable in all sheep in Trial B on Day 37 whereas they were observed only from 13% animals (2/16) in Trial C on Day 42. This indicated that antibodies may remain detectable longer amongst sheep exposed to serovar Hardjobovis than those exposed to serovar Pomona.

Compared with a previous cattle trial with Hardjobovis, in which MAT titres were first detected on Day 3 to 7 and attained maximum mostly on Day 10 to 14 (Cousins *et al.* 1985), antibodies against serovar Hardjobovis in Trial D were first detected and reached maximum later, on Day 9 and Day 20 (Table 3.5), respectively. The duration of MAT titres was shorter in Trial D (titres were not detected on Day 42 for all three cattle) than the cattle trial reported by Cousins *et al.* (1985), in which titres were detectable 12 weeks (84 days) after inoculation. Although a similar concentration of leptospires was used for challenge in Trial D and the cattle trial reported by Cousins *et al.* (1985), the different inoculating strains, inoculation routes (nasal and conjunctival versus intramuscular), and the different age of cattle (19 months old versus seven to ten months old) may explain the different immune response detected in two trials. For Trial D, results were obtained from limited number of animals (three). Therefore, any conclusion about when antibodies become detectable and attain maximum level after Hardjobovis

infection in cattle should be made with caution and further research with a larger number of animals need to be carried out to confirm the findings.

The clinical observations from this study may contribute to the understanding of clinical symptoms of Hardjobovis and Pomona infection in sheep, and Hardjobovis infection in cattle. Although anorexia, depression, haemoglobinuria, and slight jaundice of the conjunctival mucus were reported in previous sheep trials with serovar Pomona (Morse *et al.* 1957; Hodges 1974), no abnormal clinical features were found in the sheep from Trial C. For Trial B, an average weight loss of 5.2 kg was observed in five of eight sheep (except B4 died on Day 15 and not weighed on the day of sacrifice; range from 1.5 to 8 kg). No information of weight loss was found in previous sheep trials with serovar Hardjobovis to compare with this finding. For Trial D, the drop of rectal temperature in cattle from Day 0 to 7 may be due to the cold environment (the cattle were grazed on pasture *ad-libitum* outside), or the cattle were more stressed on Day 0, and gradually adapted to the handling procedure on subsequent days. Therefore, the rectal temperature detected during this period may not truly indicate a febrile status. Temperature elevation was observed from D1, 2 and 3 (inoculated with serovar Hardjobovis) on Day 9 and 14, which was in contrast with another cattle trial with servoar Hardjobovis which reported no clinical signs (Cousins *et al.* 1985).

In this study, the sampling schedule was different for each trial; especially in Trial A, samples were collected from fewer sampling days than the other trials because this trial was designed primarily to select a strain that may successfully infect the animals and be used for challenges in the future (e.g. MU K4-12, passaged through sheep A2 from Trial A was used to challenge sheep from Trial B). Also, animals of a different age were used. If consistent sampling schedules were used in all trials among animals with similar age, results from this study would have provided a better comparison of infections with different serovars, and among different animal species.

The data obtained in this study were from challenged animals that were inoculated with large doses of leptospires, rather than naturally-infected animals that are usually expose to a relatively smaller dose of leptospires (Faine *et al.* 1999). Therefore, the immune and bacteriological responses in this study may be different from those detected in naturally infected animals to some extent. In this study, samples were collected under carefully controlled conditions (especially for sheep trials that were conducted indoors)

and sent to the laboratory for testing without much delay. Therefore, test results obtained from field samples that are often collected under less than optimal conditions and sent to laboratory with delays, may be different (especially for culture, due to contamination and reduced number of viable leptospires). While comparing results from this study with those from previous published challenges, it should be noted that different concentrations of *Leptospira* inoculation and the methods used may have an effect on the results obtained. Readings of some of the MAT, culture and DFM results (Trial A and C) were carried out in different laboratories (by HLRL, NZVP, or Gribbles). Therefore, the inter-laboratory differences may have had an impact on the precision of the test results. However, the staff from NZVP had been trained at HLRL before performing the diagnostic tests, hence the results obtained from both laboratories should be reasonably consistent. An inter-laboratory comparison of MAT between HLRL and Gribbles showed almost perfect agreement for serovar Hardjobovis tests among sheep (data presented in Chapter 5), and indicated that the MAT titres reported by Gribbles on Day 19 for A2 and A4 can be used to stand for the readings by HLRL.

In this study, the systemic immune and bacteriological responses to *Leptospira* serovars Hardjobovis and Pomona in sheep and cattle were examined by both conventional tests (MAT, culture and DFM) and qPCR, adding to our understanding of leptospiral infection with these two serovars in sheep and cattle. Although limited data were obtained from sheep with Hardjobovis infection and none from cattle with Pomona infection, the results obtained indicated that the qPCR can be used to detect leptospires in blood at an early stage of infection, and in urine or kidney at the shedding and colonisation stages. This can benefit decisions about choice of diagnostic tests, and future research into leptospires in animals and potentially in humans.

5 Acknowledgements

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Chapter 4 Shedding and seroprevalence of pathogenic leptospires in sheep and cattle at a New Zealand abattoir

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1 Summary

A cross-sectional study was carried out on sheep and cattle slaughtered at a New Zealand abattoir from September to November 2010 to investigate the supplier-specific shedding rate, renal colonisation rate, and seroprevalence of leptospires. In the 2008/09 season this abattoir experienced three human leptospirosis cases from 20 staff, of which two were hospitalised. Urine, kidney and blood samples were collected from carcasses of 399 sheep (six suppliers, 17 slaughter lines) and 146 cattle (three suppliers, 22 slaughter lines). The urine and kidney samples were tested by quantitative real-time PCR (qPCR), while serum samples (from coagulated blood samples) were tested by microscopic agglutination test (MAT). In total, 27% (73/274; 95% CI: 18%-37%) of urine samples tested positive by qPCR. Species specific shedding rates (prevalence of positive urine qPCR) were 31% (95% CI: 17%-48%) for sheep and 21% (95% CI: 14%-30%) for cattle. For 545 kidney samples tested, 145 were qPCR positive (27%; 95% CI: 17%-39%). The average prevalence of kidney qPCR positivity was 29% (95% CI: 17%-45%) for sheep and 21% (95% CI: 15%-28%) for cattle. Three hundred and thirty of 542 animals had antibodies against *L. borgpetersenii* serovar Hardjobovis (Hardjobovis) and/or *L. interrogans* serovar Pomona (Pomona), based on MAT titre ≥ 48 (overall seroprevalence of 61%; 95% CI: 48%-73%). Seroprevalence was 57% (95% CI: 40%-72%) for sheep and 73% (95% CI: 59%-83%) for cattle. Among the seropositive animals, 41% (70/170; 95% CI: 30%-54%) were shedding (tested positive by urine qPCR) and 42% (137/330; 95% CI: 30%-54%) had renal colonisation (tested positive by kidney qPCR). Risk management options for abattoirs or farms to prevent human leptospirosis infections include the application of urine qPCR to detect shedding status of stock prior to the process of slaughtering, vaccination of maintenance hosts, and the use of personal protective equipment.

2 Impacts

- The findings of this study demonstrate that the shedding rates of sheep and cattle entering slaughter premises in New Zealand and the potential risk of acquiring leptospirosis infection for workers contact with the un-vaccinated livestock can be high.
- This is the first time that in addition to seroprevalence (by MAT), shedding and carrier status of leptospires was detected in slaughtered sheep and cattle (by real-time PCR).
- The association between the results of commonly used diagnosis test (MAT) and qPCR are presented, and show that the risk of shedding in Hardjobovis-positive sheep was 12.5 (95 % CI: 4.8-32.6) times the risk of that in the Hardjobovis-negative sheep for this study.

3 Introduction

Leptospirosis is a zoonotic disease of worldwide importance caused by pathogenic spirochaetes belonging to the genus *Leptospira* (Levett, 2001; Bharti et al., 2003; Dutta and Christopher, 2005). Feral and domestic mammals can be maintenance hosts to various serovars (Plank and Dean, 2000; Levett, 2001). Leptospires colonise the renal proximal tubules of carrier animals, and may persist and be shed in the urine without causing apparent illness (Faine et al., 1999; Bharti et al., 2003; Dutta et al., 2005). Humans usually serve as an incidental host and the infection occurs through direct contact with infected animal urine, or indirect contact with contaminated soil or water (Faine et al., 1999; Bharti et al., 2003).

Compared with other temperate developed countries, leptospirosis occurs at a higher frequency in New Zealand (Thornley et al., 2002; Hartskeerl et al., 2011). From 2008 to 2011 in New Zealand, the average annual notification rate was 2.0 per 100 000. Among cases with hospitalisation status recorded, around half were admitted to hospitals (Anonymous, 2009-2012a). In New Zealand, leptospirosis is the most common occupationally-acquired zoonotic disease (Vickery et al., 2006; Keenan, 2007). From 2008 to 2011, 77.3% of the notified cases with occupation records were engaged in occupations previously identified as at high risk of exposure to *Leptospira* spp. in New

Zealand (Thornley et al., 2002). Among these, meat workers and farmers constituted the highest incidence, as 22.4% and 74.1% of the cases, respectively (Anonymous, 2009-2012a). In New Zealand leptospirosis usually occurs as sporadic cases but occasionally as outbreaks (Anonymous, 2006) in occupational settings. From 2008 to 2011, there were four leptospirosis outbreaks reported in 2008 and two in 2010 involving 20 and five cases, respectively (Anonymous, 2009-2012b). The outbreaks were all from farm or abattoir settings with exposure to infected animals or carcasses either confirmed or suspected as the source of infection (Anonymous, 2009-2012b).

Cattle and sheep are important animal reservoirs of leptospirosis in New Zealand (Vickery et al., 2006). Previous surveys of farms and abattoirs have shown that 69% (59/85) of beef cattle herds and 44% (42/95) of sheep slaughter lines were seropositive for leptospirosis (Heuer et al. 2007; Dorjee et al. 2008). A sheep abattoir survey reported that 1.3% (13/1000) of slaughtered sheep were kidney culture-positive, which indicates renal colonisation and potential shedding of leptospires in the urine (Dorjee et al., 2008). In the same abattoir, estimated median daily exposures for meat workers were reported to range from 11 to 54 (depending on the working position) kidney culture-positive carcasses per day during high risk season (May to November) and three to 18 during low risk periods (Dorjee et al., 2011). Serological surveys covering eight abattoirs (sheep, beef and deer) in New Zealand reported an average seroprevalence of leptospirosis as 11% (range from 5 to 31%) in meat workers (Benschop et al., 2009; Dreyfus, 2013). These findings suggest significant exposure to leptospiroses in slaughter-plants for meat workers in New Zealand.

This paper reports the supplier-specific urinary shedding rate, renal colonisation rate, and seroprevalence of leptospires in sheep and cattle slaughtered in an abattoir in the Waikato region. This abattoir experienced three human leptospirosis cases (two hospitalised) from 20 staff in the 2008/09 killing season.

4 Materials and Methods

4.1 Study design

A cross-sectional study was conducted from 27th September to 24th November 2010. Sample sizes were calculated for infection prevalence estimates based on simple

random sampling, given an expected seroprevalence of 44% for sheep slaughter lines (Dorjee et al. 2008) and 69% for cattle slaughter lines (Heuer et al., 2007). To estimate a line prevalence of 44% for sheep and 69% for cattle, 17 and 14 lines were required from sheep and cattle, respectively, for a 90% confidence interval and 20% precision. The carcass prevalence was estimated to be 6% for lambs and 39% for cattle (Heuer et al., 2007; Dorjee et al., 2008). To estimate a prevalence of 6% with a 90% confidence interval of 3% precision, 170 sheep carcasses were required under simple random sampling. The number was doubled to account for some degree of intra-line correlation. The total number of cattle carcasses required to achieve a 90% confidence interval of 10% around the expected 39% prevalence, an intra-line correlation adjusted sample size was 138 carcasses. To achieve at least 80% power of detecting a minimum of 5% prevalence within line, 22 carcasses were sampled from a line of ≤ 30, and 30 from a line of > 30 sheep. For beef carcasses, all carcasses present in a line were sampled for adequate power of detection, due to the small number of carcasses processed at this abattoir in a single line. Several lines per supplier of origin were sampled until the specified total sample size was reached.

4.2 Recruitment of suppliers

The study abattoir is located in the Waikato region and kills approximately 250 to 300 sheep and 70 to 92 beef a day. An information sheet and a consent form were sent by the abattoir manager to all suppliers (n =7) inviting them to participate in the study. Written consent was received from suppliers before the study commenced, allowing samples to be taken and the results to be released to the plant manager and suppliers. Six of seven suppliers consented to participate in the study. All suppliers were identified by supplier ID and kept anonymous to the researchers by coding of samples by staff collecting the samples. Supplier IDs were assigned by the species of animals sent for slaughter. Supplier A, C and F were sheep-only suppliers; supplier BG (B for sheep line and G for cattle line), DH (D for sheep line and H for cattle line) and EI (E for sheep line and I for cattle line) were mixed-species suppliers. Vaccination of stock was not used by any supplier before this study started.

4.3 Sample collection and testing

Samples were collected by trained staff wearing personal protective equipment (PPE). Collection and processing of samples had been performed to avoid cross-contamination as much as possible. For each selected carcass, duplicate urine and blood (coagulated) samples, and kidney samples were taken. The coagulated blood samples were centrifuged shortly after collection at 1300 *g* for 10 minutes at the AgResearch Ltd. Laboratory (Hamilton, New Zealand), and the serum were collected. All samples were sent under refrigerated conditions to a commercial veterinary diagnostic laboratory on the day of collection. Duplicate serum and urine samples and kidney samples were subsequently sent to the Hopkirk Leptospirosis Research Laboratory (HLRL). The inter-laboratory test comparison on duplicate serum and urine samples will be reported in Chapter 5. In this study, only test results from HLRL were reported and analysed.

Urine and kidney samples were processed upon receipt at HLRL (usually the day following collection). 1.2 ml of urine was added into a 1.5 ml Eppendorf tube (Raylab, Auckland, New Zealand), and then centrifuged at 10625 *g* for 20 minutes. Supernatant was discarded by a transfer pipette (Raylab) and 200 µl of PBS was used to re-suspend the pellet. This mixture was used to extract DNA using the QIAamp DNA Mini Kit (Qiagen, Bio-Strategy Ltd., Auckland, New Zealand) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl. Kidney surfaces were sterilised by swabbing with 70% ethyl alcohol. A 10 g section of kidney, extending from the renal cortex to the medulla, was removed aseptically. The removed section was doused with 70% ethyl alcohol (using funnel and flask), followed by flaming with a Bunsen burner. It was then homogenised in 50 ml PBS, using the Colworth Stomacher 400 (AJ Seward Ltd, London, UK). A 160 µl aliquot of this suspension was used to extract DNA, using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl.

A quantitative real-time PCR (qPCR) assay was modified from that described by Subharat et al. (2011) to suit the Rotor-Gene Q machine (Qiagen) and applied to test urine and kidney samples for leptospial DNA. Primers 2For (5'-TGAGCCAAGAAGAAACAAGCTACA-3') and 504Rev (5'-MATGGTTCCRCTTTCCGAAGA-3') were used to amplify the DNA gyrase subunit B (*gyr*B) gene (Subharat et al., 2011). The 25 µl reaction mix included 2.5 µM SYTO9

(Invitrogen, Carlsbad, CA, USA), 1×PCR buffer, 1.5 mM magnesium chloride ($MgCl_2$), 200 µM dNTPs, 5 pmol of 2For and 504Rev, one unit of *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA), 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 s, 63°C for 20 s and extension at 72°C for 20 s. 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. Positive controls were *L. borgpetersenii* serovar Hardjobovis (Hardjobovis) and *L. interrogans* serovar Pomona (Pomona) (HLRL laboratory strains), and ddH_2O was used as negative control. Samples were considered positive, if a similar melting temperature as positive controls was recorded.

Serum samples were tested by the microscopic agglutination test (MAT) against Hardjobovis and Pomona at HLRL, based on the standard procedure described by Faine (1982). These two serovars were chosen because serological evidence of leptospirosis in domestic livestock is most commonly found with serovars Hardjobovis and Pomona in New Zealand (Dreyfus *et al.* 2011). A titre of ≥ 48 was considered positive for both serovars, based on previous studies of leptospirosis in sheep and cattle (Blackmore et al. 1982; Suwimonteerabutr et al. 2005). The end-point titre was the lowest dilution at which approximately 50% of the organisms were agglutinated or lysed.

4.4 Statistical analysis

Data were categorised by animal species (sheep or cattle) and suppliers. The 95% confidence interval (95% CI) of the prevalence (qPCR or MAT results) was adjusted for the effect of correlation between animals within slaughter line using generalized estimating equations (GEE) (Liang and Zeger, 1986) and robust standard errors. The GENMOD procedure in SAS 9.3 was used for this purpose (SAS Institute Inc., Cary, NC, USA) with data aggregated at slaughter line level and line-ID being the subject effect (Smith and Smith, 2006).

The prevalence of shedding/renal colonisation (detected by urine/kidney qPCR) in seropositive and seronegative animals were presented. The prevalence ratio was evaluated using logistic regression to determine the association between urinary shedding/carrier status of seropositive versus seronegative sheep and cattle. The 95% CI

of the prevalence and prevalence ratio was adjusted for the effect of correlation between animals within slaughter line as stated above using GEE and robust standard errors. The logistic model was modified to obtain the prevalence ratio with confidence intervals instead of an odds ratio, by using a poisson distribution with a log-link as described (Zou, 2004).

5 Results

Samples were collected from carcasses of 399 sheep (six suppliers, 17 slaughter lines) and 146 cattle (three suppliers, 22 slaughter lines) as described in Table 4.1. The majority of sheep were lambs (less than one year of age) (78%, 95% CI: 74%-82%), followed by hoggets (between one and two years old) (12%, 95% CI: 9%-15%) and mixed-age sheep (two-years and older) (11%, 95% CI: 8%-14%). Cattle were primarily young stock up to 18 months of age (80%, 95% CI: 72%-85%); the remainder were adults (21%, 95% CI: 15%-28%). Kidney samples were collected from all animals (545) while 542 blood samples were collected. Urine samples were collected from 156 sheep (39% of all sheep) and 118 cattle (81% of all cattle).

Table 4.1 Number of sheep and cattle sampled from each supplier (sample size), number of samples tested (N) by urine, kidney qPCR and MAT, number of samples tested positive (N pos), and percentage positive with 95% confidence intervals (CI) adjusted for the effect of correlation of animals within slaughter line.

Species	Supplier		Sample size	Urine qPCR			Kidney qPCR			MAT (Hardjobovis or Pomona)		
				N	N pos	% (95% CI)	N	N pos	% (95% CI)	N	N pos	% (95% CI)
Sheep[a]	Sheep only	A	77	43	19	44 (15-78)	77	25	32 (16-56)	77	56	73 (70-76)
		C	60	17	6	35 (3-90)	60	25	42 (6-88)	60	32	53 (8-94)
		F	90	41	2	5 (2-10)	90	4	4 (2-12)	90	35	39 (13-73)
	Mixed sheep and cattle	BG	40	13	9	69 (51-83)	40	27	68 (64-71)	39	37	95 (88-98)
		DH	42	12	8	67 (50-80)	42	27	64 (54-73)	40	28	70 (63-76)
		EI	90	30	4	13 (6-27)	90	7	8 (3-20)	90	36	40 (19-65)
		Total	399	156	48	31 (17-48)	399	115	29 (17-45)	396	224	57 (40-72)
Cattle[b]	Mixed sheep and cattle	BG	30	16	5	31 (11-63)	30	6	20 (9-40)	30	25	83 (63-94)
		DH	22	19	1	5 (1-27)	22	3	14 (4-37)	22	16	73 (51-87)
		EI	94	83	19	23 (15-33)	94	21	22 (15-32)	94	65	69 (50-83)
		Total	146	118	25	21 (14-30)	146	30	21 (15-28)	146	106	73 (59-83)

[a] Line sizes ranged from 11-39 for 17 sheep lines from six suppliers
[b] Line sizes ranged from 1-17 for 22 cattle lines from three suppliers
A, C and F: sheep-only suppliers; BG, DH and EI: mixed-species suppliers

5.1 Urine qPCR

In total, 27% (95% CI: 18%-37%) of urine samples were positive by qPCR. The average shedding rate (prevalence of positive urine qPCR) was 31% (95% CI: 17%-48%) for sheep and 21% (95% CI: 14%-30%) for cattle, with a within-supplier range of 5%-69% for sheep and 5%-31% for cattle (Table 4.1). The three sheep suppliers with the highest shedding rates, in decreasing order, were supplier B, D and A; B and D belong to mixed-species suppliers (BG and DH). In general sheep were shedding at a higher rate than cattle.

5.2 Kidney qPCR

Of 545 kidney samples tested by qPCR, 145 were PCR positive (27%, 95% CI: 17%-39%). The average prevalence of kidney qPCR positivity was 29% (95% CI: 17%-45%) for sheep (within-supplier range 4%-68%) and 21% (95% CI: 15%-28%) for cattle

(within-supplier range 14%-22%) (Table 4.1). The three sheep suppliers with the highest rates of positive kidney qPCR results, in decreasing order, were suppliers B, D and C; B and D come from mixed-species suppliers. In general, renal colonisation rate was higher in sheep than cattle.

5.3 MAT

Three hundred and thirty of 542 animals had antibodies to either serovar Hardjobovis or Pomona based on MAT titre ≥ 48 (overall seroprevalence of 61%; 95% CI: 48%-73%). Seroprevalence was 57% (224/396; 95% CI: 40%-72%) for sheep (within-supplier range 39%-95%) and 73% (106/146; 95% CI: 59%-83%) for cattle (within-supplier range 59%-83%) (Table 4.1). Of 396 sheep, 21% (95% CI: 18-26%) were seropositive against Hardjobovis-only, 20% (95% CI: 16-24%) against Pomona-only and 15% (95% CI: 12-19%) against both serovars. Of 146 cattle, 47% (95% CI: 39-55%) were seropositive against Hardjobovis-only, 14% (95% CI: 10-21%) against Pomona-only and 11% (95% CI: 7-17%) against both serovars. For both sheep and cattle samples, seroprevalence of Hardjobovis was higher than Pomona (Figure 4.1).

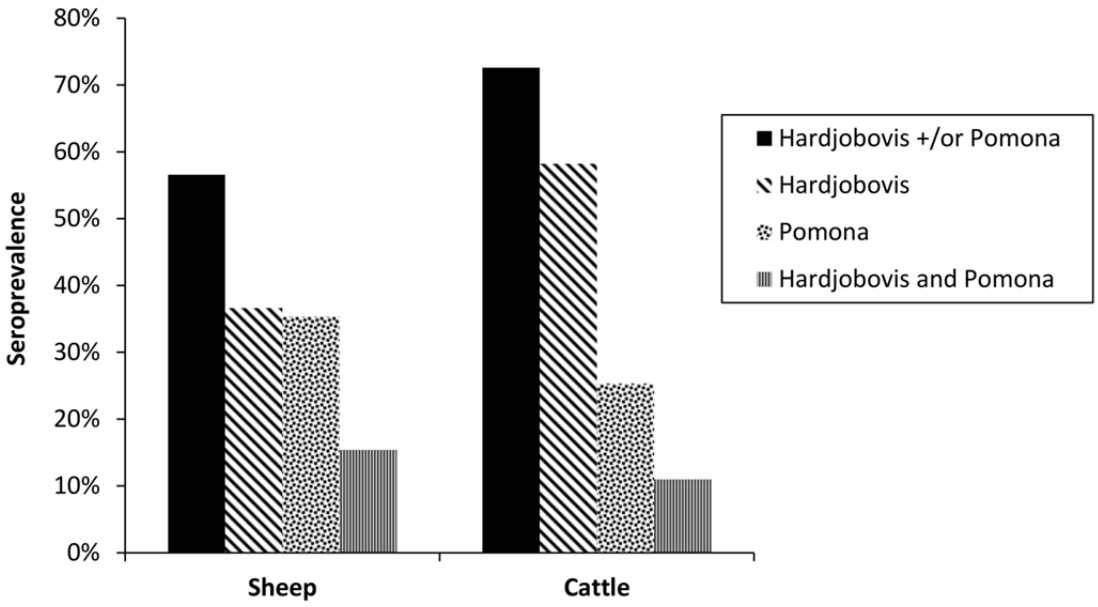

Figure 4.1 Seroprevalence of serum samples with positive MAT titre (≥ 48) from sheep and cattle by serovar.

The distribution of MAT titres to serovars Hardjobovis and Pomona among sheep and cattle is presented in Figure 4.2. For sheep and cattle samples that had positive MAT

titres (≥ 48) to serovar Hardjobovis, 97.2% and 94.1% of them had MAT titres ≥ 96 (in general an agglutinating titre of ≥ 96 is considered significant for the diagnosis of leptospiral infection (Grooms and Bolin 2005)); while for sheep and cattle samples that had positive MAT titres (≥ 48) to serovar Pomona, 76.4% and 56.8% had MAT titres ≥ 96.

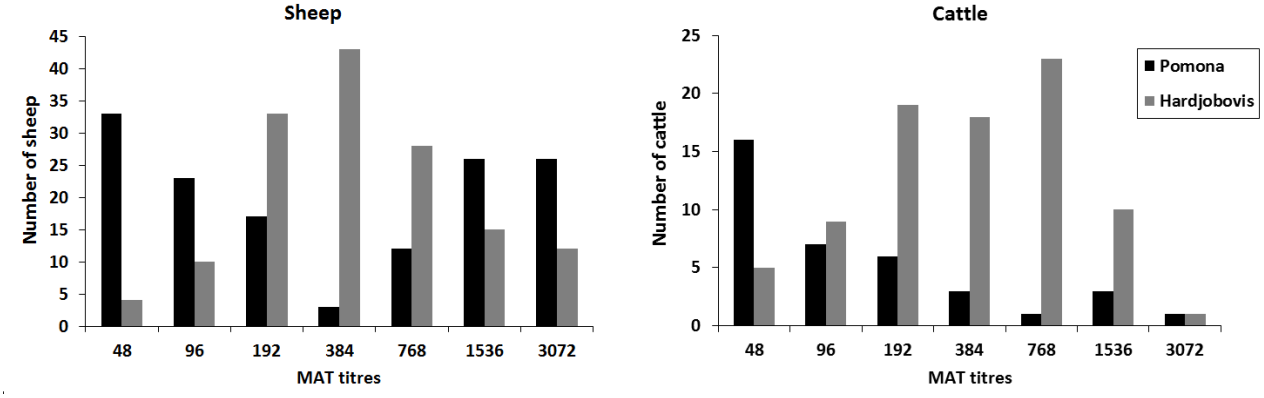

Figure 4.2 Frequency histogram of positive MAT titres (≥ 48) to serovars Pomona and Hardjobovis in sheep and cattle.

The three sheep suppliers with the highest seroprevalence (had antibodies to serovar Hardjobovis, and/or serovar Pomona), in decreasing order, were suppliers B, A and D; B and D come from mixed-species suppliers (Table 4.1). In general similar seroprevalence was found in sheep and cattle.

5.4 Urinary shedding and renal colonisation in seropositive and seronegative animals

The numbers of sheep and cattle which had positive MAT titres (≥ 48) and negative MAT titres (< 48) and also tested by urine and kidney qPCR, numbers of samples tested positive and positive rates are presented in Table 4.2. In general, the urinary shedding (tested positive by urine qPCR) and renal colonisation rates (tested positive by kidney qPCR) were higher in sheep carcasses than that in cattle carcasses, and higher in carcasses seropositive to serovar Hardjobovis than that in carcasses seropositive to serovar Pomona.

Table 4.2 Number of sheep and cattle which had positive MAT titres (≥ 48) (seropositive) and negative MAT titres (< 48) against serovar Hardjobovis and/or Pomona, Hardjobovis-only and Pomona-only and had urine or kidney samples tested by qPCR (N), number of samples tested positive by urine and kidney qPCR (N pos) and percentage positive with 95% confidence intervals (CI) adjusted for the effect of correlation of animals within slaughter line.

Type of qPCR	Species		Hardjobovis and/or Pomona			Hardjobovis-only			Pomona-only		
			N	N pos	% (95% CI)	N	N pos	% (95% CI)	N	N pos	% (95% CI)
Urine	Sheep and cattle	Seropositive	170	70	41 (30-54)	89	44	49 (36-63)	47	7	15 (6-32)
		Seronegative	104	3	3 (1-9)	151	10	7 (3-14)	193	47	24 (16-36)
	Sheep	Seropositive	85	46	54 (32-75)	33	27	82 (65-92)	29	3	10 (4-24)
		Seronegative	71	2	3 (1-11)	100	5	5 (2-12)	104	29	28 (14-48)
	Cattle	Seropositive	85	24	28 (20-38)	56	17	30 (20-44)	18	4	22 (7-52)
		Seronegative	33	1	3 (0-19)	51	5	10 (3-28)	89	18	20 (13-30)
Kidney	Sheep and cattle	Seropositive	330	137	42 (30-54)	153	79	52 (39-64)	100	13	13 (7-23)
		Seronegative	212	7	3 (1-8)	312	20	6 (3-12)	365	86	24 (15-35)
	Sheep	Seropositive	224	111	50 (33-66)	84	59	70 (58-81)	79	9	11 (6-22)
		Seronegative	172	3	2 (1-6)	251	12	5 (2-11)	256	62	24 (13-41)
	Cattle	Seropositive	106	26	25 (17-34)	69	20	29 (20-40)	21	4	19 (6-48)
		Seronegative	40	4	10 (4-25)	61	8	13 (6-27)	109	24	22 (16-30)

For sheep, the prevalence of urinary shedding in Hardjobovis-seropositive carcasses was 12.5 (95% CI: 4.8-32.6) times the prevalence in the Hardjobovis-seronegative carcasses, and the prevalence in Pomona-seropositive carcasses was 0.6 (95% CI: 0.3-1.3) times the prevalence of urinary shedding in the Pomona-seronegative carcasses. For cattle, the prevalence of urinary shedding in Hardjobovis-seropositive carcasses was 3.4 (95% CI: 0.9-13.2) times the prevalence in the Hardjobovis-seronegative carcasses, and the prevalence in Pomona-seropositive carcasses was 1.1 (95% CI: 0.4-3.4) times the prevalence of urinary shedding in the Pomona-seronegative carcasses.

For sheep, the prevalence of renal carriage in Hardjobovis-seropositive carcasses was 8.6 (95% CI: 5.6-13.3) times the prevalence in the Hardjobovis-seronegative carcasses, and the prevalence in Pomona-seropositive carcasses was 0.7 (95% CI: 0.6-0.9) times the prevalence of renal colonisation in the Pomona-seronegative carcasses. For cattle, the prevalence in Hardjobovis-seropositive carcasses was 2.2 (95% CI: 0.9-5.5) times the prevalence of renal colonisation in the Hardjobovis-seronegative carcasses, while

the prevalence in Pomona-seropositive carcasses was 0.9 (95% CI: 0.3-2.8) times the prevalence of renal colonisation in the Pomona-seronegative carcasses.

6 Discussion

This study reports the urinary shedding rate and carrier status (determined by urine and kidney qPCR) and also seroprevalence (determined by MAT) in animals slaughtered at a local abattoir. Compared to a cohort study covering eight abattoirs (sheep, beef and deer) in New Zealand that reported an annual incidence of severe clinical leptospirosis of 0.8% (3/384) among sheep abattoir workers (Dreyfus, 2013), a higher incidence rate of leptospirosis cases (15%, 3/20) was reported in the meat workers from this abattoir during the 2008/09 killing season. Hence, a higher than average rate of human exposure was expected from carcasses processed at this abattoir.

To the best of the authors' knowledge, this is the first time that multiple tests on multiple substrates have been applied to detect evidence of leptospirosis infection in slaughtered sheep and cattle. The animal-level seroprevalence found in sheep (57%, predominately lamb) and cattle (73%) was substantially higher than in previous studies of 6% in lamb carcasses (Dorjee et al., 2008) and 39% in cattle carcasses (Heuer et al., 2007). For sheep and cattle that were seropositive (MAT titres ≥ 48) against serovar Hardjobovis and Pomona, more than 95% and 67%, respectively, had MAT titres ≥ 96 which was considered significant for the diagnosis of leptospirosis infection (Grooms et al., 2005), whereas 48 was considered to indicate exposure (Blackmore et al., 1982). Among the seropositive animals, 41% and 42% had leptospires detected in urine (indicating shedding) and kidney (indicating carrier status) by qPCR, respectively. These and the reported shedding rate (27%, detected by urine qPCR) and carrier status (27%, detected by kidney qPCR) raise occupational health concerns that meat workers from this abattoir may be at risk of exposure to leptospires during their daily work routine.

In general the urinary shedding rates and carrier status were higher in sheep than cattle (Table 4.1). This may explain the findings of Dreyfus (2013) that the seroprevalence was significantly higher in workers in sheep than in cattle plants (Dreyfus, 2013), and indicated that the workers having more exposure to sheep than cattle may have higher

risk of exposure to leptospires. The association between the results of commonly used diagnosis test (MAT) and urine/kidney qPCR provided useful information for perceiving shedding/carrier status in sheep and cattle that tested positive by MAT. In general, seropositive sheep were more likely to be shedding, compared to seropositive cattle; while shedding were more likely to be found in sheep seropositive to serovar Hardjobovis than those seropositive to serovar Pomona.

Protection of meat workers against leptospirosis has focussed on the use of personal protective gear (PPE) such as goggles, face masks and gloves (Keenan, 2007). However there are issues with compliance, for example, with face masks meat workers report restricted vision, over-heating around the face and head, and the weight of the device impeding their movement (Keenan, 2007). In the cross-sectional study of 567 meat workers in New Zealand, in which seroprevalence was 10.9% (Dreyfus, 2013), the strongest risk factor among sheep and deer meat workers was 'work position', whereas wearing PPE was not found as a protective factor against leptospirosis infection. Another approach to protect meat workers against leptospirosis is to vaccinate maintenance hosts (Marshall and Manktelow, 2002). Although this does not fully prevent shedding of leptospires from already infected animals, reduction of shedding can be achieved (Ayanegui-Alcerreca et al., 2007). If vaccination is administered to animals prior to infection and combined with effective strategies for farm biosecurity, good animal husbandry, rodent control and management of other sources of new infection, immunity will be provided in most cases (Keenan, 2007). Vaccination of cattle has been considered as an efficient way of preventing leptospirosis infection (Marshall, 1987), whereas the efficacy in sheep has not been established but is the subject of current work within the Massey leptospirosis research group. However, in New Zealand only dairy cattle and pigs are routinely vaccinated. Approximately 10% of beef herds and 9% of deer herds practice vaccination for leptospirosis (Hodges and Day, 1987; Keenan, 2007; Wilson et al., 2008). Vaccination of sheep is rare (Dorjee et al., 2008).

A third and novel approach to the protection of meat workers is to identify lines/suppliers of stock that are actively shedding leptospires and take action accordingly (Keenan, 2007). For example, lines with a high proportion of shedding carcasses can be processed at the end of a shift, and the workers are warned to take particular care (Brown, 2005). Rapid field tests that do not require special laboratory

equipment and trained personnel have been developed for detecting *Leptospir*a-specific immunoglobulin M antibodies in human or animal sera (Gussenhoven et al., 1997; Ramadass et al., 1999; Smits et al., 2000b; Smits et al., 2001). However, the use of these tests to screen pre-slaughter stock would likely be hampered by false positive or negative reactions, especially for animals at early stage of infection or animals that have been vaccinated (Smits et al., 2000a; O'Keefe, 2002; Sehgal et al., 2003). In practice, identifying lines of shedding stock on the day of slaughtering requires an accurate and rapid animal-side test on urine samples, which is yet to be developed. In this study, the average number of urine samples collected from different slaughter lines was small, ranged from four to 14 for sheep suppliers and three to 10 for cattle suppliers. The variations of shedding rates between different slaughtering lines/days from the same supplier were large (data not shown here). Shedding rates determined by our urine qPCR based on small line sizes may not be reliable enough to show line-specific shedding stocks. Therefore, this paper investigated the utility of qPCR to identify suppliers of shedding stocks, and the risk of exposure to leptospires for meat workers as a tool for surveillance and alert for the workers, rather than the daily practice.

Quantitative real-time PCRs (qPCRs) on urine and kidney samples were performed in this study in addition to the commonly used MAT. Sheep and cattle infected with leptospires usually do not show any clinical signs, but may develop persistent infection with organisms colonising the renal proximal tubules leading to permanent or intermittent excretion of the organisms in urine (Gerritsen et al., 1994; Magajevski et al., 2005). This may cause the spread of infection to other susceptible animals and eventually to human populations at risk (Çetinkaya et al., 2000). Therefore, detecting leptospires in kidney or urine is important for the identification of carrier animals as a source of human infections (Çetinkaya et al., 2000; Lilenbaum et al., 2009). The MAT is the standard serological test for the diagnosis of leptospirosis (Faine et al., 1999; Lilenbaum et al., 2009). However, the assay is time-consuming and requires significant expertise to perform and to interpret the data (Levett, 2001). More importantly, results from the MAT are based on the infected host's response but not the presence of leptospires *per se* and therefore not able to differentiate actual infection from the increased antibodies caused by vaccination (Bomfim et al., 2008) and identify most chronic shedders (Thiermann, 1984). Available techniques for identifying carriers directly are techniques that detect leptospire (e.g. culture) or its DNA (e.g. PCR) in

urine and other tissues. Culture has been well established for diagnosis of leptospirosis through the recovery of bacteria from a variety of samples (Smith et al., 1994; Faine et al., 1999). However, culture is laborious and time consuming (Smith et al., 1994), and often complicated by overgrowth of other bacterial and fungal contaminants (Fearnley et al., 2008), and hence lacks sensitivity. PCR protocols have been reported as reliable and rapid methods for the diagnosis of leptospirosis (Wagenaar et al., 2000; O'Keefe, 2002; Hernández-Rodríguez et al., 2011), and applied to various clinical specimens as bovine urine (Magajevski et al., 2005; Bomfim and Koury, 2006) and urine/vaginal fluids/semen from goats and sheep (Lilenbaum et al., 2008; Lilenbaum et al., 2009). The qPCRs were introduced as techniques that further improved the conventional PCRs by performing faster and reducing the false positive results caused by carry-over contaminations (contamination with products from previous reactions) (Ahmed et al., 2009; Picardeau, 2013). Several qPCR protocols targeting different genes have been developed and are claimed to be specific for pathogenic *Leptospira* and appropriate for diagnostic purposes (Smythe et al., 2002; Palaniappan et al., 2005; Slack et al., 2006; Stoddard et al., 2009). The qPCR test applied in this study was based on the test evaluated by Subharat *et al.* (Subharat et al., 2011) with urine and kidney samples from deer. We validated the utility of this test in sheep and cattle. This study detected leptospires in kidney (by kidney qPCR) in 70% of seropositive sheep. In comparison, Dorjee et al. (2008) detected leptospires in kidney by culture in 21.7% of seropositive sheep (Dorjee et al., 2008). This indicates again the higher sensitivity of PCR compared to culture. However, further work on the detection limit and diagnostic sensitivity and specificity (as previously carried out on deer (Subharat et al., 2011)) is required to completely evaluate the utility of the test on sheep and cattle.

Symptoms reported from the three human leptospirosis cases included fever, headache, rigor, sore back, abdominal pain, diarrhoea, and a macular rash. One case developed meningitis in hospital. Two of the cases were associated with serovar Pomona. Of these two, one also reported low MAT titres for Hardjobovis, Ballum and Canicola which could be due to mixed infection or serological cross-reaction. In New Zealand, the serovars that have been isolated from animals are Pomona from cattle, sheep, pigs, deer and dogs; Hardjobovis from cattle, sheep and deer; Ballum from rats, mice, hedgehogs and secondarily in livestock animals (Marshall et al., 2002; Thornley et al., 2002; Ayanegui-Alcerreca et al., 2007). This is consistent with the proposition that infections

in these meat workers were likely to be occupationally acquired. However, non-work related risk factors, such as farming, home-slaughtering and hunting should be considered because these also involve close contact with possible host animals. Nevertheless, the survey of 567 meat workers from four sheep, two cattle and two deer abattoirs in New Zealand reported that these non-working related risk factors were not significantly associated with seroprevalence of leptospirosis in a multivariable model (Dreyfus, 2013).

The findings from this study not only demonstrate the risk for meat workers, but also for those handling the animals that were sent to this abattoir for slaughtering (e.g. the suppliers and transporters). Farmers and meat workers were reported to be the occupational groups at highest risk of contracting leptospirosis infections in New Zealand from 2001 to 2010, together accounting for more than 77% of the occupations recorded every year. Compared to previous period, in the past three years, farmers accounted for an increasing proportion of notified occupational cases, being more than 56% each year (Keenan, 2007; Anonymous, 2009-2012a).

After the first two weeks of livestock sampling and testing in the nine week study period, results showed recent leptospirosis infection with shedding of leptospires in the urine. The supplier-level seroprevalence ranged from 25% to 90.9%, and 36.8% of the carcasses had MAT titres $\geq$ 1536. Furthermore, the supplier-level rates of positive kidney qPCR ranged from 10% to 70%, and shedding rates as tested by urine qPCR ranged from 5.9% to 57.1%. These interim test results were discussed with the abattoir manager and a decision was made to inform the suppliers and their veterinarians about possible public health concerns. It was recommended that the meat workers and workers from supplier farms were reinforced to wear personal protective gear while working with livestock. Vaccination of stock from supplier farms was also recommended. After the human leptospirosis cases occurred in 2008 and 2009 abattoir management ensured that their employees had an understanding of what leptospirosis is, and how the use of PPE and careful hygiene (hand washing during and after work and before eating and smoking) helps them to protect against the disease. These interventions from management were reinforced after the findings of this study. Currently, no leptospirosis cases have been reported in meat workers in this abattoir since 2010.

Three of the six suppliers were mixed-species suppliers (sheep and cattle) and mixing of animals is expected to increase the risk of infection (Dorjee et al., 2008). Deer herds co- or cross-grazing with infected cattle and sheep flocks had approximately twice the likelihood of being seropositive than deer-only herds (Subharat et al., 2009). The lack of immunity to leptospirosis may also increase the risk of infection. Warm and humid weather conditions in spring provide better survival conditions for leptospires (Levett, 2001). The association between outbreaks of leptospirosis in sheep and rainfall accompanied by surface flooding has been previously reported in New Zealand (Vermunt et al., 1994; Dorjee et al., 2005). For this study, we were told that a sustained period of heavy rain occurred in the six weeks prior to 15th October 2010 (A. Cullum, personal communication, 2010), covering three weeks before sampling time and the first three weeks of sampling.

Samples were collected from one abattoir in the local area for this study, from September to November 2010, a period of relatively high rainfall. Therefore, results reflect only this space-time window and extrapolation beyond this should be done cautiously. Nevertheless, animal-level seroprevalence found in this study is consistent with a survey that sampled 2,308 beef cattle and 33,619 sheep from seven regions of both North and South islands of New Zealand from 2009 to 2010 (Dreyfus et al., 2011). The survey reported a seroprevalence of 50% for adult sheep and 58% for adult beef cattle.

Infections of *Leptospira* spp. in the studied animals may have been due to serovar/s that was not included in the MAT testing panel (e.g. *L. borgpetersenii* serovar Ballum). However, the panel included the two most commonly reported serovars found in New Zealand livestock (Hardjobovis and Pomona). Thus, the findings from this study covered the two serovars presumptively having the highest prevalence in the study population.

The findings from this study demonstrated that urinary shedding/renal colonisation rates of leptospires in livestock entering slaughter premises in New Zealand and the risk of exposure to leptospires for meat workers by contact with unvaccinated sheep and cattle can be high. The shedding status of leptospires in sheep and cattle (in either slaughter lines or farms) can be determined by urine qPCR to alert the meat workers and farm staffs. However, this approach needs to be balanced against the practicalities. The high

urinary shedding/renal colonisation rates found in this study from sheep and cattle reinforced the value of testing the practicality of the approach of vaccinating maintenance host to protect meat workers against leptospirosis in New Zealand.

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Chapter 5 Inter- and between-specimen comparisons of diagnostic tests for leptospirosis in sheep and cattle

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1 Abstract

A study was performed on blood and urine samples to investigate the inter-laboratory test agreements between a research and a commercial veterinary diagnostic laboratory, and on blood, urine and kidney samples (at the research laboratory) to investigate test agreements between specimens for leptospirosis diagnosis. Samples were sourced from 399 sheep and 146 beef cattle from a local abattoir. For all comparisons, better agreement was obtained for sheep than cattle samples. Inter-laboratory quantitative real-time PCR (qPCR) results on urine samples was in almost perfect agreement (kappa = 0.93), despite use of different gene amplification (DNA gyrase subunit B gene vs. 16s rRNA gene), chemistries (SYTO9 vs. TaqMan probe), and pre-PCR processing. The inter-laboratory agreement of microscopic agglutination test (MAT) positivity (titre ≥ 48 for the research laboratory and ≥ 50 for the commercial laboratory) was better for *L. borgpetersenii* serovar Hardjobovis (Hardjobovis) (kappa = 0.94) than *L. interrogans* serovar Pomona (Pomona) (kappa = 0.53). Among animals that had different titres recorded from two laboratories, higher Hardjobovis titres and lower Pomona titres were reported by the commercial laboratory than by the research laboratory ($p < 0.005$). These inter-laboratory comparisons can assist researchers and diagnosticians in interpreting the sometimes discrepant test results. Within the research laboratory, the comparison of qPCR results on urine and kidney showed almost perfect agreement (kappa = 0.84), suggesting that the qPCR on these two specimens can be used interchangeably. The agreement between MAT positivity and urine and kidney qPCR results was fair (kappa = 0.32 and kappa = 0.33, respectively). However, the prevalence ratio of urine and kidney qPCR positivity in Hardjobovis-seropositive versus Hardjobovis-seronegative sheep indicated that Hardjobovis-seropositivity found in sheep may be able to predict shedding/renal colonisation.

Key words: test comparison; leptospirosis; real-time polymerase chain reaction; microscopic agglutination test; sheep; cattle; urine; kidney.

2 Introduction

Leptospirosis, caused by pathogenic spirochetes of the genus *Leptospira,* is a zoonotic disease of global distribution which significantly impacts on public health and livestock production.[22,35] Infection with pathogenic leptospires causes economic losses in the

cattle and sheep industries due to reproductive failure (abortions, stillbirths and neonatal mortalities) and decreased milk production.[20,34,49] In addition, infected animals can develop chronic renal infection and excrete the organisms in the urine, disseminating leptospires to other animals and constituting a potential zoonotic threat to those engaged in animal production and related industries.[15,26,32,41] More than 200 serovars of pathogenic *Leptospira* within 20 species of leptospires have been isolated and described worldwide.[6,11]

In New Zealand, leptospirosis is the most common occupationally acquired zoonotic disease and associated mainly with meat and agricultural workers.[60] Cattle, sheep and farmed deer are important animal reservoirs in New Zealand.[4,17,42] Surveys in farms and abattoirs have shown that 69% of beef cattle herds, 44% of sheep lines, and 81% of deer herds have been infected with leptospirosis.[4,17,31] Among six serovars within two *Leptospira* species (*L. borgpetersenii* and *L. interrogans*) that had been isolated from animals in New Zealand, serological evidence of leptospirosis in domestic livestock is most commonly associated with serovars Hardjobovis and Pomona.[3,7]

Laboratory diagnosis of leptospirosis is based on direct examination of clinical specimens for organisms, isolating the organism, detecting anti-leptospiral antibodies through serological testing.[6] The microscopic agglutination test (MAT) is regarded internationally as the reference test for the serological diagnosis of leptospirosis.[21,35] However, this test does not recognize the presence of leptospires, and therefore does not directly indicate active infection or reflect the carrier or shedding status of the host.[14,59] The MAT cannot discriminate between infection- and vaccination-induced antibodies.[1] Also, positive results can be obtained due to cross-reactivity between closely related serovars.[35] In addition, it is well-known that different MAT results can be obtained from different laboratories, due to the source of antigen cultures, the maintenance of reference collections of *Leptospira* strains, and the subjective element of test interpretation.[12,13,35] Culture has been well established as a definitive diagnosis through isolation of the bacteria from a variety of samples.[22,53] However, culture is laborious and time-consuming, the result is often influenced by the overgrowth of other bacterial and fungal contaminants, and some serovars, e.g. Hardjobovis, are inherently difficult to culture.[16,23,53]

Polymerase chain reaction (PCR) assays have been used since the 1990s as reliable and rapid methods for the diagnosis of leptospirosis. There is evidence that PCRs are more sensitive than conventional tests such as culture.[10,29,63] PCR may be especially useful when the immune response of the maintenance host to the infecting serovar is poor (e.g. the MATs sensitivity is sub-optimal for detecting Hardjo infection in cattle).[48] The PCR assays have been used successfully to detect *Leptospira* spp. in clinical specimens, such as bovine urine and semen,[8,27,30,61] urine, vaginal fluids, and semen of goats and sheep.[38,39] Quantitative real-time PCR (qPCR) has improved the diagnosis of leptospirosis compared with conventional PCR, as it is less prone to carry-over contamination and is time-saving due to the redundancy of gel electrophoresis.[2,52] The qPCR assays targeted at different genes have been used to detect leptospires from reference strains and clinical isolates of human sources. Genes include *rrs* (16s),[45,55] *secY*,[2] *gyrB*,[52] *rpoB*[33], and *lipL32*.[36] However, there are limited reports of studies of qPCR assays for detecting leptospires in clinical specimens from animals. A qPCR assay based on a previously described assay[52] has been developed and validated in the Hopkirk Leptospirosis Research Laboratory (HLRL), ^{m}EpiLab, Hopkirk Research Institute, Massey University, Palmerston North, using urine and kidney samples from deer in New Zealand.[56] This assay has successfully detected serovars Hardjobovis and Pomona in deer. Gribbles Veterinary (GV), Palmerston North, has also developed a qPCR assay based on two previously described assays,[51,55] which can be used as a screening test for leptospirosis in animals.

It is common for researchers to compare results between studies with limited regard of possible inter-laboratory variation that may confound those comparisons. Inter-laboratory variation has been demonstrated for diagnosing different diseases. For example, an inter-laboratory comparison of enzyme-linked immunosorbent assays for detecting *Salmonella enteritica* serotype Enteritidis in chickens was conducted in 14 laboratories, with a variety of antigens being used. This comparison demonstrated good agreement on the interpretation of optical density readings of high and low titre sera, but differences in the interpretation of medium titre sera.[5] A study was performed on epithelial specimens to investigate the inter-laboratory comparison of qPCR and culture that were routinely performed in five European laboratories for foot-and-mouth disease. This demonstrated comparable results for qPCRs, but variable sensitivity for cultures across laboratories.[24] Knowledge of likely between laboratory differences can contribute

to more meaningful comparison of studies determining the prevalence and incidence of diseases. However, few published studies have investigated inter-laboratory agreement of serological[12,13] or PCR tests for leptospirosis to allow laboratory variation to be factored into conclusions.

The primary purpose of this study was to improve the understanding of the contribution that inter-laboratory variation may have in explaining differences in results of studies when different laboratories undertake sample testing. This was done by comparing tests for leptospirosis between our research laboratory (HLRL) and a nearby commercial veterinary diagnostic laboratory (GV), using samples from sheep and beef cattle. The inter-laboratory comparisons included qPCRs on urine samples and MATs on serum samples. Additionally, this research allowed comparisons between tests at HLRL, namely qPCR on urine and kidney, and between qPCR on both kidney and urine samples and MAT (both positivity and titres). This enhances understanding of predictive relationships between tests on different specimens for diagnostic and epidemiological purposes.

3 Materials and methods

3.1 Study design

This study used urine, blood and kidney samples from a sheep and beef cattle abattoir cross-sectional study described in the previous chapter. Samples were collected from an abattoir in the Waikato area by AgResearch Ltd. staff wearing personal protective equipment (PPE).

Coagulated blood samples were collected in two plain 10 ml blood tubes from each animal at slaughter.[a] Urine samples were collected by direct bladder puncture into two 8ml plain blood tubes.[b] The whole right-side kidney was collected from each sheep carcass, while one wedge (extending from the renal cortex to the medulla) from the right-side kidney was collected from each beef cattle carcass. The capsule of each kidney sample was removed and the sample was put into a 500 ml container.[c] The coagulated blood samples were centrifuged shortly after collection at 1300 *g* for 10 minutes at the AgResearch Ltd. Laboratory, and the sera were collected. All samples were sent under refrigerated conditions to GV on the day of collection. One set of the serum and urine samples was retained by GV whilst the other set, plus the kidney

samples, were sent to HLRL. Collection and processing of samples was undertaken in a sterile manner to avoid cross-contamination. All serum samples were MAT tested at GV and HLRL, while qPCRs were conducted in HLRL and GV as described in Figure 5.1.

If positive urine or kidney qPCR results were received from either HLRL or GV, culture was initiated on kidneys at HLRL primarily to provide strains for multi-locus sequence typing, as described in Chapter 6.

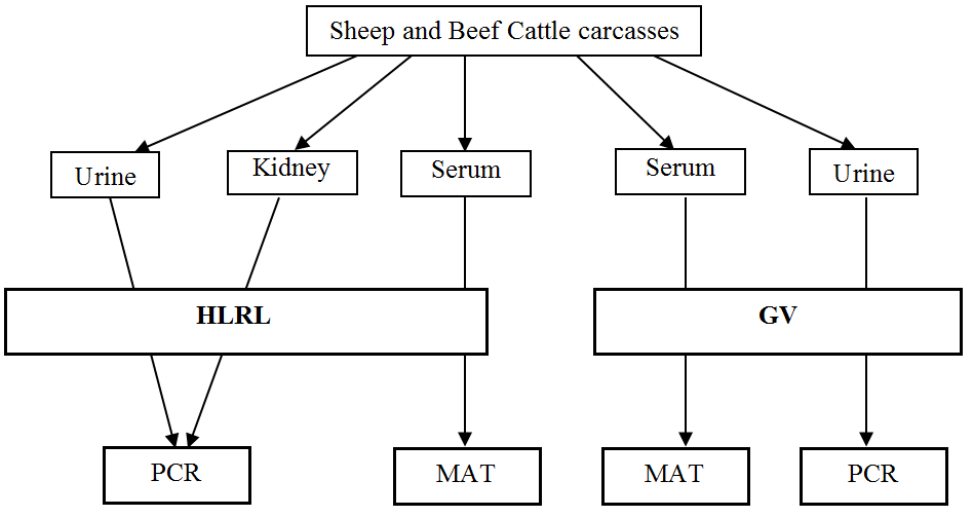

Figure 5.1 Flowchart showing sampling and testing procedure.

The sample size needed to produce robust agreement evaluation (kappa statistic inter-rater reliability) was calculated, given a relative error of 0.2 and the difference between the observed agreement and expected agreement of 0.5 (anticipating the tests will agree about 50% of the time).[28] The number of animals slaughtered at the abattoir during the 9-week sampling period was 7065 sheep and 162 beef cattle. The minimum number of samples required for calculating test agreements was 99 for sheep and 62 for cattle.

3.2 Quantitative real-time PCR

3.2.1 DNA extraction

For HLRL, urine and kidney samples were processed upon receipt, usually the day following collection. For GV, urine samples were stored at 4 °C, and processed within a week based on laboratory capacity.

3.2.1.1 Urine samples (HLRL)

A 1.2 ml urine sample was aliquoted into a 1.5 ml Eppendorf tube,[d] and then centrifuged at 10625 *g* for 20 minutes. Supernatant was discarded and 200 µl of PBS (Phosphate Buffered Saline) was used to re-suspend the pellet. This mixture was used to extract DNA using a commercial kit[e] as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl elution buffer.

3.2.1.2 Urine samples (GV)

A two hundred microlitre urine sample was used for DNA extraction using a commercial kit[f] as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl elution buffer.

3.2.1.3 Kidney samples (HLRL)

Kidney surfaces were sterilised by swabbing with 70% ethyl alcohol. A 10 g section of kidney, extending from the renal cortex to the medulla, was removed aseptically. The removed section was washed with 70% ethyl alcohol using funnel and flask, followed by flaming with a Bunsen burner. It was then homogenised in 50 ml PBS, using a stomacher.[g] A 160 µl aliquot of this suspension was used to extract DNA, using a commercial kit[h] as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl elution buffer.

3.2.2 DNA amplification

3.2.2.1 Hopkirk Leptospirosis Research Laboratory

The qPCR assay was modified based on a previously developed assay in the HLRL[56] to suit a real-time genetic analyser.[i] Green-fluorescent nucleic acid stain SYTO9[j] 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 mix included 2.5 µM SYTO9,[j] 1×PCR buffer, 1.5 mM magnesium chloride ($MgCl_2$), 200 µM dNTPs, 5 pmol of 2For and 504Rev, one unit of *Taq* DNA polymerase,[k] 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 s, 63°C for 20 s and extension at 72°C for 20 s. 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. Positive controls were serovars Hardjobovis and Pomona (HLRL laboratory strains), and ddH_2O was used as negative control. Samples were considered positive, if a similar melting temperature as positive controls was recorded.

3.2.2.2 Gribbles Veterinary

The qPCR assay was based on two previously prescribed assays,[51,55] and modified to suit a real-time genetic analyser.[l] Each 25 µl PCR reaction contained 12.5 µl of the QuantiTech Probe PCR Master Mix,[m] 0.4 mM each of the forward primer (5'-CCCGCGTCCGATTAG-3'), reverse primer (5'-TCCATTGTGGCCGRACAC-3') and the 6-carboxyfluorescein-labeled (FAM) Black Hole Quencher 1 (BHQ1) quenched TaqMan probe (5'-CTCACCAAGGCGACGATCGGTAGC- 3'),[n] 5 µl of DNA template and RNase/DNase-free water to 25 µl. Thermal cycling comprised initial denaturation at 95°C for 15 min, followed by 50 cycles of denaturation at 95°C for 20 s and annealing/extension at 60°C for 30 s. Fluorescent data acquisition was performed at the end of each extension cycle. Positive samples were defined as having a cycle-to-threshold (Ct) value less or equal to 36 cycles. Positive controls were serovar Tarassovi (GV laboratory strain), and ddH_2O was used as negative control. Negative samples were defined as having a Ct value greater than 40 cycles. Samples with Ct value >36 to ≤40 were defined as being "suspect" positive.

3.3 Microscopic agglutination test

The standard storage of sera was the same for both laboratories, being at 4 °C for one week or at -20 °C for longer periods. A titre of ≥ 48 for MAT-HLRL and ≥ 50 for MAT-GV was considered positive for both serovars, based on previous studies of leptospirosis in sheep and cattle.[7,57]

3.3.1 Hopkirk Leptospirosis Research Laboratory

Thirty microlitres of each serum sample was mixed with 150 µl standard saline to make a 1:6 dilution. MAT was performed by the same observer against serovars Hardjobovis

and Pomona (HLRL laboratory strains) based on the standard procedure.[21] The antigens used for MAT were 4 to 14-day-old cultures of Hardjobovis and Pomona containing approximately 1–2×10^8 organisms/ml (as determined by the McFarland scale). Eight, two-fold serial dilutions of serum in standard saline covering the range from 1:24 to 1:3072 were prepared in a 96-well plate.[o] A plate containing the 25 µl standard antiserum (sera) and standard saline, respectively, with 25 µl saline was prepared in the same manner as a positive and negative control. The plates were then placed in re-sealable plastic bags and incubated at room temperature (20-30°C) for 1.5-4 hours, after which the degree of agglutination was determined by examination under a dark-field microscope.[p] The end-point titre was the lowest dilution at which approximately 50% of the organisms were agglutinated.

3.3.2 Gribbles Veterinary

The same standard method for MAT was applied, and performed by one observer from GV for all of the samples. However, each serum sample was not diluted before MAT testing. The two-fold serial dilutions of sera were made with Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, and the serum-antigen mixtures used were from 1:50 onwards. The negative control was also prepared with EMJH medium. Different source of antigen (Hardjobovis and Pomona) and standard antiserum from HLRL were used.

3.4 Statistical Analysis

Agreement between HLRL and GV urine qPCR and MAT, between HLRL urine and kidney qPCR, and between HLRL urine, kidney qPCR and MAT was evaluated using the Cohen's kappa statistic[62] with Fleiss-adjusted 95% confidence intervals (95% CI),[25] for sheep and cattle samples combined, and also independently on sheep and cattle samples. The interpretation for the kappa statistic is: < 0.2 slight agreement, 0.2 - 0.4 fair agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 substantial agreement, and > 0.8 almost perfect agreement.[62]

Logistic regression analysis was used to determine the association between the continuous variable Ct value from GV urine qPCR (gct) and the binary outcome (positive HLRL urine qPCR results). The aim of this analysis was to find out whether the agreement observed between two qPCRs on urine was influenced by other factors. The Ct value was set as 50 for those samples that did not have Ct value detected by GV urine qPCR. The confounders/interactions considered included; animal species,

supplier, line ID, age, and the serological result. The serological result was included in the model both as a binary outcome and as a raw titre. Variables were considered as confounding factors if the presence of variables changed the regression coefficient of the Ct value in the model by ±15%. Interactions were evaluated and considered as present if the Likelihood Ratio Test (LRT) was statistically significant ($p \leq 0.05$). Variables showing a univariable association ($p < 0.2$) with the outcome and confounders/interactions were used to develop a multivariable model by a stepwise selection process retaining variables significant at $p \leq 0.05$ level. The goodness-of-fit of the model was assessed using the le Cessie-van Houwelingen normal test statistic, and the predictive ability of the model was assessed by plotting the receiver operating characteristic curve (ROC).

To investigate agreement of MAT titres between GV and HLRL, bubble plots were produced and examined. Among animals that had different titres reported from two laboratories (≥ one dilution difference), the null hypothesis that the probability of occurrence of higher and lower titres was equal (50%) was tested. The analysis was conducted independently on serovars (Hardjobovis and Pomona) and animal species (sheep and cattle).

To determine whether the different MAT results demonstrated from the two laboratories were associated with different serum sample storage times between the two laboratories, linear regression models stratified by serovar were used. One model set the outcome as the difference in log MAT titres reported by two laboratories. A second model set the outcome as the difference in the MAT titre recorded; for example, if titre 48 was recorded from HLRL and titre 50 was recorded from GV, the titres were treated as the same, and the outcome was zero; while if titre 96 was recorded from HLRL and titre 50 from GV, there was one level difference, and the outcome was one, etc.. The main-effect continuous predictor was the same in both models, as the difference in days of the serum sample storage times between two laboratories. Due to the non-normal distribution of intervals the continuous variables (difference of intervals) were categorised to improve model fit. The same variable selection procedure for multivariable models and confounders/interactions evaluation (animal species, suppliers, line IDs) procedure as stated above in the logistic regression model were used.

Significance was declared at $p \leq 0.05$. Analyses were implemented with the statistical software R 2.14.1.[q]

The prevalence of urine shedding and renal colonisation detected by urine/kidney qPCR in seropositive and seronegative animals was calculated. The prevalence ratio was evaluated using logistic regression to determine the association between urinary shedding/carrier status of seropositive versus seronegative animals. When stratified by serovar, animals with positive titres for both Hardjobovis and Pomona (indicating dual infection) were excluded from the analysis. The 95% confidence interval (95% CI) of the prevalence and prevalence ratio was adjusted for the effect of correlation between animals within slaughter line using generalized estimating equations (GEE)[37] and robust standard errors. The GENMOD procedure in SAS 9.3[r] was used for this purpose with data aggregated at slaughter line level and line-ID being the subject effect.[54] The logistic model was modified to obtain the prevalence ratio with confidence intervals instead of an odds ratio, by using a poisson distribution with a log-link.[65]

4 Results

Samples tested were 545 kidneys (399 sheep and 146 beef cattle), 542 sera (396 sheep and 146 beef cattle), and 274 urine samples (156 sheep and 118 cattle).

4.1 Inter-laboratory comparisons

4.1.1 Urine qPCR

The results of qPCR are presented in Table 5.1, including when suspect results obtained from GV were and were not considered as positive. When suspect results obtained from GV were considered as positive, the agreement between two qPCRs was almost perfect when sheep and cattle samples were combined; and the kappa value was higher for sheep than cattle samples, when analysed by species. When suspect results obtained from GV qPCR were considered as negative, the kappa value was higher for sheep and cattle samples combined and cattle samples alone, but similar for sheep samples.

Table 5.1 Number of urine samples tested positive (Pos) or negative (Neg) by HLRL and GV qPCR when the GV suspect results (36< Ct ≤40) were and were not considered as positive, and agreement (presented by kappa) between these two qPCRs, stratified by animal species.

HLRL qPCR	GV qPCR (Ct value ≤ 40 as cut-off)								
	Sheep and Cattle combined			Sheep			Cattle		
	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)
Pos	72	1	0.87 (0.80-0.93)	48	0	0.96 (0.91-1.00)	24	1	0.73 (0.60-0.87)
Neg	14	187		3	105		11	82	

HLRL qPCR	GV qPCR (Ct value ≤ 36 as cut-off)								
	Sheep and Cattle combined			Sheep			Cattle		
	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)
Pos	65	8	0.90 (0.85-0.96)	45	3	0.95 (0.90-1.00)	20	5	0.81 (0.68-0.95)
Neg	2	199		0	108		2	91	

A significant negative association ($p < 0.001$) was found between the continuous variable (Ct value from GV urine qPCR) and the binary outcome (HLRL urine qPCR results) in the logistic regression analysis. For one unit increase in Ct value, the odds of a sample being tested positive by HLRL urine qPCR decreased by 0.65 (95% CI: 0.45-0.84). The association was not distorted by species, supplier or line ID as none of these variables changed the regression coefficient of the Ct value by more than 15%. None of the interactions were significant. Ct value from GV urine qPCR was the only variable that was significant at $p < 0.05$ level and retained. The p value for the le Cessie-van Houwelingen normal test was 0.57 indicating a good model fit. The area under the ROC curve was 0.997, indicating that the Ct value of GV qPCR predicted the qPCR outcome of HLRL almost perfectly.

4.1.2 MAT

The agreements between HLRL and GV MAT results, stratified by serovar and animal species, are presented in Table 5.2. Agreement between two MATs, cattle and sheep combined, was almost perfect against serovar Hardjobovis and moderate against serovar Pomona. When stratified by animal species, the kappa value was higher for sheep than cattle samples.

Table 5.2 Number of serum samples that were MAT positive (Pos) or negative (Neg) by HLRL and GV, and agreement (presented by kappa) between these two MATs, stratified by serovar and animal species.

HLRL MAT		GV MAT					
		Hardjobovis			Pomona		
		Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)
Sheep and Cattle combined	**Pos**	217	13	0.93 (0.90-0.96)	81	96	0.53 (0.45-0.60)
	Neg	5	307		1	364	
Sheep	**Pos**	139	7	0.95 (0.91-0.98)	66	74	0.53 (0.45-0.62)
	Neg	2	248		1	255	
Cattle	**Pos**	79	6	0.89 (0.81-0.96)	15	22	0.50 (0.34-0.67)
	Neg	2	59		0	109	

The comparisons of MAT titres against Hardjobovis and Pomona from HLRL and GV are presented in Figure 5.2.

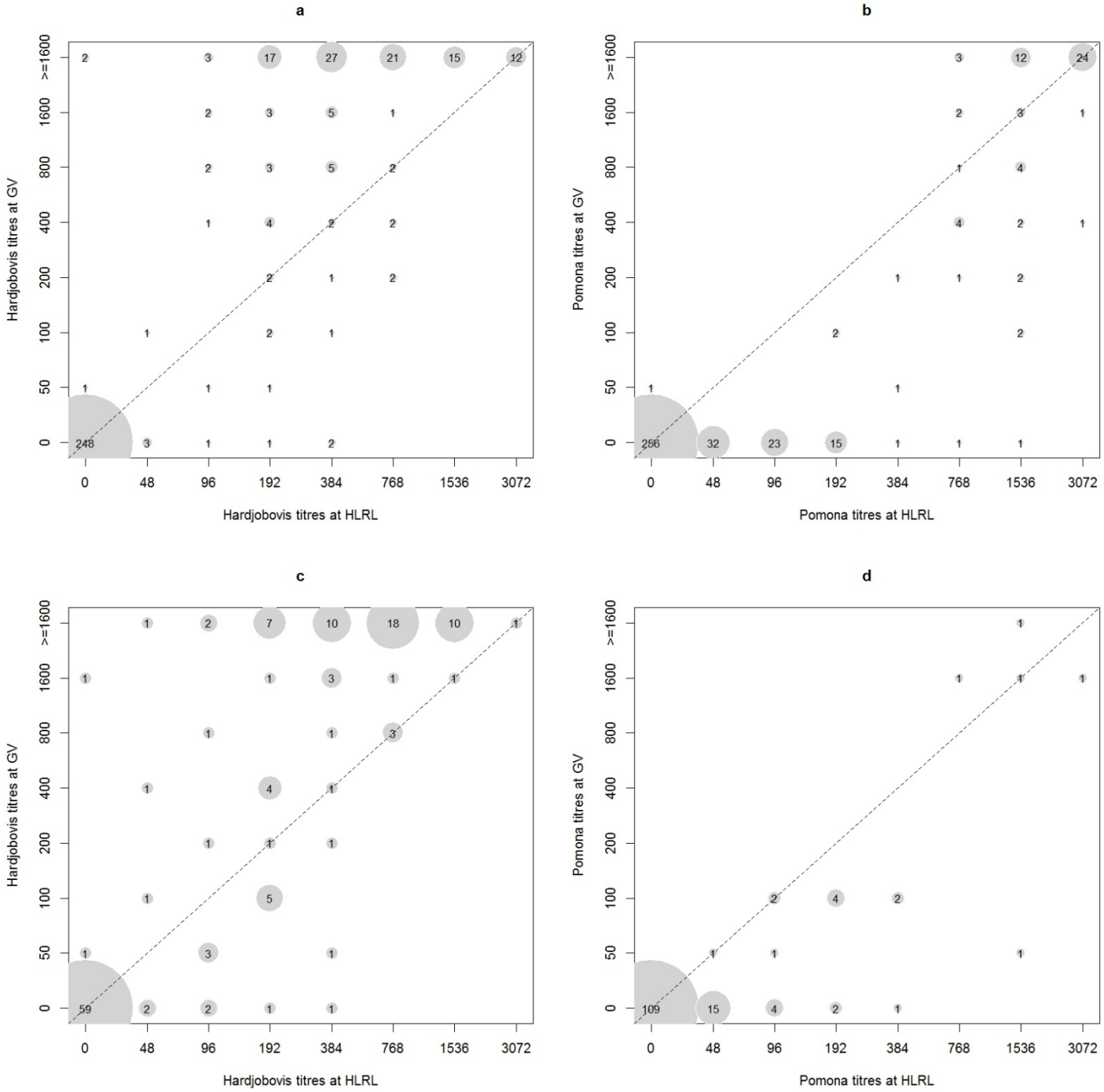

Figure 5.2 Bubble plots comparing the MAT titres for serum samples between GV and HLRL stratified by animal species: a) Hardjobovis in sheep; b) Pomona in sheep; c) Hardjobovis in cattle; d) Pomona in cattle. Bubble size is proportional to the number of samples.

For sheep and cattle combined that had different Hardjobovis titres (39%; 210/542) reported, higher titres were reported by GV than HLRL ($p < 0.005$); this occurred in 87% (113/130; 95% CI: 80%-92%) of sheep ($p < 0.005$) and 80% (64/80; 95% CI: 70%-88%) of cattle ($p < 0.005$). However, for sheep and cattle combined that had different Pomona titres (27%; 146/542) reported, lower titres were reported by GV than

HLRL (p < 0.005); this occurred in 84% (95/113; 95% CI: 76%-90%) of sheep (p<0.005) and 94% (31/33; 95% CI: 80%-99%) of cattle (p < 0.005).

The differences in serum sample storage times between the two laboratories (the storage time at HLRL minus that at GV), stratified by serovar are presented in Figure 5.3. In general, serum samples were stored longer at HLRL than GV, especially prior to testing against serovar Hardjobovis. Samples were stored for a median of 108 days (IQR, 10; Minimum, -25; Maximum, 152) longer for serovar Hardjobovis and 88 days (IQR, 46; Minimum, -25; Maximum, 116) longer for serovar Pomona at HLRL than at GV. In linear regression models for either serovar, no significant association was detected between differences of MAT results (for Hardjobovis or Pomona) and differences of serum sample storage time reported from two laboratories.

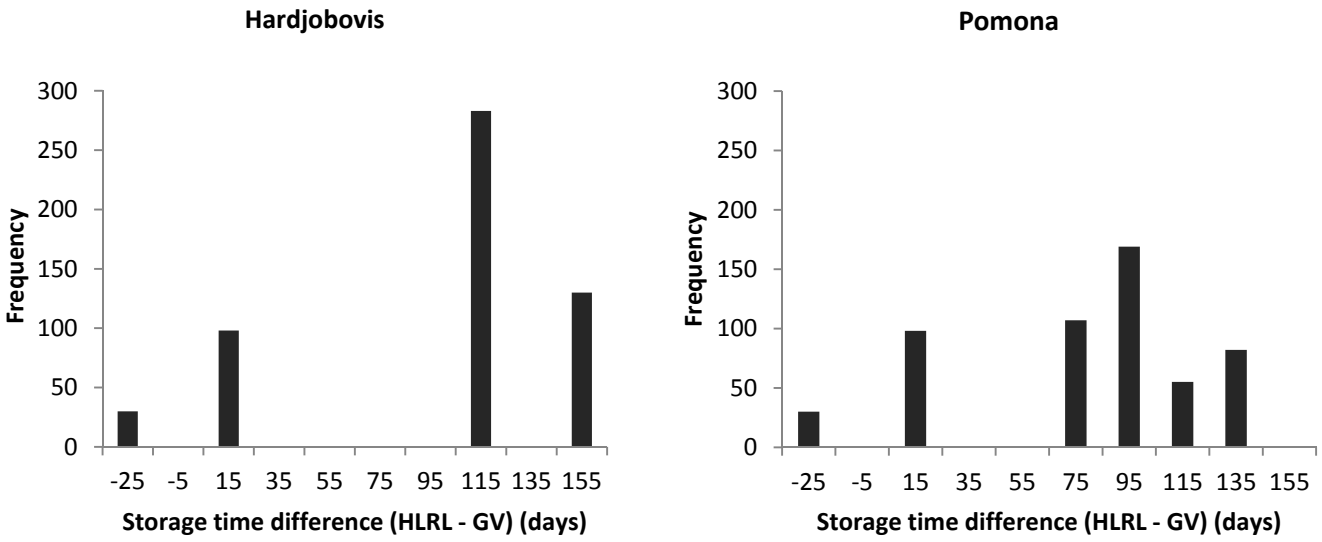

Figure 5.3 Histogram of the frequency of differences (the interval at HLRL minus the interval at GV) in serum sample storage time (between sample collection date and MAT testing date) between HLRL and GV; by serovar Hardjobovis (left) and Pomona (right).

4.2 Intra-laboratory comparisons between tests on different specimens

These comparisons were for tests performed at HLRL only.

4.2.1 Urine and kidney qPCR

The qPCR results of the 274 animals that had both kidney and urine samples collected are presented in Table 5.3. The agreement between kidney qPCR and urine qPCR

results, cattle and sheep combined, was almost perfect. When stratified by animal species, the kappa value was higher for sheep than cattle.

Table 5.3 Number of animals tested urine and kidney qPCR positive (Pos) or negative (Neg), and agreement (presented by kappa) between urine and kidney qPCR results, stratified by animal species.

Urine qPCR	Kidney qPCR								
	Sheep and Cattle combined			Sheep			Cattle		
	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)
Pos	65	8	0.84 (0.77-0.91)	44	4	0.92 (0.86-0.99)	21	4	0.71 (0.56-0.86)
Neg	9	192		1	107		8	85	

4.2.2 Urine and kidney qPCR and MAT

The urine and kidney qPCR and MAT results of the animals that had both urine or kidney and blood samples collected, and the agreement between urine or kidney qPCR results and MAT results (all animals with titres ≥48), stratified by animal species are presented in Table 5.4. For animals (sheep and cattle combined) that had both blood and urine samples collected, and that had both blood and kidney samples collected, the agreement between MAT and qPCR results was fair. When stratified by animal species, the kappa value was higher for sheep than cattle samples.

Table 5.4 Number of urine and kidney samples tested qPCR positive (Pos) or negative (Neg) by MAT results (titre ≥ 48, against Hardjobovis and/or Pomona), and agreement (presented by kappa) between urine, kidney qPCR results and MAT results, stratified by animal species.

MAT	Urine qPCR								
	Sheep and Cattle combined (n=274)			Sheep (n=156)			Cattle (n=118)		
	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)
Pos	70	100	0.32 (0.24-0.40)	46	39	0.49 (0.37-0.61)	24	61	0.16 (0.08-0.25)
Neg	3	101		2	69		1	32	

MAT	Kidney qPCR								
	Sheep and Cattle combined (n=542)			Sheep (n=396)			Cattle (n=146)		
	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)	Pos	Neg	kappa (95%CI)
Pos	137	193	0.33 (0.27-0.39)	111	113	0.45 (0.37-0.52)	26	80	0.09 (0.01-0.17)
Neg	7	205		3	169		4	36	

The percentage of urine and kidney samples that were qPCR positive in seropositive and seronegative animals and the prevalence ratio of urine and kidney qPCR positivity in seropositive versus seronegative animals were presented in Table 5.5. With sheep and cattle results combined, the prevalence of urine qPCR positivity in seropositive (either

Hardjobovis and/or Pomona) animals was 9.1 (95% CI: 3.7-22.9) times that in seronegative animals. The prevalence of kidney qPCR positivity in seropositive animals was 5.1 (95% CI: 3.0-8.8) times that in seronegative animals. When stratified by serovar, the prevalence of urine and kidney qPCR positivity in Hardjobovis-seropositive versus that in Hardjobovis-seronegative animals was higher than the prevalence of urine and kidney qPCR positivity in Pomona-seropositive versus that in Pomona-seronegative animals. The prevalence of urine and kidney qPCR positivity in Hardjobovis-seropositive versus that in Hardjobovis-seronegative animal was higher in sheep than cattle.

Table 5.5 Percentage of urine and kidney samples that were qPCR positive in seropositive (MAT titre ≥ 48) and seronegative (MAT titre < 48) animals, prevalence ratio of urine and kidney qPCR positivity in seropositive versus seronegative animals, with 95% confidence intervals (CI) adjusted for the effect of correlation of animals within slaughter line

		Seropositive (%) (95% CI)	Seronegative (%) (95% CI)	Prevalence ratio (95% CI)
Urine qPCR positive				
Hardjobovis and/or Pomona	Sheep and cattle (n=274)	41 (30-54)	3 (1-9)	9.1 (3.7-22.9)
	Sheep (n=156)	54 (32-75)	3 (1-11)	6.9 (3.5-13.4)
	Cattle (n=118)	28 (20-38)	3 (0-19)	9.3 (1.3-65.1)
Hardjobovis-only	Sheep and cattle (n=240)	49 (36-63)	7 (3-14)	6.9 (3.1-15.6)
	Sheep (n=133)	82 (65-92)	5 (2-12)	12.5 (4.8-32.6)
	Cattle (n=107)	30 (20-44)	10 (3-28)	3.4 (0.9-13.2)
Pomona-only	Sheep and cattle (n=240)	15 (6-32)	24 (16-36)	0.7 (0.3-1.3)
	Sheep (n=133)	10 (4-24)	28 (14-48)	0.6 (0.3-1.3)
	Cattle (n=107)	22 (7-52)	20 (13-30)	1.1 (0.4-3.4)
Kidney qPCR positive				
Hardjobovis and/or Pomona	Sheep and cattle (n=542)	42 (30-54)	3 (1-8)	5.1 (3.0-8.8)
	Sheep (n=396)	50 (33-66)	2 (1-6)	5.6 (4.0-7.7)
	Cattle (n=146)	25 (17-34)	10 (4-25)	2.5 (0.8-7.6)
Hardjobovis-only	Sheep and cattle (n=465)	52 (39-64)	6 (3-12)	6.0 (3.7-9.7)
	Sheep (n=335)	70 (58-81)	5 (2-11)	8.6 (5.6-13.3)
	Cattle (n=130)	29 (20-40)	13 (6-27)	2.2 (0.9-5.5)
Pomona-only	Sheep and cattle (n=465)	13 (7-23)	24 (15-35)	0.6 (0.5-0.9)
	Sheep (n=335)	11 (6-22)	24 (13-41)	0.7 (0.6-0.9)
	Cattle (n=130)	19 (6-48)	22 (16-30)	0.9 (0.3-2.8)

The percentage of urine and kidney samples that were qPCR positive in sheep and cattle for the different MAT titre values against serovar Hardjobovis or Pomona is presented in Figure 5.4. Dual infections were excluded. In general Hardjobovis-seropositive sheep had higher percentage of qPCR positivity at each MAT titre compared with Hardjobovis-seropositive cattle. In animals with Pomona titres ≥ 48, there were significant trends ($p < 0.01$) of increasing proportions of RT-PCR positive kidneys (sheep, cattle) and RT-PCR positive urine (cattle) as titres increased.

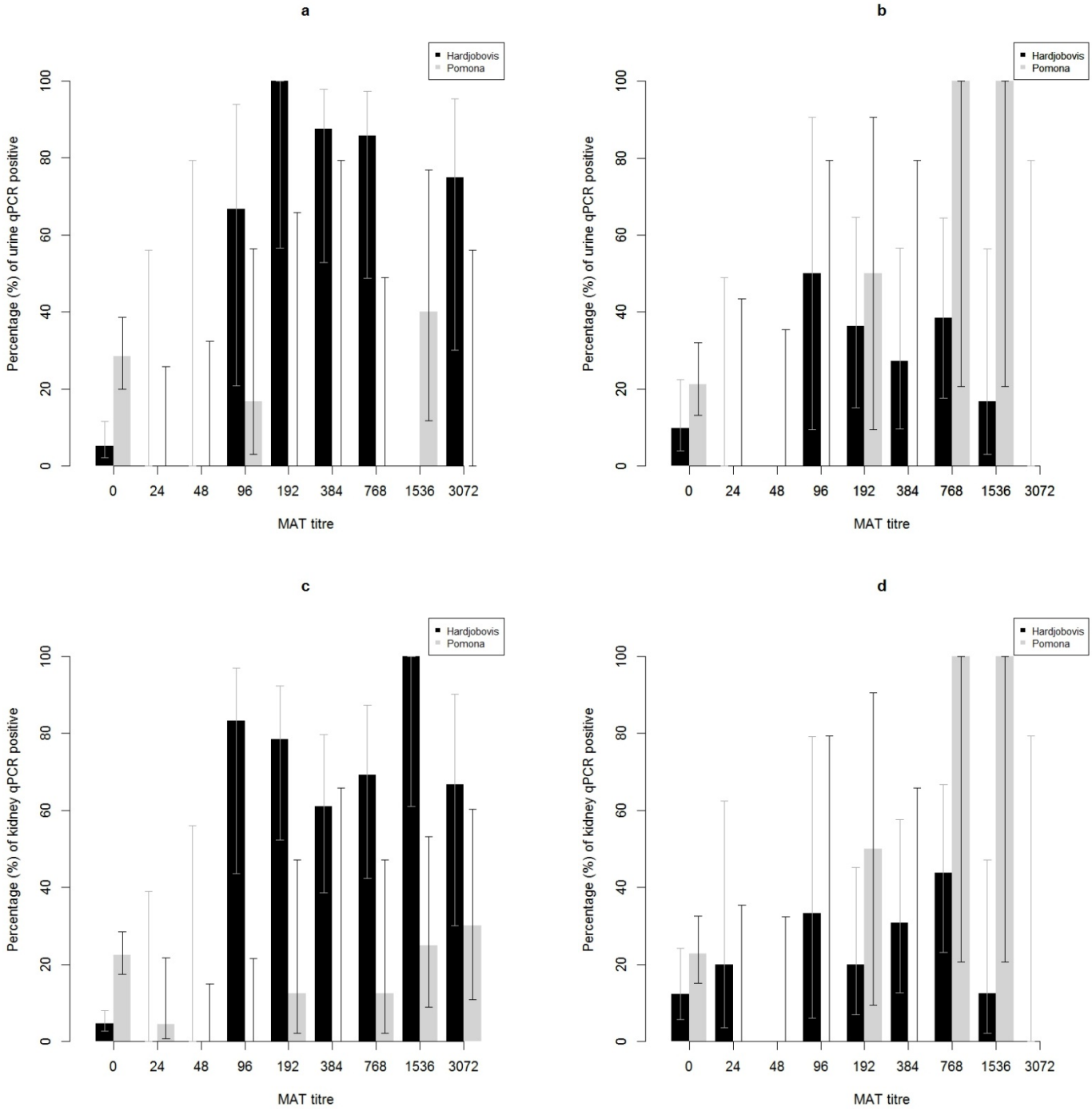

Figure 5.4 Percentage (and 95% CI) of urine and kidney samples that were qPCR positive in sheep and cattle that had different levels of MAT titres against serovar Hardjobovis or Pomona: a) percentage of urine samples that were qPCR positive in sheep; b) percentage of urine samples that were qPCR positive in cattle; c) percentage of kidney samples that were qPCR positive in sheep; d) percentage of kidney samples that were qPCR positive in cattle.

4.3 Kidney culture

Of the 144 kidneys cultured from animals that were either kidney or urine qPCR positive, leptospires were isolated from 26 (18%; 95% CI: 13%-25%). For 135 animals that were kidney qPCR positive, 26 (19%; 95% CI: 13%-27%) were culture positive. For 67 animals that were urine qPCR positive, 13 (19%; 95% CI: 12%-30%) were culture positive. All kidney culture positive animals were seropositive.

5 Discussion

This study investigated agreement between qPCRs and MATs for leptospirosis diagnosis performed in a research and a commercial veterinary diagnostic laboratory, using urine samples and sera from sheep and cattle. This can assist researchers and diagnosticians in understanding the sometimes discrepant test results between studies. Inter-laboratory agreement for urine qPCRs results was almost perfect, while the agreement for positivity to MATs was serovar-dependent (almost perfect for Hardjobovis and moderate for Pomona). Additionally, qPCR performed at the research laboratory on paired urine and kidney samples was compared. The almost perfect agreement suggested the qPCR on these two specimens can be used interchangeably. There was fair agreement between MAT positivity for Hardjobovis and/or Pomona and qPCR on both kidney and urine in sheep and cattle. However, the prevalence ratio of urine and kidney qPCR positivity in Hardjobovis-seropositive versus Hardjobovis-seronegative sheep indicated that Hardjobovis-seropositivity found in sheep may be able to predict shedding/renal colonisation at individual animal level.

In recent years, several qPCR assays targeted at various genes (e.g. *rrs*, *secY*, *gyrB* and *lipL32* gene) have been developed and used in many diagnostic, reference and research laboratories for the rapid detection of leptospires in biological fluids and tissues. Although comparison of qPCRs for human leptospirosis diagnosis in blood has been reported,[9,58] this comparison has not been performed for animal leptospirosis diagnostics on any specimen, to the best of author's knowledge. Also, no comparison of qPCRs between laboratories has been published to indicate the generalization of qPCRs results recorded from different laboratories. This study demonstrated almost perfect agreement between the qPCRs from a research and a veterinary diagnostic laboratory on urine samples from sheep and cattle, although a higher kappa value was obtained when suspect results recorded from GV were considered as negative. The significant negative

association between Ct values from GV urine qPCR and the probability of HLRL urine qPCR results being positive, and that this was not attenuated by other confounding variables (e.g. species), strengthens the finding of almost perfect agreement between laboratories for urine qPCR. The differences in the qPCR protocols from these two laboratories comprised different primers targeted at different genes (*gyrB* gene for HLRL and 16s rRNA gene for GV), different chemistries (SYTO9 for HLRL and TaqMan probe for GV), and also the different pre-PCR processing including different DNA extraction protocols and delay times between collection and processing of samples. Urine samples were centrifuged before the DNA extraction process at HLRL with QIAamp DNA Mini Kit, while at GV, urine samples were used directly for DNA extraction with BioSprint 96 One-For-All Vet Kit. HLRL processed the samples the following day of collection, while GV processed samples within a week based on laboratory capacity. Leptospires can lyse in urine during the storage and transport of samples. Also, because the urine samples were not treated with EDTA and formaldehyde or centrifuged and re-suspended as suggested to prevent the DNase activity, long storage may affect the quality and quantity of the DNA extracted.[27,61] However, whether this contributed to the different results obtained from two laboratories has to be confirmed with amplifying samples stored for different intervals.

Although it is well known anecdotally that different MAT results can be obtained from different laboratories, studies comparing MATs between laboratories are limited. Two studies have documented that MAT titres reported by different laboratories on the same sample varied.[13,46] Different MAT results between laboratories may be due to different source and maintenance of antigen cultures. Strain contamination and mislabelling or switching of strains are potential problems associated with the maintenance of antigen cultures. However, these problems are more likely to occur in laboratories in which the capacity for quality control and long-term storage of culture collections is limited.[12] Due to the subjective nature of interpretation of the MAT, variation in readings may occur among laboratories and also between different observers within the same laboratory.[35] In this study, the agreement for MAT results between laboratories was serovar-dependent, which indicated that the different results in this study may be more likely due to the different source of antigen (especially serovar Pomona) cultures used in two laboratories than variation in reporting cut-off titre values by the two laboratories. Even though, the proportion of serum samples that had variable MAT titres reported by two

laboratories showed that the MAT reading of titres can be varied between observers from two laboratories. It is possible that the differences of serum storage times between two laboratories can explain the different MAT results obtained. However, the data showed that the association between the difference of serum storage times and the different MAT titres reported by two laboratories was not significant for either serovar in this study.

Although the MAT is the serological reference test for leptospirosis diagnosis,[47] it detects antibodies in serum, rather than the presence of leptospires directly. The direct detection of leptospires or their DNA in urine and kidney is important for identifying carriers.[22] Since the MAT is still the most commonly used diagnostic test, and sampling blood from live animals is more practical than sampling urine/kidney, it is worthwhile investigating the association between serological results and shedding/renal carriage status. It is important to note that none of the diagnostic tests is perfect for all purposes, and neither MAT nor qPCRs should be treated as the gold standard. Also, there is limitation of comparing MAT and qPCR which are conducted on different specimens (MAT on sera; qPCR on urine and kidney) directly. Due to the lack of capacity for qPCR to identify the serovar, positive qPCR results may not always indicate shedding of the same serovar that were identified by MAT. However, considering Hardjobovis and Pomona are the two most commonly found *Leptospira* serovars in livestock in New Zealand,[3,7,43] it is reasonable to make the comparison between MAT (against Hardjobovis and/or Pomona) and qPCR results on urine and kidney.

When results from sheep and cattle were combined, the agreement between MAT and qPCR on urine and kidney was fair (kappa = 0.32 and kappa = 0.33, respectively). The seroprevalence found in urine or kidney qPCR negative animals was 50% (100/201) and 48% (193/398), respectively. Several factors may contribute to this finding. MAT titres may persist after leptospires were cleared from kidney and shedding had ceased. Seroprevalence found in urine qPCR negative animals may also due to the intermittent shedding of leptospires.[35] It is also possible that animals were recently infected and may not have started shedding leptospires in urine at the time of sampling. Antibodies are usually produced from around three to 10 days after infection, before the leptospires start being shed in urine from the second week after infection.[22] These findings are consistent with those that have shown that positive MAT titres provide limited

prediction of shedding and renal carriage in sheep and cattle, when determined by urine PCR and kidney culture, respectively.[17,38]

In total, 4.1% of the urine qPCR positive and 4.9% of kidney qPCR positive animals were seronegative to both serovars. This may be attributable to antibodies decreasing to low or non-detectable after a long period past exposure. In naturally infected animals, detectable MAT titres may persist for one to two years, or even at low levels throughout the life of the animal.[22,40,64] However, all sheep and cattle that were urine or kidney qPCR positive but seronegative were aged less than 12 months old and 18 months old, respectively. This suggests that the time elapsed since exposure may not be a reason for the seronegativity in these qPCR positive animals. Another possible explanation for this can be that these animals were shedding leptospires in urine before an antibody response was detected.[22] Further, infections may have been due to serovar/s that was not tested by the MAT in this study (e.g. Ballum). In total, 97.1% and 96.7% of MAT-negative animals were urine- and kidney-PCR negative, respectively. Therefore, negative MAT was a reasonable predictor of non-carrier and non-shedding state.

For urine and kidney samples that were qPCR positive, data was provided for MAT positivity for Hardjobovis and/or Pomona, and also for MAT positivity for Hardjobovis-only and Pomona-only. Data suggested that both sheep and cattle with Hardjobovis-only positive titres were more likely to be shedding/had renal colonisation than those with Pomona-only positive titres. The prevalence ratio of urine and kidney qPCR positivity in Hardjobovis-seropositive versus Hardjobovis-seronegative animals was higher in sheep than cattle. Animals infected with host-adapted strains tend to have a long kidney phase and shed leptospires in the urine for longer duration than those infected with non-host adapted strains.[50] Study findings may be a reflection of a higher probability of detecting leptospires in animals infected with host-adapted strains as opposed to those infected with non-host adapted strains and suggest that Hardjobovis was better adapted than Pomona to sheep and cattle, while the adaption of Hardjobovis was better in sheep than cattle.

When interpreting the level of MAT titres, our data suggest that the interpretation was different for serovars Hardjobovis and Pomona. For Pomona-seropositive animals, higher MAT titres predicted higher probability of shedding. However, this association was not seen for Hardjobovis-seropositve animals. In these, regardless of the level of

the MAT titre, high probability of shedding was reported. Nevertheless, the true level of shedding in a flock or herd needs to take into account the proportion of shedders that were MAT-negative. The different trends between Hardjobovis and Pomona may further indicate that the two pathogens elicit different host immune responses and/or have different effects on sheep and cattle. Data from sero-conversion ('infection') rates among abattoir workers in New Zealand also showed differences between these two serovars: infection rates with Pomona were twice as high as with Hardjobovis[19] whereas exposure rates to Pomona were lower.[18] Furthermore, data revealed that workers with 'flu-like' symptoms had significantly higher titres against Pomona than those without 'flu-like' symptoms (p = 0.02); while Hardjobovis titres of workers with 'flu-like' symptoms did not differ from those without 'flu-like' symptoms.[19] Thus it appears that the three hosts under study (humans, cattle, and sheep) show a greater response when infected by Pomona than Hardjobovis.

In this study, culture was undertaken on kidneys to provide strains for multi-locus sequence typing (Chapter 6). The isolation rate from kidney samples, which were only from animals with positive qPCR results, was 19% (95% CI: 13%-27%). PCR assays can detect both viable and dead bacteria, and culture requires sufficient numbers of viable bacteria.[48] Also, the detection limit of culture is considered to be lower than that of PCR assays.[29] Hence some negative cultures may be due to there being only dead leptospires in kidney and/or urine, or live leptospires at low concentration. In kidney, growth of leptospires usually reaches a maximum about 21 to 28 days after infection. It is possible that some of the animals had just recovered from acute infection at the time of sampling (e.g. one to two weeks after infection), while leptospires persisted in small numbers (lower than the detection limit of culture) in the proximal renal tubules following clearance from the bloodstream.[22] The low isolation rate of culture may also be attributed to the storage of kidneys at 4°C for up to two days while awaiting results from the qPCRs. It has been reported that post-mortem changes and storage can reduce the number of viable bacteria in kidneys.[44] Moreover, contamination (overgrowth by other bacteria and fungus) may also interfere with isolation of leptospires from qPCR positive kidneys.[23]

The inter-laboratory comparison of urine qPCRs on sheep and cattle in this study was novel and has yielded useful results for researchers and diagnosticians. Almost perfect agreement between urine qPCRs results from a research and a commercial veterinary

diagnostic laboratory, where different amplification genes, chemistries, and pre-PCR processing were used, was demonstrated. This confirms that studies based on these two tests for detecting leptospiral shedding in sheep and cattle in New Zealand are comparable. The inter-laboratory comparison of MATs revealed moderate agreement for the Pomona MATs. This again emphasized the well-known challenge of interpreting MAT results obtained from different laboratories, and the importance of quality control for MAT testing at each individual laboratory, and use of standards when research is relying on testing conducted by different laboratories. The comparison of HLRL qPCR on urine and kidney samples showed almost perfect agreement, indicating that results of this q on urine samples is representative useful surrogate measure for the results of qPCR on kidneys. This is useful for veterinary studies where sampling of kidney from live animals is not possible. The comparison between HLRL MAT and urine/kidney qPCR results indicated that, except for Hardjobovis in sheep, MAT was not a reliable predictor of shedding and renal carriage of Pomona in sheep or Hardjobovis and Pomona in cattle at individual animal level in New Zealand.

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7 Sources and manufacturers

a. Vacutainer® SST^{TM} II advance tubes, BD, Franklin Lakes, NJ.
b. Vacutainer® evacuated plastic conical bottom tubes, BD, Franklin Lakes, NJ.
c. Container 500ml (screwed cap), Raylab, Auckland, New Zealand.
d. Eppendorf tube, Raylab, Auckland, New Zealand.
e. QIAamp DNA Mini Kit, Qiagen, Bio-Strategy Ltd, Auckland, New Zealand.
f. BioSprint 96 One-For-All Vet Kit, Qiagen, Bio-Strategy Ltd, Auckland, New Zealand.
g. Colworth Stomacher 400, AJ Seward Ltd, London, UK.
h. High Pure PCR Template Preparation Kit, Roche, Mannheim, Germany.
i. Rotor-Gene Q machine, Qiagen, Bio-Strategy Ltd, Auckland, New Zealand.
j. Invitrogen Corp., Carlsbad, CA.
k. *Taq* PCR Kit, New England BioLabs, Ipswich, MA.
l. Corbett Rotor-Gene™ 6000, Bio-Strategy Ltd, Auckland, New Zealand.
m. QuantiTech Probe PCR Master Mix, Qiagen, Bio-Strategy Ltd, Auckland, New Zealand.
n. Integrated DNA Technologies (IDT), Custom Science, Auckland, New Zealand.
o. 96-well flat-bottom polystrene plate, Greiner Bio-one, Frickenhausen, Germany.
p. Olympus BH2 dark-field microscope, Olympus, Tokyo, Japan.
q. R Development Core Team, 2011; R Foundation for Statistical Computing, Vienna, Austria.
r. SAS Institute Inc., Cary, NC.

8 Declaration of conflicting interests

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Chapter 6 Multilocus Sequence Typing of *Leptospira* field strains isolated from sheep and cattle in New Zealand

1 Introduction

Leptospirosis is a zoonotic disease of global distribution caused by the pathogenic spirochetes of the genus *Leptospira* (Levett 2001; Bharti *et al.* 2003). Feral and domestic mammals can be maintenance hosts and excrete leptospires in the urine (Plank and Dean 2000; Levett 2007). Humans serve as an incidental host for a leptospiral infection which is usually acquired through direct contact with the urine or body fluids of infected animals, or indirect contact with a contaminated environment (Faine *et al.* 1999; Bharti *et al.* 2003). In New Zealand, leptospirosis is the most common occupationally-acquired zoonotic disease (Vickery *et al.* 2006; Keenan 2007), and the occupations identified as being at high risk of exposure to *Leptospira* spp. were meat workers, farmers, and forestry-related workers (Thornley *et al.* 2002). From 2008 to 2012, there were 456 notified cases of leptospirosis in NZ. Among 414 notified cases that had occupations recorded, meat workers and farmers constituted 22.7% (94/414) and 52.7% (218/414), respectively (The Institute of Environmental Science and Research Ltd 2009-2012).

Identification of *Leptospira* species plays an important role in understanding the epidemiology and pathogenicity of leptospirosis, and also the development of effective vaccines and preventive strategies. According to the traditional (phenotypic) classification, the genus *Leptospira* is divided into two species: *L. interrogans* including pathogenic strains and *L. biflexa* representing saprophytic (non-pathogenic) strains (Levett 2001). These two groups can be differentiated by their nutritional requirements and other phenotypic properties (Johnson and Rogers 1964; Levett 2001; Cerqueira and Picardeau 2009). Within the pathogenic and saprophytic species, numerous serovars are defined serologically by the cross agglutination absorption test (CAAT), based mainly on the diversity of structure of the surface-exposed components of the lipopolysaccharide (LPS) (de la Peña-Moctezuma *et al.* 1999; Cerqueira and Picardeau 2009). More than 60 serovars of *L. biflexa* have been recorded and more than 200

serovars that have been distributed among 24 serogroups of antigenically similar serovars are recognised within *L. interrogans* (Levett 2001). Identification of isolates to serovar level is essential for understanding the epidemiology of leptospirosis for both humans and animals. However, because the CAAT is cumbersome and time-consuming for routine typing, few laboratories are able to perform this technique (Terpstra *et al.* 1985). Therefore, most isolates are identified by the microscopic agglutination test (MAT) for serogroup information, which does not indicate taxonomic status, but is useful for both diagnosis and epidemiological purposes (Faine *et al.* 1999).

Genetic (genotypic) classification based on DNA-DNA hybridization analysis has been introduced due to the genetic variation observed within the genus *Leptospira* (Haapala *et al.* 1969; Brendle *et al.* 1974; Yasuda *et al.* 1987; Brenner *et al.* 1999). The serological classification of serovars is based on cell surface antigen (notably the LPS). However, genetically diverse strains can be serologically closely related (e.g. *L. interrogans* serovar Hardjoprajitno vs. *L. borpetersenii* serovar Hardjobovis), causing confusion. The genetic classification is based on the molecular identity of a strain and is considered a more reliable method of characterization (Ahmed *et al.* 2006; Nalam *et al.* 2010). In the genetic classification system, a number of species were demonstrated and include all serovars of both *L. interrogans* and *L. biflexa* (Levett 2001). The identified species include pathogenic species that are capable of causing humans and animals infection, saprophytic (non-pathogenic) species that are environmental *Leptospira* and do not infect mammals, and intermediate species that are able to infect humans and animals but cause no or mild symptoms (Nalam *et al.* 2010; Ricaldi *et al.* 2012). Until now at least 20 species have been recognised, while *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. kirschneri* and *L. alexanderi,* are considered as the seven main pathogenic *Leptospira* species (Nalam *et al.* 2010).

The correlation between the serological and genetic classification is poor. Serovars belonging to the same serogroup are found in different *Leptospira* species (Levett 2001), suggesting that the genes determining serotype may be transferred horizontally between species (Cerqueira and Picardeau 2009). For example, by comparing the sequences of LPS biosynthetic loci (*rfb*), it is proposed that the Hardjoprajitno *rfb* locus is derived from a strain closely related to serovar Copenhageni, and subsequently acquires specific sections of the Hardjobovis *rfb* locus by horizontal genetic transfer. Therefore, two serologically indistinguishable subtypes of serovar Hardjo are produced,

but are genetically classified as two different species (de la Peña-Moctezuma *et al.* 1999). To be compatible with the system of serogroups/serovars that routinely serves clinicians and epidemiologists, the genetic classification is now being used in conjunction with the serological classification (Levett 2001).

Because it is not practical to use DNA-DNA hybridization for routine analysis (Faine *et al.* 1999), many other molecular methods have been employed for identification and subtyping of *Leptospira*. Such methods include digestion of whole genome DNA by restriction endonucleases analysis (REA) (Robinson *et al.* 1982; Thiermann *et al.* 1986), restriction fragment length polylmorphism (RFLP) (Zuerner *et al.* 1993), ribotyping (Perolat *et al.* 1994), pulsed field gel electrophoresis (PFGE) (Herrmann *et al.* 1991; Herrmann *et al.* 1992), variable number of tandem repeats (VNTR) analysis (Majed *et al.* 2005), fluorescent amplified fragment length polymorphism (FAFLP) (Vijayachari *et al.* 2004) and a number of PCR-based approaches. However, these methods have certain limitations, such as the requirement for large quantities of purified DNA, low discriminatory power, low reproducibility, or ambiguous interpretation of data. Moreover, there is difficulty of transferring such data between different laboratories (Levett 2001; Ahmed *et al.* 2006; Maiden 2006). To overcome these drawbacks, several methods based on the analysis of PCR-amplified sections of leptospiral DNA have been employed. Sequencing of specific genes or gene fragments has been used for the molecular typing of *Leptospira spp.* including *rrs*, *rpoB*, *gyrB*, *secY* and *ligB* (La Scola *et al.* 2006; Morey *et al.* 2006; Slack *et al.* 2006; Ahmed *et al.* 2009; Cerqueira *et al.* 2009). Whole genome sequencing has been employed to analyse the genomic DNA of leptospiral serovars, primarily to better understand the mechanisms of virulence and pathogenesis in leptospirosis, rather than the identification of *Leptospira* (Ren *et al.* 2003). There are currently four published complete genome sequences of two leptospiral pathogenic species: two strains of *L. borgpetersenii* serovar Hardjobovis and two serovars (Lai and Copenhageni) of *L. interrogans* (Ren *et al.* 2003; Nascimento *et al.* 2004; Bulach *et al.* 2006).

Multilocus sequence typing (MLST) is a PCR based technique which has been used for the study of bacterial evolution and population biology of a large number of microbial species such as *Neisseria meningitidis* (Maiden *et al.* 1998), *Streptococccus pneumoniae* (Enright and Spratt 1998), *Staphylococcus aureus* (Enright *et al.* 2000), *Haemophilus influenzae* (Meats *et al.* 2003), *Escherichia coli* (Adiri *et al.* 2003),

Campylobacter jejuni (Dingle *et al.* 2001), *Listeria monocytogenes* (Salcedo *et al.* 2003) and *Candida albicans* (Cliff *et al.* 2008). MLST assigns the alleles at different targeted loci by nucleotide sequence, and the allelic profile provided by the alleles from all loci defines the sequence type (ST) of each isolate (Enright and Spratt 1999). The selected loci for MLST are generally housekeeping genes, which are not subject to diversifying selection and evolve slowly over an evolutionary time-scale, making MLST an appropriate technique for long term and global epidemiology (Maiden *et al.* 1998; Enright and Spratt 1999; Maiden 2006). The MLST scheme provides highly discriminatory, reliable, reproducible and easy-to-interpret results that can be stored in a digital form and hence, are readily exchangeable between laboratories (Maiden 2006; Cerqueira and Picardeau 2009). MLST has been applied to *Leptospira* spp. via two different sets of genes, *adk*, *icdA*, *lipL32*, *lipL41*, *rrs*, and *secY* developed by Ahmed *et al.* (Ahmed *et al.* 2006), and *pntA*, *sucA*, *pfkB*, *tpiA*, *mreA*, *glmU*, *fadD* developed by Thaipadungpanit *et al.* (Thaipadungpanit *et al.* 2007). Further modification has been made to the scheme from Thaipadungpanit *et al.* (2007) by replacing gene *fadD* with gene *caiB* (as gene *fadD* was not present in some pathogenic species) and designing new primers for the other six genes (Boonsilp *et al.* 2013).

In New Zealand, six serovars from two species of *Leptospira* have been isolated and are known to be endemic in animals. These are *L. interrogans* serovars Copenhageni and Pomona, and *L. borgpetersenii* serovars Balcanica, Hardjobovis, Tarassovi and Ballum (Table 6.1). Two serovars *L. interrogans* Australis and Canicola have been isolated rarely from humans, and cannot be ascribed endemic status in New Zealand (Marshall and Manktelow 2002). Cattle, sheep and deer are important animal reservoirs of leptospires in New Zealand. Previous surveys of farms and abattoirs have shown that 69% (59/85) of beef cattle herds, 44% (42/95) of sheep slaughter lines, and 81% (90/111) of deer herds were seropositive for leptospirosis (Heuer *et al.* 2007; Dorjee *et al.* 2008; Ayanegui-Alcérreca *et al.* 2010). Among the six serovars that had been isolated from animals reservoirs, serological evidence of leptospirosis in domestic livestock is most commonly found with serovars Hardjobovis and Pomona in New Zealand (Dreyfus *et al.* 2011).

Table 6.1 *Leptospira* species endemic in New Zealand and animal reservoirs to which the serovars are adapted (Hathaway 1981; Marshall and Manktelow 2002; Ayanegui-Alcerreca *et al.* 2007; Dorjee *et al.* 2008).

Species	Serogroup	Serovar	Animal reservoirs
L. interrogans	Icterohaemorrhagiae	Copenhageni	Brown or Norway rat (*Rattus norvegicus*)
	Pomona	Pomona	Pigs, deer
L. borgpetersenii	Sejroe	Balcanica	Brush tail possums (*Trichosurus vulpecula*)
		Hardjobovis	Cattle, sheep and deer
	Tarassovi	Tarassovi	Pigs
	Ballum	Ballum	Black rat (*Rattus rattus*), mice, hedgehogs

Since a limited number of serovars have been isolated in New Zealand and most of them belong to different serogroups (except Hardjobovis and Balcanica which both belong to the serogroup Sejroe which can be differentiated by infected animal species) (Hathaway 1981), it is considered that infection with different serovars can be differentiated serologically by MAT. However, studies have shown that the commonly found paradoxical reactions and cross-reactions between serogroups/serovars prevent the MAT from being a reliable test to predict the infecting serogroup/serovar, especially at the acute phase of infection (Levett 2003; Miller *et al.* 2011). Although genetic classification is considered to provide a more reliable characterization than serological classification, genotypic data from *Leptospira* in New Zealand is limited. In the early 1980s, REA was used for characterizing *Leptospira* isolates (mainly Hardjobovis and Balcanica) from New Zealand, and also comparing the fragment patterns of New Zealand strains to those of reference strains (provided by the Centre for Disease Control) (Marshall *et al.* 1981; Robinson *et al.* 1982). In 2009 a MLST scheme based on a previously described paper (Ahmed *et al.* 2006) was developed and feasibility tested at the Hopkirk Leptospirosis Research Laboratory (HLRL) (Platero 2009), using reference strains of six serovars that are endemic in New Zealand (Table 6.2). Sequencing of *gyrB* gene PCR product from field isolates cultured from deer kidney and urine was used for the identification of pathogenic *Leptospira* species in a recent study (Subharat *et al.* 2011).

Table 6.2 Strain type of each of the six serovars of *Leptospira spp.* reference cultures maintained by HLRL

Species	Serovar (sv.)	Strain
L. borpetersenii	Tarassovi	HLRL culture collection
	Ballum	(Mus 127) ex WHO
	Balcanica	(Burgas 1627) ex WHO
	Hardjobovis	#38, HLRL stock
L. interrogans	Pomona	# 68, HLRL stock
	Copenhageni	HLRL culture collection

HLRL = Hopkirk Leptospirosis Research Laboratory; WHO = World Health Organization Leptospirosis Reference Laboratory at Brisbane, Australia

The aim of this study was to apply the MLST scheme set up by Platero (2009) to characterise a pilot panel of field isolates of *Leptospira* from sheep and cattle. Information obtained from this work has the potential to inform mitigation strategies, such as targeting the strains present in livestock for inclusion in vaccines. Sequences of isolates from sheep and cattle from the same farm were compared to investigate the potential for inter-species transmission of *Leptospira.*

2 Materials and Methods

2.1 Cultures for *Leptospira* isolates

Workers in a sheep and cattle abattoir in the Waikato region experienced three leptospirosis cases (two hospitalised) from 20 staff in the 2008/09 killing season. Two of the cases were associated with serovar Pomona. The abattoir is a small abattoir that processes sheep and cattle provided by seven suppliers for local consumption. The six suppliers recruited in the study were either sheep-only or mixed-species (sheep and cattle) suppliers that grazed animals geographically close to the abattoir. From September to November 2010, blood, urine and kidney samples from 399 sheep and 146 beef cattle were collected at slaughter. Blood samples were centrifuged at 1300 *g* for 10 minutes and the supernatants (serum) were collected and sent with urine and kidney samples under refrigerated conditions to HLRL on the day of collection. The samples were tested to determine the supplier-specific urinary shedding rate, renal colonisation (using real-time PCR) and seroprevalence (using MAT) of leptospires (protocols were

described in Chapter 4). Culture was undertaken on kidney (n=144) samples if positive quantitative real-time PCR results were received from either urine or kidney.

Kidney surfaces were sterilised by swabbing with 70% ethyl alcohol. An approximately 10 g section of kidney, extending from the renal cortex to the medulla, was removed aseptically. The removed section was doused with 70% ethyl alcohol followed by flaming with a Bunsen burner. It was then homogenised in 50 ml phosphate buffered saline, using a Colworth Stomacher 400 (AJ Seward Ltd, London, UK). A 100 µl aliquot of this suspension was inoculated into 5 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (containing 5'-fluorouracil to help prevent contamination) prepared at the HLRL. From this inoculated medium, a serial dilution was performed by transferring a 100 µl aliquot to another 5 ml of medium, followed by further transfer of 100 µl to another 5 ml of medium. All three inoculated medium bottles were then incubated aerobically at 28–30°C, and examined every week for the presence of leptospires, using dark-field microscopy. Cultures that showed no detectable growth after 12 weeks were declared negative.

2.2 Leptospire typing by MLST

2.2.1 DNA extraction

About 1 ml of each culture isolate was added into a 1.5 ml Eppendorf tube and centrifuged at 12470 *g* for 5 min. The supernatant was discarded and the pellet was re-suspended with 400 µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8). The solution was heated at 100 ºC by using a multi-block heater for 10 min. After cooling down, the sample solution was centrifuged at 12470 *g* for 5 min. The supernatant was then used for PCR amplification.

2.2.2 PCR amplification

The six MLST target genes and primers used in this study (based on a previous described paper (Ahmed *et al.* 2006)) are presented in Table 6.3.

Table 6.3 The six MLST genes and primers used in this study

Gene	Gene size	PCR product size (bp)	Functions	Primer sequence
adk	564	531	Adenylate Kinase	F5'-GGGCTGGAAAAGGTACACAA-3' R5'-ACGCAAGCTCCTTTTGAATC-3'
icdA	674	674	Isocitrate Dehydrogenase	F5'-GGGACGAGATGACCAGGAT-3' R5-TTTTTTGAGATCCGCAGCTTT-3'
lipL41	520	520	Outer membrane Lipoprotein LipL41	F5'-TAGGAAATTGCGCAGCTACA-3' R5'-GCATCGAGAGGAATTAACATCA-3'
rrs2	541	541	16S ribosomal RNA	F5'-CATGCAAGTCAAGCGGAGTA-3' R5'-AGTTGAGCCCGCAGTTTTC-3'
secY	549	549	Translocase pre-protein secY	F5'-ATGCCGATCATTTTTGCTTC-3' R5'-CCGTCCCTTAATTTTAGACTTCTTC-3'
lipL32	474	474	Outer membrane Lipoprotein LipL32	F5'-ATCTCCGTTGCACTCTTTGC-3' R5'-ACCATCATCATCATCGTCCA-3'

The PCR amplification was performed in a Gradient Palm Cycler (Bio-Strategy Ltd, Auckland, New Zealand), according to the method described by Platero *et al.* (2009) (Platero 2009). The 25 µl reaction mix included 1×PCR buffer, 1.0 mM $MgCl_2$, 200 µM dNTPs, 5 pmol each of both the forward and reverse primer, 1.25 units of *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA), 2 µl of DNA template and double distilled water (ddH_2O). Thermal cycling involved initial denaturation at 95°C for 5 min, followed by 40 cycles of amplification consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and a final extension at 72°C for 1 min.

A 2 µl aliquot of each of the PCR products was mixed with approximately 1 µl of loading dye (Bromophenol Blue) and subjected to electrophoresis in a 1.5 % agarose gel (UltraPure™ Agarose, Invitrogen Corp., Carlsbad, CA, USA) in Tris-borate-EDTA buffer (45mM Tris-borate, 1mM EDTA). A 5 µl aliquot of DNA ladder (1 Kb Plus DNA ladder, Invitrogen) was loaded into the gel as a reference. After electrophoresis, the gel was stained with ethidium bromide and visualised using a UV illuminator (Bio-Rad laboratories, Foster City, CA, USA) and the band size of the PCR product was compared with the DNA ladder.

2.2.3 Purification of the amplified PCR products

After a sample had been checked by electrophoresis, 23 µl of each PCR amplicon was mixed with 23 µl of 20% PEG solution (polyethylene glycol, NaCl) and incubated at 30 ºC for 30 min. After incubation, the solution was centrifuged at 12470 *g* for 30 min. The supernatant was discarded and the pellet was re-suspended with 150 µl of 80% ethanol. The solution was centrifuged at 12470 *g* for 30 min again, and the supernatant was discarded. The pellet was left to completely dry overnight at room temperature. A 12 µl aliquot of distilled water was added to the purified PCR product.

2.2.4 Sequencing

The DNA concentration of each purified PCR product was determined by using the NanoDrop – 1000 Spectrophotometer (NanoDrop Technology, Inc. Wilmington, DE, USA). Each of the sequencing reactions was prepared as required (Appendix 6a) with 3.2 pmol of each forward or reverse primer, purified PCR product (2 ng/100 bp), and ddH_2O to a volume of 15 µl. The reactions were sent to the Genome Service, Institute of Molecular Biosciences, Massey University, Palmerston North for sequencing.

2.2.5 Sequence analysis

DNA sequence editing, alignment and the construction of phylogenetic trees were performed using software Geneious version 5.5.8. (Biomatters Ltd, Auckland, New Zealand). The edited nucleotide sequences of each strain on six loci were compared with the sequences of the six reference *Leptospira* serovar strains from HLRL (Platero 2009).

The sequences of the 23 isolates were aligned to those from an in-house database (Appendix 6b) constructed by inclusion of data for 271 strains from Ahmed *et al.* (Ahmed *et al.* 2006), two strains from Genebank (*L. borgpetersenii* serovar Hardjobovis strain JB197, accession number CP000350.1 and *L. borgpetersenii* serovar Hardjobovis strain L550, accession number CP000348.1) and 19 Mexican strains that typed based on the same allelic scheme (Carmona-Gasca *et al.* 2011). In this database, the amplicon sequences included the primer sequences for three genes (*secY, lipL32 and lipL41*). It is not possible to obtain high quality sequence data for bases very close to the primer ends (Ahmed *et al.* 2011). Given this unreliability, the amplicon sequences for these three genes from the 271 strains were trimmed to align with the 23 strains in this study.

Carmona-Gasca *et al.* (2011) had used variations in some of these less reliable areas of sequence reads to define allelic profiles. Restricting analysis to the trimmed amplicon sequences however, resulted in less differentiation and fewer allelic strain types and we were thus unable to directly compare our isolates with the allelic profiles that were assigned in the in-house database. Therefore, in this study, our isolates were compared with the 271 isolates in the in-house database by the grouping of sequences at each locus and presented by allelic groupings.

A phylogenetic tree was constructed for each locus and also for the concatenated sequences of six loci to show the genetic relatedness between isolates based on the distance matrices (nucleotide substitutions per site). Genetic distance model Tamura-Nei and the neighbour-joining method was used to construct the phylogenetic trees. The gene order of the 'super locus' comprising the concatenated sequences of all six loci was *adk, icdA, lipL32, lipL41, rrs2, and secY*.

3 Results

3.1 *Leptospira* isolates

For the 144 kidneys cultured, detectable growth of leptospires was obtained from 26 samples (18%; 95% CI: 13%-25%), sourced from five suppliers. Among these, the concentration of leptospires in 23 cultures reached the level required for a successful DNA extraction procedure. Supplier and animal species, MLST allelic groupings, and MAT titres against serovar Hardjobovis and Pomona for the 23 isolates in this study are presented in Table 6.4.

3.2 PCR amplification

All of the 23 isolates were amplified on six loci.

3.3 Sequence analysis

Although re-amplification (with hot-start *Taq* and a reduced annealing temperature) and re-sequencing were done, the quality of amplicon sequences from isolates D29 and E48 at loci *icdA*, and from isolate E48 at loci *lipL41* was poor and could not be read (Table 6.4). Therefore those three sequences were excluded from the final discussion.

The sizes of the fragments analysed for the six genes were 430 bp for *adk*, 557 bp for *icdA,* 435 bp for *lipL32,* 478 bp for *lipL41,* 452 bp for *rrs2, and* 504 bp for *secY*. The size of the concatenated sequences of all six loci was 2856 bp.

For 13 isolates, nucleotide sequences from all six loci were genetically identical to the *L. borgpetersenii* Hardjobovis reference strain from HLRL. For eight isolates, nucleotide sequences from all six loci were genetically identical to the *L. interrogans* serovar Pomona reference strains. These indicated that the cluster of 13 isolates was *L. borgpetersenii* serovar Hardjobovis isolates and the cluster of eight isolates was *L. interrogans* serovar Pomona isolates.

For D29 that had the sequences for only 5 genes, the sequences were identical to the cluster of Hardjobovis isolates. For E48 that had sequences of four genes, the sequences of *adk, lipL32, and rrs2* were genetically identical to the other Hardjobovis isolates, while the sequence of gene *secY* was identical to the Pomona isolates. For the amplicon sequences from E48 at loci *lipL41*, the unreadable polymorphic sites constituted 43 of the 44 non-synonymous sites between Hardjobovis and Pomona isolates (Appendix 6c), indicating sequences obtained from both Hardjobovis and Pomona bases.

3.4 Phylogenetic tree analysis

For all of the six genes tested, two distinct clusters were generated in the neighbour-joining trees based on the genome species (for example, *adk* gene, Figure 6.1). Within each cluster, strains were genetically identical. According to the comparison with reference strains from HLRL (Table 6.2), one cluster grouped Hardjobovis isolates and the other grouped Pomona isolates. The grouping of strains in the two clusters was consistent for all of the six genes, except for E48 which was grouped in the Hardjobovis cluster for genes *adk, lipL32, and rrs2,* but grouped in the Pomona cluster for gene *secY.* The percentage of sequence bases that are identical between the two clusters of isolates was 85.4% for *adk* gene, 88.0% for *icdA* gene, 95.2% for *lipL32*, 90.8% for *lipL41*, 97.8% for *rrs2*, and 81.6% for *secY*.

A neighbour-joining tree for concatenated sequences was constructed for 21 isolates (Figure 6.2). Sequences were not obtained from isolates E48 and D29 on all loci and were excluded for this analysis. For these 21 isolates, two distinct clusters were

generated and were consistent with those presented in neighbour-joining tree for each of the six genes.

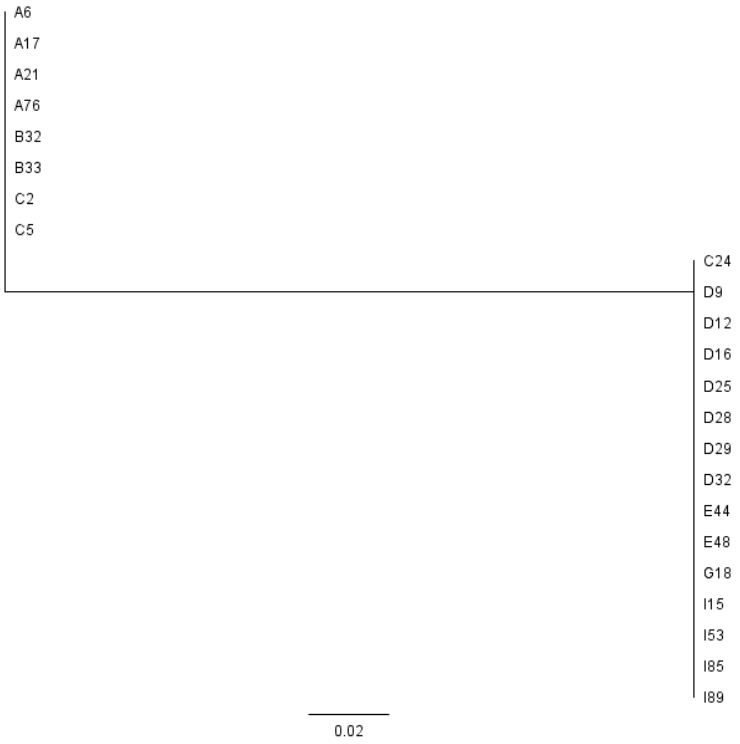

Figure 6.1 Neighbour-joining tree built on Tamura-Nei model of 23 isolates for *ad*k gene. The bar indicates 0.02 estimated substitution per sequence position

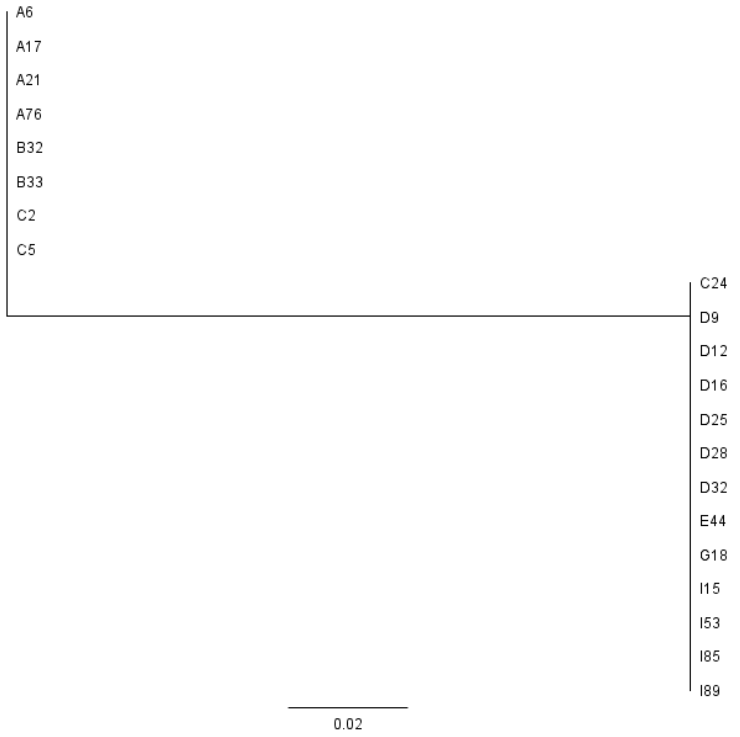

Figure 6.2 Neighbour-joining tree built on Tamura-Nei model based on concatenated sequences of six loci from 21 isolates. The bar indicates 0.02 estimated substitution per sequence position.

3.5 MLST results

Comparing the sequences of all six loci for the 23 isolates in this study with those for strains from the in-house database, the sequences for Pomona isolates were identical to a *L. interrogans* serovar Kennewicki strain LT1026 (serogroup Pomona) from USA, while the sequences for the Hardjobovis isolates were identical to the *L. borgpetersenii* serovar Hardjobovis strain L550 (serogroup Sejroe) (Table 6.4).

Table 6.4 Supplier and animal species, MLST allelic groupings for the 23 isolates, and microscopic agglutination test (MAT) titres against serovar Hardjobovis and Pomona for the animals from which the 23 isolates were obtained in this study

Supplier		**Species**	**Isolates**	**Allelic groupings**						**MAT titres** [b]	
				adk	***icdA***	***lipL32***	***lipL41***	***rrs2***	***secY***	**Hardjobovis**	**Pomona**
Sheep only	A	Sheep	A6	P	P	P	P	P	P	–	>1600
		Sheep	A17	P	P	P	P	P	P	–	>1600
		Sheep	A21	P	P	P	P	P	P	–	>1600
		Sheep	A76	P	P	P	P	P	P	–	>1600
	C	Sheep	C2	P	P	P	P	P	P	800	>1600
		Sheep	C5	P	P	P	P	P	P	>1600	>1600
		Sheep	C24	H	H	H	H	H	H	>1600	>1600
Mixed sheep and cattle	BG	Sheep	B32	P	P	P	P	P	P	>1600	800
		Sheep	B33	P	P	P	P	P	P	>1600	>1600
		Cattle	G18	H	H	H	H	H	H	>1600	–
	DH [a]	Sheep	D9	H	H	H	H	H	H	>1600	–
		Sheep	D12	H	H	H	H	H	H	>1600	–
		Sheep	D16	H	H	H	H	H	H	>1600	–
		Sheep	D25	H	H	H	H	H	H	>1600	–
		Sheep	D28	H	H	H	H	H	H	>1600	–
		Sheep	D29	H	/	H	H	H	H	>1600	–
		Sheep	D32	H	H	H	H	H	H	1600	–
	EI	Sheep	E44	H	H	H	H	H	H	>1600	–
		Sheep	E48	H	/	H	/	H	P	>1600	–
		Cattle	I15	H	H	H	H	H	H	>1600	–
		Cattle	I53	H	H	H	H	H	H	>1600	–
		Cattle	I89	H	H	H	H	H	H	>1600	–
		Cattle	I85	H	H	H	H	H	H	>1600	–

H = sequences identical to *L. borgpetersenii* serovar Hardjobovis strain L550 (serogroup Sejroe)
P = sequences identical to *L. interrogans* serovar Kenniwicki strain LT1026 (serogroup Pomona)
'/' = sequences from isolates D29 and E48 were not readable at the locus
[a] no cattle isolate was obtained from supplier DH
[b] MAT was performed at Gribbles Veterinary Pathology, Palmerston North
'–' = no MAT titres detected

4 Discussion

This is the first time the MLST technique has been applied to animal field isolates in New Zealand. The findings in this study demonstrate the utility of the MLST scheme (Platero *et al.* 2009) for genotyping *Leptospira* spp. field isolates from sheep and cattle in New Zealand. The sequence results provided sufficient genetic variability to assign the isolates to two distinct species, those being *L. borgpetersenii* and *L. interrogans*. Two dominant serovars (Hardjobovis and Kenniwicki) were identified by sequencing *Leptospira* isolates from sheep and cattle. Identical sequences found in Hardjobovis isolates from sheep and cattle provided evidence for inter-species transmission of *Leptospira* spp.. A larger number of field strains from broader areas in New Zealand now need to be genotyped to support these findings.

The sequences of all six loci from the eight Pomona isolates were the same as the *L. interrogans* serovar Kenniwicki strain from USA. Due to the close antigenic relationships between some serovars, the serological classification of serogroup Pomona is complicated (Ciceroni *et al.* 2002). On the basis of earlier REA typing results in 1982, which revealed no difference between reference strains of serovar Pomona, Kennewicki, Monjakov, and Cornelli from serogroup Pomona, these four serovars were grouped as serovar Pomona in the list of *Leptospira* serovars by the Sub-committee for the Taxonomy of the Leptospires (Stallman 1984). However, subsequent studies using REA, PFGE and arbitrarily primed PCR found that Pomona and Kennewicki strains were genetically similar, but not identical (Thiermann *et al.* 1985; Barriola and Saravi 1989; Skilbeck *et al.* 1992; Ciceroni *et al.* 2002). The findings in this study are consistent with previous studies that reported *Leptospira* strains (including from human, sheep and unrecorded sources) from New Zealand serologically classified as serovar Pomona belonged to serovar Kennewicki, based on REA results (Thiermann *et al.* 1985; Skilbeck *et al.* 1992). In New Zealand, vaccinations are formulated with serovar Pomona rather than Kennewicki, but the level of cross-protection afforded by the Pomona vaccines has not been confirmed experimentally. The characterization of serovar Kennewicki in this study suggests that further investigations with strains from a larger number of animals and more animal species are needed to determine whether serovar Pomona or serovar Kennewicki is the main serovar from serogroup Pomona that present in New Zealand. This could be important for achieving optimum vaccine efficacy in New Zealand.

Of the 13 Hardjobovis isolates, the sequences of all six loci were identical to those derived from the *L. borgpetersenii* serovar Hardjobovis strain L550 which was isolated in Australia from a human case who contracted leptospirosis from exposure to infected cattle (Bulach *et al.* 2006). The 13 Hardjobovis isolates were genetically identical to the *L. borgpetersenii* serovar Hardjobovis strain JB197 (strain isolated in the USA from a beef steer at slaughter) at four loci (*adk, lipL32, rrs2,* and *secY*), and differentiated from strain JB197 by gene *lipL41* and *icdA*, with percentage of identical sequence bases as 99.8% and 99.6%, respectively. Although the strain L550 and JB197 were found to be genetically close in this study and the complete genomic sequences of these two strains showed nearly identical genetic contents, with subtle frame shift and point mutations in another study (Bulach *et al.* 2006), these two strains are Hardjobovis strains representing two distinct clonal subtypes that have distinct phenotypes and virulence (Zuerner *et al.* 1993; Faine *et al.* 1999). Hardjobovis has a broad distribution all over the world, with different epidemiology of infections reported from different geographical regions. For example, Hardjobovis is a common cause of human leptospirosis in New Zealand and is not commonly associated with bovine abortions (Mackintosh *et al.* 1980; Carter *et al.* 1982; Faine *et al.* 1999), while it often causes reproductive failure in cattle in North America, where human infections were not commonly reported (Prescott *et al.* 1988; Miller *et al.* 1991). These observations indicate that besides the factor of different farming practices, the different epidemiology of infections reported from different regions may also be due to the Hardjobovis isolates differing at the genetic level. The previous REA studies of cattle field isolates of serovar Hardjobovis obtained from different regions (North and South American, Europe, Middle East, Africa and New Zealand) identified novel DNA patterns for New Zealand isolates, while isolates from other regions were clustered in genetic groups (e.g. isolates from North and South America, British Isles and Africa were clustered in one of the genetic groups) (Thiermann *et al.* 1986; Zuerner *et al.* 1993). The findings from this study again revealed Hardjobovis strains in New Zealand are genetically different from the strains in USA, based on the current database.

The MLST scheme from Ahmed *et al.* (2006) was selected for this study because the scheme was able to type strains that belonged to six of the seven important leptospiral species: *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. kirschneri and L. alexanderi*, and from different geographical areas and sources (Ahmed *et al.* 2006).

More importantly, the scheme was tested on the six HLRL reference strains of the most common infecting serovars of *Leptospira* in New Zealand, while all of the six reference strains were amplified and sequenced at the six loci (Platero 2009). As the MLST scheme developed by Thaipadungpanit *et al.* (2007) failed to type *L. borgpetersenii* strains at all seven loci (Thaipadungpanit *et al.* 2007), it was not used in this study. A study using both schemes for the genotyping of 48 isolates consisting of 40 *L. interrogans* and eight *L. kirschneri* found comparable discriminatory ability between two schemes; however, the isolates were resolved into a larger number of STs by the scheme described by Ahmed *et al.* (2006) than that described by Thaipadungpanit *et al.* (2007) (30 versus 21) (Ahmed *et al.* 2011).

All of the 23 isolates were amplified at six loci. However, the sequences of two isolates (D29 and E48) at gene *icdA* were of poor quality and not readable. The good sequences of these two isolates at other genes indicated that while the DNA quality was sufficient to obtain sequence reads from these alleles, the primers may not have annealed properly and the amplification may not be optimal for these two isolates at gene *icdA*. It is possible there was sequence divergence at these two alleles. The polymorphic sites of isolate E48 at gene *lipL41* matched nucleotide positions that are known to differ between the Hardjobovis and Pomona allele sequences and the conflicting signals matched either of the established Pomona or Hardjobovis base options (Appendix 6c). Therefore, the ambiguous sequence obtained from isolate E48 at gene *lipL41* was possibly due to the dual-infection of Hardjobovis and Pomona. The next logical step to confirm this will be testing culture of isolate E48, by say, the MAT. However, it is not applicable due to the poor growth of E48 in culture medium, therefore cannot be frozen and stored for further research. Since serovars Hardjobovis and Pomona are the most important serovars for both animal and human leptospirosis infections in New Zealand, more field isolates need to be obtained and sequence typed to confirm if the current MLST scheme applied is a reliable protocol for molecular characterization of *Leptospira* isolates in New Zealand. Previous studies working at antigen encoding genes *ligB* and *rpoB* showed that these two genes are potentially useful for the molecular discrimination of *Leptospira* strains (La Scola *et al.* 2006; Cerqueira *et al.* 2009). In addition, in a study that evaluated the ability of the *ligB, lipL41, secY,* and *rpoB* loci to discriminate between 38 pathogenic *Leptospira* strains from six pathogenic species, these four genes all showed optimal discriminatory power. In the same study,

the inclusion of the *rpoB* and *lipL41* loci to the MLST scheme developed by Thaipadungpanit et al. (2007) improved the level of discrimination of the original scheme (Cerqueira *et al.* 2010). Therefore, *ligB* and *rpoB* genes should be considered to be added to the MLST scheme for further evaluation.

The original concept of MLST suggested selection of housekeeping genes that are species specific, stable and evolve slowly (Enright and Spratt 1999). However, the scheme developed by Ahmed *et al.* (2006) does not conform to this concept, and includes a 16S rRNA gene (*rrs2*) and two genes encoding surface-expressed proteins (*lipL41* and *lipL32*). Although *lipL41* and *lipL32* were not under positive selection as expected, the conserved nature of two loci *lipL32* and *rrs2* was reported to reduce the between-species resolution (Ahmed *et al.* 2011). Unlike the scheme developed by Thaipadungpanit *et al.* (2007) which is accessible at the MLST website (http://leptospira.mlst.net/), the scheme described by Ahmed *et al.* (2006) is not associated with a public database and website. Individual investigators need to download the sequences from GenBank to perform comparative phylogenetic analysis. The MLST scheme developed by Thaipadungpanit *et al.* (2007) has been recently modified to allow the characterization of *Leptospira* strains for seven major pathogenic species (Boonsilp *et al.* 2013). It is worth comparing this scheme with the scheme described by Ahmed *et al.* (2006) for the molecular classification of *Leptospira* strains in New Zealand in the future.

Of the 21 isolates with sequences obtained on all loci, 16 isolates (76.2%) had the same serovar classification from both the MAT and MLST, while inconsistent classification were obtained from five sheep isolates (23.8%) (Table 6.4). Of these five, four isolates (B32, B33, C2 and C5) that were classified as serovar Pomona and one (C24) that were classified as serovar Hardjobovis by MLST had similar high MAT titres against both serovars. Since the MLST was based on PCR amplification of strains obtained from kidney cultures, the results may be influenced by the carrier status of leptospires in the animals. It is possible that these isolates came from sheep infected by both serovars. For example, one scenario is that exposure to one serovar lead to acute infection only, while following exposure to another serovar, the sheep recovered from the acute infection and had developed chronic infection by the time of sampling. A second possible scenario is the sheep had a recent infection with one serovar, but were re-infected with another serovar very shortly after. The kidney colonisation by the new serovar cleared the

colonisation by the previous one. A third scenario is that the serovar Hardjobovis is difficult to culture (Cousins *et al.* 1985), which may explain the case for isolates B32, B33, C2 and C5. The findings from this study demonstrated that agreement between the serological and genetic classification for sheep *Leptospira* isolates was poorer (31.3% (5/16) of sheep isolates had inconsistent identification) than that for cattle isolates (100% (5/5) of six isolates had consistent identification).

According to the MLST results from this study (Table 6.4), serovar Pomona was isolated from six sheep kidneys from sheep-only suppliers (except C24 from supplier C) and from two sheep kidneys (B32 and B33) from a mixed-species supplier, whereas servoar Hardjobovis was isolated from eight sheep kidneys, mostly from mixed-species suppliers (except E48 from supplier EI). For all of the five cattle isolates, serovar Hardjobovis was identified as the infecting serovar. Although supplier C only sent sheep for slaughtering, both sheep and cattle were grazed at that farm. The same genotype of serovar Hardjobovis isolated in sheep and cattle from one mixed-species supplier (EI) in this study suggests that inter-species transmission of leptospirosis does occur. Sheep and beef cattle are regarded as the maintenance hosts for serovar Hardjobovis and either accidental individual hosts or possibly maintenance hosts for Pomona in New Zealand (Subharat *et al.* 2007; Dreyfus 2013). The MLST results in this study supported the theory that sheep may be the reservoir host of both Hardjobovis and Pomona. Pomona infection in sheep was considered as sporadic in the past (Dorjee *et al.* 2008; Subharat *et al.* 2009). In contrast to the previous findings, the animal level seroprevalence of Pomona was 35.1% in sheep in this study local abattoir. One study, which included 3361 sheep farms located in eight regions of both North and South islands of New Zealand from 2009 to 2010 also showed animal-level seroprevalence of Pomona as 14.0% in sheep (Dreyfus 2013). These indicate that Pomona may have adapted to sheep in New Zealand.

The findings from this study, which is the first of its type in New Zealand, were based on 23 sheep and cattle isolates obtained from a single abattoir and therefore must be regarded as preliminary. The MLST scheme used in this study provided sufficient genetic variation to assign the isolates to two distinct species. Field isolates of more serovars need to be tested to investigate if this scheme is able to differentiate serovars from the same species. Hardjobovis and Kennewicki were the dominant serovars identified in this study. If further studies identify Kennewicki as the dominant strain

across the country rather than Pomona, the appropriateness of the strain in currently available vaccines in New Zealand may need to be re-evaluated. That Hardjobovis isolates from sheep and cattle were genetically identical provides robust evidence for cross-infection between livestock species on farms. A broader study is now needed to confirm the findings from this study and whether they can be applied to other locations in New Zealand. Further research also needs to be performed to test the applicability of *ligB* and *rpoB* genes and the modified scheme described by Boonsilp *et al.* (2013) for genotyping of *Leptospira* strains in New Zealand.

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Chapter 7 Comparison of diagnostic tests for acute leptospirosis in humans in New Zealand

1 Introduction

Leptospirosis is a zoonotic disease of global distribution caused by the pathogenic spirochetes of the genus *Leptospira* (Levett 2001; Bharti *et al.* 2003). Severe cases number approximately 300,000 to 500,000 each year worldwide, with case fatality rates of up to 30% (Tulsiani *et al.* 2010). The incidence of disease is higher in tropical than temperate regions, mainly due to the longer survival of leptospires in warm and humid environment (Levett 2001). Human infections are usually acquired through direct contact with infected animal urine or body fluids, or indirect contact with a contaminated environment (Faine *et al.* 1999; Bharti *et al.* 2003). Risk factors for infection include occupational exposures (farming, meat processing, butchering, veterinary practice), recreational activities associated with fresh water (swimming, rafting, canoeing, fresh water fishing), cultural factors (bathing in rivers, animal rearing, pets) and socioeconomic circumstances (sanitation, poverty) (Plank and Dean 2000; Bharti *et al.* 2003; Lau *et al.* 2010b). Cases have also been identified after extensive flooding. Outbreaks of leptospirosis related to heavy rainfall have been reported from diverse areas of the world (Plank and Dean 2000; Lau *et al.* 2010b).

In New Zealand, leptospirosis is the most important occupationally-acquired zoonotic disease, mainly associated with agricultural and meat workers (Thornley *et al.* 2002; Cowie and Bell 2012). Compared with other temperate developed countries, leptospirosis occurs at a higher frequency in New Zealand (Thornley *et al.* 2002). From 2008 to 2011 in New Zealand, the average annual notification rate was 2.0 per 100 000 (The Institute of Environmental Science and Research Ltd 2009-2012). Among cases with hospitalisation status recorded, around half were admitted to hospitals. In Waikato region, approximately 42% of the population live in rural or small independent urban areas, with significant numbers working in dairy and dry stock farming (Cowie and Bell 2012). In general, the Waikato District Health Board (DHB) reports higher than national rates of notified leptospirosis cases in New Zealand (The Institute of Environmental Science and Research Ltd 2009-2012).

The clinical presentation of leptospirosis is characterized by an incubation period of 2-30 days (usually 5-14 days), followed by two distinct clinical phases. The acute (first) phase lasts about a week, with leptospiraemia. The immune (second) phase is characterized by excretion of leptospires in the urine and appearance of antibodies in the serum (Faine 1999; Levett 2001). Early diagnosis is of great importance because antibiotic treatment has shown to be efficient for preventing leptospiruria and shortening the duration of clinical illness if initiated early (McClain *et al.* 1984; Edwards *et al.* 1988; Guidugli *et al.* 2000; Katz *et al.* 2001).

Leptospirosis cases can present with severe complications such as renal failure, liver failure, pulmonary haemorrhage, and myocarditis (Bharti *et al.* 2003). However, the majority of cases present relatively mild symptoms and they may be misdiagnosed as having influenza or a fever of an unknown origin (Faine *et al.* 1999; Plank and Dean 2000). It is widely believed that there is considerable under-reporting of leptospirosis in New Zealand (Keenan 2007; Heuer *et al.* 2008). The underreporting may be due to the lack of awareness of the disease, the presentation with influenza-like illness symptoms in less severe cases, and the currently accepted diagnostic methods to confirm leptospirosis in the acute setting (Levett *et al.* 2001; Bharti *et al.* 2003; Vickery *et al.* 2006). These can have significant financial implications. Misdiagnosis of cases with subsequent failure to treat may lead to absenteeism in the workforce (for example, an average of 4.4 days away from work per newly infected meat workers (Dreyfus 2013)), and may also result in severe complications (e.g. renal failure) leading to high hospital costs associated with admission to intensive care (Vickery *et al.* 2006).

The microscopic agglutination test (MAT), is regarded as the “gold standard” for detecting leptospiral antibody and is used as the definitive test in reference laboratories worldwide (O'Keefe 2002; Bharti *et al.* 2003; Shivakumar and Krishnakumar 2006). However, the MAT is time-consuming and requires significant expertise to perform and interpret (Cole *et al.* 1973; Levett 2001). The antibody response detected by the MAT generally occurs at around six to ten days after first appearance of clinical symptoms (Faine *et al.* 1999; Dutta and Christopher 2005). Therefore, when applied to serum from patients in the early stage of disease, the MAT results tend to be of limited sensitivity (Levett *et al.* 2001). MAT also has a number of other disadvantages, as the interpretation of test results is subjective (O'Keefe 2002; Bajani *et al.* 2003), the maintenance of a panel of live leptospires representative of local serovars is potentially

hazardous for laboratory workers (Levett 2001; Smythe *et al.* 2002), and antibiotic treatment has been reported to cause false negative results (Babudieri 1961). In addition, convalescent serum are often not collected from patients with presumed mild or resolved leptospirosis (personal communication with Chris Mansell, Clinical Microbiologist, Waikato Hospital).

Due to the complexity of MAT, the enzyme-linked immunosorbent assay (ELISA) has been developed and used widely as a rapid screening test for leptospirosis, with several assays commercially available (Winslow *et al.* 1997; Smits *et al.* 1999; Levett and Branch 2002; Bajani *et al.* 2003). Detection of immunoglobulin M (IgM) antibodies by ELISA has been shown to be more sensitive than MAT in the acute phase of infection in several studies (Ribeiro *et al.* 1994; Cumberland *et al.* 1999; Levett and Branch 2002). However, because false positive results may be obtained due to the persistence of antibodies from previous infections (Lupidi *et al.* 1991; Cumberland *et al.* 2001), it is recommended that the ELISA result based on a single sample should be confirmed by testing a convalescent sample by MAT (Winslow *et al.* 1997).

Leptospires can be isolated by culture from blood and cerebrospinal fluid samples during the first few days of illness (Faine 1982; Bharti *et al.* 2003). However, the sensitivity of culture depends on the limited survival of leptospires in the blood fluid or tissue sample, immune system responses, exposure to administered antibiotics, and overgrowth by other bacterial and fungal contaminants (Smythe *et al.* 2002; Fearnley *et al.* 2008; Boonsilp *et al.* 2011). Culture also has drawbacks as it is laborious and requires a long period of incubation. Even if optimal conditions are provided, organisms grow slowly and cultures cannot be reported negative until 12-13 weeks after inoculation (Levett 2001).

Polymerase Chain Reaction (PCR) assays can detect *Leptospira* in blood taken from patients within the first week of clinical symptoms, and precludes the need for isolation and culture, making it the test of choice for the rapid diagnosis of leptospirosis in the acute phase (Ooteman *et al.* 2006; Ahmed *et al.* 2009; Thaipadunpanit *et al.* 2011). A number of conventional and real-time PCRs have been described (Mérien *et al.* 1992; Smythe *et al.* 2002; Levett *et al.* 2005; Slack *et al.* 2006; Stoddard *et al.* 2009). The quantitative real-time PCRs (qPCRs) were introduced as being more sensitive, time-

saving and reducing the risk of false positive results caused by carry-over contamination (contamination with products from previous reactions) (Ahmed *et al.* 2009).

In New Zealand the diagnosis of leptospirosis considered in the occupational disease claims by Accident Compensation Corporation (ACC) is based on a clinically compatible illness and at least one of the following laboratory results: (1) *Leptospira* isolation from a clinical specimen; (2) a four-fold or greater rise in MAT titre between acute and convalescent serum; (3) a single high MAT titre of ≥ 400; and (4) detection of leptospiral nucleic acid from a clinical specimen, in line with the standard applied by Ministry of Health (Annonymous 2012). The difficulties in culturing leptospires and the incomplete or ambiguous MAT results make it extremely difficult to confirm leptospirosis cases in acute settings. The primary aim of this study is to determine the value of PCR in analysing blood samples, in comparison with those of the MAT and culture, for the early diagnosis of human leptospirosis. An additional aim was to compare the performance of PCR methods in use at the Hopkirk Leptospirosis Research Laboratory (HLRL), ^{m}EpiLab, Massey University, Institute of Environmental Science and Research Ltd (ESR), and the Canterbury Health Laboratory (CHL). Results were intended to establish the best diagnostic test or combination of tests for acute cases of leptospirosis to satisfy the need for rapid diagnosis, prompt appropriate treatment, and also meet ACC requirements.

2 Materials and Methods

2.1 Study design

This study was conducted from 1st August 2011 to 31st December 2012, using cluster sampling to recruit 100 suspect leptospirosis patients via rural general practitioners (GPs), hospital doctors and phlebotomists within the Waikato DHB area.

2.2 Ethical approval

This study received ethical approval from the Northern Y Ethics Committee; reference number NTY/10/10/083. Māori consultation was sought through Waikato DHB's Kaumatua Kaunihera Research Sub-committee.

2.3 Recruitment

2.3.1 GP recruitment

GP selection and recruitment was facilitated by Population Health, Waikato DHB. A Medical Officer of Health (MOoH) provided a list of 18 medical centres from seven rural townships (Taumarunui, Te Kuiti, Te Awamutu, Cambridge, Otorohanga, Matamata, and Morrinsville). The medical centres were selected based on the high number of leptospirosis notifications reported from these centres in past years (EpiSurv). A covering letter and a GP information sheet (Appendix 7a, 7b) were sent to each GP from these medical centres to invite them to participate in the study. After the letters were sent, the medical centres (practice manager, research nurse or main practice GP) were contacted by telephone to determine if they were interested in participating. Following this, the author (F Fang) and J Benschop (the supervisor) visited 13 of the 18 medical centres to further explain the study and provide detailed study documents. Within one month after the visits, the 13 centres were contacted again to confirm their willingness to participate. In total, 42 GPs from 11 medical centres within seven towns (Figure 7.1) were recruited for this study. Patient enrolment through rural GPs commenced in August 2011.

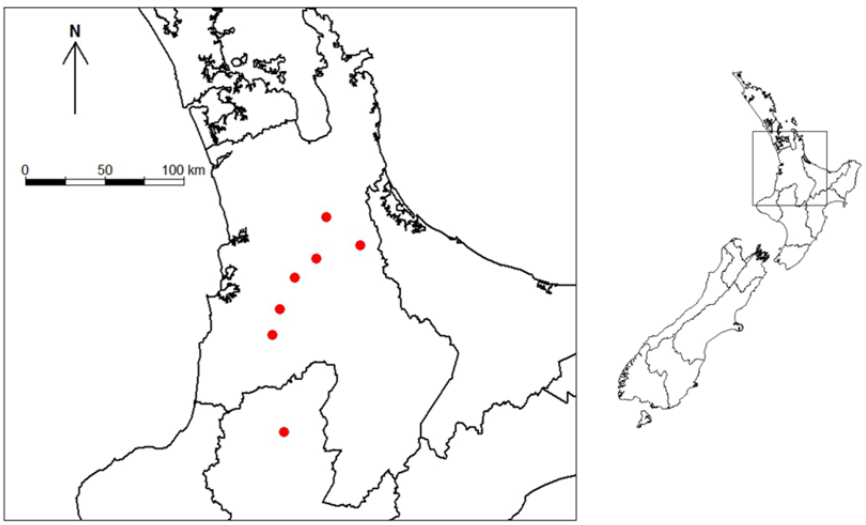

Figure 7.1 Map showing the seven towns in Waikato region of North Island, New Zealand, from which the 11 medical centres were recruited for this study. Insert: map of New Zealand showing the location covering the study area (Waikato region).

2.3.2 Hospital doctors/phlebotomists recruitment

To augment recruitments through rural GPs, the doctors from four 'T' hospitals (Taumarunui, Te Kuiti, Tokoroa and Thames) were recruited by the Clinical Microbiologist, Waikato Hospital, and the recruitment of patients through hospital doctors commenced from February 2012.

From April 2012, laboratory-based recruitment was also set up to augment GP recruitments. The phlebotomists from four 'T' hospital laboratories and the phlebotomists from six main collection centres (Te Awamutu, Leamington, Cambridge, Morrinsville-Dallas, Matamata and Thames) of PathLab Waikato laboratories were recruited for this study.

2.3.3 Patient recruitment procedure

The GPs/hospital doctors/phlebotomists were provided with a protocol to guide the patient recruitment procedure (Appendix 7c, 7d). Adult patients who GPs/hospital doctors suspected may have had leptospirosis as their provisional diagnosis were invited to enrol in the study using the Information Sheet for Study Participants (Appendix 7e). The following suggestions were provided to assist in the identification of suspected cases: the patients may report animal contact and their clinical signs may include fever, chills, myalgia and conjunctivitis (based on main clinical features reported in Faine *et al.* (1999) and experience of the Clinical Microbiologist, Waikato Hospital). Adult patients who were referred to blood collection centres by GPs to have blood samples taken for leptospirosis testing (e.g. MAT or ELISA) were invited by phlebotomists to participate in the study. Exclusion criteria were that the patients must not have had antibiotic treatment within the last three weeks and were no less than 16 years of age.

Once the patients consented, the GP filled in the Study Participant Identification Form (including demographic, clinical signs and treatment information) (Appendix 7f) and provided them with the special purpose laboratory test request forms to take to the blood collection centre. For patients recruited by phlebotomists, the consent form and Study Participant Identification Form were filled in at blood collection centres, before blood sampling. The GPs/phlebotomists communicated the recruitment to the MOoH who arranged notification and the follow up of participants with questionnaires (EpiSurv form). The Waikato Leptospirosis Questionnaire (Appendix 7g) that included more detailed questions about exposures to risk factors for leptospirosis infections

(during the three weeks before the patients became unwell), was also filled in by the MOoH.

2.3.4 Data and serum samples sourced from ESR

In addition to the recruitment of patients as above, from June to December 2012, the serum samples and data from suspected leptospirosis cases from the whole New Zealand were sent from ESR to HLRL, aiming to increase the sample size for test comparison. The selection criterion was suspected cases that had serum samples sent to ESR for MAT as diagnosis of leptospirosis (if enough volume of serum was left from the standard tests). The cases were not contacted for further information. The basic demographic information of the cases (age, sex, region), based on the records from the ESR system, and the sampling dates and test results of MAT/ELISA (if performed in local laboratories) on the serum samples was provided by ESR.

2.4 Sample collection and processing

Sample collection was facilitated through collaboration with PathLab and Waikato Hospital Laboratories (WHL). At the first visit, 15 ml of blood from each patient were collected, including one 2 ml of heparin blood in BD Vacutainer® Heparin Tubes (BD, Franklin Lakes, NJ, USA), two 3.5 ml of coagulated blood in BD Vacutainer® SST™ II Advance Tube (BD, Franklin Lakes, NJ, USA) and three 2 ml of whole blood in BD Vacutainer® EDTA tubes (BD, Franklin Lakes, NJ, USA). After blood collection, two drops of heparin blood (approximately 100 μl) were added into two glass vials of 5 ml EMJH culture medium (previously prepared and supplied by HLRL to the participating centres) and 5 ml Fort Richard culture medium (ESR) at room temperature, respectively. At the second visit, one 3.5 ml of coagulated blood in BD Vacutainer® SST™ II Advance Tube (BD, Franklin Lakes, NJ, USA) was collected. Coagulated blood samples were centrifuged at 1300 *g* for 10 min for serum separation. Blood cultures at ambient temperature, and serum and whole blood samples (at around 4ºC) were sent to three laboratories (ESR, HLRL and CHL) for MAT, serum and whole blood PCR and blood culture (according to the schedule in Figure 7.2).

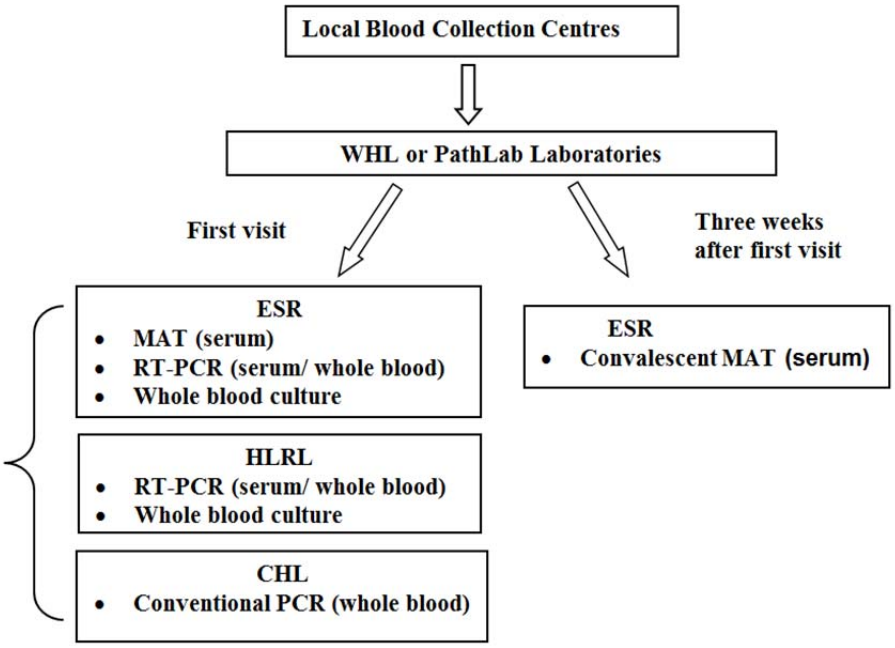

Figure 7.2 Flow chart for blood sample collection and dispatch to laboratories (WHL= Waikato Hospital laboratory; Pathlab= PathLab Waikato laboratories; ESR = Institute of Environmental Science and Research Ltd, Wellington; HLRL = Hopkirk Leptospirosis Research Laboratory, Massey University; CHL = Canterbury Health Laboratory, Christchurch), and tests performed.

The serum samples sourced through ESR had been stored at -20°C and were sent in batches to HLRL (at around 4°C). The samples were qPCR tested by both ESR and Massey (CHL tested PCR only on whole blood samples, therefore was not included in this comparison).

2.5 Diagnostic tests

2.5.1 MAT

The MAT was performed by ESR on serum and tested against an antigen battery of eight reference serovars: *L*. Hardjobovis, *L*. Pomona, *L*. Ballum, *L*. Tarassovi, *L*. Copenhageni, *L*. Canicola, *L*. Grippotyphosa, and *L*. Australis. One hundred microlitres of each serum sample was mixed with phosphate buffered saline (PBS, PH 7.2) to make a 1:12.5 dilution for testing. Twelve two-fold serial dilutions of serum in PBS, covering the range from 1:25 to 1:25600, were prepared in 96-well flat-bottom polystyrene plate (Greiner Bio-one, Frickenhausen, Germany). Positive controls using standard reference antisera against the serovars tested and, a negative control using PBS, were prepared in a similar manner. The plates were sealed with a lid to reduce evaporation and shaken on

a microtitre shaker for approximately 45 sec, then incubated at 30°C for one and a half to two hours. The degree of agglutination or lysis was determined by examination under Olympus BH2 dark-field microscopes (Olympus, Tokyo, Japan). The end-point titre was the lowest dilution at which approximately 50% of the organisms were agglutinated or lysed.

2.5.2 Culture

2.5.2.1 ESR

The medium used for culture was Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Fort Richard Laboratories Ltd, Otahuhu, New Zealand). Five hundred microliter of inoculated medium (sent from blood collection centre) was transferred into one 10 ml semi-solid medium on the day the blood culture was received, and both cultures were incubated at 30°C for two weeks, then 500ul of each culture were transferred into two 10 ml aliquots of medium. These four cultures were incubated for another 10 weeks. All of the cultures were examined every week using dark-field microscopy. If no *Leptospira* were observed, results were reported as negative.

2.5.2.2 HLRL

The medium used for culture was EMJH medium prepared at the HLRL, and contained 5'-fluorouracil to help prevent contamination, as described by Ellinghausen and McCullough (1965), Johnson and Harris (1967) and Faine *et al*. (1999). A serial dilution of culture was performed by adding 100 µl of the inoculated medium (sent from blood collection centre) into 5 ml of medium, followed by further transfer of 100 µl to another 5 ml of medium. All three inoculated medium bottles were then incubated aerobically at 28–30°C and examined every week for the presence of leptospires, using dark-field microscopy. Cultures which showed no detectable growth after 12 weeks were declared negative.

2.5.3 PCR

2.5.3.1 ESR qPCR on serum and whole blood

One hundred microlitres of the supernatant (serum) from the centrifuged coagulated blood samples and 100 µl of whole blood were used to extract DNA, using the MagNA

Pure isolation system (Roche Diagnostics, Auckland, New Zealand) as per manufacturer's instructions. DNA was eluted in a final volume of 100 µl each.

The qPCR assay was modified based on the assay described by Slack *et al.* (2007) using the Roche LightCycler version 1. Each PCR reaction contained 12.5 µl of the PerfeC$_{Ta}$ qPCR ToughMix, Low ROX (Quanta BioSciences, Gaithersburg, MD, USA), 3 pmol of the forward (5'^{171}-CCCGCGTCCGATTAG-3') and reverse primers (5'^{258} TCCATTGTGGCCGR$^{A/G}$ACAC3') based on *rrs* (16S) gene, 1 pmol of the 6-carboxyfluorescein-labeled (FAM) Black Hole Quencher 1 (BHQ1) quenched Taqman probe (Biosearch Technologies, Petaluma, CA, USA), 5 µl of DNA template and 6.1 µl of the DNase and RNase free water to 20 µl. Thermal cycling was as follows: an initial denaturation/hot-start *Taq* activation at 95°C for 8 min, followed by 50 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 1 min. Fluorescent data acquisition was performed at the end of the annealing/extension cycle of the PCR in Channel F1. Positive control was serovar Pomona (ESR laboratory strains), and DNase and RNase free water was used as negative control. Positive samples were defined as having a cycle-to-threshold (Ct) value less or equal to 38 cycles.

2.5.3.2 HLRL qPCR on serum and whole blood

Two hundred microlitres of the supernatant (serum) from the centrifuged coagulated blood samples and 200 µl of whole blood were used to extract DNA, using the QIAamp DNA Mini Kit (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand.) as per manufacturer's instructions. DNA was eluted in a final volume of 200 µl each.

The real-time PCR assay was modified based on the assay described by Subharat *et al.,* 2011 (Subharat *et al.* 2011). Green-Fluorescent Nucleic Acid Stain SYTO9 (Invitrogen Corp., Carlsbad, CA, USA) was used as the intercalating dye. The assay was performed in a Rotor-Gene Q machine (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand.). Primers 2For (5'-TGAGCCAAGAAGAAACAAGCTACA-3') and 504Rev (5'-MATGGTTCCRCTTTCCGAAGA-3') were used to amplify *gyr*B gene. The 25 µl reaction mix comprised 2.5 µM SYTO9 (Invitrogen Corp., Carlsbad, CA, USA), 1×PCR buffer, 1.5 mM Magnesium Chloride (MgCl2), 200 µM dNTPs, 5 pmol of 2For and 504Rev, one unit of *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA), 2 µl of DNA elution and double distilled water (ddH2O). Thermal cycling was as follows: 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. Positive controls were serovar Ballum, Hardjobovis and Pomona (HLRL laboratory strains), and ddH2O was used as negative control. Samples were considered positive, if a similar melting temperature as positive controls was recorded.

2.5.3.3 CHL conventional PCR on whole blood

DNA was extracted from 200 µl of whole blood, using the the gDNA extraction kit on the SPRI-TE nucleic acid extractor (Beckman Coulter, Auckland, New Zealand). Five microlitres of an internal control DNA (in-house assay) was added to the sample and processed as per manufacturer's instructions. The DNA was eluted in 60 µl of elution buffer.

The DNA amplification was performed using the adapted 16S rRNA gene PCR (Mérien *et al.* 1992) in a hemi-nested format with primer Lept-F4 designed at Canterbury Health Laboratories. It includes a first round amplification with primers Lept-A/B followed by a second round with Lept-F4/B. Ten microlitres of eluted sample DNA was added to PCR reaction mixture to give a final reaction volume of 50 µl for the first PCR step. One microlitre of the PCR amplicon was then added to the second round PCR reaction mixture to give a final reaction volume of 25 µl. The PCR reaction mixtures contained (final concentrations), 1xPCR buffer, 2.0 mM $MgCl_2$, 150 µM of each deoxynucleoside triphosphates (Roche Diagnostics, Auckland, New Zealand), and 1.25 units of FastStart *Taq* DNA polymerase (Roche Diagnostics, Auckland, New Zealand). Primers were added to each PCR master mixture at 0.1 µM for first round PCR primers Lept-A (5'-GGCGGCGCGTCTTAAACATG-3') and Lept-B (5'-TTCCCCCCATTGAGCAAGATT -3') and 0.3 µM of each primer Lept-F4 (5'-ACTTTCCGAAAGGGRAGCTAATA-3') and Lept-B for second round PCR. All PCR amplifications were performed on the GeneAmp® PCR system 9700 (Applied Biosystems, Life Technologies, CA, USA) thermal cycler with first round PCR thermal cycling performed as follows: 94°C for 6 min, followed by 35 cycles of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 45 sec, with the last cycles concluding with 72°C for 5

min, and 4°C for 5 min. Second round PCR thermal cycling was as follows: 94°C for 6 min, followed by 25 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec, with the last cycles concluding with 72°C for 5 min, and 4°C for 5 min. PCR products were electrophoresed through a 2% agarose gel and visualised by UV illumination after SYBR safe (Life Technologies, Carlsbad, CA, USA) staining. PCR positive and negative controls were included in each run and a valid negative result was confirmed by a positive internal control PCR. Positive results were detected by the presence of expected fragment size of 234 bp product.

2.6 Database set up and data analysis

The information obtained from the study participation form, EpiSurv and Waikato Leptospirosis Questionnaire was entered into a database set up by the MOoH. The researcher stored the data from these forms and the test results in a password protected electronic database. Descriptive analysis was performed for each variable using statistical software R 2.14.1 (R Development Core Team 2011).

3 Results

3.1 Recruited patients

In total, 14 patients were recruited into the study, either through GPs (medical centres/hospitals, n=10) or phlebotomists (n=4). All patients were tested by PCR and MAT, and all but one (Patient 2) were tested by culture (Table 7.1). Ten patients (71%) had a second serum sample collected for convalescent MAT. According to the case definition of leptospirosis by MAT (a four-fold or greater rise in leptospiral MAT titre between acute and convalescent serum, or a single high MAT titre ≥ 400), *Leptospira* isolation, or detection of leptospiral nucleic acid from a clinical specimen in New Zealand (Annonymous 2012), five cases (Patient 6, 10, 12, 13, and 14) were confirmed.

Table 7.1 PCR (q- and conventional), culture and MAT results for the 14 patients recruited.

Patient ID	ESR qPCR		HLRL qPCR		CHL PCR	Culture		MAT (serovar/titre)		
	serum	blood	serum	blood	blood	ESR	HLRL	Acute serum	Convalescent serum	Interval of two serum were taken (in days)
1	–	–	–	–	–	–	–	– (< 25)	–	22
2	–	–	–	–	–	ND	ND	– (< 25)	–	21
3	–	–	–	–	–	–	–	– (< 25)	–	23
4	–	–	–	–	–	–	–	– (< 25)	– [a]	24
5	–	–	–	–	–	–	–	**E (Pomona/50)**	**E (Pomona/50)**	12
6	–	–	–	–	–	–	–	**+ (Pomona/800)**	**+ (Pomona/800)**	24
7	–	–	–	–	–	–	–	– (< 25)	ND	/
8	–	–	–	–	–	–	–	– (< 25)	ND	/
9	–	–	–	–	–	–	–	– (< 25)	ND	/
10	+	+	–	+	+	+	–	– (< 25)	**+ (Ballum/200)** [a]	60
11	–	–	–	–	–	–	–	– (< 25)	ND	/
12	–	–	–	–	–	–	–	– (< 25)	**+ (Pomona/800)**	21
13	–	–	–	+	+	–	–	– (< 25)	**+ (Tarassovi/800)**	21
14	–	–	–	–	–	–	–	– (< 25)	**+ (Hardjobovis and Pomona/400)**	38

ND= Tests were not performed
'/'= information was not applicable
'+'= positive results, '–'= Negative results, 'E'= detectable MAT titres ≤ 200, and ≥ 25, without four-fold sero-conversion detected (indicates previous exposure to leptospires)
Acute serum = serum samples taken on recruitment date; convalescent serum= serum samples taken around three weeks later
ESR= Institute of Environmental Science and Research Ltd, Wellington; HLRL= Hopkirk Leptospirosis Research Laboratory, Massey University; CHL= Canterbury Health Laboratory, Christchurch
[a] MAT was performed by Waikato District Health Board laboratory

Clinical symptom information was obtained from 13 patients, including four of the five confirmed cases (Patient 10, 12, 13 and 14). The number of cases and non-cases that reported each symptom is presented in Figure 7.3. Most common symptoms in confirmed cases were fever (4/4; 100%) and nausea (4/4; 100%); followed by myalgia, headaches, sweating, tiredness, and sore eyes, reported with the same frequency (3/4;75%). In non-cases, the most common symptoms were fever, sweating, and tiredness (8/9; 89%); followed by myalgia, headaches, nausea (7/9; 78%), and sore eyes (5/9; 56%). Stomach pains was reported only for non-cases (4/9; 44%), while jaundice

and conjunctival suffusion were not reported for any patient. One non-case reported chest pains as 'other symptoms'.

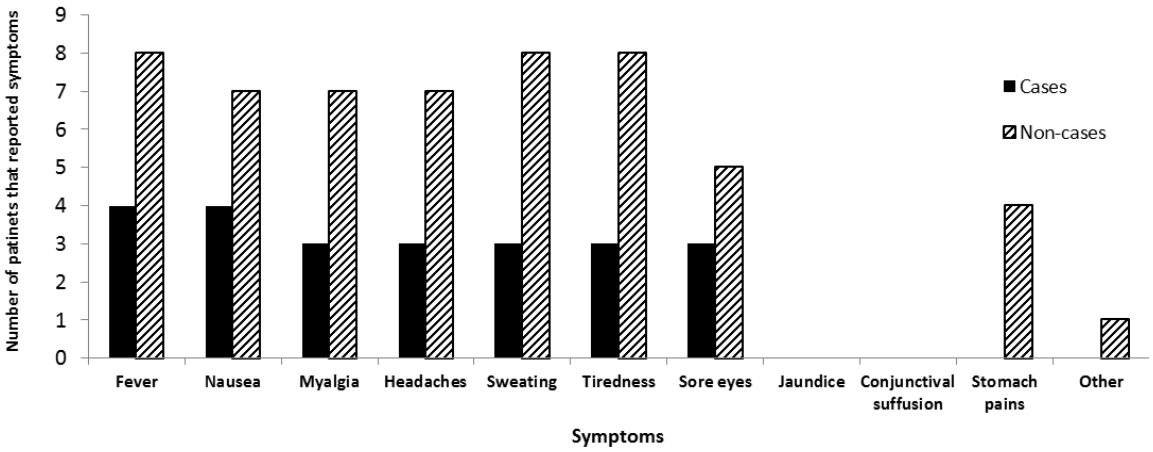

Figure 7.3 Number of patients that reported each symptom stratified by cases (n=4) and non-cases (n=9); 13 of 14 patients had recorded symptom information.

For the five confirmed cases, the median age was 49 years (IQR, 5; Minimum, 33; Maximum, 73), while all but one case was male (4/5; 80%). As comparison, for the nine non-cases, the median age was 39 years (IQR, 21; Minimum, 11; Maximum, 54), while all of them were male (Table 7.2). Five of the 14 patients were hospitalised, including 60% (3/5) of cases and 22% (2/9) of non-cases. For those who had date of onset of clinical signs recorded (n=12, data for patient 8 was excluded due to record error), the median interval between onset date and recruitment date was marginally shorter in non-cases (2.0 days, IQR, 1.0; Minimum, 1.0; Maximum, 9.0) than in cases (2.5 days, IQR, 2.2; Minimum, 1.0; Maximum, 53.0). For 12 patients who had antibiotic treatment records, 11 (92%) of them received antibiotic treatment, with ten being prescribed doxycycline and for the other one no antibiotic was prescribed.

Ten of 14 (71%) patients had EpiSurv data available (sourced by the MOoH), providing demographic, occupational, and animal contact information in detail (Table 7.2 and 7.3). Most of the patients reported ethnicity as NZ European (9/10, 90%). All of the ten patients were from the rural territorial authorities of Waitomo (n=5), Ruapehu (n=4) and Otorohanga (n=1). No apparent differences were found between cases and non-cases for ethnicity and location.

Table 7.2 Demographic, clinical course, and treatment information for the 14 patients.

	Patient ID	Sex	Age	Ethnicities	Territorial authorities	Onset date	Date recruited	Hospitalised	Antibiotics treatment	Type of antibiotics given
Confirmed-cases	6	Male	49	NZ European	Ruapehu	15/03/2012	7/05/2012	+	+	Doxycycline
	10	Male	73	NZ European	Ruapehu	14/06/2012	18/06/2012	+	+	Doxycycline
	12	Female	49	NZ European	Otorohanga	6/08/2012	10/08/2012	+	+	Doxycycline
	13	Male	54	NZ European	Ruapehu	28/10/2012	30/10/2012	–	+	Doxycycline
	14	Male	33	NZ European	Waitomo	4/11/2012	9/11/2012	–	+	Doxycycline
Non-cases	1	Male	39	NZ European	Waitomo	16/08/2011	18/08/2011	+	+	Doxycycline
	2	Male	54	NZ European	Ruapehu	2/09/2011	5/09/2011	–	+	Doxycycline
	3	Male	29	NZ European	Waitomo	23/10/2011	25/10/2011	–	+	Doxycycline
	4	Male	54	/	/	3/02/2012	4/02/2012	+	+	Doxycycline
	5	Male	36	/	/	/	27/04/2012	–	/	/
	7	Male	18	/	/	22/05/2012	23/05/2012	–	+	Doxycycline
	8	Male	50	NZ European	Waitomo	29/12/2012[a]	25/05/2012	–	+	/
	9	Male	47	/	/	13/06/2012	15/06/2012	–	/	/
	11	Male	11	Maori	Ruapehu	11/07/2012	20/07/2012	–	–	–

'+'= Yes; '–'= No; '/'= not recorded

[a] Onset date of patient 8 was wrongly recorded as later than the date of recruitment

Two of the five confirmed-cases (40%) worked in a farming environment, while other occupations recorded were meat workers (2/5; 40%) and retired farmer (1/5; 20%). For non-cases, occupations recorded as farmers in 80% (4/5) patients, and as school child who lived on a farm in one patient. During the three weeks before their illness, all 10 patients had been exposed to farm animals. Beef cattle (5/5; 100%) and sheep (3/5; 60%) were the two most frequently reported species in cases; while beef cattle (3/5; 60%), dairy cattle (2/5; 40%), and sheep (2/5; 40%) were the three most frequently reported species in non-cases. The five leptospirosis confirmed-cases reported that the farm animals they had exposure to were either not vaccinated or with unknown immunised status against leptospirosis; while four (4/5; 80%) non-cases reported that the farm animals were vaccinated. Exposure to fresh water (river swims) was reported in one non-case; no patient had travelled overseas and eight identified their exposures as part of their employment.

Table 7.3 Information of occupation and exposure to risk factors of leptospirosis infection for the 14 recruited patients

	Patient ID	Occupation	Animal Contact					Water exposure	Travel overseas	Exposure part of employment
			Beef	Dairy	Deer	Sheep	Immunised (vaccinated)			
Confirmed-cases	6	Farm worker	+	–	–	+	Unknown	–	–	+
	10	Retired Farmer	+	–	–	+	–	–	–	Unknown
	12	Meat worker	+	+	–	–	–	–	–	+
	13	Stock truck driver	+	–	+	–	Unknown	–	–	+
	14	Meat worker	+	+	–	+	Unknown	–	–	+
Non-cases	1	Farmer	–	+	–	–	+	–	–	+
	2	Farmer	+	–	+	–	+	–	–	+
	3	Farmer	+	–	–	–	+	–	–	+
	4	/	/	/	/	/	/	/	/	/
	5	/	/	/	/	/	/	/	/	/
	7	/	/	/	/	/	/	/	/	/
	8	Farmer	–	+	–	+	+	–	–	+
	9	/	/	/	/	/	/	/	/	/
	11	school child lives on farm	+	–	–	+	–	+	–	–

'+'=Yes; '–'= No; '/'= not recorded

The Waikato Leptospirosis Questionnaire was filled in by the MOoH with detailed information of exposure to other risk factors for nine of the ten patients for which EpiSurv information was available (Table 7.4). None of them had underlying health problems. Smoking and processing of meat at home or work were reported by two confirmed cases (2/5; 40%) who were meat workers, but not in other cases or non-cases. Contacting with pets and drinking water from risk source (e.g. surface stream, lake, and spring) was reported by one case (1/5; 20%) and two non-cases (2/4; 50%). Other risk factors were also not frequently reported in both cases and non-cases. No patient was aware of contact with people who have suffered from similar symptoms within the 10 days before his/her illness.

Table 7.4 Information of exposure to other risk factors of leptospirosis infection for nine patients who had the Waikato Leptospirosis Questionnaire filled in by the MOoH

	Patient ID	Smoking	Process meat at home/work	Contact with pets	Drink water from risk source	Work near water	Hunting	Clear bush around property	Other maintenance of property
Confirmed-cases	6	–	–	–	–	–	+	+	–
	10	–	–	+ (Dogs)	+	–	–	–	–
	12	+	+	–	–	–	–	–	–
	13	–	–	–	–	–	–	–	–
	14	+	+	–	–	–	–	–	–
Non-cases	1	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	+	–
	3	–	–	+ (Dogs, cats, chicken)	+	–	–	–	–
	8	–	–	+ (Dogs, cats)	+	+	–	–	+

'+'=Yes; '–'= No

3.2 Serum samples sourced from ESR

In total, 23 serum samples from 17 suspected leptospirosis patients were recruited into the study. The median interval between sample collection date and testing date was 32 days (IQR, 42; Minimum, 15; Maximum, 118). The majority of the patients were male (71%), and the median age was 47 years (IQR, 18; Minimum, 24; Maximum, 63) (Table 7.5). For six patients recruited, both the acute and convalescent serum samples were received. For the other patients (n=11), nine of them had one serum received without the status (acute vs. convalescent) known, while two had one convalescent serum received. Samples from nine patients (53%, 95% CI: 31%, 74%) tested positive by MAT and/or ELISA (if applicable). All of the 23 serum samples were tested negative by both ESR and HLRL qPCR.

Table 7.5 Demographic and sampling information of the 17 cases sourced from ESR, MAT from ESR, ELISA screening from local laboratories, and qPCR (ESR and HLRL) results for the 23 serum samples from these 17 cases.

Case ID	Age	Sex	DHB	Serum sample date		Sample test date	Reference test results			qPCR
							MAT (serovar/ titre)		ELISA	ESR and HLRL
				Acute serum	Convalescent serum		Acute serum	Convalescent serum		
ESR1	28	Male	Manawatu	2/03/2012	28/03/2012	28/06/2012	– (<25)	**+ (Pomona/400)**	+	–
ESR 2	29	Male	Hawkes Bay	13/03/2012	2/04/2012	28/06/2012	– (<25)	**+ (Ballum/3200)**	+	–
ESR 3	25	Male	Northland	19/05/2012	13/06/2012	28/06/2012	– (<25)	**+ (Ballum/200)**	ND	–
ESR 4	40	Male	Greymouth	16/04/2012	1/05/2012	26/07/2012	**E (Tarassovi/200)**	**E (Tarassovi/200)**	+	–
ESR 5	51	Male	Southland	19/06/2012	2/07/2012	26/07/2012	– (<25)	– (<25)	ND	–
ESR 6	59	Male	Wellington	28/06/2012[a]		26/07/2012	– (<25) [a]		ND	–
ESR 7	51	Male	Hawkes Bay	22/06/2012[a]		26/07/2012	– (<25) [a]		+	–
ESR 8	45	Male	Manawatu	14/08/2012[a]		21/09/2012	– (<25) [a]		+	–
ESR 9	50	Male	Otago	20/08/2012[a]		21/09/2012	– (<25) [a]		ND	–
ESR 10	49	Female	Waikato	10/08/2012	31/08/2012	21/09/2012	– (<25)	**+ (Pomona/800)**	ND	–
ESR 11	33	Male	Otago	3/09/2012[a]		21/09/2012	– (<25) [a]		ND	–
ESR 12	37	Female	Otago	3/09/2012[a]		21/09/2012	– (<25) [a]		ND	–
ESR 13	24	Female	Otago	4/09/2012[a]		21/09/2012	– (<25) [a]		ND	–
ESR 14	63	Male	Northland	6/09/2012[a]		21/09/2012	– (<25) [a]		ND	–
ESR 15	47	Female	Hawkes Bay	5/11/2012		29/11/2012	/ [b]	**+ (Ballum/1600)**	+	–
ESR 16	47	Female	Otago	5/11/2012		29/11/2012	/ [b]	**+ (Ballum/1600)**	ND	–
ESR 17	59	Male	Northland	30/10/2012[a]		29/11/2012	– (<25) [a]		ND	–

ND= test was not performed
'+'= positive results, '–'= Negative results, 'E'= detectable MAT titres ≤ 200, and ≥ 25, without four-fold sero-conversion detected (indicates previous exposure to leptospires)
DHB= District Health Board district; HLRL= Hopkirk Leptospirosis Research Laboratory, Massey University; acute serum= serum samples taken within five days of symptom onset; convalescent serum= serum samples taken two to three weeks later
[a] "acute" or "convalescent" status was not recorded on the sample
'/ [b]' volume of serum samples left from the standard tests were not enough for qPCR testing, therefore not recruited; MAT titres were recorded to be <25

4 Discussion

Although a low number of patients (n=14) were recruited and five were cases, we provided evidence that PCR can detect leptospirosis at an earlier stage of infection, compared to the MAT. If the findings could be extended the the general population, the combination of PCR and MAT (with acute and convalescent serum) would provide rapid diagnosis enabling prompt treatment that can reduce hospitalisation burden and the severe spectrum of the disease and, also confirmation of the diagnosis.

This study provides information about the utility of the different tests. There were two leptospirosis cases (Patients 10 and 13) that were confirmed by a four-fold rise in MAT

titre. The interval between onset date and recruitment date was four days and two days for Patient 10 and Patient 13, respectively, indicating that these two patients were recruited at the early stage of infection. For both of these patients, leptospires were detected in blood by PCR prior to the antibody response being detected by the MAT; while qPCRs results from HLRL were positive on whole blood, but negative on serum.

Blood culture was positive for one (Patient 10) of these two patients. The limited survival of the organism in blood and the amount of blood inoculated into the medium (too much can be inhibitory to growth) plus the fact that dilution of original culture by subculture was not able to be performed until two to four days later, may be the reason for the failure of culture for the other patient (Faine *et al.* 1999; Smythe *et al.* 2002). That no positive PCR results were obtained from non-leptospirosis cases (confirmed by MAT), attests to the specificity of the PCR assays.

MAT result indicates previous exposure to leptospires and positive MAT result were obtained from Patients 5 and 6 for both acute and convalescent phase serum. The interval between onset date and recruitment date was 53 days for Patient 6 indicating that this patient was recruited a long time after exposure and had developed immune response. The onset date information was missing for Patient 5. Leptospiraemia usually occurs during the first week of illness. After this period, the leptospires disappear from the bloodstream due to the development of antibodies, except for the massive leptospiraemia due to the severe infections (Faine *et al.* 1999; Levett 2001). This can explain why no leptospires were detected in blood by both PCRs and culture for these two patients.

Sero-conversion from negative to a MAT titre of 800 against serovar Pomona and 400 against Hardjobovis and Pomona was detected in Patients 12 and 14, respectively. These two patients tested negative by all other methods, although the patients were recruited within the acute phase of infection (four and five days after onset date). The number of leptospires present in blood samples is small and density as low as 10^1 leptospires/ml were reported in cases (Truccolo *et al.* 2001; Merien *et al.* 2005). The negative PCR and culture results may be due to the small number of leptospires circulating in the blood, and thus were under the limit of detection of PCRs and culture.

For the 23 serum samples from 17 cases sourced through ESR, qPCR results from both ESR and HLRL were negative. Recent studies showed that serum from clotted and

centrifuged blood was not the most appropriate specimen for detection of leptospires by PCR, compared to other fractions of blood (Kositanont *et al.* 2007; Stoddard *et al.* 2009). Although not quantified, leptospiral DNA was detected in the clot from the SST and serum tubes, which may have reduced the positivity rate of qPCR on serum samples (Stoddard *et al.* 2009; Bourhy *et al.* 2011). In this study, test results from two patients recruited by GPs/phlebotomists also indicated that PCRs on whole blood had higher sensitivity than on serum. Future studies involving a larger number of clinical samples may benefit the evaluation of PCRs on serum versus whole blood samples, since serum is more often collected for standard tests and available. The negative results on the 23 serum samples (especially for those 11 with unknown serum type) may also be due to the phase of the disease in which the samples were collected. The average persistence of leptospiral DNA in serum was reported to be 12 days (Merien *et al.* 1995). Therefore, PCRs on serum samples collected at later stage of the diseases may not be able to detect leptospiral DNA. The negative results on the 23 serum samples may also due to the long storage time, which can cause the degradation of samples (the median interval between sample collection date and testing date was 32 days). This may explain why six samples had positive ELISA screening tests. However, a study reported performed PCR on serum frozen at -20°C for the maximum of two years obtained positive amplification in 32% of the samples, indicating that the time of storage may not be the main reason for the negative results (Ooteman *et al.* 2006).

The common onset symptoms recorded for leptospirosis are headache, fever, myalgia, conjunctival suffusion, nausea, and a transient skin and mucosal rash (Faine *et al.* 1999; Vinetz 2000). The symptoms that follow depend on the type of leptospirosis (anicteric or icteric), which is due to the serovars of leptospires the patients were infected with (e.g. the serovars of the Icterohaemorrhagiae serogroup frequently cause severe leptospirosis) (Faine *et al.* 1999; Levett 2001). Three confirmed leptospirosis cases (Patient 12, 13 and 14) in this study showed the same clinical symptoms, while for Patient 10, only fever and nausea were recorded. No apparent difference was found between the symptoms in leptospirosis cases and those in non-cases in this study (Figure 7.3). Although the frequently reported symptoms for confirmed acute leptospirosis cases (e.g. headache, myalgia and nausea (Levett 2001)) were found in the leptospirosis cases (3/4, 3/4, and 4/4, respectively) in this study, they were also reported from 78% (7/9) of the non-cases. On the other hand, symptoms not considered as

typical symptoms for leptospirosis (while compared to other diseases with similar febrile syndromes) such as sweating, tiredness and sore eyes (Vinetz 2000), were commonly reported from both non-cases and confirmed cases in this study. Although this study is based on records from a small number of patients (n=13), these again indicate the difficulties of differentiating leptospirosis from fever of an unknown origin by clinical symptoms.

Although it is considered that majority of leptospirosis cases may show very mild symptoms, and do not require medical treatment (Plank and Dean 2000), hospitalisation was reported with higher frequency in leptospirosis cases (60%, 3/5) than in non-cases (22%, 2/9) in this study. Although this difference is not statistically significant, this suggests that the clinical course of leptospirosis cases is more likely to be severer than other undifferentiated febrile illness.

According to the surveillance data for leptospirosis cases in New Zealand from 2008 to 2011, the average age-specific rates was found to be highest in the age group 40 to 49 years, and the majority of cases were males and reported ethnicity as NZ European (The Institute of Environmental Science and Research Ltd 2009-2012). The differences in median age between cases and non-cases in this study suggested that the risk of getting leptospirosis may be higher in age group 40 to 49 years than 30 to 39 years. All but one patient recruited was male and most of them reported ethnicity as NZ European. Therefore, the comparison between cases and non-cases cannot indicate whether 'gender' and 'ethnicity' are risk factors for leptospirosis infection in this study.

From 2008 to 2011, occupations previously identified as at high risk of exposure to *Leptospira spp.* in New Zealand (Thornley *et al.* 2002) were reported for 76.8% of the 310 notified cases who had occupations recorded. Among these, farmers and meat workers constituted 67.2% and 30.3% of the cases, respectively. Other occupations which involved direct animal contacts (e.g. stock truck driver, park ranger) were also reported (The Institute of Environmental Science and Research Ltd 2009-2012). The findings from this study were consistent with the situation of the New Zealand population, and indicated that farm workers, meat workers, and people working as occupations associated with contacting livestock (especially livestock with unknown immunisation status) can be at risk of contracting leptospirosis (Table 7.3). Nine of 14 patients were able to be followed up with the Waikato Leptospirosis Questionnaire and

this provided information on exposure to other risk factors (e.g. water, hunting); however, these risk factors were infrequently reported by both cases and non-cases. The limited data from a small number of cases and non-cases in this study cannot provide solid interpretation of these risk factors for leptospirosis infection.

In this study, despite multiple efforts to improve recruitment, including initial personal visits to each practice by the researchers, regular phone and email contacts with participating GPs/practice managers, reminders and on-going support from Waikato DHB staff, and setting up recruitment through phlebotomists, 14 patients were recruited. The limited number of patients recruited for this study is consistent with other studies that involved recruitment through GPs, although considerable time and efforts had been dedicated to improve the referral rates. Time constraints, fading awareness of the study, and few eligible patients treated are the main factors that affect GPs' recruitment of patients into research studies (Peto *et al.* 1993; Page *et al.* 2011). The weak research culture which has been found in GPs from different countries (Askew *et al.* 2002) and the participating GPs' lack of interest in particular research topics can also be the reasons for the barriers of obtaining sufficient recruitments (Williamson *et al.* 2007). In this study, the researchers sent an invitation letter of introduction first and then initiated the visits for personal discussion and further explanation of the project to recruit the GPs. The study protocol has been designed to be as simple as possible and the recruitment steps were presented in a flowchart, which was intended to be easy to comply with. After the study commenced, the contact was maintained with participating GPs/practice managers who were reminded of the study by phone calls, newsletters of project updates, faxes and emails. Although these approaches are reported in a previous study to be effective in encouraging the ownership of the GPs for the study and improving recruitment (Bell-Syer and Moffett 2000), the recruitment through GPs was poor in this study. Recruitment through phlebotomists was achieved to augment the recruitment. However, only a slight increase in recruitments was achieved. The small number of recruitments may be also due to a limited number of leptospirosis cases occurring during the research period. Follow up with one practitioner reports few patients with suspect leptospirosis symptoms visiting the medical centres, and some of these had antibiotics, thus did not meet the selection criteria (personal communication, Dr. Keith Buswell, April 2013). From the start of study to December 2012, there were 32 leptospirosis cases notified around Waikato area. Among these, five cases were

recruited for this study. The other cases were missed either because the GPs were not participating in the study (n= 20, 74%) or the failure of participating GPs to recruit the patients (n= 7, 26 %) (data provided by MOoH). In the meantime, as this thesis chapter is drafted, the recruitment of patients is still continuing, at least until the end of 2013. The recruitment through more pathways (e.g. recruit meat workers with suspect leptospirosis symptoms through clinical physician) or covering more areas (e.g. areas covered by the Mid-Central DHB) may also be considered to recruit more patients and help achieve robust conclusions from the study.

The diagnosis of leptospirosis by MAT is based on a single high titre (≥ 400) or a four-fold or greater rise in MAT titres between acute and convalescent serum samples. However, the difficulty of obtaining the convalescent serum sample is considered as a drawback of MAT in confirming diagnosis. Based on ESR's records of serum samples submitted for MAT, about half of the patients who had acute serum samples taken did not submit the convalescent serum samples, especially those with positive results on first MAT (personal communication with Karen Cullen, senior technician, ESR). In this study, four of 14 patients did not have the convalescent serum sample collected, although patients had been reminded by the phlebotomists. The difficulty of collecting both an acute and a convalescent serum for MAT again emphasizes the usefulness of PCRs to diagnose leptospirosis at the early stage of infection. Antibodies are often not able to be detected by MAT on the acute serum samples, and the convalescent samples are often not taken.

This study was carried out in a purposely-selected area (Waikato) with patients recruited through the GPs who were willing to participate in the study. The sample size for this study was not determined by a formal assessment of study power or precision, but was considered the approximately the number of samples that could be ascertained in the study period (personal communication, Dr. Keith Buswell, Te Kuiti Practice and Michael Addidle, Clinical Microbiologist, PathLab) and given the budget restrictions. One of the patients was aged less than 16 years old; however, the information from this patient was included in the discussion due to the limited number of patients recruited in this study. Therefore, the findings of this study may be unrepresentative and the extrapolation should be done cautiously. The performance of the different tests was presented with results from 14 recruited patients (in four of whom the diagnosis was not

confirmed by MAT). Definitive comparison of tests and validation of each test should be carried out with a larger number of samples.

Although it is difficult to make conclusions based on findings from 14 patients recruited from one region, this is the first attempt to compare different diagnostic tests for acute leptospirosis cases in New Zealand. The information of clinical symptoms, demographic, and exposure to risk factors can contribute to the GPs' provisional diagnosis on suspected leptospirosis cases. The experience of conducting this research study with GPs' involvement in New Zealand may benefit the development of patient recruitment strategies in future studies, which should emphasize GP's interests in the studies and also minimise the effects of time constraints in recruiting patients.

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Chapter 8 Seroprevalence and exposure to risk factors for leptospirosis among veterinary students at Massey University

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1 Abstract

AIMS: To determine the seroprevalence and quantify putative risk factors for exposure to leptospirosis both within and outside veterinary curriculum among undergraduate veterinary students at Massey University, New Zealand.

METHODS: A cross-sectional study was conducted from September 2010 to November 2011. In total, 302 students were blood sampled, with serum tested by microscopic agglutination test (MAT) for *Leptospira borgpetersenii* serovars Hardjobovis, *Leptospira interrogans* Pomona and *Leptospira borgpetersenii* Ballum. Information on demographic characteristics, potential exposure within and outside veterinary curriculum in the previous 18 months, and previous leptospirosis-like clinical history were recorded using an online questionnaire.

RESULTS: All students were microscopic agglutination test negative for each serovar, using a cut-point of ≥ 48. Exposure to animal urine within and outside veterinary curriculum was reported by 85.8% (259/302) and 49.7% (150/302) of the students, respectively. The median number of potential exposures to animal urine within the veterinary curriculum was 63 (min 1, max 155). The other potential exposures among respondents included home slaughter (20.9%; 63/302), hunting (14.2%; 43/302) and outdoor activities involving exposure to fresh water (79.8%; 241/302).

CONCLUSIONS: This study demonstrated that these veterinary students were at low risk of contracting leptospirosis, despite frequent exposure to potential sources of infection. The findings in this study contribute to a broader understanding of the occupational risk of leptospirosis. Data described the level of animal exposure in veterinary students, which can support other zoonotic disease studies in this group.

KEY WORDS: *Leptospirosis, veterinary students, seroprevalence, risk factors*

ESR Institute of Environmental Science and Research

MAT Microscopic agglutination test

PPE Personal protective equipment

2 Introduction

Leptospirosis is a zoonotic disease of worldwide importance caused by pathogenic spirochaetes belonging to the genus *Leptospira* (Levett 2001; Bharti *et al.* 2003; Dutta and Christopher 2005). Feral and domestic mammals can be maintenance hosts for

various serovars, while humans usually serve as an incidental host (Plank and Dean 2000; Levett 2001). Human infection with *Leptospira* spp. results from direct exposure if the source of infection is animal tissue, body fluids or urine, or indirect exposure if the source is environmental, e.g. soil or water contaminated with urine of carrier animals (Bharti *et al.* 2003; Sharma and Yadav 2008). Therefore, humans in occupations involving contact with animals are at significant risk of infection (Levett 2001). In temperate countries such as New Zealand, such occupations are livestock farming, veterinary practice, meat processing, butchering and forestry (Faine *et al.* 1999; Thornley *et al.* 2002). Fresh water exposures, including recreational activities, e.g. swimming, rafting, and exposure to flooding are also risk factors for human infection (Plank and Dean 2000; Levett 2001; Jansen *et al.* 2005).

The majority of leptospirosis cases, particularly in New Zealand, show mild symptoms and medical treatment is not required (Keenan 2007). However, a diverse range of severe clinical manifestations including jaundice, aseptic meningitis, renal failure and rarely, lethal pulmonary haemorrhage and cardiac involvement, have been reported in infections with *Leptospira* spp. (Bharti *et al.* 2003). In New Zealand, leptospirosis is the most common occupationally-acquired zoonotic disease (Vickery *et al.* 2006; Keenan 2007). From 2009 to 2011, there were 222 notified cases of leptospirosis in New Zealand. Meat workers and farmers constituted 22.5% (36/160) and 73.8% (118/160) of those with occupations reported and previously identified as high risk for exposure to *Leptospira* spp. (Thornley *et al.* 2002), respectively (The Institute of Environmental Science and Research Ltd 2009-2012). For the 62 of 222 notified cases during this period that either reported low risk occupations or did not have an occupation recorded, 37 (59.7%) reported animal or environmental risk exposure (The Institute of Environmental Science and Research Ltd 2009-2012). *Leptospira* species and serovar/s were recorded for 181 (81.5%) cases from 2009 to 2011 with *Leptospira borgpetersenii* serovar Ballum, *Leptospira borgpetersenii* serovar Hardjobovis and *Leptospira interrogans* serovar Pomona, in decreasing order, reported as the most commonly identified serovars (The Institute of Environmental Science and Research Ltd 2009-2012).

Undergraduate veterinary students have close contact with animals during on-farm practical, extramural veterinary clinical practice, and intramural teaching experiences during the curriculum. Furthermore, they are likely to have contact with animals in their

leisure time, e.g. pet owning, lifestyle farming, and hunting. This group is therefore likely at high risk of infection with zoonotic agents (Venables and Allender 2006). Examples for zoonotic exposures in veterinary students included rabies (Russell *et al.* 1977), *Brucella abortus* (Duclos *et al.* 1989), and cryptosporidiosis (Grinberg *et al.* 2011).

Studies of leptospirosis in veterinary students are limited in the international literature and have not been conducted in New Zealand. A repeated cross-sectional study conducted in veterinary students from Spain (Simon *et al.* 1999) reported 8.1% (39/479) seroprevalence of leptospirosis at the beginning of the study and 11.4% (54/472) one year later. A cross-sectional study of veterinary students in Peru, using the microscopic agglutination test (MAT) reported overall seroprevalence of leptospirosis as 11.9% (14/118) (Dammert *et al.* 2009).

The aim of the present study was to determine the seroprevalence of leptospirosis among veterinary students at Massey University, New Zealand, and to better understand the level of exposure to putative risk factors both within the veterinary curriculum, including intramural and extramural components and personal life, that may be associated with infections with *Leptospira* spp.. Results from this population could be used to determine the need or otherwise for targeted prevention strategies, and for comparison with other occupational groups at risk (such as meat workers and practising veterinarians) as part of on-going research and understanding of leptospirosis in New Zealand human and animal populations.

3 Materials and methods

3.1 Study design

The study was a cross-sectional study of veterinary students at Massey University, New Zealand conducted from September 2010 to November 2011. This study was reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 10/44.

3.2 Student recruitment

Massey University is the only university in New Zealand that offers a Bachelor of Veterinary Science degree course. It is a five-year full time programme and is registrable with the Veterinary Council of New Zealand. For each academic year, entry is restricted to around 100 students. The recruitment for this study followed consultation with the programme director and head of undergraduate teaching. All veterinary students (notionally 500) were invited to participate in the study either by the research nurse or academic staff at the beginning or after classes. Sampling of students for this study was conducted throughout the study period based on the students' availability. A blood sample from each participant was taken by the research nurse at a room set up for this study. Subsequently, participants were asked to go on-line with their study ID and complete a four-section questionnaire (Appendix 8) either using computers provided in the same room, or independently at a later time. The procedure took around 20 to 30 minutes. If completed surveys were not received, a reminder was sent to increase the response rate. Blood samples were kept at room temperature for the first 30 minutes then transported to the Hopkirk Leptospirosis Research Laboratory, IVABS, Massey University (HLRL) while maintained at cool conditions.

3.3 Survey development

The questionnaire was established on-line using Surveygizmo. The first section obtained demographic information, including gender, age, ethnic affiliation, academic year, speciality track (fifth-year students only) and residential location in the past 18 months. The second section was specific to the risk of exposure to animal urine within the veterinary curriculum, both intramural and extramural, in the past 18 months. Questions were stratified by animal species. Students were also asked what percentage of the time they used personal protective equipment (PPE) during veterinary curriculum. The third section asked about the potential for exposure in the student's personal life in the past 18 months, i.e. other than during the veterinary curriculum. Questions included ownership of animals, regular (at least weekly) contact with animals that may lead to exposure or risk of exposure to animal urine, contact with wildlife, home-slaughter or helping with home slaughter of livestock, and hunting, all stratified by animal species. Questions also covered outdoor activities that involved exposure to fresh water, and flooding around residences. The fourth section asked whether the

students had been diagnosed with leptospirosis any time previously or had influenza-like symptoms in the past 18 months.

3.4 Serological test

Blood samples were tested using the MAT procedure based on Faine (1982) for titres against *Leptospira* serovars Hardjobovis, Pomona and Ballum. A titre of ≥ 48 was considered positive for any serovar and used to indicate past infections with *Leptospira* spp., as used elsewhere for serosurvey in asymptomatic high risk groups (Faine *et al.* 1999; Shivakumar and Krishnakumar 2006). If MAT titres were found in any samples, they were then to be sent to the *Leptospira* Reference Laboratory (ESR) in New Zealand for confirmation (a panel of eight serovars representative of New Zealand *Leptospira* serovars were included). Test results were reported to students by post within three months after sampling. It was intended that seropositive students would be advised to consult a medical practitioner, in compliance with ethical approval.

3.5 Statistical analyses

Descriptive analysis was performed for all variables with statistical software R 2.14.1 (R Development Core Team 2011). The association between academic year and the outcomes animal urine exposure frequency (total number of exposures) and count (total number of animals associated with all exposures) within the veterinary curriculum in the past 18 months were investigated with box plots and linear regression models.

4 Results

In total, 327 of (notional 500) veterinary students agreed to participate in the study. Blood samples were collected from 311 and completed questionnaires were returned from 318. Sixteen students either declined to be blood sampled or difficulty was experienced with sampling. Overall, 302 students supplied both complete questionnaires and blood samples, forming the final study population with a response rate of 60.4% (95% CI: 56.0%-64.6%). MAT titre against serovar Pomona (24) was detected in one of the 327 students. Serum from this student was sent to ESR and tested negative against all of the eight serovars. No MAT titres were detected in the other students against any of the three serovars.

4.1 Demographic characteristics

Demographic characteristics are presented in Table 8.1. The population was predominately female which closely reflected the gender balance within the veterinary programme. The number of students recruited was similar between the academic years.

Table 8.1 Demographic characteristics for the 302 veterinary students who completed the questionnaire and provided blood samples

Variables	Category	No. (%)
Gender	Male	70 (23.2)
	Female	232 (76.8)
Age (years)	<20	38 (12.6)
	20-30	231 (76.5)
	30-40	28 (9.3)
	40-50	5 (1.7)
Ethnicity	Asian	44 (14.6)
	NZ-European	196 (64.9)
	NZ-Maori	7 (2.3)
	Other	55 (18.2)
Residential location (in past 18months)	Rural	5 (1.7)
	Urban	108 (35.8)
	Lifestyle[a]	7 (2.3)
	Variable/Mixed styles	182 (60.3)
Academic year	First	67(22.2)
	Second	53 (17.5)
	Third	58 (19.2)
	Fourth	52 (17.2)
	Fifth	72 (23.8)

[a] i.e. small block with grazing animals

4.2 Potential exposure within the veterinary curriculum

All of the 302 students reported usage of recommended PPE during the veterinary curriculum, and 259 students (85.8%) stated that they had used PPE ≥ 80% of the time.

Among respondents, 259 (85.8%) had been exposed to animal urine during the veterinary curriculum in the past 18 months. Within this group, 245 (94.6%) reported

the animal species. Potential exposure within the veterinary curriculum, by animal species, is presented in Table 8.2. Dairy cattle and dogs were the two most frequently reported animal species students were exposed to.

Table 8.2 The number (and %) of students reporting exposure to animal urine within the veterinary curriculum (n=245) and outside the veterinary curriculum (n=143) by species within the previous 18 months

Animal species	No. (%) exposed	
	Within veterinary curriculum	Outside veterinary curriculum
Beef cattle	129 (52.7)	13 (9.1)
Dairy cattle	196 (80.0)	29 (20.3)
Sheep	174 (71.0)	27 (18.9)
Deer	100 (40.8)	2 (1.4)
Horses	152 (62.0)	15 (10.5)
Goats	44 (18.0)	8 (5.6)
Pigs	58 (23.7)	4 (2.8)
Dogs	186 (75.9)	95 (66.4)
Cats	154 (62.9)	106 (74.1)
Laboratory animals	80 (32.7)	NA[a]
Other[b]	23 (9.4)	18 (12.6)

[a] laboratory animals were not included in the questions for exposure outside the veterinary curriculum

[b] other animals students reported exposures to within veterinary curriculum included alpacas, birds, and reptiles; other animals students reported exposures to outside veterinary curriculum included rabbits, birds, and rats

For the 245 students who reported animal species, 159 (64.9%) gave precise answers of the animal urine exposure frequency (total number of exposures) and count (total number of animals associated with all exposures). Taking all of the animal species into account, the median number of exposures in the past 18 months for a student was 63 (min 1, max 155) times and the median number of animals was 400 (min 1, max 1,0721). Broadly similar associations were seen in the box plot of exposure frequency vs. year of study and the box plot of exposure count vs. year of study. The lowest median exposures were in students from year one and there was little variation about this median. Students from year two to five had higher median exposures with greater variation than those from year one. Similar variation about the medians were seen in students from year two to four with the variation in exposures in year five students substantially larger than those in students from other years. The linear regression

models showed significant associations ($p < 0.05$) between the log of animal exposures (frequency and count) and the year of study for all years when compared with year one as a baseline. However there was no evidence of increasing trend of exposures in students from year two to five.

4.3 Potential exposure outside the veterinary curriculum

Among the 302 students, 282 (93.4%) students owned or had frequent contact with animals outside the veterinary curriculum. One hundred and fifty (49.7%) students reported that they might have had exposure to animal urine outside veterinary curriculum in the past 18 months. Within this group, 143 (95.3%) reported the animal species. Potential exposure outside the veterinary curriculum, by animal species, is presented in Table 8.2. Cats and dogs were the two most frequently reported animal species students were exposed to.

Other exposures in the past 18 months that could be associated with infections with *Leptospira* spp. are listed in Table 8.3. Home slaughter or helping with home slaughter was mainly associated with sheep, then cattle, deer and pigs. Hunting was mainly for small game, then deer, wild pig and goats, while 53.5% (23/43) of the students butchered the carcass of hunted animals. For the 202 students that reported having seen rats, mice, possums, rabbits or hedgehogs at their dwelling places, 79 (39.1%) of them set traps or laid poison for the wildlife.

Table 8.3 The number (and %) of students (n=302) reporting exposure to potential sources of leptospirosis outside the veterinary curriculum in the past 18 months

Variables	No. (%)
Home slaughter	63 (20.9)
Hunting	43 (14.2)
Outdoor activities providing exposure to fresh water	241 (79.8)
Seen rats, mice, possums, rabbits or hedgehogs at dwelling place	202 (66.9)
Residence had been flooded	72 (23.8)

4.4 Previous leptospirosis cases and Influenza-like symptoms

One fifth-year student reported having been diagnosed with leptospirosis 13 years previously by a doctor in New Zealand. There was laboratory confirmation (blood test),

but no record of the serovars tested. The student was away from school for one to two weeks because of the illness with fever, headache, sore muscles/bones, sore eyes, and sweating. The student could not recall whether any treatment was performed. No other student reported previous history of leptospirosis.

Influenza-like symptoms in the past 18 months were reported by 135 (44.7%) students. Of the 133 students who specified the symptoms, those symptoms reported by most, in order of frequency were: sore throat by 112 (84.2%), headache by 111 (83.5%), coughing by 98 (73.7%), fever by 88 (66.2%), and sore muscles by 85 (63.9%).

5 Discussion

This is the first reported study to estimate the seroprevalence of leptospirosis among veterinary students in New Zealand. A cross-sectional study was performed as this is an appropriate design for investigating a group with an unknown leptospirosis status. Serovars Ballum, Hardjobovis and Pomona were investigated in this study because they are the three *Leptospira* serovars reported most frequently in leptospirosis notifications in New Zealand from 2007 to 2011 (The Institute of Environmental Science and Research Ltd 2009-2012). Blood samples from all 302 students were MAT negative for all three serovars.

The questionnaire results confirmed that students had experienced both direct and indirect potential exposure to *Leptospira* spp. The potential exposure variables associated with infection with *Leptospira* spp. analysed in this study were similar to those investigated in similar studies of veterinary students elsewhere (Simon *et al.* 1999).

The seroprevalence of *Leptospira* antibodies was reported to be 11.4% (54/472) from veterinary students at Zaragoza in Spain (Simon *et al.* 1999). That study used an enzyme-linked immunosorbent assay (ELISA) testing *Leptospira* serovars Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, Hardjo and Pomona, but data on prevalence for each serovar was not given. Working with live animals and owning pets were reported as risk factors for infection with *Leptospira* spp. in that study, with odds ratios 1.97 and 4.33, respectively. In the present study, 85.8% (259/302) of students reported possible exposure to animal urine during the curriculum, similar to the 76.5%

(361/472) reported by Simon (Simon *et al.* 1999). However, comparing frequency of exposure to animals and/or urine between these two studies is challenging given the differences in the wording of the questions. In this study, students were specifically asked about their exposure to animal urine while in the previous study the question was not about urine *per se* but about working with live animals.

Most students in this study (93.4%, 282/302) owned or had frequent contact with pets, higher than the 28.8% (136/472) reported by Simon *et al.* (1999). However, definition of 'frequent contact' was not given in the latter study, which limits inferences from the comparison. The definition of 'pets' was also different in the two studies, with this study covering a broader range. While dogs and cats, birds, hamsters, fish or tortoises were listed in previous study (Simon *et al.* 1999), the present study also included horses, goats and small mammals, e.g. rabbits, guinea pigs.

The zero seroprevalence in veterinary students here was despite exposure to potential sources of infection, including sheep, beef cattle and deer, reported in a PhD thesis to have animal-level seroprevalence of 50.5% (1698/3361), 58.3% (1346/2308) and 33.8% (674/1992), respectively (Dreyfus 2013). This contrasts with results from a cross-sectional study of meat workers in New Zealand, in which seroprevalence was 10.9% (62/567) (Dreyfus 2013). The strongest risk factor among sheep and deer meat workers in New Zealand was 'work position' in relation to potential exposure to urine (Dreyfus 2013). Comparing the frequency of exposure to the analysed variables between these two groups could contribute to understanding of risk factors for infection with *Leptospira* spp. in at-risk groups in New Zealand. In the meat worker study (Dreyfus 2013), the non-work related questions were similar to those used in the present study, although questions were asked with different timeframes, namely the past three years for meat workers and past 18 months for veterinary students. Antibodies against *Leptospira* spp. usually become detectable by MAT by the end of the first week after development of symptoms, with persistence for months or even years (Lupidi *et al.* 1991; Cumberland *et al.* 1999; Faine *et al.* 1999). Titres persist for longer and at higher levels in cases who had higher titres initially (Cumberland *et al.* 2001). Contrary to meat workers working where exposure to *Leptospira* spp. is frequent, veterinary students might only have sporadic and low level exposure, which may cause silent infection and a low antibody response that lasting for shorter periods.

The frequency of exposure to animals was different between veterinary students and meat workers. For the 159 students who reported exposure to animal urine during the veterinary curriculum in the past 18 months and gave precise answers about the exposure time and number of animals associated with the exposures, the median number of exposure times per day for a student was 0.1 times and the median number of animals they were exposed to was 0.7. By comparison, meat workers were at higher risk of getting clinical leptospirosis and/or seroconverting because of their continuous and therefore cumulative contact with animals. For example, at a sheep-only slaughterhouse in New Zealand the median number of sheep processed by a meat worker per day ranged from 225 to 1123, depending on the work position (Dorjee *et al.* 2011). Median daily exposure to animals that shed *Leptospira* spp. organisms for meat workers in that slaughterhouse was estimated to be 11 to 54 per day during high-risk periods, and three to 18 during low-risk periods (Dorjee *et al.* 2011). However, it should be noted that compared to the constant (daily) exposures to animals for the meat workers, the veterinary students have sporadic exposures to animals, depending on the veterinary curriculum. Therefore, it is likely to be more difficult for them to recall the number of exposures times and animals associated with the exposures.

A further reason for differences in sero-conversion between meat workers and veterinary students might be differences in knowledge of hygiene practices, which underpin preventive measures for infection with *Leptospira* spp. (Faine *et al.* 1999). Given hygiene instruction and knowledge of zoonotic diseases, veterinary students might pay more attention to protecting themselves from infection while in contact with animals. Alternatively, conditions inherent in working environment make consistent use of PPE and other protective measures difficult to adhere to, regardless of the awareness and intentions of the meat workers (Keenan 2007). In this study, all students used recommended PPE and 85.8% (259/302) reported use no less than 80% of the time, which was more frequent than that reported in meat workers. The frequency of wearing PPE in meat workers differed by species of slaughterhouse and types of PPE. In total, 17-67%, 43-71% and 4-12% of workers always or often wear gloves, glasses and facemask, respectively (Dreyfus 2013).

Participation in non-work related exposures such as ‘hunting’ and ‘home slaughter’ were similar between veterinary students and meat workers (18.0% and 30.0%, respectively, in meat workers), as were frequencies of ‘had influenza-like-symptoms’

(43.6% in meat workers), nevertheless these two groups had different level of seroprevalence. In the study of meat workers, these three variables were not significantly associated with prevalence of infection with *Leptospira* spp. Combined, these observations concur with the conclusion of Dreyfus (2013) that out-of-work activities are not a significant risk factor for leptospirosis in people in at-risk workplace environments.

In this study, the exposures to potential risk factors were investigated by an online questionnaire. Compared with a face-to-face delivery method, online delivery can save time for researchers, save money and reduce the risk of transcription errors by moving from a paper format to an electronic version (Wright 2005). Other reasons for choosing an online delivery method included: students could finish the questionnaire online then there was no requirement for large number of staff to arrange the interviews; if the students did not have time to finish the questionnaire on the day of sampling, they could do this later with their own internet access, which gives more flexibility; and since the questionnaire included a question about previous medical history of leptospirosis, and the students may have felt more comfortable to answer it online than in face-to-face communication.

While this study has provided useful information about exposure to potential sources of infection with *Leptospira* spp. among veterinary students, it contains potential limitations. First of all, as we wish to make inference for all of the veterinary students, it is important to consider that voluntary participation potentially introduced selection bias. Students with a high level of interest in leptospirosis or who had been exposed to animal urine within/outside veterinary curriculum may be more likely to participate in the study. This is likely to over-estimate the seroprevalence and thus affect the external validity of the study. However, as no seropositive result was obtained, this is not a concern for our study. Secondly, only one serum was sampled from each student in this study and this may miss the recently exposed and currently infected students who have not developed antibodies, thus under-estimating the seroprevalence. The antibody response (detected by MAT) is delayed for around 10 to 24 days after exposure. The usual incubation period for infection after exposure varies from three to 14 days in human cases (Faine *et al.* 1999), and the detectable antibody MAT titres occur approximately seven to 10 days after onset of leptospirosis symptoms (Dutta and Christopher 2005). Furthermore, because leptospires usually penetrate the human body

through skin lesions or mucous membranes (Picardeau 2013), it may have been more appropriate to include questions regarding animal urine and broken skin/mucous membranes. However, these questions were not included as this would have made the questionnaire overly-long and potentially reduced the response rate.

The finding that all students were seronegative in this study can be used to provide comparison of risk factors with other occupationally exposed groups, e.g. meat workers, farmers and veterinarians, and has assisted a broader understanding of the epidemiology of infection in at-risk groups. This study has also provided a better understanding of the level of animal exposure in veterinary students, thus providing information to support other zoonotic disease studies in this group. It also suggests that instruction within the curriculum at Massey University to prevent exposure to *Leptospira* spp. appears to be effective.

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Chapter 9 General Discussion

1 Introduction

The studies presented in this thesis were designed to answer some key questions about leptospirosis diagnostics for both animals (sheep and cattle) and humans in New Zealand (NZ): how do different diagnostic tests perform on various specimens collected at different stages of infection; how do tests from a commercial and a research laboratory compare; how do serological test results and urine/kidney quantitative real-time PCR (qPCR) compare; and what is the utility of PCRs on blood from acute human cases? The utility of multi-locus sequence typing (MLST) for the classification of leptospiral animal field strains was also investigated. The shedding/renal colonisation rates and seroprevalence of *Leptospira* spp. were examined in slaughtered animals to raise awareness of the potential risk of contracting leptospirosis in meat workers. The seroprevalence of leptospirosis among veterinary students at Massey University was investigated as an example of an animal-human transmission investigation, and provided an opportunity to investigate exposures to putative risk factors both within the veterinary curriculum and personal life and for use as a comparative baseline for other occupation groups.

In this thesis, a number of diagnostic tests and types of specimens were evaluated. Diagnostic tests included the microscopic agglutination test (MAT), PCRs (both conventional and real-time), culture and dark-field microscopy (DFM). Specimens used included serum, whole blood, urine, and kidney. The serological tests for sheep and cattle focused on *Leptospira borgpetersenii* serovar Hardjobovis (Hardjobovis) and *Leptospira interrogans* serovar Pomona (Pomona) since they are the two most common *Leptospira* serovars found in beef cattle and sheep and in notified cases of disease in meat workers in NZ. The serological tests for veterinary students focused on three serovars (Hardjobovis, Pomona and *Leptospira borgpetersenii* serovar Ballum (Ballum)) since they were the three most common leptospiral serovars notified from human infection in NZ in the past five years.

This chapter places the research findings in context, identifies lessons learnt, reflects critically on study design and methodology (beyond that covered in the individual chapters) and suggests areas for future research.

2 Research findings in context

2.1 Leptospirosis diagnosis

2.1.1 Choice of diagnostic tests and specimen at different stages of infection

One of the most influential factors determining performance of diagnostic tests for leptospirosis is the stage of the illness (Jouglard *et al.* 2006). To mimic natural infection, experimental trials can be used. Trials performed before the 1990s on sheep and cattle used DFM, culture, MAT, or ELISA at various stages post-challenge in animals (Morse *et al.* 1957; Hodges 1974; Hathaway and Marshall 1979; Cousins *et al.* 1985; Bolin *et al.* 1989b). PCRs have been used to detect *Leptospira* spp. in subsequent experimental trials; however, mainly for urine samples from cattle that were inoculated with serovar Hardjobovis (Gerritsen *et al.* 1991; Taylor *et al.* 1997; Wagenaar *et al.* 2000). Work in Chapter 3 has provided new information for diagnostic test selection and interpretation post-infection using DFM, culture, MAT and qPCR with various specimens (serum, whole blood, urine, and kidney) on both sheep and cattle and with serovars Hardjobovis and Pomona in NZ.

Results from Trial C (16 sheep inoculated with serovar Pomona) are an example to illustrate the importance of choosing the right type of specimen and test for leptospirosis diagnosis at different stages post-infection. Predictive models based on the test results from Trial C can be used to demonstrate the performance of tests that detect leptospires/leptospiral DNA in blood and urine, and MAT that detect antibodies at different stages post-infection. This helps refine test usage and interpretation for future studies in animals and, potentially in humans. Logistic regression models were used to predict the probability of detecting leptospires in blood and urine from Day 0 to 42 after infection. For these models, positive blood test results obtained from either culture on whole blood or qPCR (on serum or whole blood) were treated as positive; while positive urine test results obtained from either culture or qPCR were treated as positive (DFM positive results were not included, due to the poor specificity and sensitivity of this technique). A linear regression model was used to predict the relationship between log of MAT titres and days post-infection. As shown in Figure 9.1, the predicted probability of leptospires being detectable by blood tests was more than 50% between Days 3 and 7 post-infection, with the highest probability (86.9%) occurring on Day 4. Prior to 12 days after infection, the predicted probability of leptospires being detectable

by urine tests was less than 50%; from Day 21, the probability was greater than 90%, and reached maximum on Day 28 (94.6%). From the predicted model the MAT titre became detectable (log of titre 24 = 3.178) from Day 5, with the highest log titre detected on Day 20.

Although these predictions were based on data from 16 sheep, and leptospirosis in challenged animals will be different from natural infections in animals and humans, some important inferences can be made about test selection at various stages following infection. Firstly, the observation shows that blood tests are likely to be sensitive only in the early stage of infection. Secondly, the probability of urine tests being positive at early stages was relatively low (less than 50% prior to 12 days post-infection). By contrast, detection rate increased to more than 90% from three weeks after infection and maintained this level until 37 days post- infection. Lastly, MAT titres can be detected from Day 5, and reach maximum around Day 20. Considering these patterns, and the higher sensitivity (p = 0.197) of PCR (87.5%, 14/16) compared with culture on blood samples (68.8%, 11/16) (Chapter 3), a combination of blood PCR and MAT on serum is recommended for diagnosis at an early stage (acute phase) of leptospirosis infection, followed by confirmation by MAT on convalescent phase sera collected two to three weeks apart. For cases that have demonstrated symptoms for more than two weeks before sampling, urine rather than blood samples should be considered for testing. Sensitivity of culture for detecting urinary shedding in sheep was higher than PCR in Chapter 3, although these were data obtained from challenged animals under controlled conditions. Considering the performance of urine culture is influenced by such factors as overgrowth of other bacterial and fungal contaminants (Fearnley *et al.* 2008) and limited survival time of viable leptospires in urine (Quinn *et al.* 1994), which are both difficult to reduce in the field, urine PCR is recommended.

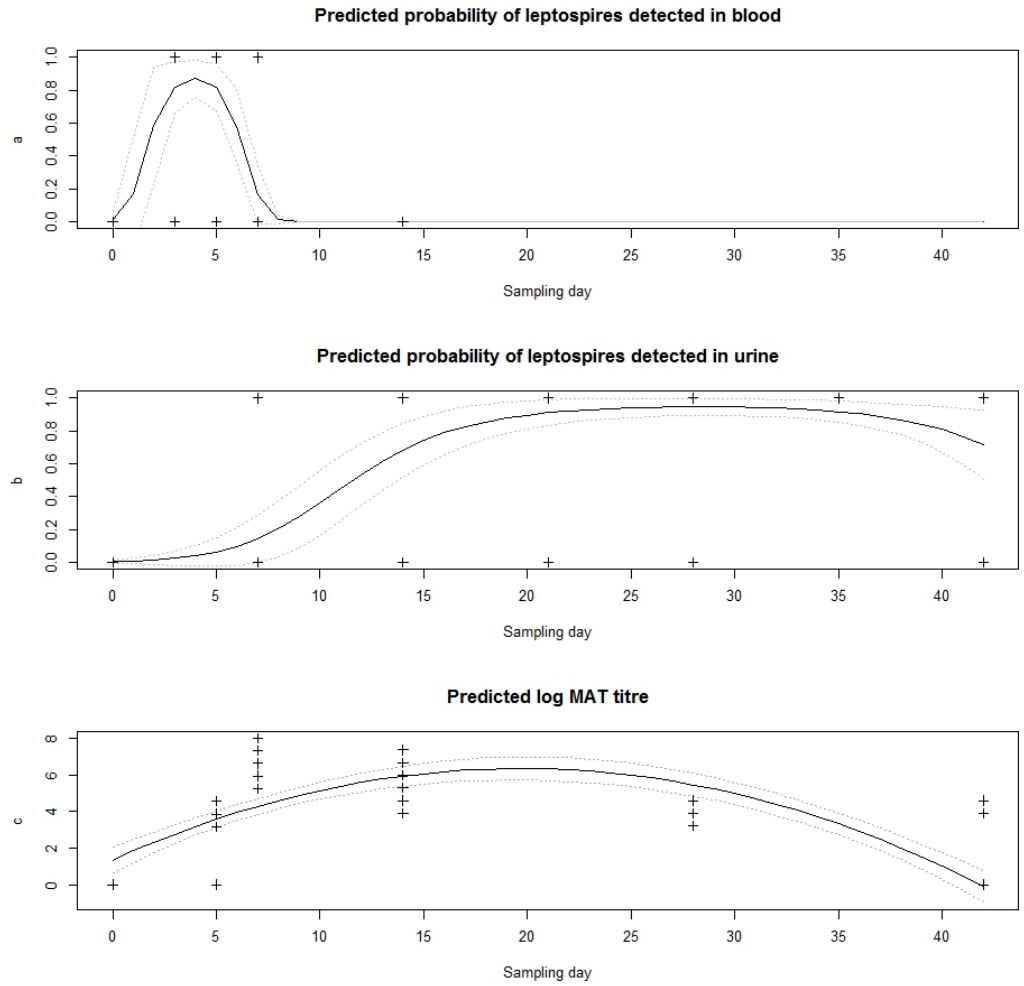

Figure 9.1 Predicted synopsis with 95% confidence interval (represented by dashed lines) of clinical Pomona infection in sheep, from Day 0 to Day 42 after infection; a) predicted probability of leptospires being detected in blood; b) predicted probability of leptospires being detected in urine; c) predicted log MAT titre against serovar Pomona; based on data from 16 sheep that were inoculated with serovar Pomona in Trial C; raw data are represented by '+'.

Which blood fraction should be used for PCRs to diagnose acute phase infection of leptospirosis is still an unsettled question. Conflicting findings about serum PCR versus whole blood PCR were reported in human studies. Serum PCRs were found to be less sensitive than whole blood for the detection of *Leptospira* DNA in blood by three studies in humans (Kositanont *et al.* 2007; Stoddard *et al.* 2009; Bourhy *et al.* 2011). Although the amount was not quantified, leptospiral DNA was detected in the clot from the SST and serum tubes, that may have impacted on the positive rate of PCR on serum samples (Stoddard *et al.* 2009). However, another human study, in Sri Lanka, reported that the sensitivity for quantitative PCR of serum was higher than whole blood (51.0% versus 18.4%, respectively) (Agampodi *et al.* 2012). In this thesis, serum qPCR had a

higher sensitivity (87.5%, 14/16) than whole blood qPCR (56.3%, 9/16) (p = 0.057) in sheep challenged with serovar Pomona (see Trial C Chapter 3). However, in the HRC study (Chapter 7), both of the confirmed human leptospirosis cases (Patients 10 and 13) who were recruited two and four days, respectively, after onset of symptoms tested positive by whole blood qPCR, but tested negative by serum qPCR. Although these conflicting findings could be explained by host factors and/or possible difference between natural and experimental infections, because of the limited number of samples tested in both studies, future studies involving larger number of clinical samples are needed to evaluate the qPCR on serum versus whole blood samples.

2.1.2 Evaluation of gyrB gene based qPCR with veterinary and human clinical samples

In the last decade, qPCRs were introduced for leptospirosis diagnosis and have advantages over the conventional PCRs by taking less time and reducing the false positive results caused by carry-over contaminations (contamination with products from previous reactions) (Ahmed *et al.* 2009; Picardeau 2013). Compared with the wide use of qPCR assays in diagnosis of leptospirosis in humans, the use of these assays in animal studies worldwide is relatively limited but includes studies in deer (Subharat *et al.* 2011), dogs (Rojas *et al.* 2010; Ahmed *et al.* 2012), pigs (Fearnley *et al.* 2008) and cows (Mughini-Gras *et al.* 2013). Prior to work done in this thesis, the use of qPCR had not been reported in sheep.

When compared to culture, which requires sufficient numbers of viable bacteria, PCR assays can detect both viable and dead bacteria (O'Keefe 2002), and can provide higher sensitivity in clinical settings. Leptospires remain viable in clinical specimens for a limited time, especially in urine (Quinn *et al.* 1994), as they require a pH range 6.8 to 7.4 for survival (Bharti *et al.* 2003). In clinical settings, samples are often collected and handled under less than optimal conditions and there can be delays in submission to laboratories. Therefore, qPCR is a useful diagnostic tool in the clinical medical and veterinary setting. In addition, Chapter 4 has demonstrated the utility of qPCRs in epidemiological studies in animals (e.g. screening of populations of animals to investigate the prevalence of leptospirosis and the potential risk to other animals and humans).

In this thesis, the *gyrB* gene based qPCR was used to detect leptospires in blood, urine and kidney samples from sheep and cattle. The utility of using this assay in the diagnosis of leptospirosis infection, shedding and renal carrier status of leptospires in these two species was evaluated (Chapters 3). Although no statistically significant differences, the sensitivity of serum qPCR was higher than blood culture for detecting early stage infection. In sheep the sensitivity of urine culture was higher than urine qPCR for detecting shedding, while in cattle higher sensitivity was achieved using urine qPCR. Comparable sensitivity was found between kidney qPCR and kidney culture for detecting renal colonisation in sheep.

After the utility of this assay on sheep blood was evaluated (by Trial C from Chapter 3), this was extended to the clinical blood samples from humans (Chapter 7). Unlike the qPCR assays based on 16S rRNA gene and *lipL32* gene that have been validated on human blood/serum samples (Slack *et al.* 2007; Ahmed *et al.* 2009; Thaipadungpanit *et al.* 2011) and urine samples (Villumsen *et al.* 2012), the *gyrB* gene based qPCRs have not been validated with clinical samples from human. Although limited confirmed cases were recruited for the HRC study (Chapter 7), results showed that among four cases that were recruited, at an early stage of infection (onset date two to five days before recruitment), two were tested positive by this assay, before sero-conversion was detected by MAT. Also, no positive results were obtained from nine non-leptospirosis cases (non-reactive MAT), attesting to the specificity of this assay.

The findings from this study demonstrated the value of using the *gyrB* gene based qPCR for diagnosis of leptospirosis, although further evaluation (proposed in section 4.1) needs to be done on more samples, both from animals and humans.

2.1.3 Clinical symptoms and leptospirosis diagnosis in humans

The common symptoms recorded in humans for leptospirosis include fever, headache and myalgia (Vinetz 2000). However, similar symptoms are reported with other infectious diseases such as malaria, dengue fever, influenza, or influenza-like diseases (Cermakova *et al.* 2013). Due to the diverse symptoms that mimic other infectious diseases that are often endemic and epidemic in the same locations, as well as the asymptomatic or sub-clinical infections that occur in some cases, leptospirosis is often an under-recognized or misdiagnosed febrile illness (Plank and Dean 2000; Vinetz

2000; Hoffmeister *et al.* 2010). Recent studies in several developing countries showed that leptospirosis is a common cause of undifferentiated febrile illness (El Jalii and Bahaman 2004; Murdoch *et al.* 2004; Suttinont *et al.* 2006; Biggs *et al.* 2011).

Although a low number of patients (n=14) were recruited and five were cases, the HRC study (Chapter 7) provided information about clinical symptoms in leptospirosis in NZ. As presented in Table 9.1, three confirmed leptospirosis cases in this study showed the same clinical symptoms, while for Patient 10, only fever and nausea were recorded. No apparent difference in symptoms was found between confirmed leptospirosis cases and non-cases. Jaundice and conjunctival suffusion were not reported in any of these cases, and this is likely due to the lack of severity of the cases, which is highly related to the infecting serovar. In a previous review of 15 patients that were admitted to the intensive care of a regional hospital in NZ from June 1999 to May 2005, conjunctival suffusion was recorded in nine (60%) patients (Vickery *et al.* 2006). Serovars of the Icterohaemorrhagiae serogroup, e.g. Copenhageni in NZ, frequently cause severe leptospirosis (Faine *et al.* 1999; Levett 2001). Findings from Chapter 7 again show that it is difficult to diagnose leptospirosis based on clinical symptoms alone, and emphasize the importance of laboratory confirmation. However, it is important for clinical workers (e.g. general practitioners, hospital doctors) to have a high index of suspicion for leptospirosis when seeing patients who have 'flu-like' symptoms, particularly those with animal contact (especially farm animals).

Table 9.1 Clinical symptoms and diagnostic results from the 13 patients recruited for HRC study (Chapter 7) who had clinical symptoms recorded; first serum sample was collected on recruitment date; second serum sample was collected 3 weeks apart from the recruitment date;

Patient ID	Clinical symptoms [a]									Diagnostic results [b]			
										MAT		PCR*	Culture
	Fever	Myalgia	Headaches	Sweating	Tiredness	Sore eyes	Stomach pains	Nausea	Other	Acute serum	Convalescent serum		
10	**+**	**–**	**–**	**–**	**–**	**–**	**–**	**+**	**–**	**–**	**+ (Ballum)**	**+**	**+**
12	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**+**	**–**	**–**	**+ (Pomona)**	**–**	**–**
13	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**+**	**–**	**–**	**+ (Tarassovi)**	**+**	**–**
14	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**+**	**–**	**–**	**+ (Hardjobovis and Pomona)**	**–**	**–**
1	+	+	+	–	+	+	–	+	–	–	–	–	–
2	+	–	+	+	+	+	–	+	–	–	–	–	ND
3	+	+	+	+	+	–	–	+	chest pains	–	–	–	–
4	+	+	–	+	+	–	+	+	–	–	–	–	–
5	–	–	–	+	–	–	–	–	–	**E**	**E**	–	–
7	+	+	+	+	+	+	+	+	–	–	ND	–	–
8	+	+	+	+	+	+	+	+	–	–	ND	–	–
9	+	+	+	+	+	+	–	–	–	–	ND	–	–
11	+	+	+	+	+	–	+	+	–	–	ND	–	–

information for patients confirmed as leptospirosis cases are shaded in grey.

[a] + = Yes; – = No

[b] + = positive results; – = Negative results; E = detectable MAT titres ≤ 200, and ≥ 25, without four-fold sero-conversion detected (indicates previous exposure to leptospires); ND=Tests were not performed

* PCR results were treated as positive, if one of the three laboratories reported positive results on whole blood or serum

In the veterinary student study (Chapter 8), 'flu-like' illness was reported by 44.7% (135/302) of the students in the past 18 months prior to sampling. All students were MAT negative for all tested serovars (Hardjobovis, Pomona and Ballum). In comparison, in a cross-sectional study of 567 abattoir workers in NZ that reported seroprevalence as 10.9% (seropositive against either serovar Hardjobovis or Pomona, or both), a similar proportion (43.6%) of the participants had 'flu-like' illness in the three years prior to the sampling (Dreyfus 2013). No statistically significant association was found between having had 'flu-like' illness and sero-status in that study (Dreyfus 2013). The similar proportion of those reporting 'flu-like' illness recorded in these two groups again demonstrating that 'flu-like' symptoms are non-specific for leptospirosis infection. Notwithstanding, in a cohort study that sampled 384 sheep abattoir workers between 50 and 61 weeks apart to investigate annual risk of contracting leptospirosis infection, new infection (sero-conversion against serovar Hardjobovis or Pomona)

increased the risk of 'flu-like' illness 1.9-fold (95% CI 1.3-2.7, p=0.007) (Dreyfus 2013).

2.2 Test comparison

2.2.1 Inter-laboratory comparison of urine qPCR

In recent years, several qPCR assays targeted at various genes (e.g. 16s rRNA (*rrs*), *secY*, *gyrB* and *lipL*32 gene) have been developed and used in many reference, commercial and research laboratories for the detection of leptospires in biological fluids. Although comparison of qPCRs targeted at different genes for human leptospirosis diagnosis in blood and urine has been reported (Bourhy *et al.* 2011; Thaipadungpanit *et al.* 2011; Villumsen *et al.* 2012), such comparison has not been performed for animal leptospirosis diagnostics on any specimen, to the best of the author's knowledge.

In the abattoir study (Chapter 5), notwithstanding the protocols of urine qPCR from two laboratories (Hopkirk Leptospirosis Research Laboratory (HLRL) and Gribbles Veterinary (GV)) that included different DNA extraction kits, different primers targeted at different genes (*gyrB* gene for HLRL and 16s rRNA gene for GV) and different chemistries (SYTO9 for HLRL and TaqMan probe for GV), almost perfect agreement was recorded (with better agreement obtained when suspected results ($36<Ct\leq40$) obtained from GV qPCR were considered as negative rather than as positive). This is likely explained by the similar sensitivity and specificity (currently being estimated by latent class analysis, see details in section 4.1) of these two qPCRs on urine samples. It can also be explained by the limited genetic diversity within Hardjobovis and Pomona strains in NZ. In Chapter 6, 21 isolates from sheep and cattle kidneys sourced from one abattoir were genotyped by MLST at six loci (Ahmed *et al.* 2006). Concatenated sequences of the six loci clustered as two groups, those being, Hardjobovis (n=13) and Kenniwicki (n=8) isolates. The allelic profiles of the isolates within each cluster were identical. As these findings were based on a limited number of sheep and cattle samples collected from a small abattoir, a larger number of samples from more animal species (e.g. deer), and from broader area of NZ should be considered in a future study to more fully explore the genetic diversity within each *Leptospira* serovar.

As an initial investigation into the hypothesis that different processing of samples prior to conducting the PCR reaction may influence discordant qPCR results between the two

laboratories, a panel of 16 DNA extracts that had discordant urine qPCR results were exchanged and tested. In Chapter 5, when comparing test results, the suspect results (Ct value >36 and ≤40) from GV potentially had dual classification as positive or negative by adjusting the cut-off. However, in this chapter, no reclassification was done and suspect results remain classified as suspect. Therefore, the panel of 16 extracts that were exchanged includes, but is not limited to, all suspects from GV. The urine qPCR results from both laboratories before and after DNA samples were exchanged are presented in Table 9.2.

At GV, eight samples that initially tested as negative or suspect on GV DNA extracts subsequently tested positive on HLRL DNA extracts. One sample that initially tested as suspect on GV DNA extracts subsequently tested negative on HLRL DNA extracts. Compared to the mean GV Ct value of the 16 samples on GV DNA extract, the mean Ct value reduced by 2.7 on HLRL DNA extracts ($p < 0.05$, paired *t* test). At HLRL, five samples that initially tested positive on HLRL DNA extracts subsequently tested negative on GV DNA extracts; while other samples that initially tested negative on HLRL DNA extracts remained tested negative on GV DNA extracts. Although these findings are based on a small number of samples selected by having discordant results between laboratories, this suggests different DNA yields were obtained from the two laboratories. This was most likely due to the different pre-PCR processing steps performed, including different DNA extraction protocols and the different delays times between the collections and processing of samples (HLRL processed the samples the day following collection, while GV processed samples within a week of collection). The most important influence on the effectiveness of PCR assays come from the inhibitors originating either from the biological samples or from sample preparation prior to PCR, or from both (Rådström *et al.* 2004). Efforts have been made in human and animal studies of leptospirosis to reduce the inhibitors in the DNA extracts from urine samples (Gerritsen *et al.* 1991; Lucchesi *et al.* 2004; Ahmed *et al.* 2009). Although a small number of DNA extracts were included in the comparison in our abattoir study, the results from the DNA exchanged between laboratories again emphasize the importance of consideration of pre-PCR processing when evaluating the sensitivity of PCR testing.

Table 9.2 GV (Ct value included) and HLRL urine qPCR results before and after DNAs were exchanged for the 16 samples that had different urine qPCR results originally.

Sample ID	GV Urine qPCR[a]				HLRL Urine qPCR[b]	
	GV DNA Extraction (Ct value)		HLRL DNA Extraction (Ct value)		HLRL DNA Extraction	GV DNA Extraction
A7[c]	–	40.26	+	35.37	–	–
G8	S	38.5	S	36.74	+	+
G9	S	37.89	S	37.04	+	–
H5	S	36.86	–	45.84	–	–
I20	–	44.45	S	37.88	+	–
F26	S	37.45	+	34.22	–	–
I35	S	37.31	S	37.2	–	–
I38	S	38.46	S	37.22	–	–
I41	S	36.08	S	37.49	–	–
A48	S	36.81	+	28.08	+	–
I49	S	37.23	S	36.48	–	–
C19	S	37.09	+	31.32	+	–
I64	S	36.03	+	31.48	+	–
G20	S	37.84	+	33.64	+	+
E36	S	37.01	+	29.84	+	+
G29	S	39.09	+	35.37	–	–

+ = positive results; S = suspect results; - = Negative results

[a] Positive: defined as having a Ct value less or equal to 36; Negative: defined as having a Ct value greater than 40; Suspect: defined as having a Ct value between 36 and 40

[b] Positive: defined as having a similar melting temperature (± 0.03) as positive control

[c] A7 was included as the Ct value was close to 40

2.2.2 Inter-laboratory comparison of MAT

The inter-laboratory comparison of the MAT in a veterinary setting in NZ, reported in Chapter 5, was novel, to the best of the author's knowledge. It is well-known that different MAT results for the same sample can be obtained from different laboratories (Levett 2001). Reasons for this include variation in the source of antigen cultures, the maintenance of reference collections of *Leptospira* strains, and the effect of subjective observer variation (Levett 2001; Chappel *et al.* 2004; Cerqueira *et al.* 2010b).

For the 542 serum samples (396 sheep and 146 beef cattle) tested, the agreement between two MATs (from HLRL and GV, at a cut-off of ≥ 48 and ≥ 50, respectively) against serovar Hardjobovis was almost perfect (kappa = 0.94). The agreement between the two MATs against serovar Pomona was moderate (kappa = 0.53). This serovar-dependent difference found between two laboratories suggests that the different MAT results in this study may be more likely due to the different source of antigen (especially serovar Pomona) cultures used in two laboratories than observer variation from the two laboratories. HLRL used a laboratory stock of antigen culture, while the Gribbles used antigen culture sourced from the *Leptospira* Reference Laboratory (ESR) in NZ. It is worth performing a comparison (especially for serovar Pomona) between these two laboratories, using the same stock of antigen culture to investigate whether different source of antigen was the main reason for different results obtained between laboratories.

Previous work comparing MAT results between HLRL with the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, was conducted by Subharat on deer serum (Subharat 2010). Using the same cut-off (≥ 48) at HLRL as in Chapter 5, and cut-off ≥ 50 at the WHO/FAO/OIE reference laboratory, the comparison revealed moderate agreement for Hardjobovis (kappa = 0.41) and substantial agreement for Pomona (kappa = 0.68) at the individual animal level. Variation between two MATs was expected due to the subjectivity of test interpretation (Subharat 2010).

Observer variation was not considered as the main reason for the different MAT results (positive/negative classification based on a cut-off) obtained between two laboratories in the comparison of MATs in Chapter 5. However, when considering the raw data (actual dilution of MAT titres recorded), the proportion of serum samples that had different reciprocal MAT titres reported by two laboratories among seropositive sheep (83.3% for Hardjobovis, 91.8% for Pomona) and seropositive cattle (81.4% for Hardjobovis, 96.6% for Pomona) suggested that the MAT reading can be subjective. In order to access the degree of variation for MAT results between different observers, an intra-laboratory comparison of readings from different observers on the same samples had been conducted at HLRL before. Ten serum samples of unknown serological status, positive control (serovar Pomona) and negative control (saline) were included in the testing panel (Table 9.3). The readings using different methods of diluting serum

samples (by micro diluter or multichannel pipettes) were also compared. In general, there was a tendency (p = 0.006) for Observer 2 to report one to two dilution higher titres than Observer 1 when using the Micro Diluter to dilute the serum samples. When using a multichannel pipette, the titres read for the same serum were higher for Observer 1 (p = 0.01); whereas no obvious trend (p = 0.83) was observed for the readings from Observer 2. These again indicated that the variation of readings from different observers can lead to discordant readings of MAT titres. This example also showed that using different equipment to dilute serum samples, which is laboratory specific, may have an impact on the MAT results.

Table 9.3 Reading of MAT titres from Observer 1 and Observer 2 (experienced leptospirosis researchers, HLRL) on ten serum samples of unknown serological status, positive control (serovar Pomona (pom +ve)) and negative control (saline), stratified by different methods of diluting the serum samples in the MAT process (Micro Diluter/multichannel pipettes).

Serum ID	Micro diluter		Multichannel pipette	
	Observer 1	Observer 2	Observer 1	Observer 2
23	48	192	48	96
28	192	192	384	192
31	96	96	384	768
70	192	384	192	192
94	96	192	96	96
96	192	384	384	192
117	24	192	48	192
124	48	96	96	192
161	192	384	384	192
169	96	384	384	384
Pom +ve	1536	768	3072	1536
Neg	Neg	Neg	Neg	Neg

In summary, MATs performed at different laboratories, or at the same laboratory by different persons, can yield different results on the same sample. There have been a number of sero-surveys of prevalence of leptospirosis in NZ in different species that showed varied results (Heuer *et al.* 2012). In addition to random variation and that in source populations and seasonal influences, based on our findings, the between-laboratory variation in reporting MAT results is another important factor that should be taken into account when interpreting comparison between different studies. To improve the generalisation of MAT results obtained from different laboratories, quality control at each individual laboratory is important (proposed in section 4.2). To avoid the variation of titre reading between different observers within laboratory, it is preferably to have the

same person read MAT results for all samples of a given study to ensure consistency and validity of results. Otherwise, appropriate training and blind testing against known results are necessary to ensure the greatest consistency between personnel.

2.2.3 Association between serological test and testing for shedding/renal colonisation

Although the MAT is the most widely used test for leptospirosis diagnosis (Adler and de la Peña Moctezuma 2010), it detects antibodies in serum rather than the presence of leptospires directly. The direct detection of leptospires or their DNA in urine or kidney is important for identifying carrier animals (Faine *et al.* 1999). Previous studies have generally shown that MAT was a poor predictor of shedding and renal carriage in sheep and cattle, when determined by urine PCR and kidney culture, respectively (Thiermann 1984; Magajevski *et al.* 2005; Suwimonteerabutr *et al.* 2005; Lilenbaum *et al.* 2009). However, in our abattoir study (Chapter 5), the prevalence ratios of shedding and renal colonisation in Hardjobovis-positive versus Hardjobovis-negative sheep was reported as 12.5 (95% CI: 4.8-32.6) and 8.6 (95% CI: 5.6-13.3), respectively. These results suggest that Hardjobovis-seropositivity (at titre cut-off $\geq$ 48) detected in sheep resonably indicate shedding/renal colonisation in NZ.

Since the MAT remains the most commonly used test by veterinarians, and often only MAT results are available, linking the shedding status with MAT titres can inform animal disease control and management. The question of whether higher MAT titres (e.g. $\geq$ 800 which is indicative of more recent infection) are associated with higher shedding rates was addressed in Chapter 5, by investigating the association between MAT titres and urine qPCR results from HLRL. In Hardjobovis or Pomona-seropositive sheep, no obvious association was found between level of MAT titres and probability of shedding (Figure 5.4). However, in Pomona-seropositve sheep, there were significant trends ($p < 0.01$) between the level of MAT titres and probability of renal colonisation.

Another question is whether higher MAT titres are associated with a higher concentration of leptospires shed in the urine. The Ct value of qPCR is an indication of the concentration of leptospires in the urine samples (the lower Ct value, the higher concentration of leptospires in the urine). Linear regression models were used to determine the association between the MAT titres (provided by GV) and the continous

outcome, Ct value from GV urine qPCR among seropositive animals, stratified by species. Animals which had positive titres for both serovars Hardjobovis and Pomona (indicating dual infection) were excluded from the analysis. Among Hardjobovis-seropositive (titre cut-off 50) sheep, for every one unit increase in the log of MAT titre, the Ct value decreased by 1.75 (95% CI: 0.04-3.45) ($p < 0.05$), suggesting that sheep with higher Hardjobovis titres have higher concentration of leptospires shed in the urine.

Whether Hardjobovis seropositivity detected in sheep can indicate shedding/renal colonisation, and the association between shedding rate, concentration of leptospires being shed in the urine and level of MAT titres should be investigated with a larger number of samples, and from wider area of NZ. It should be noted that majority (77.9%) of the sheep sampled in our abattoir study were lambs aged less than 12 months, and the finding may not represent sheep from all age groups. Animals maintained in the flock for breeding have different exposures; therefore it is worth conducting longitudinal studies in sheep from all age groups (lambs, hoggets, and ewes) to further investigate the association between MAT and urine qPCR results. This can also contribute to the knowledge of age-specific endemics and epidemics in sheep.

2.3 Host-serovar relationship between Hardjobovis/Pomona and sheep/cattle in NZ

Historically, cattle were considered as a reservoir host for serovar Hardjobovis in NZ, while infections by serovar Pomona in cattle and by both serovars in sheep were considered to be of a sporadic nature (Hathaway 1981). However, the high seroprevalence of Hardjobovis found in sheep in recent nationwide farm studies (Subharat *et al.* 2009; Dreyfus *et al.* 2011), along with the findings from Dorjee *et al.* (2008) who isolated *Leptospira* by culture from kidneys of 22% of lamb carcasses seropositive against Hardjobovis (although this was based on data from lambs, which are more likely to be shedding than older sheep), suggest that sheep may have become a reservoir host of Hardjobovis in NZ. It was also proposed by Dreyfus (2013) that Pomona may also be well adapted to sheep, considering the seroprevalence (14%) of Pomona found in the sheep from the nationwide farm study (Dreyfus *et al.* 2011), and also the annual incidence rate of 7.3% (sero-conversion against serovar Pomona) found

in sheep abattoir workers (n=384) from a cohort abattoir study, which indirectly indicated that the sheep were shedding Pomona (Dreyfus 2013).

A maintenance host of a particular host-adapted serovar can be characterized by the relatively low pathogenicity of the serovar to the host (usually seen as a chronic rather than an acute infection), and the persistence of the serovar in the kidney (Faine *et al.* 1999; Radostitis *et al.* 2007). The persistence of the organism in the kidney is best investigated with experimental trials or cohort studies (with sequential sampling of urine) over a prolonged observation period, although the urine tests may not provide 100% accurate indication of kidney colonisation, due to the intermittent shedding of leptospires in animals. In the abattoir study, the percentage of urine and kidney samples that were qPCR positive (indicating shedding and renal colonisation rates) was reported in clinically healthy animals with variable MAT titres. This cross-sectional "snapshot" can indicate whether the animals infected with particular serovars (as determine by MAT) are likely to have renal colonisation and shed the organism in the urine. As shown in Chapter 5, for sheep, the percentage of urine qPCR positives detected in Hardjobovis-positive and Pomona-positive (MAT titre ≥ 48) carcasses was 82% and 10%, respectively; while the percentage of kidney qPCR positives detected in Hardjobovis-positive and Pomona-positive carcasses was 70% and 11%, respectively. Moreover, both Hardjobovis and Pomona isolates (identified by MLST) were obtained from sheep kidney cultures in the same abattoir (Chapter 6). These findings permit the proposal that sheep may be or have become a reservoir host for both serovars; while serovar Hardjobovis is better adapted to sheep, compared to serovar Pomona. Also, the readily established Hardjobovis and Pomona infections in some sheep from the challenge trials (see Trial A and C results from Chapter 3), with shedding/renal colonisation detected supported this hypothesis.

The animal level seroprevalence of Pomona (at titre cut-off ≥ 48) in beef cattle was reported to be 25% in the nationwide farm study (Dreyfus *et al.* 2011). In our abattoir study (Chapter 4), the same seroprevalence (25%; 95% CI: 17-36%) of Pomona (using same titre cut-off, and performed at the same laboratory) was reported in beef cattle carcasses. These data suggest that Pomona infection in NZ beef cattle may happen more often than previously found (e.g. 5.5% in 273 cattle sampled from lower North Island (Subharat *et al.* 2007) and 4% in 591 dairy cattle sampled in the district of Taranaki, North Island (Bahaman *et al.* 1984)), and beef cattle may also be reservoir host for

serovar Pomona. However, data obtained from this thesis only partially supports this statement. Although in the abattoir study 22% and 19% of the Pomona-positive beef cattle tested positive by urine qPCR and kidney qPCR respectively (Chapter 5), only isolates of serovar Hardjobovis were obtained from beef cattle kidney cultures (Chapter 6). Moreover, Pomona infections were not established in cattle from the challenge trial (see Trial D results from Chapter 3), although this may also be due to the low virulence of the inoculating culture.

2.4 Reflection on occupational exposure to leptospires

In NZ, the majority of notified leptospirosis cases are occupationally acquired (Vickery *et al.* 2006; Keenan 2007), and the occupations identified as being at high risk of exposure to *Leptospira* spp. were meat workers, farmers, and forestry-related workers (Thornley *et al.* 2002). From 2008 to 2012, there were 456 notified cases of leptospirosis in NZ. Among 414 notified cases that had occupations recorded, meat workers and farmers constituted 22.7% (94/414) and 52.7% (218/414), respectively (The Institute of Environmental Science and Research Ltd 2009-2012). In presenting data from both animals and humans, the findings from this thesis are in concordance with previous findings on occupational risk for leptospirosis in NZ.

In response to the concerns that the occupational exposure to leptospirosis is high in NZ, previous studies have been undertaken to investigate the epidemiology of leptospirosis in livestock from abattoirs and farms (Heuer *et al.* 2007; Dorjee *et al.* 2008; Dreyfus 2013). Compared with those earlier studies, our abattoir study (Chapter 4) reported higher animal-level seroprevalence (57% for sheep and 73% for cattle) than the 6% for sheep from an abattoir study (Dorjee *et al.* 2008) and 39% for cattle from farms (Heuer *et al.* 2007); while work in this thesis reported comparable seroprevalence to those (50% for sheep and 58% for cattle) reported in the farm survey from 2009 to 2010 (Dreyfus 2013). In addition to seroprevalence, shedding rates and renal carriage status in the slaughter-ready sheep and cattle were also investigated by urine/kidney qPCR in our abattoir study. Across all suppliers shedding rates were 31% (95% CI: 17%-48%) for sheep and 21% (95% CI: 14%-30%) for cattle, while the renal carriage rate was 29% (95% CI: 17%-45%) for sheep and 21% (95% CI: 15%-28%) for cattle. This high urinary shedding/renal carriage rate and seroprevalence of leptospires

reported in our abattoir study emphasise the on-going risk of exposure to *Leptospira* spp. in meat workers.

Compared with a cohort study that covered eight abattoirs (sheep, beef and deer) in New Zealand and reported an annual incidence of severe clinical leptospirosis of 0.8% (3/384) among sheep abattoir workers (Dreyfus 2013), a higher annual incidence rate of leptospirosis cases (15%, 3/20) was reported in the meat workers from the abattoir of this thesis during the 2008/09 killing season. The samples collected in our abattoir study were sourced only around Waikato area, while the abattoir workers in the cohort study (Dreyfus 2013) processed sheep from several other regions besides Waikato (e.g. Hawke's Bay, Wanganui). It is possible that there was a regional difference in prevalence of leptospirosis infection in livestock, thus leading to different exposures rates in meat workers. However, a recent farm survey from eight geographical regions in both North and South Islands (Dreyfus 2013), reported no statistically significantly ($p < 0.05$) seroprevalence difference regionally for either Hardjobovis or Pomona in beef cattle herds, or Hardjobovis in sheep flocks. By contrast, sheep flocks in Hawke's Bay and East Coast had 2.7 times the odds (95% CI 1.26-5.61) ($p = 0.01$) of being Pomona-seropositive than those in Waikato. Also, similar seroprevalence was found between our abattoir study and the farm survey (Dreyfus 2013) which sampled livestock from both North and South Islands. These indicated that the region of livestock being processed may not be associated with the level of exposure to leptospires for those who handle the animals.

In the abattoir study conducted by Dorjee *et al.* (2008) in the southern North Island, significantly higher seroprevalence to Hardjobovis was reported in lambs sampled in May and June 2004 than those sampled in the corresponding months in 2005. High rainfall accompanied by widespread surface flooding in February 2004 and the relatively less rainfall in 2005 was considered as a contributing factor for this between-year difference (Dorjee *et al.* 2008). Other reports of an association between rainfall and surface flooding and outbreaks of leptospirosis in lambs have previously been reported in New Zealand (Vermunt *et al.* 1994; Dorjee *et al.* 2005). For our abattoir study, we were told that a sustained period of heavy rain occurred in the six weeks prior to 15th October 2010 (A. Cullum, personal communication, 2010), covering three weeks before sampling time and the first three weeks of sampling. The high seroprevalence and shedding rates detected in our study again supported the association between heavy

rainfall and increased infection rate in livestock, which can increase the risk of exposure to *Leptospira* spp. in staff who handle the animals (e.g. suppliers, transporters, meat workers).

Since shedding of leptospires is considered more likely to occur in younger animals (Heath and Johnson 1994), the high shedding rate detected in the abattoir study is likely to be partially attributable to the young age of the sampled animals. However, in both sheep and cattle sampled in the abattoir study, no statistically significant association was found between age group and the animals tested positive by urine and kidney qPCR (based on logistic regression models, data not shown). Due to the fact that the majority of sheep (77.9%) and cattle (79.5%) were from young stocks in our abattoir study, further research with more samples from older age groups should be considered to investigate the association between age of animals and shedding rates, thus contributing to the better understanding of whether staff handling animals of younger age are at higher risk of exposure to leptospires.

The occupational information for the leptospirosis laboratory-confirmed cases in the HRC study (Chapter 7) showed that people who have exposure to livestock as part of their employment are at risk of contracting a clinical leptospirosis infection. Among the five confirmed cases, one was a farm worker, the others were meat workers (n=2), a stock truck driver (n=1), and a retired farmer (n=1, who was reported to have continued exposure to animals).

In the veterinary student study (Chapter 8), the comparison of the frequency of exposure to putative risk factors between this low-risk group and a high-risk group (meat workers) contributed to the understanding of risk factors for leptospirosis infection in NZ. The results from the veterinary students were compared with those from a recent cross-sectional study of meat workers in NZ, which reported seroprevalence as 10.9% (62/567) (Dreyfus 2013). The frequency of exposure to non-work activities (hunting and home slaughtering) was found to be similar in these two groups, adding strength to the finding of Dreyfus *et al.* (2013) that non-work activities are less important risk factors than within-work activities. By contrast, in a more recent cross-sectional study of veterinarians in NZ that reported seroprevalence as 4.7% (13/277), in addition to the risk factor of working in mixed practice, home slaughtering cattle or pigs, which is an non-work activity, was found to be a significant risk factor for being seropositive

(Sanhueza *et al.* 2013). Whether the non-work related activities have impact on seroprevalence found in high-risk groups in NZ can be further evaluated with a study of leptospirosis in farmers, which is currently being conducted by another PhD student from our leptospirosis research group. However, it is important to be aware that what constitutes a "non-work" activity changes across occupations. For example, home-slaughtering is a non-work activity for veterinarians, veterinary students and meat workers; however, for farmers it is part of their work. Home-slaughtering was reported more frequently in farmers (by 86.0% (153/178) of farmers, data sourced from a cross-sectional study that sampled 127 farms from both North and South Island) (Juan Sanhueza, PhD candidate, unpublished data, 2013), compared to 20.9% and 30.0% reported in veterinary students (Chapter 8) and meat workers (Dreyfus 2013), respectively.

2.5 The value and challenge of multi-disciplinary research

Due to the development of specialisation (knowledge of scientists restricted to one subject area) in the past, one of the biggest challenges of the 21st century is the breaking-down of this hierarchical structure, and the setting-up of collaboration with a cross-functional approach (Mullner 2009). Most scientists work in government or university research facilities and thus there is a concern that scientific knowledge or research results developed may not be broadly applicable in the real world (Busch 1984). Also, the understanding of problems and processes may be biased through the eyes of highly specialised researchers. Inter-disciplinary collaboration can overcome these concerns. Collaboration is a mutually beneficial and well-defined relationship set up by two or more organizations to achieve common goals with a jointly developed structure and shared responsibility (Mattessich *et al.* 2001). To manage complex health issues associated with zoonotic diseases, inter-disciplinary collaboration and exchange of knowledge among a diverse group of practitioners (from veterinary, medical and environmental sciences) is considered as effective approaches, depending on the good understandings of key attributes of successful collaboration (Anholt *et al.* 2012).

In the studies in this thesis, we collaborated with multiple stakeholders, including those of the same discipline (research institutes of veterinary settings) and those of different disciplines (veterinary diagnostics laboratory and human health settings covering public

health researchers, medical workers, hospitals, and diagnostic laboratories). We worked closely with staff from Estendart Ltd. and New Zealand Veterinary Pathology Ltd., Palmerston North, for the Estendart Trial project (Chapter 3). Samples for the abattoir study (Chapter 4, 5) were collected and processed by staff from AgReserach Ltd., Ruakura Research Centre, and delivered through Gribbles Pathology Hamilton/Palmerston North, while dual testing (MAT and urine qPCR) were performed by staff from Gribbles Veterinary Palmerston North. The veterinary student study (Chapter 8) is part of a Veterinary Student Public Health Study, with collaboration set up with Prof. Jeroen Douwes and staff from Centre for Public Health Research, Wellington, Massey University, for the ethics application, study design and on-line survey development. The project that relied most heavily on collaboration was Chapter 7, the Health Research Council (HRC) study, which involved considerable time and effort to set up and maintain the partnerships with General Practices (GPs) from Waikato area, Waikato District Health Board (WDHB), PathLab Hamilton (PathLab), Institute of Environmental Science and Research Ltd (ESR), and the Canterbury Health Lab (CHL) (Figure 9.2)

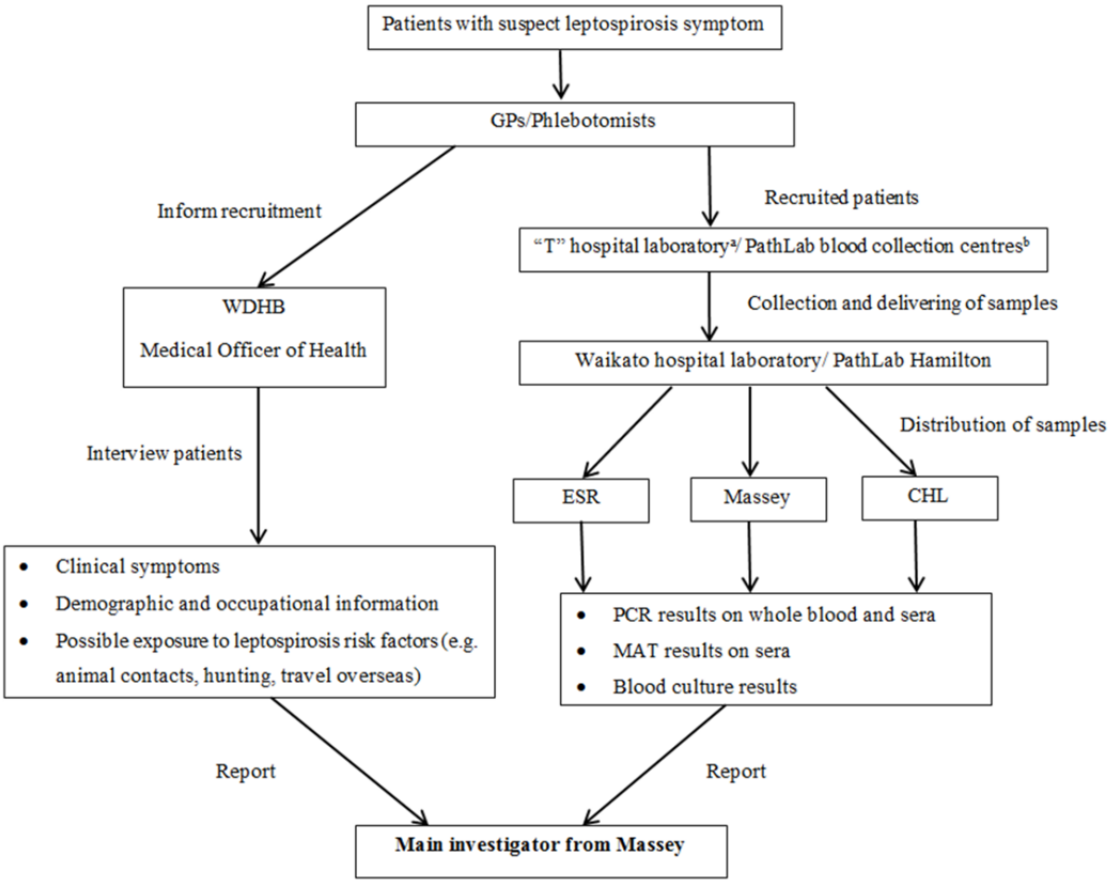

Figure 9.2 Framework of the multidisciplinary collaborative approach for the Health Research Council study; "T" hospital laboratory[a] = Taumarunui, Te Kuiti, Tokoroa and Thames hospital laboratory; PathLab blood collection centres[b] = Path Lab Te Awamutu, Leamington, Cambridge, Morrinsville-Dallas, and Matamata blood collection centres; ESR = Institute of Environmental Science and Research Ltd, Wellington; Massey = Hopkirk Leptospirosis Research Laboratory, Massey University; CHL = Canterbury Health Laboratory, Christchurch; WDHB = Waikato District Health Board.

Despite the importance of multi-disciplinary collaboration for particular research, the practice is challenging and may involve a steep learning curve for researchers. In the HRC study, recruitment rate of leptospirosis suspect patients through GPs was poor; although considerable time and effort had been dedicated to improve this (the participating GPs/practice managers were kept in contact and reminded of the study by phone calls, newsletters of project updates, faxes and emails). From August 2011 to December 2012, there were 32 leptospirosis cases notified around the Waikato area; however, our study captured only five of these. Among the cases that were not captured, seven (27%) of cases were missed due to the failure of participating GPs to recruit the patients (data provided by Dr Anita Bell, WDHB medical officer of health). The others were missed due to the incomplete coverage by GPs from the Waikato region. Time constraints and GPs' lack of interest in research topics, as have been reported by others (Peto *et al.* 1993; Williamson *et al.* 2007; Page *et al.* 2011) may be reasons for the barriers to recruiting all of the eligible patients. For this study, three laboratories needed to treat the cases as urgent cases, and report test results within five working days to make sure the GPs could have the results as soon as possible for the patients' treatment. However, for the first two cases recruited, results were not released from one laboratory within 5 working days due to the workload of their routine practice. We negotiated with the senior scientist and reinforced the importance of reporting on time for this study.

Multi-disciplinary research for this thesis involved the main investigator working at a distance from collaborators. For this and for other diverse reasons, it was challenging to make sure the field staff adhered strictly to the protocol which is essential for the quality of research (Tanaka *et al.* 2008). Examples that can be taken from the HRC project involve the sampling/distribution procedure and the paper work regarding the study participation form and the consent form. For the first three cases recruited, culture, whole blood and serum samples were not sent under the correct transport conditions. Considering the impact of this may have on the test results, we contacted Dr Chris Mansell, Clinical Microbiologist, Waikato Hospital to see how we could better enforce

the study protocol. The clinical microbiologist set up a spread sheet, recorded the irregularities, and we sent a diagram to help clarify the procedure for staff who were in charge of distributing samples at Waikato Hospital Laboratory. Furthermore, there were some issues around the study participation form that need to be filled in by GPs to record patients' clinical symptoms. Among 14 patients recruited for this study, five of them had this information missing. We contacted the hospital laboratory managers and they assisted by asking the GPs to fill in the forms. In total, this form was filled in for all of the patients except one that was recruited by a GP who was on maternity leave and not contactable.

Last but not least, for multi-disciplinary research, communication is the most important factor for setting up and maintaining the partnership. The close communication with collaborators is the key point for solving multifaceted problems that inevitably happen due to the complexity of this kind of study. For example, due to the close relationship that had been set up with Dr Anita Bell, we had access to test results, clinical symptoms, demographic factors and putative risk factors for leptospirosis infection drawn from the with EpiSurv form and the Waikato Leptospirosis Case Form and Questionnaire. The close contact between Drs Bell and Mansell and us was very influential in ensuring all of the procedures had been performed correctly for each patient, and we were well-supported in attempts to improve the patient recruitment (e.g. set up the pathway of recruiting patients from hospital doctors and phlebotomists).

According to our experience and experiences from other researchers (Mattessich *et al.* 2001; Tanaka *et al.* 2008; Mullner 2009), the following are some recommendations for consideration about multi-disciplinary team research:

- Establish clear and measurable goals for the collaboration, and clarify the responsibilities associated with the research program at the onset.
- Partners need to respect each other's expertise and contributions, understand the capabilities of each individual, communicate and function as a true team. A demonstration of respecting expertise across disciplines was our consultations with medical practitioners with regard to expected clinical signs and the interval between acute and convalescent MATs.
- The main researchers need to confirm that the protocol is clear to all partners and strictly adhered to. A demonstration of this was our consultation with the

laboratories with regard to sample receipt and onward distribution resulting in the easy-to-understand documentation including flowcharts.

- The main researchers need to set up a regular review mechanism and provide on-going feedback to all partners regarding success and challenges in meeting the research goals, and the opportunities for them to have meaningful involvement. A demonstration of this was the provision of newsletter to GPs and regular contact with WDHB.
- Negotiations and compromises may be necessary, and partners who usually work within a specialty area may need to move outside their comfort zone to solve problems.
- Identify partner groups that are under-represented in the collaboration and implement strategies to increase their involvement in the partnership.

2.6 Implication of thesis findings for vaccination of sheep and cattle in NZ

The key for controlling leptospirosis infection in animals and humans is to limit direct and indirect transmission of *Leptospira* spp. between susceptible hosts and carriers (Heath and Johnson 1994). Occupational hygiene such as personal protective equipment (PPE) and the avoidance of splashes from urine or contaminated water are useful for reducing the transmission of *Leptospira* spp. to humans (Faine *et al.* 1999). However, it is challenging to implement PPE as it may impede work: for example, for meat workers, wearing face masks is reported to restrict vision. Vaccinating livestock prior to exposure is considered as an effective approach to reduce carrier state, and thereby breaking the infection cycle. Previous studies in NZ showed that vaccination could reduce the risk of urinary shedding of leptospires in deer by 11% (95% CI: 4%-18%) (Subharat *et al.* 2012), and has a significant degree of protection on urinary shedding in cattle (detection of serovar Hardjobovis in urine from 6/10 non-vaccinated calves versus 0/9 vaccinated calves (Marshall *et al.* 1979)). Although vaccination does not fully prevent shedding of leptospires from already infected animals, reduction of shedding (e.g. by 44% in the infected deer herds after one vaccination course (Ayanegui-Alcerreca 2006)) can be achieved. Since human infections are derived mostly from domestic livestock in NZ, vaccination of domestic livestock is an efficient approach to control animal infection and concurrently reduce the exposure of people (especially those in high-risk occupations) (Marshall and Manktelow 2002). A dramatic fall in the number of notified

human leptospirosis cases was found in NZ, concurrent with widespread use of a bivalent animal vaccine against serovars Hardjobovis and Pomona in dairy cattle, since it was introduced to the market in 1979 (Marshall and Manktelow 2002). Notifications of human infection dropped from 677 in 1979, to 325 in 1981. In 1982, the number of notified cases was 179, and remained at less than 200 for another five years (Marshall and Chereshsky 1996).

In the abattoir study (Chapter 4), high seroprevalence and shedding rates were reported in sheep and cattle that were not vaccinated. The exposures to these unvaccinated stock during daily work routine likely contributed to leptospirosis cases (15%, 3/20) reported in the meat workers from this abattoir during the 2008/09 killing season. In the HRC study (Chapter 7), ten participants reported livestock exposures and vaccination status of the animals. Among them, the five laboratory-confirmed leptospirosis cases reported exposure to livestock with 'unknown' or 'not vaccinated' status. In contrast, four (80%) of the five non-cases reported that the livestock they were exposed to were vaccinated. Although from one region and with a small number of cases, and the exposure to wildlife and other domestic animals (e.g. dogs) that can be sources of leptospires transmissions were not covered, the findings from the abattoir study and HRC study, combined with existing knowledge of vaccine efficacy on shedding, reinforce that contact with unvaccinated livestock presents a greater risk of leptospirosis, and reinforces the importance of vaccinating livestock to protect humans from leptospirosis.

For the 21 isolates that were obtained from sheep and cattle in the abattoir study, multi-locus sequence typing showed two dominant serovars (*L. borgpetersenii* Hardjobovis and *L. interrogans* serovar Kennewicki) (Chapter 6). The vaccines registered in NZ all include serovar Hardjobovis and Pomona, while some include *L. interrogans* serovar Copenhageni (Benschop *et al.* 2012). As serovar Kennewicki and serovar Pomona are indistinguishable serologically, cross-protection may be provided by the vaccine formulated with serovar Pomona. However, although an experimental study in NZ has showed the effectiveness of Pomona vaccine in preventing Pomona infection in cattle (Marshall *et al.* 1982), this has not been experimentally confirmed for sheep (which is the animal species that had Kennewicki isolated in Chapter 6). The characterization of serovar Kennewicki in the abattoir study highlights the importance of getting more isolates from more areas of NZ to confirm whether serovar Pomona or serovar Kennewicki is the main serovar from serogroup Pomona that is present in livestock

populations. This could be important for achieving optimum vaccine efficacy in NZ. If serovar Kennewicki is found to be the main serovar that present, and limited cross-protection of Pomona vaccine for Kennewicki infection in NZ livestock is experimentally confirmed, Kennewicki field strain would need to be considered for the production of vaccine for livestock in NZ.

3 Reflective critique of study methodologies

3.1 Design of challenge trials

The designs of the challenge trials (Chapter 3) were determined by Estendart Ltd. in conjunction with their client to meet their needs for collecting data and contributing to the study design of vaccination efficacy trials in the future. As our involvement was opportunistic, we had no input into the protocols hence the design did not specifically fit our aims (for example, the sampling timing and frequency).

To provide more detailed information about the performance of each leptospiral diagnostic test at various known times post-challenge, sampling would be optimised by including more days. For example, blood samples should be taken every day, from Day 0 until Day 15, to evaluate the qPCR for detecting early stage infection. Since duration of MAT titres and shedding after infection is also of interest, observation periods should be extended to suit this purpose. For example, instead of ceasing sampling on Day 42, serum and urine samples would ideally be collected weekly from the animals until week 13 or later after challenge (a similar schedule has been used in previous trials (Morse *et al.* 1957; Cousins *et al.* 1985)).

As the major aim of the challenge trial study was to evaluate leptospiral diagnostic tests at various known times post-infection, it was very important to make sure animals are leptospirosis-free before challenge. The conduct for all trials was that after being screened and identified as MAT-negative, animals were grazed with other stock with unknown leptospirosis status (for 12 to 40 days, varied by trials) before challenge. The co-grazing with other stock increased the chance of exposure to leptospires for these animals (as shown in sheep B6, resulting in deletion from analysis). It is therefore recommended that the animals be grazed separately from stock of unknown

leptospirosis status, and preferably on areas that have not been stocked for the previous six weeks before challenge (Mackintosh *et al.* 1981).

Only female sheep/cattle were selected in an attempt to increase the ease of collecting urine samples and reduce the risk of contamination in the urine. Human studies have been conducted to investigate the association between leptospirosis infection and gender as a factor. In a German study of laboratory-confirmed leptospirosis cases during 1997-2005, male patients were reported to be more likely to exhibit severe outcomes than female patients (Jansen *et al.* 2007). The gender-related differences were not associated with the type of exposure or time from onset of symptoms to treatment (Jansen *et al.* 2007). In a Sri Lankan study that analysed the leptospiral load in serum/blood samples from 58 leptospirosis cases by quantitative PCR, the median leptospiral load in male patients was significantly higher than that of female patients (Mann-Whitney U test, $p = 0.022$) (Agampodi *et al.* 2012). Given these findings, it is reasonable to assume that the biology of susceptibility and the pathogenesis for leptospirosis can be different between human males and females. Literature investigating the association between the gender and leptospirosis status in livestock animals are limited. In a previous serological survey of seven deer farms in the North Island of NZ, gender was reported to have significant effect ($p < 0.001$) on the seroprevalence of Pomona in deer (at a cut-off titre of ≥ 48). The odds ratio of being seropositive in the male groups was 1.63 (95% CI: 1.21-2.19) times that of the female groups; whereas the seroprevalence of Hardjobovis and Copenhageni were similar in both gender groups (Ayanegui-Alcerreca 2006). Conflicting results were found in our abattoir study. The majority of the sheep and cattle sampled were female (70% and 64%, respectively). No significant association ($p > 0.05$) was found between the factor gender and the seroprevalence of both serovar Hardjobovis and Pomona (at a cut-off titre of ≥ 48), or the shedding/renal carriage status for both sheep and cattle. However, it needs to be noted that our abattoir study was based on the limited number of sheep and cattle samples collected from a small abattoir in the local area, and with more female than male animals involved. Also, findings from both Ayanegui-Alcerreca (2006) and our abattoir study can be confounded by the factor that management practices may differ between male and female animals within herds. Since findings in human studies suggested that the susceptibility for leptospirosis has a sex difference, while no solid evidence has been reported from studies in livestock (e.g. in experimental trials), it is worth testing this hypothesis in future research. If male

animals are proved to be more susceptible to leptospirosis infection, this needs to be considered in study design and also used to interpret study results accordingly.

3.2 Utility of the microscopic agglutination test

Whether the MAT can provide accurate estimation of serological prevalence or incidence of exposure to specific *Leptospira* serovars depends on adequate coverage of the serovars in the battery of test antigens, and also on the MAT titre cut-off chosen to define a sample positive. In NZ, serovars Hardjobovis and Pomona are the two serovars most commonly tested in veterinary settings, based on the assumption that no new serovars have been introduced into the populations since earlier studies. In ESR, for diagnosis of human cases, a panel of eight serovars representative of NZ *Leptospira* serovars (Hardjobovis, Pomona, Ballum, Tarassovi, Copenhageni, Canicola, Grippotyphosa, and Australis) is routinely tested.

It needs to be noted that the present understanding of leptospiral serovars present in animals and humans in NZ is still based on the reports of isolation of leptopires in 1980s (Hathaway 1981). Considering the importation of livestock/goods and humans travelling internationally, consideration needs to be taken that exotic serovar/s may have been introduced into NZ. For example, the screening of deer sera for a panel of 23 reference serovars at the Brisbane WHO/FAO/OIE reference laboratory demonstrated 14 samples from 11 NZ farms were seroreactive to serovar Arborea (belonging to serogroup Ballum) (Subharat *et al.* 2009). A limited but unsuccessful attempt was made to culture the organism. Although further serological screening, and ultimately culture, is required to confirm that this, or any other serovar, is indeed present, the observation confirms the importance of periodic screening of animal and human population in NZ to insure the adequate coverage of serovars in the battery of test antigens for MAT. It is well known that MAT results can be influenced by the cross-reactivity between serovars (Levett 2001). Therefore, it is possible that exotic serovars may have been introduced, but are not recognised serologically, due to the cross-reaction with serovars that are endemic in NZ. To investigate this hypothesis, culture and/or genotyping methods (e.g. MLST) should be applied.

For the abattoir study (Chapter 4), two serovars (Hardjobovis and Pomona) were chosen for the detection of seroprevalence of leptospirosis in sheep and cattle by MAT, because

they are the two most commonly found serovars in cattle, deer and sheep in NZ (Dreyfus *et al.* 2011). *L. borgpetersenii* Ballum is maintained in rodents/hedgehogs and occurs secondarily among livestock animals in NZ (Hathaway 1981; Thornley *et al.* 2002). Previous studies of serological responses to leptospiral serovars in livestock animals showed low prevalence of Ballum (Hellstrom 1978; Blackmore *et al.* 1982; Wilson *et al.* 1998) and indicated that the serological responses of livestock animals to this serovar may result from occasional contact with the maintenance hosts. The relationship between animal contacts and infecting serovars among notified human cases from 2001 to 2003 in NZ showed that among 75 cases that had contact with cattle, serovar Ballum constituted 18.7% of the infections (Baker and Lopez 2004). In the same review, 14 Ballum cases reported exposure to cattle, while two reported exposure to rodents (Baker and Lopez 2004). Although the records of exposure to cattle may be confounded by the exposure to rodents that were easily neglected or hard to appreciate, this still showed the possibility that the prevalence of Ballum in livestock animals may have changed in NZ. Notified data (The Institute of Environmental Science and Research Ltd. 2003-2013) showed an increasing proportion of notified Ballum human cases in NZ in the past 10 years, and this rise is considered to be coincident with an increase in the proportion of farmers represented among notified cases from 2006 to 2011 (Benschop *et al.* 2012). The data from a recent retrospective review of human cases in the Waikato region between 2004 and 2010 show that 20% (5/25) of the dairy farmer cases and 17.6% (6/34) of the dry stock farmer/farm manager cases were notified with Ballum infection (Cowie and Bell 2012). In a current Massey University survey that looks to estimate the seroprevalence of Hardjobovis, Pomona, and Ballum in livestock, preliminary results (covering three beef, 11 sheep and seven deer farms from the South Island) show seroprevalence of Ballum as 7.7% in beef cattle, 9.2% in sheep, and 0.6% in deer (Juan Sanhueza, PhD candidate, unpublished data, 2013). These results indicate that for future research of leptospirosis in livestock animals, it may be important to include serovar Ballum in the MAT panel. Other serovars, such as Copenhageni should also be considered, if sampling is conducted in mixed-species farms that include deer, beef cattle and sheep on the same property (Ayanegui-Alcérreca *et al.* 2010).

For the study of leptospirosis among veterinary students (Chapter 8), three serovars (Hardjobovis, Pomona and Ballum) were investigated because they were the three most

common leptospiral serovars notified from human infection in NZ from 2008 to 2011 (The Institute of Environmental Science and Research Ltd 2009-2012). According to the online-survey results, after livestock, dogs were the most frequently reported animal species students were exposed to, both within and outside the veterinary curriculum. Dogs can act as a maintenance host for pathogenic *Leptospira* serovars and excrete leptospires via urine (Rojas *et al.* 2010). Leptospiruria has been demonstrated by PCR in 7.1% of dogs in Ireland, 8.8% of dogs in the United States, and 22% of dogs in Iran (Harkin *et al.* 2003; Rojas *et al.* 2010; Zakeri *et al.* 2010). These results highlighted the significance of canine leptospirosis and its zoonotic potential. In NZ, the four serovars most likely to cause leptospirosis in dogs are Copenhageni, Pomona, Hardjobovis and Ballum. This raises the question as to whether serovars associated with canine leptospirosis should be included in the testing panel to investigate if the students have been exposed to leptospires through contacting dogs. In a serological survey of leptospiral antibodies in dogs in the lower half of the North Island and the South Island of NZ, serovar Copenhageni was the most common serovar and found in 10.3% of dogs, followed by serovar Hardjobovis (3.5%), Pomona (1.1%) and Ballum (0.8%) (Harland *et al.* 2012). However, it is difficult to correlate the serological findings with the prevalence of chronic infection and urinary shedding (Ellis *et al.* 1981; Faine *et al.* 1999) and limited information was available for *Leptospira* spp. isolated from dogs in NZ (except serovars Pomona and Tarassovi isolated from dogs in one study (Mackintosh *et al.* 1980)). In a study in progress that has included 117 dogs from 28 farms (with up to eight dogs sampled on each farm), 16% dogs tested positive by urine qPCR (Alison Harland, companion animal veterinarian, unpublished data, 2013). This indicates that dogs may contribute to the transmission of leptospires to humans in NZ. Identification of the shedding serovars is important future work to ensure adequate coverage of *Leptospira* serovars in the MAT testing panel for detecting leptospirosis infection in humans/animals that have frequent contacts with dogs.

The titre cut-off chosen for determining exposure to leptospires is different from that for determining clinical disease due to leptospirosis. It is suggested that a titre cut-off of ≥ 48/50 should be used to indicate exposure to *Leptospira spp.* for both humans and animals in the absence of clinical disease (Blackmore *et al.* 1982; Faine *et al.* 1999; Shivakumar and Krishnakumar 2006). Since in both the abattoir study and the veterinary student study, the aims were to investigate previous exposure to leptospires, a

MAT titre of ≥ 48 was chosen. Using this cut-off also makes the results comparable with previous sero-surveys of leptospirosis conducted by HLRL in livestock (Dorjee *et al.* 2008; Subharat *et al.* 2009; Dreyfus *et al.* 2011) and humans (Dreyfus 2013) in NZ. Although we believe that we chose a valid cut-off to investigate previous exposure to leptopsires, there were other studies in NZ (e.g. (Wilson *et al.* 1998; Matthews *et al.* 1999; Benschop *et al.* 2009)) that used higher or lower titre cut-off. Therefore, when comparing seroprevalence with these studies, the use of different titre cut-off needs to be taken into account for interpretation.

4 Suggested areas for future work

4.1 Reporting the accuracy of diagnostic tests

The qPCR assay used in the challenge trials (Chapter 3) and abattoir study (Chapter 4, 5) was a modification of the assay set up and validated by Dr Supatsak Subharat using deer urine and kidney samples (Subharat *et al.* 2011). In this thesis, the sensitivity and specificity of this assay has not been determined with sheep and cattle samples. The most important infecting serovars for sheep and cattle NZ are Hardjobovis and Pomona, the same as for deer (Ayanegui-Alcérreca *et al.* 2010). Also, these animals have similar metabolisms, which lead to a similar pH level in urine/kidney samples, a determinant in the survival of leptospires in the samples. It therefore seems reasonable to assume that the accuracy of PCRs should be similar in the urine and kidney samples from sheep and cattle as in those from deer. However, additional work on the limits of detection and diagnostic sensitivity and specificity (as previously carried out on deer samples (Subharat *et al.* 2011)) is required to completely evaluate the utility of the test on sheep and cattle specimens.

Bayesian latent class analysis (LCA) is a useful tool for evaluating diagnostic tests where a "gold standard" does not exist. No assumption is made that any test or combination of tests is perfect, and the disease status remains latent (Boelaert *et al.* 1999; Bajani *et al.* 2003). For leptospirosis diagnosis, LCA has been used to evaluate tests on human blood samples and indicated that the commonly applied reference test (MAT and/or culture) may not be valid to be treated as "gold standard" (Bajani *et al.* 2003; Limmathurotsakul *et al.* 2012). The abattoir study (Chapter 5) provided results from a panel of different tests for detecting previous exposure and shedding of

leptospires in sheep and cattle. This panel has provided an opportunity for applying LCA, and is currently being analysed by a master student Shen He from our leptospirosis research group. The 'latent state (disease status)' in the LCA model is defined on the basis of the overall frequency of the possible combinations of test results (Joseph *et al.* 1995; Dendukuri and Joseph 2001), for this study will be MAT, urine and kidney qPCR. The accuracy of each test will then be estimated based on the defined 'latent state'.

The utility of our qPCR assay on whole blood and serum samples was investigated in the challenge trial study, showing that the assay was able to detect leptospires in serum (given the time post inoculation) from 14 of 16 sheep inoculated with serovar Pomona. The accuracy of the assay on human blood has the potential to be evaluated in the HRC project, for example, by setting the 'latent state' as overall frequency of the combinations of MAT, blood culture, and blood PCRs results in the LCA model. However, it has not yet been performed due to the limited number of patients recruited thus far.

The standards for reporting of diagnostic accuracy (STARD) statement (www.stard-statement.org, accessed by October 2013) were published in 2003 to improve the accuracy and completeness of reporting diagnostic accuracy studies, and to allow readers to assess the generalizability and utility of the results for the tests' intended purpose (Bossuyt *et al.* 2004). The STARD statement includes a checklist of 25 items and recommends the use of a flow diagram to describe the design of the study (Bossuyt *et al.* 2004). Until February 2013, 200 biomedical journals have encouraged the use of STARD in their instructions for authors (Ochodo and Bossuyt 2013). Adherence to standards is considered to benefit end-users of tests including doctors, veterinarians and other healthcare professionals and the human and animal patients the tests are used for (Gardner 2010). Compared to human medicine journals, veterinary journals infrequently recommend adoption of the STARD statement. Considering that the most common application of STARD for animal diseases (classification of disease status) is different from that for human diseases (prognosis and prediction), a study discussing the possible modification of STARD for animal disease studies has been recently published (Gardner 2010). Future research regarding accuracy of the qPCR assay on livestock and human samples is recommended to be conducted following the STARD guidelines. Since LCA analysis is allowed in the STARD with proper reports in particular items

(Item 12 or 21) of the 25 check lists, this can be used to evaluate the accuracy of the assay. This approach will add weight to the sensitivity and specificity values by reporting more information about how the value was obtained and whether the test (applied for a specified purpose) is appropriate for the target population.

4.2 Quality control of microscopic agglutination test

The use of proficiency schemes can have a positive impact on the performance of the MAT. One proficiency scheme has been provided by the International Leptospirosis Society (ILS) (http://www.med.monash.edu.au/microbiology/staff/adler/proftemp.html, accessed October 2013). The number of participants (laboratories) for this proficiency testing scheme had grown from 37 from 23 countries in round one (2002) to 60 from 34 countries in round two (2003) (Chappel *et al.* 2004), and there had been ten rounds of testing up until 2012 when 97 laboratories from 49 countries participated. Laboratories performing MAT for the diagnosis of leptospirosis in the medical or veterinary settings, or both were involved. The growth of participant numbers and test rounds indicate that many participants find it to be of value (Chappel *et al.* 2011). The impact of the proficiency testing scheme was not easily demonstrated; however, when that author compared two groups of participants who participated in round two, significantly more false-negative MAT results were reported from the laboratories participating for the first time than from laboratories that had participated in both rounds (Chappel *et al.* 2004). A false-negative result was defined by reporting a negative result (a titre of 100 or 80) for any serovar from the serogroup against which a sample was raised. The new participants also reported overall significantly more variable results (Chappel *et al.* 2004). Therefore, participation in this proficiency scheme may be of value in controlling the quality of MAT performance in the two laboratories involved in this thesis (HLRL and GV) in the future.

4.3 Assessment of occupational exposure to leptospirosis

To more fully understand occupational exposures to leptospirosis (e.g. in meat worker, farmers), it will be of advantage to collect blood and urine samples from both livestock and humans, and also companion animals (e.g. working dogs). The prevalence of *Leptospira* serovars in animals and humans can be inferred from MAT results, with

urinary shedding detected by qPCR in seropositive animals to indicate potential transmission. However, considering the accuracy of MAT for predicting the infecting serovars can be influenced by many factors (e.g. cross-reactions, difficulty of designing serovars panel), it is better to obtain strains from animals and humans (through culturing urine samples) and use them for genotyping (e.g. MLST). The same genotype of strains obtained from livestock and humans can indicate inter-species transmission. A farm study which is currently in progress by our leptospirosis research group includes blood samples from livestock and farmers, and blood and urine from working dogs from the same farm. Samples will be tested against three serovars (Hardjobovis, Pomona, and Ballum) as a first attempt to investigate the association between seroprevalence in animals and human seroprevalence on the same farm (Juan Sanhueza, PhD candidate, personal communication, 2013).

4.4 Inclusion of urine testing in animal studies

Although detection of leptospires or their DNA in urine is considered important for identifying carrier animals (Faine *et al.* 1999), in most of the animal leptospirosis literature, urine samples were not included (due to budget/difficulty of handling urine samples). In general, there was poor correlation between MAT and urine qPCR results for sheep and cattle at individual animal level in Chapter 5. The exception was the good correlation with Hardjobovis-seropositivity and qPCR in sheep. Therefore, it is reasonable to generally recommend that in addition to serum samples, urine samples should be included in future studies of leptospirosis in animals to provide reliable indication of shedding. This can help to indicate carriage status in the sampled animals, which is important to inform management strategies to prevent the spread of leptospirosis infection (Faine *et al.* 1999). Also, it is recommended that urine samples be collected from animals of different age groups to investigate the epidemiology of leptospirosis at different stages of the infectious cycle within a flock. More information on shedding in seropositive animals can also contribute to the knowledge of the host-adaption relationship between the endemic serovars and animals (e.g. help to solve the unsettled question of relationship between serovar Pomona and cattle) in NZ.

4.5 The ability of qPCR to distinguish serovars using melting temperature

For the qPCR results on urine and kidney samples from sheep and cattle in the abattoir study, in general, the melting temperature (*T*m) of Pomona samples was higher than Hardjobovis samples (serovars identified by MAT). To investigate the potential of using this qPCR to distinguish serovars directly by *T*m, DNA extracts from cultures of six HLRL laboratory strains that are endemic in NZ (Hardjobovis, Pomona, Ballum, Tarassovi, Copenhageni, and Balcanica) were tested by qPCR, with three repetitions.

Consistent differences of *T*m were found between Hardjobovis and Pomona strain, with higher median *T*m reported as 84.0 °C ± 0.3 for Pomona strain than that reported for Hardjobovis strain (83.5 °C ± 0.3). The median *T*m of Balcanica and Tarassovi strains was the same, 83.3 °C ± 0.4. However, *T*m was not able to differentiate two species; for example, the Copenhageni strain that belongs to species *L. interrogans* had the same median *T*m (83.5 °C ± 0.4) as Hardjobovis strain, while Ballum strain that belongs to species *L. borgpetersenii* had similar *T*m (83.9 °C ± 0.4) as Pomona strain.

At this stage, and within the NZ context, using *T*m for this qPCR is shown to be only valuable for differentiating serovars Hardjobovis and Pomona in conjunction with MAT. However, it indicates the value of further investigation/evaluation (i.e. including alternate genes that may be more discriminatory) for analysing amplification PCR products by *T*m to provide a fast alternative identification to MAT.

4.6 Genotyping of *Leptospira* isolates in New Zealand

A larger number of field strains from more animal species (livestock, wildlife, and companion animals) and human species should be collected to evaluate the current MLST scheme for molecular characterization of *Leptospira* isolates in NZ, and also identify serovars that were present, which is important for understanding the epidemiology of leptospirosis in NZ. Genes *ligB* and *rpoB* which have been shown to have good discriminatory power can be considered to be added to the MLST scheme for further evaluation (La Scola *et al.* 2006; Cerqueira *et al.* 2009). Since the MLST scheme developed by Thaipadungpanit *et al.* in 2007 has been modified to allow the characterization of *Leptospira* strains for *L. borgpetersenii* and *L. interrogans* (Boonsilp *et al.* 2013), and the scheme has the advantage of having data accessible at the MLST

website, it is worth comparing this modified scheme with the scheme used in this thesis for the molecular classification of *Leptospira* strains in NZ.

To avoid the challenges of choosing the correct genes for the MLST scheme, whole genome sequencing of field isolates can be used as an alternative for epidemiology studies. Besides providing classification of *Leptospira* isolates in NZ, whole genome analysis can enhance our understanding of the mechanisms of virulence (e.g. by analysing chemotaxis/motility) and pathogenesis (e.g. by analysing lipopolysaccharide synthesis and adhesion and/or surface protein of leptospiral gene) in leptospirosis (Ren *et al.* 2003; Nascimento *et al.* 2004; Picardeau *et al.* 2008).

5 Conclusion

Leptospirosis is an important zoonotic disease locally and internationally. The complexities in the epidemiology and transmission of leptospirosis found both in NZ and elsewhere, hinder the full understanding of this disease. Symptoms of leptospirosis in both animals and humans are not disease-specific, which leads to the lack of clinical suspicion and consequently under-reporting of this disease. The challenge of diagnostic testing also contributes to the under-estimation of this disease. Currently, no diagnostic technique is completely satisfactory for all diagnosis purposes.

Studies in both animals and humans have been included in this thesis to investigate leptospirosis at the human-animal interface in NZ, and as a first attempt to understand some key questions about diagnosis and occupational risks in this country. These included: the first report of using qPCR along with other diagnostic tests on various specimens collected at different stages of infection in challenged sheep and cattle; the first report of a comparison of leptospiral qPCRs from two laboratories on animal samples and showed the general concordance of these two tests results on urine in NZ; the first report of comparison of MAT between laboratories in veterinary settings in NZ; the first report of applying a MLST scheme on animal field isolates and contributing to the limited genotypic data of leptospires in NZ; and also, the first report of seroprevalence of leptospirosis in veterinary students in NZ. Besides the conventional test (MAT), qPCRs were applied to examine the leptospirosis status in slaughtered animals, and raised the awareness of the potential risk of contracting leptospirosis in meat workers.

Finally, additional research needs to be done to answer the questions raised by this thesis. For example, the reconsideration of the traditional reservoir/spill-over host model for leptospiral transmission and a deeper understanding of the host-pathogen dynamics gained through both phenotypic and genotypic characterisation.

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Appendix

Appendix 3a Challenge and sampling schedule for all four trials

Table 1. Challenge and sampling schedule for sheep Trial A

Study Day	Event	Blood Collection			Urine Collection			Kidney Collection		
		Plain for MAT	Plain for PCR	EDTA for PCR	PCR	Culture	DFM	PCR	Culture	DFM
-40	MAT screening	✓								
-1	Transfer sheep into pens in APU according to allocation plan									
0	First challenge	✓	✓							
1	Second challenge	✓	✓							
2	Third challenge	✓	✓							
3		✓	✓							
19		✓			✓	✓	✓			
22				✓	✓	✓	✓			
27					✓	✓	✓			
30					✓	✓	✓			
33					✓	✓	✓			
42	Sacrifice sheep and kidney recovery	✓	✓	✓				✓	✓	✓

Table 2. Challenge and sampling schedule for sheep Trial B

Study Day	Event	Blood Collection				Urine Collection			Kidney Collection		
		Plain for MAT	Plain for PCR	EDTA for culture	EDTA for PCR	PCR	Culture	DFM	PCR	Culture	DFM
-29 &-16	MAT screening	✓									
-2	Transfer sheep into pens in APU according to allocation plan										
0	First challenge	✓	✓	✓	✓	✓	✓				
1	Second challenge										
2	Third challenge										
5		✓	✓	✓	✓	✓	✓	✓			
8		✓	✓	✓	✓	✓	✓	✓			
12		✓	✓	✓	✓	✓	✓	✓			
14		✓	✓	✓	✓	✓	✓	✓			
21		✓	✓	✓	✓	✓	✓	✓			
28		✓	✓	✓	✓	✓	✓	✓			
37		✓	✓	✓	✓	✓	✓	✓			
42	Sacrifice sheep and kidney recovery								✓	✓	✓

Table 3. Challenge and sampling schedule for sheep Trial C

Study Day	Event	Blood Collection				Urine Collection			Kidney Collection		
		Plain for MAT	Plain for PCR	EDTA for culture	EDTA for PCR	PCR	Culture	DFM	PCR	Culture	DFM
-14	MAT screening	✓									
-2	Transfer sheep into pens in APU according to allocation plan										
0	First challenge	✓	✓	✓	✓	✓	✓				
1	Second challenge										
2	Third challenge										
3			✓	✓	✓						
5		✓	✓	✓	✓						
7		✓	✓	✓	✓	✓	✓	✓			
14		✓	✓		✓	✓	✓	✓			
21						✓	✓	✓			
28		✓				✓	✓	✓			
35						✓	✓	✓			
42	Sacrifice sheep and kidney recovery	✓				✓	✓	✓	✓	✓	✓

Table 4. Challenge and sampling schedule for cattle Trial D

Study Day	Event	Blood Collection				Urine Collection			Rectal Temperature
		Plain for MAT	Plain for PCR	EDTA for Culture	EDTA for PCR	PCR	Culture	DFM	
-40	MAT screening	✓							
0	First challenge	✓	✓		✓				✓
1	Second challenge								✓
2	Third challenge								✓
7		✓	✓		✓				✓
9		✓	✓	✓	✓				✓
14		✓	✓	✓	✓				
20		✓	✓		✓	✓	✓	✓	
23		✓	✓		✓	✓	✓	✓	
26						✓	✓	✓	
28						✓	✓	✓	
35						✓	✓	✓	
42		✓	✓		✓	✓	✓	✓	

Appendix 3b Count of challenge cultures for all four challenge trials

Table 1. Count of challenge culture (organisms/ml) for challenge Trial A

Organism	**1st Challenge (Day 0)**	**2nd Challenge (Day 1)**	**3rd Challenge (Day 2)**
	10th March 2010	**11th March 2010**	**12th March 2010**
Pomona (HLRL)	~ 5.4×10^7	~ 6.1×10^7	~ 9×10^7
Hardjobovis (HLRL)	~ 4.4×10^7	~ 5.4×10^7	~ 5.3×10^7
Pomona (Gribbles)	~ 6.3×10^7	~ 5.3×10^7	~ 6.6×10^7
Hardjobovis (Gribbles)	~ 6.2×10^7	~ 5.1×10^7	~ 8.9×10^7

* 2 ml of culture were used for challenge

Table 2. Count of challenge culture (organisms/ml) for challenge Trial B

Organism	**1st Challenge (Day 0)**	**2nd Challenge (Day 1)**	**3rd Challenge (Day 2)**
	7th July 2010	**8th July 2010**	**9th July 2010**
Hardjobovis (HLRL, passaged from Trial A)	~ 5×10^8	~ 5×10^8	~ 4.5×10^8

* 2 ml of culture were used for Day 0 and 1, 2.3mls were used on Day 2

Table 3. Count of challenge culture (organisms/ml) for challenge Trial C

Organism	**1st Challenge (Day 0)**	**2nd Challenge (Day 1)**	**3rd Challenge (Day 2)**
	8th December 2010	**9th December 2010**	**10th December 2010**
Pomona (LPC04/4)	~ 6.6×10^8	~ 7.4×10^8	~ 6.4×10^8
Pomona (LPC04/8)	~ 7.6×10^8	~ 7.6×10^8	~ 6.2×10^8

* 2 ml of culture were used for challenge

Table 4. Count of challenge culture (organisms/ml) for challenge Trial D

Organism	**1st Challenge (Day 0)**	**2nd Challenge (Day 1)**	**3rd Challenge (Day 2)**
	14th April 2010	**15th April 2010**	**16th April 2010**
Pomona (HLRL) [a]	~5×10^8	~5×10^8	~5×10^8
Hardjobovis (HLRL) [a]	~5×10^8	~5×10^8	~5×10^8

* 2 ml of culture were used for challenge

[a] Same culture as Trial A were applied in this Trial for challenge

Appendix 3c Test results for all four challenge trials

(HLRL= Hopkirk Leptospirosis Research Laboratory, Massey University, GV= Gribbles Veterinary Pathology, Palmerston North, NZVP= New Zealand Veterinary Pathology, Palmerston North; Culture, qPCR, and DFM results: N= negative results, P= positive results; MAT results: N= MAT titres were not detected; / = samples were not from those sampling days)

1. Sheep trial A

1.1. Culture results from HLRL

ID	Urine					Kidney
	Day 19	Day 22	Day 27	Day 30	Day 33	Day 42
A1	N	N	N	N	N	N
A2	N	**P**	**P**	N	N	**P**
A3	N	N	N	N	N	N
A4	**P**	**P**	**P**	**P**	N	**P**
A5	N	N	N	N	N	N
A6	N	N	N	N	N	N
A7	N	N	N	N	N	N
A8	N	N	N	N	N	N

1.2. MAT results from HLRL and GV

ID	Serovar	Day 0	Day 1	Day 2	Day 3	Day 19	Day 42
A1	Pomona	N	/	/	/	N	N
	Hardjobovis	N	/	/	/	N	N
A2	Pomona	N	N	N	N	N	N
	Hardjobovis	N	N	N	N	**100**	**192**
A3	Pomona	N	/	/	/	N	N
	Hardjobovis	N	/	/	/	N	N
A4	Pomona	N	N	N	N	N	N
	Hardjobovis	N	N	N	N	**1600**	**768**
A5	Pomona	N	**24**	N	N	N	**24**
	Hardjobovis	N	N	N	N	N	N
A6	Pomona	N	/	/	/	N	N
	Hardjobovis	N	/	/	/	N	N
A7	Pomona	N	/	/	/	N	N
	Hardjobovis	N	/	/	/	N	N
A8	Pomona	N	N	N	N	N	N
	Hardjobovis	N	N	N	N	N	N

1.3. qPCR results from HLRL

ID	Specimen	Day 0	Day 1	Day 2	Day 3	Day 19	Day 22	Day 27	Day 30	Day 33	Specimen	Day 42
A1	Urine	/	/	/	/	N	N	N	N	N	Kidney	N
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N
A2	Urine	/	/	/	/	P	P	N	P	N	Kidney	P
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N
A3	Urine	/	/	/	/	N	N	N	N	N	Kidney	N
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N
A4	Urine	/	/	/	/	N	N	N	N	N	Kidney	P
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N
A5	Urine	/	/	/	/	N	N	N	N	N	Kidney	P
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N
A6	Urine	/	/	/	/	N	N	N	N	N	Kidney	N
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N
A7	Urine	/	/	/	/	N	N	N	N	N	Kidney	N
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N
A8	Urine	/	/	/	/	N	N	N	N	N	Kidney	N
	Serum	N	N	N	N	/	/	/	/	/	Serum	N
	Whole blood	/	/	/	/	/	N	/	/	/	Whole blood	N

1.4. DFM results from Massey University

ID	Urine					Kidney
	Day 19	Day 22	Day 27	Day 30	Day 33	Day 42
A1	N	N	**P**	N	N	N
A2	N	N	N	N	N	**P**
A3	N	N	N	N	N	N
A4	N	N	N	N	N	N
A5	N	N	N	N	N	**P**
A6	N	N	N	N	N	N
A7	N	N	**P**	N	N	N
A8	N	N	N	N	N	N

2. Sheep trial B

2.1. Culture results from HLRL

ID	Urine								Kidney[a]
	Day 0	Day 5	Day 8	Day 12	Day 14	Day 21	Day 28	Day 37	Day 42
B1	N	N	N	N	N	N	N	N	N
B2	N	N	N	N	N	N	N	N	N
B3	N	N	N	N	N	N	N	N	N
B4	N	N	N	N	N	/	/	/	N
B5	N	N	N	N	N	N	N	N	N
B6	**P**	**P**	**P**	**P**	**P**	**P**	N	**P**	N
B7	N	N	N	N	N	N	N	N	N
B8	N	N	N	N	N	N	N	N	N

[a] died on Day 15 at which time the kidney was sampled

Blood Culture results								
ID	Day 0	Day 5	Day 8	Day 12	Day 14	Day 21	Day 28	Day 37
B1	N	N	N	N	N	N	N	N
B2	N	N	N	N	N	N	N	N
B3	N	N	N	N	N	N	N	N
B4	N	N	N	N	N	/	/	/
B5	N	N	N	N	N	N	N	N
B6	N	N	N	N	N	N	N	N
B7	N	N	N	N	N	N	N	N
B8	N	N	N	N	N	N	N	N

2.2. MAT results from HLRL

ID	Serovar	Day 0	Day 5	Day 8	Day 12	Day 14	Day 21	Day 28	Day 37
B1	**Pomona**	N	N	N	N	N	N	N	N
	Hardjobovis	N	N	N	**384**	**192**	**384**	**48**	**96**
B2	**Pomona**	N	N	N	N	N	N	N	N
	Hardjobovis	N	N	**96**	**1536**	**768**	**384**	**192**	**48**
B3	**Pomona**	N	N	N	N	N	N	N	N
	Hardjobovis	N	N	**48**	**1536**	**768**	**96**	**48**	**24**
B4	**Pomona**	N	N	N	N	N	/	/	/
	Hardjobovis	N	N	**96**	**768**	**768**	/	/	/
B5	**Pomona**	N	N	N	N	N	N	N	N
	Hardjobovis	N	N	**48**	**3072**	**1536**	**384**	**192**	**192**
B6	**Pomona**	N	N	N	N	N	N	N	N
	Hardjobovis	**24**	**48**	**192**	**96**	N	**192**	**96**	**24**
B7	**Pomona**	N	N	N	N	N	N	N	N
	Hardjobovis	N	N	**384**	**768**	**1536**	**384**	**384**	**96**
B8	**Pomona**	N	N	N	N	N	N	N	N
	Hardjobovis	N	N	**768**	**1536**	**768**	**192**	**96**	**96**

2.3. qPCR results from HLRL

ID	Specimen	Day 0	Day 5	Day 8	Day 12	Day 14	Day 21	Day 28	Day 37
B1	Urine	N	N	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	N	N
	Whole blood	N	N	N	N	N	N	N	N
B2	Urine	N	N	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	N	N
	Whole blood	N	N	N	N	N	N	N	N
B3	Urine	N	N	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	N	N
	Whole blood	N	N	N	N	N	N	N	N
B4	Urine	N	N	N	N	N	/	/	/
	Serum	N	N	N	N	N	/	/	/
	Whole blood	N	N	N	N	N	/	/	/
B5	Urine	N	N	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	N	N
	Whole blood	N	N	N	N	N	N	N	N
B6	Urine	**P**	**P**	**P**	**P**	**P**	**P**	**P**	**P**
	Serum	N	N	N	N	N	**P**	N	N
	Whole blood	N	N	N	N	N	N	N	N
B7	Urine	N	N	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	N	N
	Whole blood	N	N	N	N	N	N	N	N
B8	Urine	N	N	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	N	N
	Whole blood	N	N	N	N	N	N	N	N

Kidney qPCR Results		
ID	**Day 42**	**Day 15**
B1	**P**	/
B2	N	/
B3	N	/
B4	/	N
B5	N	/
B6	**P**	/
B7	N	/
B8	N	/

(Animal B4 died on Day 15, at which time the kidney was sampled)

2.4. DFM results from HLRL

ID	Urine							Kidney	
	Day 5	Day 8	Day 12	Day 14	Day 21	Day 28	Day 37	Day 42	Day 15
B1	N	N	N	N	N	N	N	N	/
B2	N	N	N	N	N	N	N	**P**	/
B3	N	N	N	N	N	N	**P**	**P**	/
B4	N	N	N	N	/	/	/	/	N
B5	N	N	N	N	N	N	**P**	**P**	/
B6	N	N	N	**P**	**P**	N	**P**	**P**	/
B7	N	N	N	N	**P**	N	N	**P**	/
B8	N	N	N	N	N	N	**P**	**P**	/

2.5. Weight records (kg)

ID	Day 0	Day 42
B1	27	19
B2	26.5	29.0
B3	26.5	25.0
B4	23	/
B5	26.5	21.5
B6	27	21.5
B7	32.5	35.0
B8	32	26.0

(Animal B4 died on Day 15 and were not weighed on Day 42)

3. Sheep trial C

3.1. Urine and kidney culture results from NZVP

ID	Urine							Kidney
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 42
C1	P	N	N	P	P	N	N	N
C2	N	N	N	N	N	N	N	N
C3	P	N	N	P	N	N	N	N
C4	P	N	P	P	P	P	P	P
C5	P	N	P	P	P	P	P	P
C6	P	N	P	P	P	P	N	P
C7	P	N	P	P	P	P	P	P
C8	N	N	P	P	P	P	P	P
C9	P	N	P	P	P	P	P	P
C10	P	N	P	P	P	P	P	P
C11	P	N	N	P	P	P	P	P
C12	P	N	P	P	P	P	P	P
C13	N	N	P	P	P	P	P	N
C14	P	N	N	P	P	P	P	P
C15	P	N	P	P	P	P	P	P
C16	P	N	N	N	P	P	N	P

3.2. Blood culture results from HLRL

ID	Day 0	Day 3	Day 5	Day 7
C1	N	N	N	N
C2	N	N	N	N
C3	N	N	N	N
C4	N	P	N	N
C5	N	P	N	N
C6	N	P	P	N
C7	N	P	N	N
C8	N	P	N	N
C9	N	P	N	N
C10	N	P	P	N
C11	N	P	N	N
C12	N	P	N	N
C13	N	P	N	N
C14	N	N	N	N
C15	N	P	N	N
C16	N	N	N	N

3.3. MAT results from HLRL and NZVP

ID	Serovar	Day 0	Day 5	Day 7	Day 14	Day 28	Day 42
C1	Pomona	N	96	3072	800	50	N
	Hardjobovis	N	N	N	N	N	N
C2	Pomona	N	96	192	50	25	N
	Hardjobovis	N	N	N	N	N	N
C3	Pomona	N	24	768	100	50	N
	Hardjobovis	N	N	N	N	N	N
C4	Pomona	N	48	3072	800	25	N
	Hardjobovis	N	N	N	N	N	N
C5	Pomona	N	24	1536	800	50	N
	Hardjobovis	N	N	N	N	N	N
C6	Pomona	N	N	1536	800	25	N
	Hardjobovis	N	N	N	N	N	N
C7	Pomona	N	48	3072	800	100	N
	Hardjobovis	N	N	N	N	N	N
C8	Pomona	N	48	3072	200	100	N
	Hardjobovis	N	N	N	N	N	N
C9	Pomona	N	24	384	400	50	N
	Hardjobovis	N	N	N	N	N	N
C10	Pomona	N	N	384	400	25	N
	Hardjobovis	N	N	N	N	N	N
C11	Pomona	N	48	1536	400	25	N
	Hardjobovis	N	N	N	N	N	N
C12	Pomona	N	24	768	800	100	50
	Hardjobovis	N	N	N	N	N	N
C13	Pomona	N	48	1536	400	100	N
	Hardjobovis	N	N	N	N	N	N
C14	Pomona	N	48	768	1600	100	100
	Hardjobovis	N	N	N	N	N	N
C15	Pomona	N	N	384	800	50	N
	Hardjobovis	N	N	N	N	N	N
C16	Pomona	N	48	768	400	25	N
	Hardjobovis	N	N	N	N	N	N

3.4. qPCR results from HLRL

ID	Urine							Kidney
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 42
C1	N	N	P	P	N	P	N	N
C2	N	N	N	N	N	P	N	N
C3	N	N	P	N	N	P	N	N
C4	N	N	P	P	P	P	P	P
C5	N	N	P	P	P	P	P	P
C6	N	P	N	N	P	P	N	P
C7	N	N	P	P	P	P	P	P
C8	N	N	N	P	P	P	P	P
C9	N	N	N	N	P	P	P	P
C10	N	N	P	P	P	P	P	P
C11	N	N	N	P	P	P	P	P
C12	N	N	N	P	P	P	P	P
C13	N	N	P	P	P	P	P	P
C14	N	P	N	N	P	P	N	N
C15	N	N	N	P	P	P	P	P
C16	N	N	N	N	N	N	N	N

ID	Blood qPCR					
	Specimen	Day 0	Day 3	Day 5	Day 7	Day 14
C1	Serum	/	P	P	N	N
	Whole blood	N	N	N	N	N
C2	Serum	/	P	P	N	N
	Whole blood	N	N	P	P	N
C3	Serum	/	N	N	N	N
	Whole blood	N	N	N	N	N
C4	Serum	/	P	P	N	N
	Whole blood	N	N	N	N	N
C5	Serum	/	P	P	N	N
	Whole blood	N	N	P	N	N
C6	Serum	/	P	P	N	N
	Whole blood	N	N	N	N	N
C7	Serum	/	P	N	N	N
	Whole blood	N	P	P	N	N
C8	Serum	/	P	P	N	N
	Whole blood	N	N	P	N	N
C9	Serum	/	P	P	P	N
	Whole blood	N	P	N	N	N
C10	Serum	/	P	P	N	N
	Whole blood	N	N	N	P	N
C11	Serum	/	P	P	N	N
	Whole blood	N	N	P	N	N
C12	Serum	/	P	N	N	N
	Whole blood	N	P	N	N	N
C13	Serum	/	P	P	N	N
	Whole blood	N	P	P	N	N
C14	Serum	/	P	N	N	N
	Whole blood	N	N	N	N	N
C15	Serum	/	P	P	N	N
	Whole blood	N	N	N	N	N
C16	Serum	/	N	N	N	N
	Whole blood	N	N	N	N	N

3.5. DFM results from NZVP

ID	Urine						Kidney
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 42
C1	N	N	N	N	N	N	N
C2	N	N	N	N	N	N	N
C3	P	N	N	N	P	P	N
C4	N	N	N	P	P	N	P
C5	N	N	P	N	P	N	N
C6	N	N	N	N	N	N	N
C7	P	N	N	P	P	N	N
C8	N	N	N	N	N	N	N
C9	N	N	N	N	N	N	N
C10	N	N	N	P	N	N	N
C11	N	N	P	N	N	N	N
C12	N	N	N	P	N	N	N
C13	N	N	N	N	P	N	N
C14	N	P	N	N	P	N	N
C15	N	N	N	N	N	N	P
C16	N	N	N	N	N	N	N

4. Cattle trial D

4.1. Culture results from HLRL

Urine culture results						
ID	Day 20	Day 23	Day 26	Day 28	Day 35	Day 42
D1	N	N	N	N	N	N
D2	N	N	N	N	P	N
D3	N	N	N	P	N	N
D4	N	N	N	N	N	N
D5	N	N	N	N	N	N
D6	N	N	N	N	N	N

Whole Blood culture results		
ID	Day 9	Day 14
D1	N	N
D2	N	N
D3	N	N
D4	N	N
D5	N	N
D6	N	N

4.2. MAT results from HLRL

ID	Serovar	Day 0	Day 7	Day 9	Day 14	Day 20	Day 23	Day 42
D1	Hardjobovis	N	N	N	192	3072	384	N
	Pomona	N	N	N	N	N	N	N
D2	Hardjobovis	N	N	384	192	1536	192	N
	Pomona	N	N	N	N	N	N	N
D3	Hardjobovis	N	N	384	384	384	96	N
	Pomona	N	N	N	N	N	N	N
D4	Hardjobovis	N	N	N	N	N	N	N
	Pomona	N	N	N	N	N	N	N
D5	Hardjobovis	N	N	N	N	N	N	N
	Pomona	N	N	N	N	N	N	N
D6	Hardjobovis	N	N	N	N	N	N	N
	Pomona	N	N	N	N	N	N	N

4.3. qPCR results from HLRL

ID	Specimen	Day 0	Day 7	Day 9	Day 14	Day 20	Day 23	Day 26	Day 28	Day 35	Day 42
D1	Urine	/	/	/	/	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	/	/	/	N
	Whole blood	N	N	N	N	N	N	/	/	/	N
D2	Urine	/	/	/	/	N	N	N	P	P	P
	Serum	N	N	N	N	N	N	/	/	/	N
	Whole blood	N	N	N	N	N	N	/	/	/	N
D3	Urine	/	/	/	/	N	P	P	N	N	N
	Serum	N	N	N	N	N	N	/	/	/	N
	Whole blood	N	N	N	N	N	N	/	/	/	N
D4	Urine	/	/	/	/	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	/	/	/	N
	Whole blood	N	N	N	N	N	N	/	/	/	N
D5	Urine	/	/	/	/	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	/	/	/	N
	Whole blood	N	N	N	N	N	N	/	/	/	N
D6	Urine	/	/	/	/	N	N	N	N	N	N
	Serum	N	N	N	N	N	N	/	/	/	N
	Whole blood	N	N	N	N	N	N	/	/	/	N

4.4. Urine DFM results from HLRL

ID	Day 20	Day 23	Day 26	Day 28	Day 35	Day 42
D1	N	N	N	N	N	N
D2	N	N	N	N	N	N
D3	N	N	N	**P**	N	N
D4	N	N	N	N	N	N
D5	N	N	N	N	N	N
D6	N	N	N	N	N	N

4.5. Rectal temperature record

ID	Day 0	Day 1	Day 2	Day 7	Day 9	Day 14
D1	39.6°C	39.0 °C	38.9 °C	38.7 °C	39.3 °C	39.3 °C
D2	39.8 °C	39.8 °C	38.8 °C	38.6 °C	39.6 °C	39.2 °C
D3	39.5 °C	38.6 °C	38.8 °C	38.6 °C	38.7 °C	39.1 °C
D4	39.3 °C	38.5 °C	38.6 °C	38.6 °C	38.4 °C	38.8 °C
D5	39.3 °C	38.7 °C	38.9 °C	38.5 °C	38.5 °C	38.5 °C
D6	39.5 °C	39.7 °C	38.7 °C	38.5 °C	38.7 °C	38.3 °C

Appendix 4 Test results for the abattoir study

(Species: 1=sheep, 0=cattle; Sex: 1=male, 0= female; Age: for sheep, L=Lamb, H=Hogget, M= Mixed-age, for cattle, Y= young stocks (≤ 18 months old), A= adult stock; "/" = samples were not collected from those animals; for test results, 0= Negative, 1=Positive; Hurine= urine qPCR results from HLRL; Gurine= urine qPCR results from Gribbles (suspect results (36 < Ct ≤40) is considered as negative); Hkidney= kidney qPCR results from HLRL; Hhardjo= MAT results against serovar Hardjobovis from HLRL; Hpomona= MAT results against serovar Pomona from HLRL; Ghardjo= MAT results against serovar Hardjobovis from Gribbles; Gpomona= MAT results against serovar Pomona from Gribbles)

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
A1	1	A	0	L	27/09/2010	/	/	0	0	1536	0	200
A2	1	A	0	L	27/09/2010	/	/	0	0	24	0	0
A3	1	A	0	L	27/09/2010	/	/	0	0	0	0	0
A4	1	A	1	L	27/09/2010	0	0	0	0	48	0	0
A5	1	A	1	L	27/09/2010	0	0	0	3072	3072	>1600	>1600
A6	1	A	1	L	27/09/2010	1	1	1	0	1536	0	>1600
A7	1	A	0	L	27/09/2010	0	1	0	0	768	0	800
A8	1	A	0	L	27/09/2010	/	/	0	0	3072	0	>1600
A9	1	A	0	L	27/09/2010	/	/	0	0	1536	0	200
A10	1	A	1	L	27/09/2010	/	/	0	0	24	0	0
A11	1	A	1	L	27/09/2010	0	0	0	0	3072	0	>1600
A12	1	A	0	L	27/09/2010	/	/	0	0	192	0	100
A13	1	A	1	L	27/09/2010	0	0	0	0	3072	0	>1600
A14	1	A	1	L	27/09/2010	0	0	0	0	0	0	0
A15	1	A	1	L	27/09/2010	0	0	0	0	24	0	0
A16	1	A	1	L	27/09/2010	0	0	0	0	3072	0	>1600
A17	1	A	1	L	27/09/2010	/	/	1	0	3072	0	>1600
A18	1	A	0	L	27/09/2010	/	/	0	0	3072	0	>1600
A19	1	A	1	L	27/09/2010	0	0	0	0	384	0	200
A20	1	A	1	L	27/09/2010	0	0	0	0	768	0	0
A21	1	A	1	L	27/09/2010	/	/	1	0	3072	0	>1600
A22	1	A	0	L	27/09/2010	/	/	0	0	0	0	0
A23	1	A	0	L	27/09/2010	/	/	0	0	24	0	0
A24	1	A	0	L	27/09/2010	0	0	0	0	24	0	0
A25	1	A	1	L	27/09/2010	0	0	0	0	0	0	0
A26	1	A	1	L	27/09/2010	0	0	0	0	1536	0	>1600
A27	1	A	0	L	27/09/2010	/	/	0	0	768	0	1600
A28	1	A	0	L	27/09/2010	0	0	0	0	1536	0	800
A29	1	A	0	L	27/09/2010	0	0	0	0	768	0	400
A30	1	A	1	L	27/09/2010	0	0	0	0	768	0	200
B1	1	B	0	L	27/09/2010	1	1	0	3072	48	>1600	0
B2	1	B	0	L	27/09/2010	0	0	0	0	0	0	0
B3	1	B	1	L	27/09/2010	1	1	1	3072	0	>1600	0
B4	1	B	1	L	27/09/2010	/	/	1	384	1536	400	>1600
B5	1	B	1	L	27/09/2010	0	0	0	384	1536	800	1600
B6	1	B	1	L	27/09/2010	1	1	1	384	1536	800	>1600
B7	1	B	1	L	27/09/2010	0	0	1	768	0	200	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
B8	1	B	1	L	27/09/2010	/	/	1	384	0	200	0
B9	1	B	1	L	27/09/2010	1	1	1	384	0	800	0
B10	1	B	1	L	27/09/2010	/	/	1	192	0	400	0
B11	1	B	1	L	27/09/2010	/	/	1	768	24	800	0
G1	0	G	1	Y	27/09/2010	0	0	0	0	48	0	50
G2	0	G	1	Y	27/09/2010	0	0	1	0	0	0	0
G3	0	G	1	Y	27/09/2010	0	0	0	0	96	0	100
G4	0	G	1	Y	27/09/2010	0	0	0	0	48	0	0
H1	0	H	1	Y	28/09/2010	0	0	0	96	24	50	0
I1	0	I	0	Y	29/09/2010	0	0	0	0	24	0	0
I2	0	I	0	Y	29/09/2010	0	0	0	384	24	1600	0
I3	0	I	0	Y	29/09/2010	0	0	0	96	96	50	50
I4	0	I	0	Y	29/09/2010	1	1	1	192	24	400	0
I5	0	I	0	Y	29/09/2010	0	0	0	0	24	0	0
I6	0	I	0	Y	29/09/2010	0	0	0	0	48	0	0
I7	0	I	0	Y	29/09/2010	0	0	0	0	0	0	0
I8	0	I	0	Y	29/09/2010	0	0	0	0	24	0	0
I9	0	I	0	Y	29/09/2010	0	0	0	0	0	0	0
I10	0	I	0	Y	29/09/2010	0	0	0	0	0	0	0
I11	0	I	1	Y	29/09/2010	1	1	1	96	0	50	0
I12	0	I	1	Y	29/09/2010	0	0	0	0	48	0	0
I13	0	I	1	Y	29/09/2010	0	0	0	0	0	0	0
I14	0	I	1	Y	29/09/2010	0	0	0	0	0	0	0
G5	0	G	0	Y	4/10/2010	0	0	0	0	384	0	100
G6	0	G	0	Y	4/10/2010	1	1	1	0	768	0	1600
G7	0	G	0	Y	4/10/2010	0	0	0	0	48	0	0
G8	0	G	0	Y	4/10/2010	1	0	1	0	192	0	100
G9	0	G	0	Y	4/10/2010	1	0	1	0	1536	0	>1600
D1	1	D	1	L	5/10/2010	/	/	1	1536	0	>1600	0
D2	1	D	0	L	5/10/2010	1	1	1	3072	0	>1600	0
D3	1	D	0	L	5/10/2010	/	/	1	384	0	>1600	0
D4	1	D	1	L	5/10/2010	/	/	0	192	0	>1600	0
D5	1	D	0	L	5/10/2010	/	/	0	0	0	0	0
D6	1	D	0	L	5/10/2010	/	/	1	96	0	1600	0
D7	1	D	0	L	5/10/2010	/	/	1	0	24	50	0
D8	1	D	0	L	5/10/2010	1	1	1	96	24	800	0
D9	1	D	0	L	5/10/2010	/	/	1	1536	48	>1600	0
D10	1	D	1	L	5/10/2010	0	0	0	0	0	0	0
D11	1	D	0	L	5/10/2010	/	/	1	192	0	800	0
D12	1	D	0	L	5/10/2010	/	/	1	3072	0	>1600	0
D13	1	D	1	L	5/10/2010	/	/	0	0	0	0	0
D14	1	D	0	L	5/10/2010	/	/	0	384	24	>1600	0
D15	1	D	0	L	5/10/2010	/	/	1	192	0	400	0
D16	1	D	0	L	5/10/2010	/	/	1	1536	0	>1600	0
D17	1	D	0	L	5/10/2010	0	0	0	0	0	0	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
D18	1	D	1	L	5/10/2010	/	/	0	0	0	0	0
D19	1	D	0	L	5/10/2010	/	/	1	384	0	>1600	0
D20	1	D	0	L	5/10/2010	/	/	0	0	0	0	0
H2	0	H	1	Y	5/10/2010	0	0	1	24	0	1600	0
H3	0	H	1	Y	5/10/2010	/	/	0	384	24	>1600	0
H4	0	H	1	Y	5/10/2010	1	1	1	192	0	400	0
H5	0	H	1	Y	5/10/2010	0	0	0	48	24	100	0
I15	0	I	0	Y	6/10/2010	1	1	1	1536	24	>1600	0
I16	0	I	0	Y	6/10/2010	0	0	0	384	24	>1600	0
I17	0	I	0	Y	6/10/2010	0	0	0	192	0	>1600	0
I18	0	I	0	Y	6/10/2010	0	0	0	0	24	0	0
I19	0	I	0	Y	6/10/2010	1	1	1	384	0	>1600	0
I20	0	I	0	Y	6/10/2010	1	0	1	0	0	0	0
I21	0	I	0	Y	6/10/2010	/	/	0	0	0	0	0
I22	0	I	0	Y	6/10/2010	0	0	0	0	192	0	100
I23	0	I	0	Y	6/10/2010	0	0	0	0	48	0	0
I24	0	I	0	Y	6/10/2010	0	0	0	0	0	0	0
G10	0	G	0	Y	11/10/2010	/	/	0	192	3072	200	1600
G11	0	G	0	Y	11/10/2010	/	/	0	24	96	0	0
G12	0	G	0	Y	11/10/2010	/	/	0	192	0	400	0
G13	0	G	0	Y	11/10/2010	/	/	0	0	384	0	100
G14	0	G	0	Y	11/10/2010	/	/	0	96	96	200	0
E1	1	E	0	L	13/10/2010	/	/	0	0	96	0	0
E2	1	E	0	L	13/10/2010	/	/	0	0	192	0	0
E3	1	E	0	L	13/10/2010	/	/	0	0	192	0	0
E4	1	E	1	L	13/10/2010	0	0	0	0	48	0	0
E5	1	E	1	L	13/10/2010	/	/	0	0	0	0	0
E6	1	E	0	H	13/10/2010	0	0	0	0	0	0	0
E7	1	E	0	H	13/10/2010	/	/	0	768	0	>1600	0
E8	1	E	0	H	13/10/2010	/	/	0	0	48	0	0
E9	1	E	0	H	13/10/2010	/	/	0	0	192	0	0
E10	1	E	0	H	13/10/2010	0	0	0	0	24	0	0
E11	1	E	0	H	13/10/2010	0	0	0	96	0	0	0
E12	1	E	0	H	13/10/2010	/	/	1	3072	48	>1600	0
E13	1	E	0	H	13/10/2010	/	/	0	0	0	0	0
E14	1	E	0	H	13/10/2010	/	/	0	0	0	0	0
E15	1	E	0	H	13/10/2010	1	1	1	384	0	>1600	0
E16	1	E	0	H	13/10/2010	/	/	0	0	0	0	0
E17	1	E	0	H	13/10/2010	/	/	0	0	0	0	0
E18	1	E	0	H	13/10/2010	/	/	0	0	48	0	0
E19	1	E	0	H	13/10/2010	0	0	0	0	0	0	0
E20	1	E	0	H	13/10/2010	/	/	0	0	48	0	0
E21	1	E	0	L	13/10/2010	0	0	0	0	96	0	0
E22	1	E	0	L	13/10/2010	/	/	0	0	48	0	0
E23	1	E	0	L	13/10/2010	0	0	0	0	24	0	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
E24	1	E	0	L	13/10/2010	/	/	0	0	0	0	0
E25	1	E	0	L	13/10/2010	0	0	0	0	96	0	0
E26	1	E	0	L	13/10/2010	/	/	0	0	48	0	0
E27	1	E	0	L	13/10/2010	/	/	0	0	96	0	0
E28	1	E	0	L	13/10/2010	0	0	0	0	0	0	0
E29	1	E	0	L	13/10/2010	/	/	0	0	48	0	0
E30	1	E	0	L	13/10/2010	0	0	0	0	0	0	0
F1	1	F	0	M	13/10/2010	/	/	0	0	0	0	0
F2	1	F	0	M	13/10/2010	0	0	0	0	0	0	0
F3	1	F	0	M	13/10/2010	/	/	0	0	24	0	0
F4	1	F	0	M	13/10/2010	/	/	0	0	384	0	50
F5	1	F	0	M	13/10/2010	0	0	0	0	48	0	0
F6	1	F	0	M	13/10/2010	/	/	0	0	0	0	0
F7	1	F	0	M	13/10/2010	/	/	0	0	48	0	0
F8	1	F	0	M	13/10/2010	/	/	0	0	48	0	0
F9	1	F	0	M	13/10/2010	/	/	0	0	0	0	0
F10	1	F	0	M	13/10/2010	/	/	0	0	96	0	0
F11	1	F	0	M	13/10/2010	/	/	0	0	24	0	0
F12	1	F	0	M	13/10/2010	/	/	0	192	24	1600	0
F13	1	F	0	M	13/10/2010	0	0	0	0	192	0	0
F14	1	F	0	M	13/10/2010	/	/	0	24	192	0	0
F15	1	F	0	M	13/10/2010	0	0	0	0	192	0	0
F16	1	F	0	M	13/10/2010	0	0	0	96	96	50	0
F17	1	F	0	M	13/10/2010	0	0	0	48	0	0	0
F18	1	F	0	M	13/10/2010	/	/	1	192	192	1600	0
F19	1	F	0	M	13/10/2010	/	/	0	48	0	100	0
F20	1	F	0	M	13/10/2010	/	/	0	384	0	>1600	0
F21	1	F	0	H	13/10/2010	0	0	0	0	48	0	0
F22	1	F	0	H	13/10/2010	0	0	0	3072	0	>1600	0
F23	1	F	0	H	13/10/2010	/	/	0	3072	0	>1600	0
F24	1	F	0	H	13/10/2010	/	/	0	1536	24	>1600	0
F25	1	F	0	H	13/10/2010	/	/	1	1536	768	>1600	400
F26	1	F	1	L	13/10/2010	0	1	0	0	0	0	0
F27	1	F	1	L	13/10/2010	0	0	0	0	96	0	0
F28	1	F	1	L	13/10/2010	0	0	0	0	48	0	0
F29	1	F	1	L	13/10/2010	0	0	0	0	96	0	0
F30	1	F	0	L	13/10/2010	1	1	1	768	0	>1600	0
I25	0	I	0	Y	13/10/2010	0	0	0	24	0	0	0
I26	0	I	0	Y	13/10/2010	0	0	1	768	0	>1600	0
I27	0	I	0	Y	13/10/2010	0	0	0	24	0	0	0
I28	0	I	1	Y	13/10/2010	0	0	0	48	1536	>1600	50
I29	0	I	1	Y	13/10/2010	0	0	0	24	48	0	0
I30	0	I	1	Y	13/10/2010	0	0	0	24	48	0	0
I31	0	I	0	Y	13/10/2010	0	0	0	0	0	0	0
I32	0	I	0	Y	13/10/2010	0	0	0	1536	0	>1600	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
I33	0	I	0	Y	13/10/2010	0	0	0	192	0	100	0
I34	0	I	0	Y	13/10/2010	1	1	1	24	192	0	0
I35	0	I	0	Y	13/10/2010	0	0	0	0	0	0	0
I36	0	I	1	Y	13/10/2010	0	0	0	1536	24	1600	0
I37	0	I	1	Y	13/10/2010	1	1	0	768	0	>1600	0
I38	0	I	1	Y	13/10/2010	0	0	1	768	24	>1600	0
I39	0	I	1	Y	13/10/2010	0	0	0	24	192	0	0
I40	0	I	1	Y	13/10/2010	1	1	1	192	0	100	0
I41	0	I	1	Y	13/10/2010	0	1	0	0	0	0	0
A31	1	A	1	L	18/10/2010	/	/	0	0	768	0	>1600
A32	1	A	1	L	18/10/2010	/	/	0	0	1536	0	100
A33	1	A	1	L	18/10/2010	/	/	1	0	768	0	>1600
A34	1	A	1	L	18/10/2010	/	/	1	0	1536	0	>1600
A35	1	A	1	L	18/10/2010	0	0	0	0	0	0	0
A36	1	A	0	L	18/10/2010	/	/	1	0	3072	0	>1600
A37	1	A	0	L	18/10/2010	/	/	0	0	0	0	0
A38	1	A	0	L	18/10/2010	0	0	0	0	48	0	0
A39	1	A	0	L	18/10/2010	/	/	0	0	1536	0	>1600
A40	1	A	1	L	18/10/2010	1	1	1	192	1536	>1600	>1600
A41	1	A	0	L	18/10/2010	/	/	0	0	0	0	0
A42	1	A	1	L	18/10/2010	/	/	0	0	192	0	0
A43	1	A	1	L	18/10/2010	/	/	0	0	768	0	400
A44	1	A	0	L	18/10/2010	0	0	0	0	1536	0	1600
A45	1	A	0	L	18/10/2010	/	/	0	0	24	0	0
A46	1	A	1	L	18/10/2010	/	/	0	0	3072	0	>1600
A47	1	A	1	L	18/10/2010	0	0	0	0	0	0	0
A48	1	A	1	L	18/10/2010	1	1	1	0	0	>1600	0
A49	1	A	1	L	18/10/2010	1	1	1	192	0	>1600	0
A50	1	A	1	L	18/10/2010	1	1	1	192	0	>1600	0
A51	1	A	0	L	18/10/2010	1	1	1	384	0	>1600	0
A52	1	A	0	L	18/10/2010	1	1	1	768	96	>1600	0
A53	1	A	1	L	18/10/2010	/	/	1	96	0	>1600	0
A54	1	A	1	L	18/10/2010	1	1	1	96	0	>1600	0
A55	1	A	1	L	18/10/2010	1	1	1	192	96	>1600	0
B12	1	B	1	L	18/10/2010	1	1	1	384	384	>1600	0
B13	1	B	1	L	18/10/2010	/	/	1	768	96	>1600	0
B14	1	B	1	L	18/10/2010	/	/	1	1536	0	>1600	0
B15	1	B	1	L	18/10/2010	1	1	1	384	1536	>1600	>1600
B16	1	B	1	L	18/10/2010	/	/	1	192	96	>1600	0
B17	1	B	0	L	18/10/2010	/	/	1	192	3072	>1600	>1600
B18	1	B	1	L	18/10/2010	/	/	0	768	0	>1600	0
B19	1	B	0	L	18/10/2010	/	/	0	192	0	>1600	0
B20	1	B	0	L	18/10/2010	/	/	1	192	0	>1600	0
B21	1	B	1	L	18/10/2010	/	/	0	768	24	>1600	0
B22	1	B	1	L	18/10/2010	1	1	1	384	0	>1600	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
B23	1	B	1	L	18/10/2010	/	/	1	384	192	>1600	0
B24	1	B	1	L	18/10/2010	/	/	0	192	192	>1600	0
B25	1	B	0	L	18/10/2010	0	0	0	192	192	0	0
G15	0	G	0	Y	18/10/2010	0	0	0	48	96	0	0
G16	0	G	0	Y	18/10/2010	0	0	0	384	0	>1600	0
H6	0	H	1	Y	19/10/2010	0	0	0	768	24	800	0
H7	0	H	1	Y	19/10/2010	0	0	0	96	0	>1600	0
H8	0	H	1	Y	19/10/2010	/	/	0	96	0	800	0
H9	0	H	1	Y	19/10/2010	0	0	0	0	0	0	0
I42	0	I	0	Y	20/10/2010	0	0	0	192	0	1600	0
I43	0	I	0	Y	20/10/2010	/	/	0	384	1536	>1600	1600
I44	0	I	0	Y	20/10/2010	0	0	0	48	48	400	0
I45	0	I	0	Y	20/10/2010	/	/	0	768	192	>1600	100
I46	0	I	0	Y	20/10/2010	0	0	0	768	384	>1600	0
I47	0	I	0	Y	20/10/2010	/	/	0	768	48	>1600	0
I48	0	I	0	Y	20/10/2010	0	0	0	768	0	>1600	0
I49	0	I	0	Y	20/10/2010	0	0	0	384	0	50	0
I50	0	I	0	Y	20/10/2010	1	1	0	1536	0	>1600	0
I51	0	I	0	Y	20/10/2010	1	1	1	768	0	>1600	0
C1	1	C	0	L	26/10/2010	/	/	1	384	3072	>1600	1600
C2	1	C	0	L	26/10/2010	1	1	1	96	3072	800	>1600
C3	1	C	0	L	26/10/2010	/	/	1	384	1536	>1600	>1600
C4	1	C	0	L	26/10/2010	/	/	1	768	48	>1600	0
C5	1	C	0	L	26/10/2010	/	/	1	384	3072	>1600	>1600
C6	1	C	0	L	26/10/2010	/	/	0	192	3072	>1600	>1600
C7	1	C	0	L	26/10/2010	/	/	1	384	96	>1600	0
C8	1	C	0	L	26/10/2010	/	/	1	96	768	>1600	>1600
C9	1	C	0	L	26/10/2010	/	/	1	384	1536	>1600	800
C10	1	C	0	L	26/10/2010	1	1	1	768	1536	>1600	0
C11	1	C	0	L	26/10/2010	/	/	1	384	192	>1600	0
C12	1	C	0	L	26/10/2010	/	/	1	768	24	>1600	0
C13	1	C	0	L	26/10/2010	/	/	0	192	48	1600	0
C14	1	C	0	L	26/10/2010	1	1	1	384	3072	>1600	>1600
C15	1	C	0	L	26/10/2010	/	/	0	384	192	>1600	100
C16	1	C	0	L	26/10/2010	/	/	1	768	3072	200	>1600
C17	1	C	0	L	26/10/2010	/	/	0	384	1536	>1600	>1600
C18	1	C	0	L	26/10/2010	/	/	1	384	1536	>1600	100
C19	1	C	0	L	26/10/2010	1	1	1	384	3072	>1600	400
C20	1	C	0	L	26/10/2010	/	/	1	384	48	>1600	0
C21	1	C	0	L	26/10/2010	/	/	1	192	1536	>1600	400
C22	1	C	0	L	26/10/2010	/	/	1	384	3072	>1600	>1600
C23	1	C	0	L	26/10/2010	/	/	1	384	3072	>1600	>1600
C24	1	C	0	L	26/10/2010	/	/	1	192	3072	>1600	>1600
C25	1	C	0	L	26/10/2010	/	/	1	768	24	>1600	0
C26	1	C	0	L	26/10/2010	/	/	1	384	192	1600	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
C27	1	C	0	L	26/10/2010	/	/	0	384	48	1600	0
C28	1	C	0	L	26/10/2010	/	/	1	1536	48	>1600	0
C29	1	C	0	L	26/10/2010	1	1	1	768	24	>1600	0
C30	1	C	0	L	26/10/2010	1	1	1	192	3072	>1600	>1600
D21	1	D	1	L	26/10/2010	/	/	1	192	0	>1600	0
D22	1	D	0	L	26/10/2010	/	/	0	0	0	0	0
D23	1	D	0	L	26/10/2010	/	/	1	192	24	>1600	0
D24	1	D	0	L	26/10/2010	/	/	1	1536	0	>1600	0
D25	1	D	0	L	26/10/2010	/	/	1	768	24	>1600	0
D26	1	D	0	L	26/10/2010	/	/	1	96	0	1600	0
D27	1	D	0	L	26/10/2010	0	0	0	0	0	0	0
D28	1	D	1	L	26/10/2010	1	1	1	768	0	>1600	0
D29	1	D	0	L	26/10/2010	/	/	1	768	0	>1600	0
D30	1	D	1	L	26/10/2010	1	1	0	768	0	>1600	0
D31	1	D	1	L	26/10/2010	/	/	1	0	0	0	0
D32	1	D	0	L	26/10/2010	1	1	1	384	0	1600	0
D33	1	D	0	L	26/10/2010	1	1	1	384	0	>1600	0
H10	0	H	0	Y	26/10/2010	0	0	0	384	0	0	0
H11	0	H	1	Y	26/10/2010	0	0	0	0	0	50	0
I52	0	I	0	Y	27/10/2010	1	1	1	96	24	>1600	0
I53	0	I	0	Y	27/10/2010	1	1	1	192	48	>1600	0
I54	0	I	0	Y	27/10/2010	1	1	1	768	0	>1600	0
I55	0	I	1	Y	27/10/2010	0	0	0	96	48	0	0
I56	0	I	1	Y	27/10/2010	0	0	0	768	0	>1600	0
I57	0	I	1	Y	27/10/2010	0	0	0	384	0	>1600	0
I58	0	I	1	Y	27/10/2010	0	0	0	192	0	400	0
I59	0	I	1	Y	27/10/2010	0	0	0	768	0	>1600	0
I60	0	I	1	Y	27/10/2010	0	0	0	1536	0	>1600	0
I61	0	I	0	Y	27/10/2010	0	0	0	192	0	100	0
I62	0	I	0	Y	27/10/2010	0	0	1	1536	0	>1600	0
I63	0	I	0	Y	27/10/2010	1	1	1	384	0	>1600	0
I64	0	I	0	Y	27/10/2010	1	1	0	192	0	>1600	0
I65	0	I	0	Y	27/10/2010	1	1	1	768	0	>1600	0
I66	0	I	0	Y	27/10/2010	0	1	0	1536	0	>1600	0
G17	0	G	0	Y	1/11/2010	/	/	0	192	0	100	0
G18	0	G	1	Y	1/11/2010	1	1	1	1536	96	>1600	0
G19	0	G	1	Y	1/11/2010	/	/	0	192	0	>1600	0
G20	0	G	1	Y	1/11/2010	1	1	0	384	192	1600	100
G21	0	G	1	Y	1/11/2010	0	0	0	192	0	100	0
H12	0	H	1	Y	2/11/2010	0	0	0	384	0	1600	0
H13	0	H	1	Y	2/11/2010	0	0	0	1536	0	>1600	0
H14	0	H	1	Y	2/11/2010	0	0	0	768	0	>1600	0
E31	1	E	0	L	3/11/2010	/	/	1	3072	24	>1600	0
E32	1	E	0	L	3/11/2010	0	0	0	0	0	0	0
E33	1	E	0	L	3/11/2010	1	1	1	768	0	>1600	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
E34	1	E	0	L	3/11/2010	/	/	0	3072	96	>1600	0
E35	1	E	0	L	3/11/2010	/	/	0	0	0	0	0
E36	1	E	0	H	3/11/2010	1	1	0	0	96	0	0
E37	1	E	0	H	3/11/2010	0	0	0	0	96	0	0
E38	1	E	0	H	3/11/2010	/	/	0	0	0	0	0
E39	1	E	0	H	3/11/2010	/	/	0	0	48	0	0
E40	1	E	0	H	3/11/2010	0	0	0	0	0	0	0
E41	1	E	0	H	3/11/2010	/	/	0	384	96	800	0
E42	1	E	0	H	3/11/2010	0	0	0	0	0	0	0
E43	1	E	0	H	3/11/2010	/	/	0	0	48	0	0
E44	1	E	0	H	3/11/2010	/	/	1	384	0	>1600	0
E45	1	E	0	H	3/11/2010	/	/	0	0	48	0	0
E46	1	E	0	L	3/11/2010	/	/	1	0	192	0	0
E47	1	E	0	L	3/11/2010	/	/	0	0	96	0	0
E48	1	E	0	L	3/11/2010	1	1	1	1536	48	>1600	0
E49	1	E	0	L	3/11/2010	/	/	0	0	96	0	0
E50	1	E	0	L	3/11/2010	0	0	0	0	48	0	0
E51	1	E	1	L	3/11/2010	0	0	0	0	24	0	0
E52	1	E	1	L	3/11/2010	0	0	0	0	0	0	0
E53	1	E	1	L	3/11/2010	/	/	0	0	0	0	0
E54	1	E	1	L	3/11/2010	0	0	0	0	0	0	0
E55	1	E	1	L	3/11/2010	0	0	0	0	0	0	0
E56	1	E	0	L	3/11/2010	/	/	0	0	0	0	0
E57	1	E	0	L	3/11/2010	/	/	0	0	0	0	0
E58	1	E	1	L	3/11/2010	0	0	0	0	0	0	0
E59	1	E	0	L	3/11/2010	/	/	0	0	0	0	0
E60	1	E	0	L	3/11/2010	/	/	0	0	0	0	0
F31	1	F	0	M	3/11/2010	0	0	0	0	0	0	0
F32	1	F	0	M	3/11/2010	0	0	0	0	24	0	0
F33	1	F	0	M	3/11/2010	0	0	0	0	0	0	0
F34	1	F	0	M	3/11/2010	0	0	0	0	0	0	0
F35	1	F	0	M	3/11/2010	0	0	0	0	24	0	0
F36	1	F	0	M	3/11/2010	/	/	0	0	48	0	0
F37	1	F	0	M	3/11/2010	/	/	0	24	24	0	0
F38	1	F	0	M	3/11/2010	/	/	0	192	192	50	0
F39	1	F	0	M	3/11/2010	/	/	0	0	0	0	0
F40	1	F	0	M	3/11/2010	/	/	0	768	0	1600	0
F41	1	F	0	M	3/11/2010	/	/	0	0	0	0	0
F42	1	F	0	M	3/11/2010	0	0	0	0	0	0	0
F43	1	F	0	M	3/11/2010	/	/	0	0	96	0	0
F44	1	F	0	M	3/11/2010	0	0	0	0	0	0	0
F45	1	F	0	M	3/11/2010	/	/	0	0	0	0	0
F46	1	F	0	M	3/11/2010	/	/	0	0	48	0	0
F47	1	F	0	M	3/11/2010	/	/	0	0	0	0	0
F48	1	F	0	H	3/11/2010	0	0	0	0	0	0	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
F49	1	F	0	H	3/11/2010	0	0	0	384	0	400	0
F50	1	F	0	H	3/11/2010	0	0	0	768	48	>1600	0
F51	1	F	0	H	3/11/2010	0	0	0	0	48	0	0
F52	1	F	0	H	3/11/2010	/	/	0	0	0	0	0
F53	1	F	0	H	3/11/2010	0	0	0	0	0	0	0
F54	1	F	0	H	3/11/2010	1	1	1	1536	96	>1600	0
F55	1	F	0	H	3/11/2010	/	/	0	0	0	0	0
F56	1	F	0	H	3/11/2010	/	/	0	192	0	800	0
F57	1	F	0	H	3/11/2010	0	0	0	192	24	200	0
F58	1	F	0	H	3/11/2010	0	0	0	0	0	0	0
F59	1	F	0	H	3/11/2010	/	/	0	0	0	0	0
F60	1	F	1	L	3/11/2010	/	/	0	0	0	0	0
I67	0	I	1	Y	3/11/2010	0	0	0	0	0	0	0
I68	0	I	1	Y	3/11/2010	0	0	0	0	0	0	0
I69	0	I	1	Y	3/11/2010	0	0	0	0	0	0	0
I70	0	I	1	Y	3/11/2010	0	0	0	0	0	0	0
I71	0	I	1	Y	3/11/2010	0	0	0	0	0	0	0
I72	0	I	0	Y	3/11/2010	/	/	0	384	0	800	0
I73	0	I	0	Y	3/11/2010	/	/	0	0	0	0	0
I74	0	I	0	Y	3/11/2010	0	0	0	768	0	>1600	0
I75	0	I	0	Y	3/11/2010	0	0	0	768	48	>1600	0
I76	0	I	0	Y	3/11/2010	/	/	0	1536	0	>1600	0
A56	1	A	0	L	8/11/2010	/	/	0	0	1536	0	800
A57	1	A	0	L	8/11/2010	/	/	0	0	0	0	0
A58	1	A	1	L	8/11/2010	1	1	1	384	0	>1600	0
A59	1	A	0	L	8/11/2010	/	/	0	0	3072	0	>1600
A60	1	A	1	L	8/11/2010	1	1	1	192	0	100	0
A61	1	A	1	L	8/11/2010	1	1	1	192	0	400	0
A62	1	A	0	L	8/11/2010	/	/	1	24	768	>1600	400
A63	1	A	0	L	8/11/2010	1	1	1	3072	0	>1600	0
A64	1	A	0	L	8/11/2010	/	/	0	0	1536	0	>1600
A65	1	A	0	L	8/11/2010	1	1	0	0	0	0	0
A66	1	A	0	L	8/11/2010	/	/	0	24	0	0	0
A67	1	A	1	L	8/11/2010	0	0	0	192	3072	200	>1600
A68	1	A	0	L	8/11/2010	0	0	0	24	0	0	0
A69	1	A	1	L	8/11/2010	0	0	0	0	0	0	0
A70	1	A	0	L	8/11/2010	/	/	0	0	0	0	0
A71	1	A	0	L	8/11/2010	0	0	0	48	24	0	50
A72	1	A	1	L	8/11/2010	1	1	1	1536	24	>1600	0
A73	1	A	1	L	8/11/2010	1	1	1	192	0	100	0
A74	1	A	0	L	8/11/2010	/	/	0	24	768	0	1600
A75	1	A	1	L	8/11/2010	1	1	1	1536	1536	>1600	1600
A76	1	A	1	L	8/11/2010	1	1	1	0	1536	0	>1600
A77	1	A	1	L	8/11/2010	1	1	1	384	24	800	0
B26	1	B	1	L	8/11/2010	/	/	1	1536	0	>1600	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
B27	1	B	0	L	8/11/2010	/	/	1	1536	0	>1600	0
B28	1	B	0	L	8/11/2010	/	/	1	768	24	>1600	0
B29	1	B	0	L	8/11/2010	/	/	1	1536	24	>1600	0
B30	1	B	0	L	8/11/2010	/	/	1	192	0	400	0
B31	1	B	0	L	8/11/2010	/	/	1	192	1536	800	400
B32	1	B	0	L	8/11/2010	1	1	1	768	1536	>1600	800
B33	1	B	0	L	8/11/2010	/	/	1	3072	3072	>1600	>1600
B34	1	B	0	L	8/11/2010	/	/	1	192	24	>1600	0
B35	1	B	0	L	8/11/2010	/	/	0	384	0	1600	0
B36	1	B	0	L	8/11/2010	1	1	1	768	0	400	0
B37	1	B	0	L	8/11/2010	/	/	0	384	0	1600	0
B38	1	B	0	L	8/11/2010	/	/	0	/	/	/	/
B39	1	B	0	L	8/11/2010	/	/	0	24	24	0	0
B40	1	B	0	L	8/11/2010	/	/	0	768	3072	800	>1600
G22	0	G	0	A	8/11/2010	/	/	0	0	24	0	0
G23	0	G	0	A	8/11/2010	/	/	0	0	24	0	0
G24	0	G	0	A	8/11/2010	/	/	0	384	0	>1600	0
G25	0	G	0	A	8/11/2010	0	0	0	24	96	0	100
H15	0	H	0	Y	9/11/2010	/	/	0	0	48	0	0
H16	0	H	1	Y	9/11/2010	0	0	1	0	0	0	0
H17	0	H	1	Y	9/11/2010	0	0	0	0	48	0	0
H18	0	H	0	Y	9/11/2010	0	0	0	0	24	0	0
H19	0	H	1	Y	9/11/2010	0	0	0	24	0	0	0
I77	0	I	0	A	10/11/2010	/	/	0	192	0	0	0
I78	0	I	0	A	10/11/2010	0	0	0	96	24	0	0
I79	0	I	0	A	10/11/2010	0	0	0	384	0	200	0
I80	0	I	0	A	10/11/2010	/	/	0	768	0	>1600	0
I81	0	I	0	A	10/11/2010	0	0	0	3072	0	>1600	0
I82	0	I	0	A	10/11/2010	0	0	0	768	0	1600	0
I83	0	I	0	A	10/11/2010	/	/	1	768	0	800	0
I84	0	I	0	A	10/11/2010	0	0	1	768	0	800	0
G26	0	G	0	A	15/11/2010	/	/	0	24	0	0	0
G27	0	G	0	A	15/11/2010	/	/	0	768	0	>1600	0
G28	0	G	0	A	15/11/2010	/	/	0	0	0	0	0
G29	0	G	0	A	15/11/2010	0	0	1	384	0	400	0
G30	0	G	0	A	15/11/2010	/	/	0	1536	0	>1600	0
D34	1	D	1	L	16/11/2010	1	1	1	96	0	400	0
D35	1	D	0	L	16/11/2010	/	/	0	/	/	/	/
D36	1	D	1	L	16/11/2010	/	/	1	/	/	/	/
D37	1	D	1	L	16/11/2010	/	/	1	768	0	>1600	0
D38	1	D	0	L	16/11/2010	/	/	0	24	0	0	0
D39	1	D	0	L	16/11/2010	/	/	1	768	0	400	0
D40	1	D	0	L	16/11/2010	1	1	1	768	0	>1600	0
D41	1	D	0	L	16/11/2010	/	/	0	384	0	100	0
D42	1	D	1	L	16/11/2010	0	0	0	0	0	0	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
C31	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C32	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C33	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C34	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C35	1	C	0	L	16/11/2010	0	0	0	24	0	0	0
C36	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C37	1	C	0	L	16/11/2010	/	/	0	0	24	0	0
C38	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C39	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C40	1	C	1	L	16/11/2010	/	/	0	0	24	0	0
C41	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C42	1	C	0	L	16/11/2010	/	/	0	0	24	0	0
C43	1	C	1	L	16/11/2010	/	/	0	48	0	0	0
C44	1	C	1	L	16/11/2010	0	0	0	0	24	0	0
C45	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C46	1	C	1	L	16/11/2010	/	/	0	0	0	0	0
C47	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C48	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C49	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C50	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C51	1	C	1	L	16/11/2010	/	/	0	0	0	0	0
C52	1	C	1	L	16/11/2010	0	0	0	24	0	0	0
C53	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C54	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C55	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C56	1	C	1	L	16/11/2010	/	/	0	24	0	0	0
C57	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
C58	1	C	0	L	16/11/2010	/	/	0	0	48	0	0
C59	1	C	1	L	16/11/2010	0	0	0	0	0	0	0
C60	1	C	0	L	16/11/2010	/	/	0	0	0	0	0
H20	0	H	0	A	16/11/2010	0	0	0	384	0	>1600	0
H21	0	H	0	A	16/11/2010	0	0	0	192	0	>1600	0
H22	0	H	0	A	16/11/2010	0	0	0	192	24	>1600	0
I85	0	I	0	A	17/11/2010	1	1	1	768	0	>1600	0
I86	0	I	0	A	17/11/2010	0	0	0	0	0	0	0
I87	0	I	0	A	17/11/2010	0	0	0	24	24	0	0
I88	0	I	1	A	17/11/2010	/	/	0	0	0	0	0
I89	0	I	0	A	17/11/2010	1	1	1	192	0	>1600	0
I90	0	I	0	A	17/11/2010	1	1	1	384	0	>1600	0
I91	0	I	1	A	17/11/2010	0	0	0	48	48	0	0
I92	0	I	1	A	17/11/2010	0	0	0	0	0	0	0
I93	0	I	1	A	17/11/2010	0	0	0	0	0	0	0
I94	0	I	1	A	17/11/2010	0	0	0	0	0	0	0
E61	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E62	1	E	0	L	24/11/2010	/	/	0	0	0	0	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
E63	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E64	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E65	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E66	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E67	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E68	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E69	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E70	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E71	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E72	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E73	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E74	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E75	1	E	1	L	24/11/2010	0	0	0	0	0	0	0
E76	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E77	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E78	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E79	1	E	1	L	24/11/2010	0	0	0	0	0	0	0
E80	1	E	0	L	24/11/2010	0	0	0	0	0	0	0
E81	1	E	0	L	24/11/2010	/	/	0	0	0	0	0
E82	1	E	0	M	24/11/2010	/	/	0	0	24	0	0
E83	1	E	0	M	24/11/2010	/	/	0	0	0	0	0
E84	1	E	0	M	24/11/2010	0	0	0	0	24	0	0
E85	1	E	0	M	24/11/2010	/	/	0	0	0	0	0
E86	1	E	0	M	24/11/2010	/	/	0	0	96	0	0
E87	1	E	0	H	24/11/2010	0	0	0	0	24	0	0
E88	1	E	0	H	24/11/2010	/	/	0	384	0	0	0
E89	1	E	0	H	24/11/2010	0	0	0	0	0	0	0
E90	1	E	0	H	24/11/2010	/	/	0	384	0	0	0
F61	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F62	1	F	1	L	24/11/2010	/	/	0	0	0	0	0
F63	1	F	1	L	24/11/2010	0	0	0	0	24	0	0
F64	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F65	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F66	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F67	1	F	1	L	24/11/2010	0	0	0	0	0	0	0
F68	1	F	1	L	24/11/2010	0	0	0	0	0	0	0
F69	1	F	1	L	24/11/2010	/	/	0	0	0	0	0
F70	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F71	1	F	1	L	24/11/2010	0	0	0	0	0	0	0
F72	1	F	1	L	24/11/2010	0	0	0	0	0	0	0
F73	1	F	1	L	24/11/2010	/	/	0	0	0	0	0
F74	1	F	0	L	24/11/2010	0	0	0	0	0	0	0
F75	1	F	0	L	24/11/2010	0	0	0	0	0	0	0
F76	1	F	1	L	24/11/2010	/	/	0	0	0	0	0
F77	1	F	0	L	24/11/2010	0	0	0	0	0	0	0

ID	Species	Supplier	Sex	Age	Sampling date	Hurine	Gurine	Hkidney	Hhardjo	Hpomona	Ghardjo	Gpomona
F78	1	F	1	L	24/11/2010	/	/	0	0	96	0	0
F79	1	F	0	L	24/11/2010	0	0	0	0	0	0	0
F80	1	F	1	L	24/11/2010	/	/	0	0	0	0	0
F81	1	F	1	L	24/11/2010	0	0	0	0	0	0	0
F82	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F83	1	F	1	L	24/11/2010	/	/	0	0	0	0	0
F84	1	F	1	L	24/11/2010	/	/	0	0	0	0	0
F85	1	F	1	L	24/11/2010	0	0	0	0	0	0	0
F86	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F87	1	F	0	L	24/11/2010	/	/	0	0	0	0	0
F88	1	F	0	L	24/11/2010	0	0	0	0	0	0	0
F89	1	F	0	L	24/11/2010	0	0	0	0	0	0	0
F90	1	F	0	L	24/11/2010	/	/	0	0	0	0	0

Appendix 6a Template and Primer Concentration Requirements for Full Sequencing Service

The Massey Genome Service requires customers who are using the 'Full Sequencing Service' to send your template and primers premixed in 0.2 ml individual PCR tubes or 0.2 ml strip tubes.

Template Type	**Template Total Quantity** in final volume	**Primer Total Quantity** in final volume	**Final volume required #**
PCR product: 100-200 bp 200-500 bp 500-1000 bp 1000-2000 bp >2000 bp **Rule**: For PCR products use 2 ng of template for every 100 bp.	 1-3 ng 3-10 ng 5-20 ng 10-40 ng 40-100 ng	3.2 pmol	15 µl

#Make the template/primer premix up to the final volume with filtered water.

Example:

You have a 500 bp PCR product at a concentration of 5.0 ng/µl and your primer is at a concentration of 1.6 pmol/µl. For PCR products use 2 ng of template for every 100 bp. So for a 500 bp product you will need to add 10 ng to the template/primer premix. At a concentration of 5.0 ng/ µl you will need to add 2 µl template to the premix. For the primer you need 3.2 pmol total amount in the template/primer premix. So at a concentration of 1.6 pmol/µl you will need to add 2 µl primer to the premix. You then need to add 11 µl of filter water to the premix to get the final volume of 15 µl.

Appendix 6b Details of the 292 *Leptospira* strains in the MLST database provided by Carlos Alfredo Carmona–Gasca (Carmona-Gasca *et al.* 2011)

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
Reference 1	L.interrogans	Icterohaemorrhagiae	Copenhageni	FiocurZ	Unknown	Unknown
Reference 2	L.interrogans	Icterohaemorrhagiae	Lai	56601	Unknown	Unknown
Ref1	L.interrogans	Sehgali	Portblari	DS2	Andaman	Unknown
Ref9	L.interrogans	Australis	Australis	ballico	Unknown	Unknown
Ref11	L.interrogans	Ictero	Ictero	RGA	Unknown	Unknown
UK3	L.interrogans	Canicola	Sumneri	Sumner	Malaysia	Unknown
UK4	L.interrogans	Canicola	Portlandvere	MY1039	Jamaica	Unknown
UK5	L.interrogans	Pomona	Pomona	Pomona	Australia	Unknown
UK6	L.interrogans	Pomona	Proechimys	1161 U	Panama	Unknown
UK7	L.interrogans	Pomona	Kenniwicki	LT1026	USA	Unknown
UK9	L.interrogans	Grippotyphosa	Grippotyphosa	Moskva Y	Unknown	Unknown
UK10	L.interrogans	Grippotyphosa	Muelleri	RM2	Malaysia	Unknown
UK16	L.interrogans	Hebdomadis	Goiano	Unknown	Unknown	Unknown
UK17	L.interrogans	Sejroe	Roumanica	LM 294	Roumanica	Unknown
UK18	L.interrogans	Sejroe	Saxkoebing	Mus24	Denmark	Unknown
UK19	L.interrogans	Sejroe	Hardjoprajitno	Hardjoprajitno	Indonesia	Unknown
UK20	L.interrogans	Icterrohaemorrhagiae	Lai	Lai	China	Unknown
UK24	L.interrogans	Icterrohaemorrhagiae	Copenhageni	M 20	Denmark	Unknown
UK25	L.interrogans	Grippotyphosa	Valbuzzi	Valbuzzi	Australia	Unknown
UK29	L.interrogans	Pyrogenes	Manilae	LT 398	Phillipines	Unknown
QSL2	L.interrogans	Australis	Fugis	Fudge	Malaysia	Human
QSL3	L.interrogans	Australis	Hawain	LT 62-68	New Guinea	Bandicoot

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
QSL4	L.interrogans	Australis	Lora	Lora	Italy	Human
QSL5	L.interrogans	Australis	Muenchen	Munchen C 90	Germany	Human
QSL8	L.interrogans	Autumnalis	Bangkinang	Bangkinang I	Indonesia	Human
QSL11	L.interrogans	Autumnalis	Carlos	C 3	Phillipins	Toad
QSL15	L.interrogans	Autumnalis	Mooris	Moores	Malaysia	Human
QSL17	L.interrogans	Autumnalis	Nanla	A 6	China	Human
QSL18	L.interrogans	Autumnalis	Weerasinghe	Weerasinghe	Sri Lanka	Human
QSL22	L.interrogans	Bataviae	Bataviae	Swart	Indonesia	Human
QSL27	L.interrogans	Bataviae	Losbanos	LT 101-69	Phillipins	Rat
QSL28	L.interrogans	Bataviae	Paidjan	Paidjan	Indonesia	Humam
QSL32	L.interrogans	Canicola	Benjamini	Benjamin	Indonesia	Human
QSL33	L.interrogans	Canicola	Bindjei	Bindjei	Indonesia	Human
QSL34	L.interrogans	Canicola	Broomi	Patane	Australia	Human
QSL35	L.interrogans	Canicola	Jonsis	Jones	Malaysia	Human
QSL36	L.interrogans	Canicola	Malaya	H 6	Malaysia	Human
QSL45	L.interrogans	Djasiman	Djasiman	Djasiman	Indonesia	Human
QSL46	L.interrogans	Djasiman	Gurungi	Gurung	Malaysia	Human
QSL47	L.interrogans	Djasiman	Huallaga	M 7	Peru	Opossum
QSL48	L.interrogans	Djasiman	Sentot	Sentot	Indonesia	Human
QSL51	L.interrogans	Grippotyphosa	Muelleri	RM 2	Malaysia	Rattus muelleri
QSL54	L.interrogans	Ictero	Gem	Simon	Sri Lanka	Human
QSL55	L.interrogans	Ictero	Hongchon	18R	Korea	Apedemus agrarius (field mouse)

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
QSL56	L.interrogans	Ictero	Smithi	Smith	Malaysia	Human
QSL58	L.interrogans	Ictero	Yeonchon	HM 3	Korea	Human
QSL65	L.interrogans	Javanica	Kalimantani	Amos	Indonesia	Unknown
QSL72	L.interrogans	Louisiana	Lanka	R 740	Sri Lanka	Human
QSL86	L.interrogans	Pyrogenes	Abramis	Abraham	Malaysia	Human
QSL88	L.interrogans	Pyrogenes	Biggis	Biggs	Malaysia	Human
QSL89	L.interrogans	Pyrogenes	Camlo	LT 64-67	Vietnam	Human
QSL90	L.interrogans	Pyrogenes	Guaratuba	An 7705	Brazil	Opossum
QSL94	L.interrogans	Pyrogenes	Pyrogenes	Salinem	Indonesia	Human
QSL95	L.interrogans	Pyrogenes	Robinsoni	Robinson	Australia	Human
QSL96	L.interrogans	Pyrogenes	Zanoni	Zanoni	Australia	Human
QSL105	L.interrogans	Sejroe	Geyaweera	Geyaweera	Sri Lanka	Human
QSL107	L.interrogans	Sejroe	Haemolytica	Marsh	Malaysia	Human
QSL109	L.interrogans	Sejroe	Ricardi	Richardson	Malaysia	Human
QSL110	L.interrogans	Sejroe	Saxkoebing	Mus 24	Denmark	Necked field mouse
QSL112	L.interrogans	Sejroe	Wolffi	3705	Indonesia	Human
R5	L.interrogans	Canicola	Canicola	M12/90	Brazil	Canis familiaris (dog)
R7	L.interrogans	Icterohaemorrhagiae	Copenhageni	M9/99	Brazil	Rattus norvegicus (rat)
R8	L.interrogans	Australis	Rushan	L01	Brazil	Canis familiaris (dog)
R9	L.interrogans	Canicola	Canicola	L02	Brazil	Canis familiaris (dog)
R10	L.interrogans	Canicola	Canicola	L03	Brazil	Sus scrofa (swine)
R11	L.interrogans	Canicola	Canicola	L09	Brazil	Bos Indicus (cow)
R12	L.interrogans	Icterohaemorrhagiae	Copenhageni	L10	Brazil	Bos Indicus (cow)
R13	L.interrogans	Canicola	Canicola	L14	Brazil	Bos Indicus (cow)

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
R17	L.interrogans	Lyme	Lyme	K30B	United Kingdom	Mouse
R18	L.interrogans	Australis	Australis	K9H	United Kingdom	Mouse
R19	L.interrogans	Icterohaemorrhagiae	Copenhageni	K13A	United Kingdom	Unknown
R27	L.interrogans	Unknown	Putative new servar(Arenal)	Isolate 7	Costa Rica	Human
R28	L.interrogans	Shermani	Shermani/Babudieri/Aguaruna	Isolate 8	Costa Rica	Human
R29	L.interrogans	Icterohaemorrhagiae	Copenhageni	Isolate 9	Costa Rica	Human
R30	L.interrogans	Unknown	Unknown (Varela???)	Isolate 10	Costa Rica	Human
R41	L.interrogans	Australis	Lora	1992	Tanzania	Mastomys
R43	L.interrogans	Australis	Lora	2324	Tanzania	Crocedura
R45	L.interrogans	Australis	Lora	2364	Tanzania	Mastomys
R46	L.interrogans	Australis	Lora	2366	Tanzania	Mastomys
R50	L.interrogans	Ballum	Kenya	4885	Tanzania	Crocedura
R51	L.interrogans	Ballum	Kenya	4883	Tanzania	Crocedura
Ind2	L.interrogans	Hebdomadis	Hebdomadis	Hebdomadis	Unknown	Unknown
Ind4	L.interrogans	Grippotyphosa	Grippotyphosa	CH31	Unknown	Unknown
Ind6	L.interrogans	Ballum	Ballum	Mus127	Unknown	Unknown
Ind8	L.interrogans	Grippotyphosa	Unknown	DS15	Unknown	Unknown
Ind9	L.interrogans	Grippotyphosa	Unknown	DS18	Unknown	Unknown
Ind10	L.interrogans	Grippotyphosa	Unknown	D22	Unknown	Unknown
Ind11	L.interrogans	Unknown	Unknown	DCHCF30	Unknown	Unknown
Ind15	L.interrogans	Grippotyphosa	Valbuzzi	Duyster-H2	Unknown	Unknown
Ind25	L.interrogans	Grippotyphosa	Unknown	ICI pod 179	Unknown	Unknown

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
VC1	L.interrogans	Grippotyphosa	Ratnapura	GC-1	Andaman(AD)	Pulmonary
VC2	L.interrogans	Icterohaemorrhegia	Copenhageni	GC-3	Andaman(AD)	Unknown
VC3	L.interrogans	Grippotyphosa	Ratnapura	TB-6	Andaman(AD)	Unknown
VC4	L.interrogans	Grippotyphosa	Ratnapura	TB-19	Andaman(AD)	Unknown
VC5	L.interrogans	Grippotyphosa	Valbuzzi	JAMES	Andaman(AD)	Pulmonary
VC6	L.interrogans	Icterohaemorrhegia	Copenhageni	YASUODAMMA	Andaman(AD)	Hepato-renal
VC7	L.interrogans	Grippotyphosa	Valbuzzi	DS-18	Andaman(AD)	Unknown
VC8	L.interrogans	Grippotyphosa	Valbuzzi	DCHCF-30	Andaman(AD)	Pulmonary
VC9	L.interrogans	Grippotyphosa	Ratnapura	MG-11	Andaman(AD)	Unknown
VC10	L.interrogans	Grippotyphosa	Ratnapura	MG-17	Andaman(AD)	Unknown
VC11	L.interrogans	Grippotyphosa	Ratnapura	MG-23	Andaman(AD)	Unknown
VC12	L.interrogans	Hebdomadis	Hebdomadis	MG-37	Andaman(AD)	Unknown
VC13	L.interrogans	Grippotyphosa	UnKnown	MG-47	Andaman(AD)	Unknown
VC14	L.interrogans	Sejroe	Saxkoebing	MG-73	Andaman(AD)	Unknown
VC15	L.interrogans	Pomona	UnKnown	MG-90	Andaman(AD)	Pulmonary
VC16	L.interrogans	Grippotyphosa	Ratnapura	MG-100	Andaman(AD)	Hepato-renal and pulmonary
VC17	L.interrogans	Australis	Ramisi	MG-347	Andaman(AD)	Unknown
VC18	L.interrogans	Grippotyphosa	UnKnown	MG-79	Andaman(AD)	Unknown
VC19	L.interrogans	Grippotyphosa	Valbuzzi	MG-342	Andaman(AD)	Pulmonary
VC20	L.interrogans	Grippotyphosa	Valbuzzi	MG-373	Andaman(AD)	Unknown
VC21	L.interrogans	Australis	Australis	MG-375	Andaman(AD)	Unknown
VC22	L.interrogans	Australis	Australis	MG-392	Andaman(AD)	Unknown
VC23	L.interrogans	Grippotyphosa	Valbuzzi	MG-472	Andaman(AD)	Unknown
VC24	L.interrogans	Canicola	Canicola	H-12	South India(KR)	Unknown

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
VC25	L.interrogans	Autumanalis	UnKnown	AUT(N)	South India(TN)	Unknown
VC26	L.interrogans	Canicola	UnKnown	PAI	South India(TN)	Unknown
VC27	L.interrogans	Icterohaemorrhegia	UnKnown	THAHKCHAN	South India(KE)	Unknown
VC28	L.interrogans	Canicola	UnKnown	G-1	Central India(MD)	Hepatic
VC29	L.interrogans	Canicola	UnKnown	G-2	Central India(MD)	Hepatic
VC30	L.interrogans	Canicola	UnKnown	G-3	Central India(MD)	Hepatic
VC31	L.interrogans	Djasmin	UnKnown	G-4	Central India(MD)	Hepatic
VC32	L.interrogans	Bataviae	UnKnown	G-5	Central India(MD)	Unknown
VC33	L.interrogans	Canicola	UnKnown	G-6	UnKnown	Hepatic
VC34	L.interrogans	Canicola	UnKnown	G-7	Central India(MD)	Hepatic
VC35	L.interrogans	Canicola	UnKnown	G-8	Central India(MD)	Hepatic
VC36	L.interrogans	Canicola	UnKnown	G-10	Central India(MD)	Hepatic
VC37	L.interrogans	Grippotyphosa	UnKnown	ALC-10	South India(KE)	Hepato-renal
VC38	L.interrogans	Hebdomadis	UnKnown	ALC-10	South India(KE)	Unknown
VC39	L.interrogans	Pomona	UnKnown	H-3	South India(KR)	Hepatic
VC40	L.interrogans	Pomona	UnKnown	H-41	South India(KR)	not known
VC41	L.interrogans	Pomona	UnKnown	H-61	South India(KR)	Unknown
VC42	L.interrogans	Pomona	UnKnown	H-518	South India(KR)	Renal
VC43	L.interrogans	Pomona	UnKnown	H-578	South India(KR)	Unknown
VC44	L.interrogans	Pomona	UnKnown	289-M.C.CALICUT	South India(KE)	Unknown
Ref2	L.kirschneri	Grippotyphosa	Grippotyphosa	Moskva V	Unknown	Unknown
Ref4	L.kirschneri	Grippotyphosa	Ratnapura	Wumalasena	Unknown	Unknown

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
Ref14	L.kirschneri	cynopteri	cynopteri	3522C	Unknown	Unknown
UK1	L.kirschneri	Canicola	Kuwait	136/2/2	Kuwait	Unknown
UK2	L.kirschneri	Canicola	Schueffneri	Vleermuis 90C	Indonesia	Unknown
UK8	L.kirschneri	Pomona	Mozdok	5621	Soveit Union (Russia)	Unknown
UK11	L.kirschneri	Grippotyphosa	Vanderhoedeni	Kipod 179	Isreal	Unknown
UK27	L.kirschneri	Pomona	Tsaratsovo	B 81/7	Bulgarica	Unknown
QSL9	L.kirschneri	Autumnalis	Bulgarica	Nikolaevo	Bulgaria	Human
QSL10	L.kirschneri	Autumnalis	Butembo	Butembo	Zaire	Human
QSL12	L.kirschneri	Autumnalis	Erinaceiauriti	Erinaceus auritius 670	Russia	Hedgehog
QSL14	L.kirschneri	Autumnalis	Lambwe	Lambwe	Kenya	Unstriped grass rat
QSL16	L.kirschneri	Autumnalis	Mujunkumi	Yezsh 237	Kzakhastan	Hedgehog
QSL25	L.kirschneri	Bataviae	Djatzi	HS 26	Peurto Rico	Human
QSL31	L.kirschneri	Canicola	Bafani	Bafani	Zaire	Human
QSL44	L.kirschneri	Djasmin	Agogo	Agogo	Ghana	Human
QSL53	L.kirschneri	Ictero	Bogvere	LT 60-69	Jamaica	Rat
QSL59	L.kirschneri	Ictero	Zimbabwe	SBF 23	Zimbabwe	?likely cattle
QSL85	L.kirschneri	Pomona	Kunming	K 5	China	Apodema chevieri
Ind14	L.kirschneri	Grippotyphosa	Valbuzzi	Duyster	UnKnown	Unknown
R34	L.kirschneri	Icterohaemorrhagiae	Sokoine	745	Tanzania	Cricetomys
R36	L.kirschneri	Icterohaemorrhagiae	Sokoine	771	Tanzania	Cricetomys
R37	L.kirschneri	Icterohaemorrhagiae	Mwogolo	826	Tanzania	Cricetomys
R38	L.kirschneri	Icterohaemorrhagiae	Mwogolo	845	Tanzania	Cricetomys
R48	L.kirschneri	Canicola	Qunjian	2980	Tanzania	Cricetomys
R49	L.kirschneri	Icterohaemorrhagiae	Sokoine	4602	Tanzania	Cricetomys

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
R57	L.kirschneri	Sejroe	Ricardi /Saxkoebing	1499	Ireland	Bank Vole (Clethrionomys glaredus)
R58	L.kirschneri	Sejroe	Ricardi /Saxkoebing	1501	Ireland	Wood Mouse (Apodemus Sylvaticus)
R65	L.kirschneri	UnKnown	Kenya	Njenga	UnKnown	Unknown
Ref10	L.santarosai	Grippotyphosa	Canalzonae	CZ188	UnKnown	Unknown
Ref15	L.santarosai	shermani	shermani	1342K	UnKnown	Unknown
UK12	L.santarosai	Mini	Georgia	LT117	USA	Unknown
UK13	L.santarosai	Sejroe	Caribe	UnKnown	UnKnown	Unknown
UK26	L.santarosai	Pyrogenes	Guaratuva	An 7705	Brazil	Unknown
UK28	L.santarosai	Sejroe	Recreo	380	Nicaraguva	Unknown
UK31	L.santarosai	Pyrogenes	Varella	1019	Nicaraguva	Unknown
QSL7	L.santarosai	Autumanalis	Alice	Alice	Sri Lanka	Human
QSL19	L.santarosai	Ballum	Peru	MW 10	Peru	Opossum
QSL21	L.santarosai	Bataviae	Balboa	735 U	Panama	Spiny rat
QSL23	L.santarosai	Bataviae	Brasiliensis	An 776	Brazil	Opossum
QSL26	L.santarosai	Bataviae	Kobbe	CZ 320	Panama	Spiny rat
QSL29	L.santarosai	Bataviae	Rioja	MR 12	Peru	Opossum
QSL43	L.santarosai	cynopteri	Tingomaria	M 13	Peru	Opossum
QSL50	L.santarosai	Grippotyphosa	Huanuco	M 4	Peru	Opossum
QSL63	L.santarosai	Javanica	Fluminense	Aa 3	Brazil	Field mouse
QSL81	L.santarosai	Mini	Tabaquite	TRVL 3214	Trinidad	Human
QSL87	L.santarosai	Pyrogenes	Alexi	HS 616	Puerto Rico	Human
QSL93	L.santarosai	Pyrogenes	Princestown	TRVL 112499	Trinidad	Human

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
QSL99	L.santarosai	Sarmin	Machiguenga	MMD 3	Peru	Opossum
QSL100	L.santarosai	Sarmin	Rio	Rr 5	Brazil	Rat
QSL103	L.santarosai	Sarmin	Weaveri	CZ390	Panama	Human
QSL106	L.santarosai	Sejroe	Gorgas	1413 U	Panama	Spiny rat
QSL114	L.santarosai	shermani	Babudieri	CI 40	Peru	Pig
QSL115	L.santarosai	shermani	Luis	M 6	Peru	Opossum
QSL117	L.santarosai	Tarassovi	Atchafalaya	LSU 1013	USA	Opossum
QSL118	L.santarosai	Tarassovi	Atlantae	LT 81	USA	Opossum
QSL119	L.santarosai	Tarassovi	Bakeri	LT 79	USA	Opossum
QSL121	L.santarosai	Tarassovi	Chagres	1913 K	Panama	Spiny rat
QSL122	L.santarosai	Tarassovi	Darien	637 K	Panama	Opossum
QSL123	L.santarosai	Tarassovi	Gatuni	1473 K	Panama	Opossum
QSL130	L.santarosai	Tarassovi	Rama	316	Nicaragua	Opossum
QSL135	L.santarosai	Javanica	Vargonicas	24	Peru	Rodent
R1	L.santarosai	Bataviae	Brasiliensis	An 776	Brazil	Didelphis marsupialis (opossum)
R2	L.santarosai	Sejroe	Guaricura	Bov.G	Brazil	Bos Indicus (cow)
R6	L.santarosai	Sejroe	Guaricura	M4/98	Brazil	Bubulus bubalis (buffalo)
R14	L.santarosai	Grippotyphosa	Bananal	2ACAP	Brazil	Hydrochaeris hydrochaeris (capybara)
R15	L.santarosai	Grippotyphosa	Bananal	16CAP	Brazil	Hydrochaeris hydrochaeris (capybara)
R21	L.santarosai	Pyrogenes	Alexi/Guaratuba/Princestown	Isolate 1	Costa Rica	Human
R22	L.santarosai	Sarmin	Weaveri/Rio	Isolate 2	Costa Rica	Human
R23	L.santarosai	Tarassovi	Rama	Isolate 3	Costa Rica	Human
R25	L.santarosai	Tarassovi	Rama	Isolate 5	Costa Rica	Human
R26	L.santarosai	Bataviae	Claytoni	Isolate 6	Costa Rica	Human

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
Ref7	L.borgpetersenii	javanica	Poi	poi	UnKnown	Unknown
Ref8	L.borgpetersenii	mini	Mini	sari	UnKnown	Unknown
UK14	L.borgpetersenii	Sejroe	Istrica	Bratislava M 84	Slovakia	Unknown
UK15	L.borgpetersenii	Sejroe	Sejroe	M84	Denmark	Unknown
UK21	L.borgpetersenii	Javanica	Dehong	De 10	China	Unknown
UK22	L.borgpetersenii	Javanica	Javanica	Veltrat Batavia	Indonesia	Unknown
UK23	L.borgpetersenii	Javanica	Zhenkang	L 82	China	Unknown
QSL6	L.borgpetersenii	Australis	Pina	LT 932	Panama	Opossum
QSL41	L.borgpetersenii	cellodoni	Whitcombi	Whitcomb	Malyasia	Human
QSL57	L.borgpetersenii	Ictero	Tonkini	LT96-68	Vietnam	Human
QSL61	L.borgpetersenii	Javanica	Ceylonica	Piyasena	Sri Lanka	Human
QSL64	L.borgpetersenii	Javanica	Javanica	Veldrat Batavia 46	Indonesia	Field rat
QSL67	L.borgpetersenii	Javanica	Menoni	Kerala	India	Bandicoot
QSL71	L.borgpetersenii	Javanica	Sorexjalna	Sorex Jalna	Czeckoslovakia	Shrew
QSL91	L.borgpetersenii	Pyrogenes	Kwale	Julu	Kenya	Human
QSL104	L.borgpetersenii	Sejroe	Balcanica	1627 Burgas	Bulgaria	Human
QSL108	L.borgpetersenii	Sejroe	Nyanza	Kibos	Kenya	Human
QSL124	L.borgpetersenii	Tarassovi	Gengma	M 48	China	Pig
QSL126	L.borgpetersenii	Tarassovi	Kisuba	Kisuba	Zaire	Human
QSL131	L.borgpetersenii	Tarassovi	Tarassovi	Perepelitsin	Russia	Human
QSL132	L.borgpetersenii	Tarassovi	Tunis	P 2/65	Tunesia	Pig
QSL134	L.borgpetersenii	Tarassovi	Yunxian	L 100	China	Pig

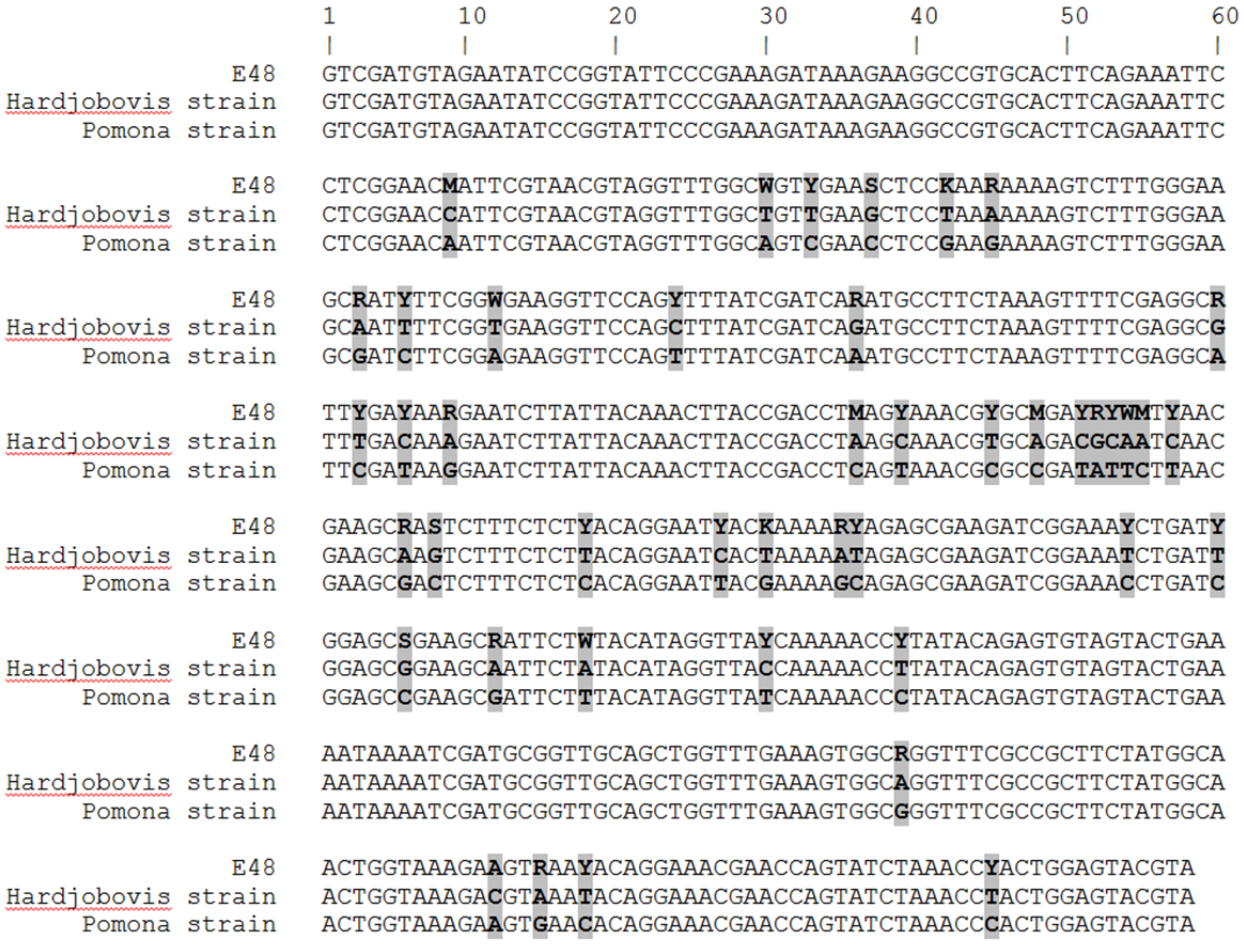
Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
Ind5	L.borgpetersenii	Canicola	Canicola	Hond utrecht IV	Unknown	Unknown
R31	L.borgpetersenii	Ballum	Kenya	153	Tanzania	Mastomys
R32	L.borgpetersenii	Ballum	Kenya	159	Tanzania	Crocedura
R33	L.borgpetersenii	Ballum	Kenya	723	Tanzania	Crocedura
R35	L.borgpetersenii	Ballum	Kenya	766	Tanzania	Cricetomys
R39	L.borgpetersenii	Ballum	Kenya	1605	Tanzania	Crocedura
R40	L.borgpetersenii	Ballum	Kenya	1610	Tanzania	Crocedura
R42	L.borgpetersenii	Ballum	Kenya	2062	Tanzania	Crocedura
R44	L.borgpetersenii	Ballum	Kenya	2348	Tanzania	Crocedura
R47	L.borgpetersenii	Ballum	Kenya	2447	Tanzania	Crocedura
R52	L.borgpetersenii	Ballum	Kenya	4880	Tanzania	Mastomys
R53	L.borgpetersenii	Ballum	Kenya	4787	Tanzania	Crocedura
R54	L.borgpetersenii	Hebdomadis	Kremastos/Hebdomadis)	873	Ireland	Dog
R55	L.borgpetersenii	Hebdomadis	Kremastos/Hebdomadis)	871	Ireland	Pygmy Shrew
R56	L.borgpetersenii	Sejroe	Saxkoebing	1498	Ireland	Gemsbuck (aborted fetus)
R59	L.borgpetersenii	Sejroe	Ricardi /Saxkoebing	1522	Ireland	Wood Mouse
R60	L.borgpetersenii	Sejroe	Ricardi/Saxkoebing	1525	Ireland	Dog (foxhound)
R61	L.borgpetersenii	Pomona	Kunming	RIM 139	IRL (Portugal)	Mus musculus
R62	L.borgpetersenii	Pomona	Kunming	RIM 201	IRL (Portugal)	Mus musculus
R63	L.borgpetersenii	Sejroe	Ricardi /Saxkoebing	RIM 156	IRL (Portugal)	Mus musculus
R64	L.borgpetersenii	Unknown	Sokoine	RM1	Unknown	Unknown
Ref3	L.noguchii	Louisiana	Louisiana	LSU 1945	Unknown	Unknown
Ref12	L.noguchii	panama	Panama	CZ214k	Unknown	Unknown
UK30	L.noguchii	Pyrogenes	Myocastoris	LSU 1551	USA	Unknown

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
QSL13	L.noguchii	Autumnalis	Fortbragg	Fort Bragg	USA	Human
QSL20	L.noguchii	Bataviae	Argentiniensis	Peludo	Argentina	Armadillo
QSL24	L.noguchii	Bataviae	Claytoni	1348 U	Panama	Spiny rat
QSL74	L.noguchii	Louisiana	Orleans	LSU 2580	USA	Nutria
QSL82	L.noguchii	panama	Cristobali	1996 K	Panama	Opossum
R24	L.noguchii	Pyrogenes	Guaratuba (on DNA info)	Isolate 4	Costa Rica	Human
Ref13	L.weilli	cellodoni	Cellodoni	cellodoni	Unknown	Unknown
QSL39	L.weilii	cellodoni	Hainan-Whitcombi	6712	China	Human
QSL62	L.weilii	Javanica	Coxi	Cox	Malaysia	Human
QSL66	L.weilii	Javanica	Mengma	S 590	China	Human
QSL68	L.weilii	Javanica	Menrun	A 102	China	Human
QSL76	L.weilii	Manhao	Qingshui ('Manhao 2)	L105	China	Human
QSL78	L.weilii	Mini	Hekou	H 27	China	Human
QSL129	L.weilli	Tarassovi	Ngavi	SBF 16	Zimbabwe	?, likely cattle
QSL75	L.inadai	Manhao	Lincang	L 14	China	Human
QSL83	L.inadai	panama	Mangus	TRVL/CAREC 137774	Trinidad	Mongoose
QSL113	L.inadai	shermani	Aguaruma	MW 4	Peru	Opossum
QSL80	L.meyeri	Mini	Perameles	Bandicoot 343	Australia	Perameles
Ref6	L.allexanderi	Manhao	Manhao	L60	Unknown	Unknown
BA1	L.borgpetersenii	Ballum	Castellonis	Castellon-3	Brisbane	Unknown
BA2	L.borgpetersenii	Ballum	Ballum	Mus 127	Brisbane	Unknown
BA3	L.interrogans	Canicola	Canicola	Hond Utrecht	Brisbane	Unknown

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
BA4	L.weilii	Celledoni	Celledoni	Celledoni	Brisbane	Unknown
BA5	L.kirschneri	Cynopteri	Cynopteri	3522-C	Brisbane	Unknown
BA6	L.kirschneri	Grippotyphosa	Grippotyphosa	Moskva V	Brisbane	Unknown
BA7	L.interrogans	Sejroe	Hardjo	Hardjo prajitno	Brisbane	Unknown
BA8	L.interrogans	Icterohaemorrh	Icterohaemorrhagiae	RGA	Brisbane	Unknown
BA9	L.borgpetersenii	Javanica	Javanica	(Veldrat Batavia 46	Brisbane	Unknown
BA10	L.noguchii	Australis	Müenchen	Müenchen C90	Brisbane	Unknown
BA11	L.noguchii	Panama	Panama	(CZ214K)	Brisbane	Unknown
BA12	L.santarosai	Shermani	Shermani	1342-K	Brisbane	Unknown
MX1	L.kirschneri	Grippotyphosa	Unknown	CAL4	Mexico	Bovine
MX2	L.interrogans	Sejroe	Hardjo	CAL6	Mexico	Bovine
MX3	L.santarosai	Mini	Unknown	CAL7	Mexico	Bovine
MX4	L.interrogans	Canicola	Canicola	CEL60	Mexico	Dog
MX5	L.borgpetersenii	Ballum	Mus	R-13	Mexico	Mus musculus
MX6	L.borgpetersenii	Ballum	Mus	R-14	Mexico	Mus musculus
MX7	L.borgpetersenii	Ballum	Mus	R-28	Mexico	Mus musculus
MX8	L. interrogans	Canicola	Canicola	LOCAS 28	Mexico	Dog
MX9	L.interrogans	Canicola	Canicola	LOCAS 31	Mexico	Dog
MX10	L.interrogans	Canicola	Canicola	LOCAS 34	Mexico	Dog
MX11	L.interrogans	Canicola	Canicola	LOCAS 46	Mexico	Dog
MX12	L.interrogans	Canicola	Canicola	LOCAS 59	Mexico	Dog
MX13	L.interrogans	Icterohaemorrh	Icterohaemorrhagiae	Palo Alto	Mexico	Dog
MX14	L.interrogans	Canicola	Portland vere	Sinaloa ACR	Mexico	Pig
MX15	L.interrogans	Sejroe	Hardjo	H89	Mexico	Bovine

Labels	Genome Species	Serogroup	Serovar	Strain	Geographical area	Source
MX16	L.interrogans	Canicola	Canicola	UADY22	Mexico	Dog
MX17	L.interrogans	Icterohaemorrh	Icterohaemorrhagiae	LOVe30	Mexico	Rattus norvegicus (rat)
MX18	L.santarosai	Tarassovi	Unknown	VH45	Mexico	Bovine
MX19	L.interrogans	Canicola	Canicola	Citlalli	Mèxico	Dog
GB1	L.borgpetersenii	Sejroe	Hardjobovis	L550	Unknown	GeneBank
GB2	L.borgpetersenii	Sejroe	Hardjobovis	BJ197	Unknown	GeneBank

Appendix 6c Sequence from isolate E48, Hardjobovis isolate (D9), and Pomona isolate (A6) at loci *lipL41*



Appendix 7a Cover letter for GP

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Leptospirosis in New Zealand: diagnostics, strain typing and transmission.

To whom it may concerned

I am writing to ask if you would be willing to participate in a study to compare the PCR-based tests for leptospirosis against the standard serology test. The Health Research Council (HRC) funded study is based in the Waikato, as this is a DHB area with high rates of leptospirosis notifications (please find details of this study in the attached Information Sheet for Rural GPs). Ethics application for this study has been approved by Northern Y Regional Ethics Committee (NTY/10/10/083).

We do expect that there will be a slight increase in your workload required. The addition would be to recruit the patient into the study, request the extra test for leptospirosis on the lab form to the patient, and send a "probable" notification form to Dr Anita Bell (MoH, Waikato DHB).

We are working with Anita Bell (MoH). Your medical centre was identified by her, as being in an area that has a high number of leptospirosis notifications within the WDHB.

We hope that you may be willing to take part in the study or at least find out more about what it may entail for you and your practice. We would like to visit your practice to talk about this study in detail, and/or address a practice group meeting. We shall be following up this letter with a telephone call in the next few weeks. Please don't hesitate to contact us before then if you have any questions about this study.

If you do not want to be contacted further, please email Fang and we will not approach you again

Thank you for your time.

Dr. Jackie Benschop	Fang Fang
BVSc (Dist), PhD, MACVSc	BVM, MVS
Senior Lecturer	PhD candidate

Appendix 7b GP information sheet

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Leptospirosis in New Zealand: diagnostics, strain typing and transmission.

INFORMATION SHEET FOR RURAL GPs

Introduction

We are writing to invite you to participate in a Health Research Council funded study. The chief investigator is Dr. Jackie Benschop; named investigators are Fang Fang (PhD candidate), Dr. Julie Collins-Emerson, Prof. Nigel French and Assoc. Prof. Michael Baker (Wellington School of Medicine, Otago University). We are also working closely with Pinnacle Group Limited, Drs Chris Mansell and Anita Bell from Waikato DHB.

Aim of study

The first objective of this study is to identify the best diagnostic test or combination of tests for acute cases of leptospirosis that will satisfy the need for early diagnosis, treatment, and also meet ACC requirements. The second objective is to characterise the population of pathogenic leptospires in New Zealand. We will try to culture and genotype leptospiral isolates from patients acutely ill with leptospirosis-like-illness. Genotyping human isolates and comparing them with animal isolates will potentially identify the species-specific source of human infections, which may help to set up specific risk reduction strategies.

Study Plan

We ask you to assist us by identifying adult patients that you suspect may have leptospirosis as your provisional diagnosis, invite them to enrol in the study using the information and consent form that will be provided. They may report animal contact and their clinical signs may include fever, chills, myalgia and conjunctivitis. <u>They must not have had antibiotic treatment within the last three weeks and no less than 16 years of age.</u>

Once the patient has consented then send the patient for blood sampling at your local collection centre as usual. We will provide you a special purpose laboratory test request form for the patient to take to the collection centre. This will request the following:

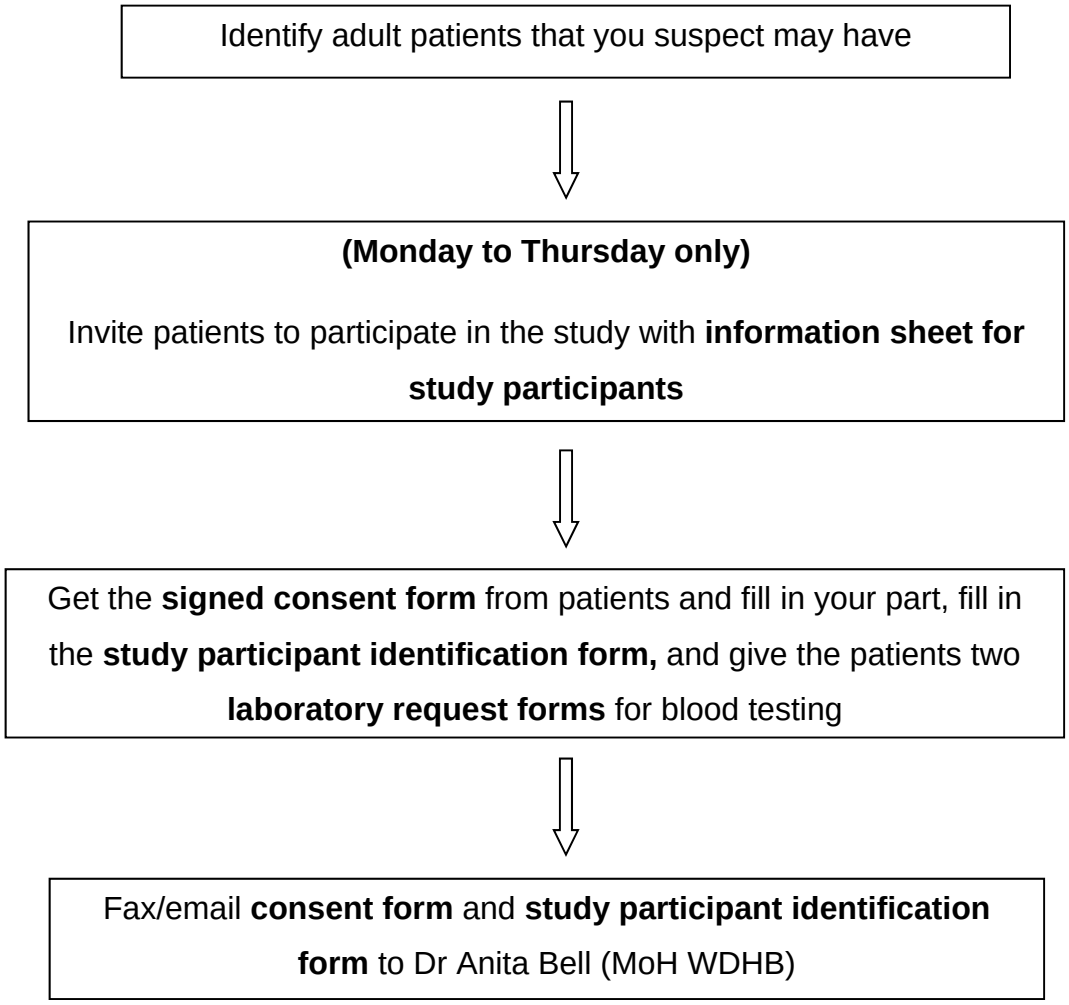
1. Microscopic agglutination test (MAT) at first presentation and for the patient to return three weeks later for a convalescent MAT (please make sure 100% of the participants will provide the convalescent blood samples).
2. PCR testing from whole blood/ serum.
3. Culture from whole blood.

Please fax or email the leptospirosis study participant identification form (including demographic, clinical signs and treatment information) and the consent form to Dr Anita Bell (MOH WDHB), fax: 07 838 2382, email: Anita.Bell@waikatodhb.health.nz. She will follow up with the patients and fill in the EpiSurv form for surveillance.

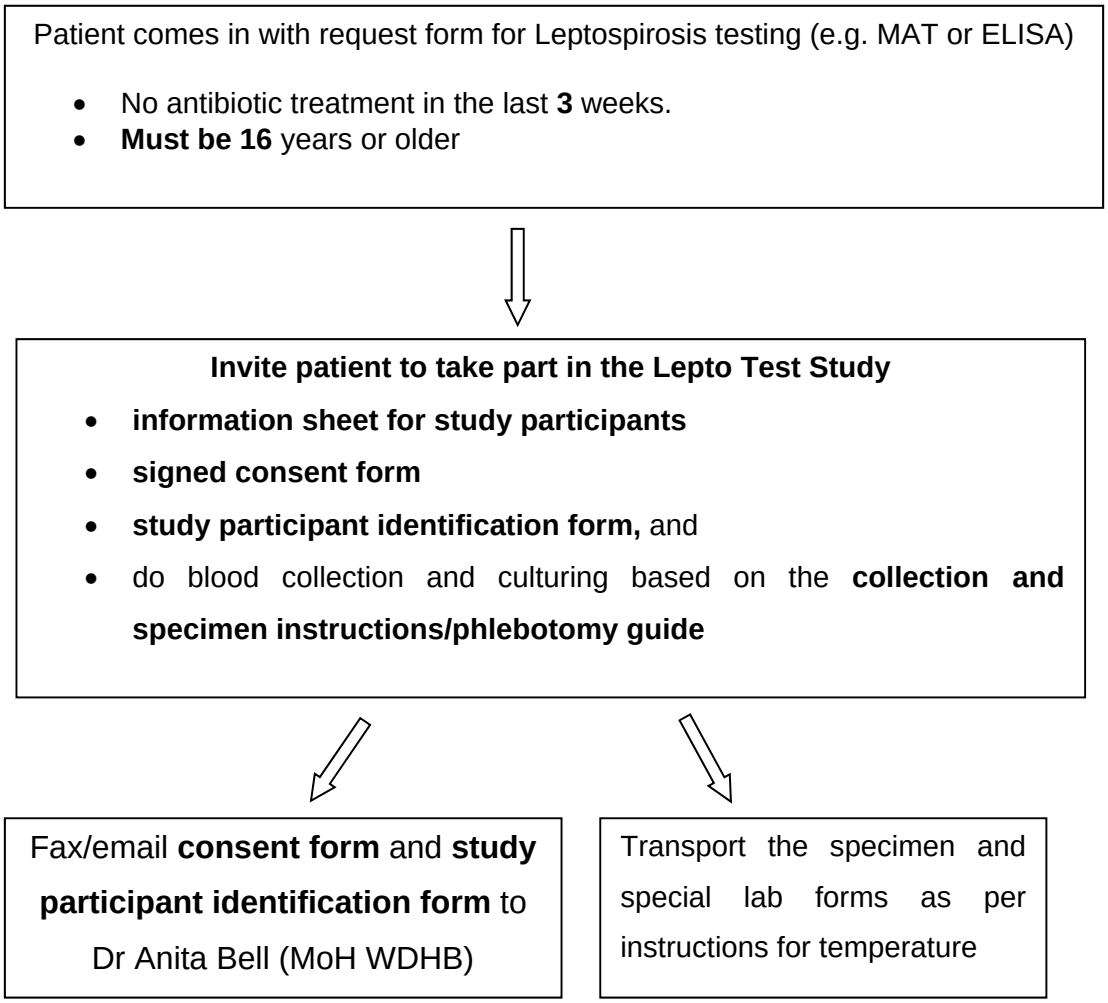
All test results will be returned to you to assist management during the patient's illness.

Thank you very much.

Appendix 7c Study protocol for GPs/hospital doctors about patient recruitment



Appendix 7d Study protocol for phlebotomists about patient recruitment



Appendix 7e Information Sheet for Study Participants

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Private Bag 11 222
Palmerston North
New Zealand

Dr. Jackie Benschop
Direct Telephone: + 64 (6) 350 4528
Email: j.benschop@massey.ac.nz

Fang Fang
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Leptospirosis in New Zealand: diagnostics, strain typing and transmission.

INFORMATION SHEET FOR STUDY PARTICIPANTS

Principal Investigators
Dr. Jackie Benschop (contact person) – Senior Lecturer, EpiCentre, Massey University
Fang Fang – PhD candidate, Molecular Epidemiology and Public Health Laboratory, Massey University
Dr. Julie Collins-Emerson – Research Officer, Molecular Epidemiology and Public Health Laboratory, Massey University
Professor Nigel French – Director, Molecular Epidemiology and Public Health Laboratory, Massey University
Dr. Michael Baker – Associate Professor, Department of Public Health, Wellington School of Medicine, Otago University.

What is this study about?
Leptospirosis (lepto) is an important infectious disease in New Zealand and often associated with animal contact. You are invited to take part in a study to determine the best way to test for leptospirosis. Your GP has identified you as suitable for this study as he/she suspects you may have lepto.

Your participation is entirely your choice. If you have any queries or concerns regarding your rights as a participant in this study, you may wish to contact an independent health and disability advocate:

Free phone: 0800 555 050
Free fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz
If you choose not to take part, this will not affect any future care or treatment. If you do agree to take part in the study, you are free to withdraw from the study at any time, without having to give a reason, and this will in no way affect your future health care.

We are investigating some new tests (PCR) to see if they perform better in people like you, in the early stages of illness. Usually, the diagnosis of lepto is based on having two blood tests three weeks apart (called MAT) and looking at the differences between these test results. This test will be done for you as routine diagnosis. However, it takes about a

week after you first become ill for it to become positive, and often people don't come back for the second test.

The PCR may allow earlier diagnosis with earlier treatment and better outcomes for you. The cost of new tests will be charged from our research funding, but results will still be used to assist your GP's diagnosis. We will run PCR tests from three different labs: ESR's *Leptospira* Reference lab, Canterbury Health lab, and Massey's Hopkirk research lab.

Also in this study we will try and grow the lepto bacteria from your blood. Comparing different strains of the bacteria in both humans and animals can help better identify the source of human lepto infection, allowing us to advise on ways to reduce the risk of infection.

Over a two-year period we will enrol 100 people that GPs suspect of having leptospirosis. We are recruiting in the Waikato area as it has a rural population, there have been cases of leptospirosis diagnosed here and we have tremendous support for our study with your district health board and rural GPs.

What participation means for you
Once you have read this information sheet and if you agree to take part in this study your doctor will ask you to sign the consent form and arrange for blood samples to be taken. It is routine for the doctor to request blood tests anyway to help with your diagnosis and for ACC requirements. Participating in our study will mean that a little more of your blood is taken from you than would have been done otherwise. When you consent your GP will record your contact details and your symptoms, arrange for the testing and ask if we can contact you by phone for further information on your risk factors for leptospirosis. Three weeks after the first blood, you will be asked to visit the lab for sampling again, this is for the usual MAT follow up test.

Results and Confidentiality
Results from the blood tests will be sent to your GP and she/he will contact you as appropriate. All of the information you give us is confidential and it will not be re-used for other research. No material that could personally identify you will be used in any report on this study. Data will be kept in a protected electronic database for 10 years. After that, all data will be destroyed. The blood samples will be stored until test results are finalised, then samples will be destroyed.

Statement of Approval
This study has received ethical approval from the Northern Y Ethics Committee, ethics reference number NTY/10/10/083.

Compensation
In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation, and Compensation Act 2001. ACC cover is not automatic, and your case will need to be assessed by ACC according to the provisions of the Injury Prevention, Rehabilitation, and Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors, such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses, and there may be no lump sum compensation payable. There is no cover for

mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators.
If you have any questions about ACC, contact your nearest ACC office or the investigator.
You are also advised to check whether participation in this study would affect any indemnity cover you have or are considering, such as medical insurance, life insurance and superannuation.

Thank you very much for your time in considering this study.

Please feel free to contact the researcher if you have any questions about this study.

Appendix 7f Leptospirosis study participant identification form

Leptospirosis study participant identification form

Date of consultation ____/____/____

Contact details

First name _____ Last name_____

NHI number _____

Current address: Number_____ Street _____Suburb_____

Town/City_____ postcode _____

Phone (day) _____ Phone (night) _____

Clinical Course and treatment

Date of onset ____/____/____ ☐ Approximate ☐ Unknown

What symptoms did you experience?

Fever	☐ Yes	☐ No
Muscle aches and pains	☐ Yes	☐ No
Headaches	☐ Yes	☐ No
Sweating	☐ Yes	☐ No
Severe debility (tiredness)	☐ Yes	☐ No
Sore eyes	☐ Yes	☐ No
Jaundice (Yellow eyes)	☐ Yes	☐ No
Conjunctival suffusion	☐ Yes	☐ No
Stomach pains (cramps)	☐ Yes	☐ No
Nausea/vomitting or loss of appetite	☐ Yes	☐ No

Other (please state) _____

Treatment prescribed at this consultation: _____

Appendix 7g Waikato Leptospirosis questionnaire

Leptospirosis Case Report Form

Interview date: __/__/__ **Interviewer name:** _____

Case EpiSurv No: _____ **Serovar (specify type)**_____

Standard privacy information given ***Y / N***

Notifier Identification

Reporting Source: □ General practitioner □ Hospital-based practitioner □ Laboratory

□ Self notification □ Outbreak investigation □ Other

Name of reporting source: _____Contact phone: _____

Date reported: ____/____/____ Name of usual GP:_____

Case Identification and Demography

First name:_____ Last name:_____

Street name and number: _____ Suburb: _____

Town:_____TLA:_____DHB:_____

Day & night phone no's: _____

DOB: ___/___/___ Age:_____ Sex:_____ NHI number:_____

Occupation: _____

Name of work/school/creche:_____Address:_____

_____

Ethnicity: NZ European □ Maori □ Pacific Island □ European □

Other, specify: _____

Clinical Course and Outcome

When did your symptoms first begin? __/__/__

How long did your symptoms last? _____days

The exposure period is **2-30** days before onset date **__/__/__ to __/__/__**

What symptoms did you experience?

Fever	□ No	□ Yes
Muscle aches and pains	□ No	□ Yes
Headaches	□ No	□ Yes
Tiredness	□ No	□ Yes
Stomach pains (cramps)	□ No	□ Yes
Nausea or loss of appetite	□ No	□ Yes
Other: _____	□ No	□ Yes

How long did your symptoms last? _____days

General Health

Do you have any underlying health problems? □ Yes □ No

If yes, give details _____

Were you antibiotics in the month before onset of your symptoms? □ Yes □ No

If yes, which antibiotics? _____

Dose/Frequency/Duration? _____/_____/_____

Do you smoke? □ Yes □ No

Contacts During the 30 days before you became unwell did you…

Have any contact with **other** people with similar symptoms? □ Yes □ No

Name of other ill person(s), relationship to person and contact details

_____ Onset date: __ / __ / __
_____ Onset date: __ / __ /
_____ Onset date: __ / __ / __

Risk Factors – Animals *During the 30 days before you became unwell did you*

Have any exposure to animals? (Farm/Domestic/Wild) □ Yes □ No

	Contact Time at Work	Contact Time Elsewhere	Vaccinated against leptospiries
Cattle – dairy			
Cattle – beef			
Pigs			
Deer			
Sheep			
Rodents			
Goats			
Horses			
Dogs			
Other (specifiy)			

Risk Factors – Animals *During the 30 days before you became unwell did you*

If vaccinated how many, with what, when and by whom? _____

If a vet, may we contact them for further information? □ Yes □ No

Vets name, address and telephone _____

Has your farm or the farm you visited had any new animals in the last few months? □ Yes □ No

If yes, which animal and when did they arrive?_____

Were they vaccinated? □ Yes □ No □ Don't know

Were any unwell? □ Yes □ No □ Don't know

For dairy farms is your milking shed a: □ Walk through □ Herringbone □ Rotary □ Don't know

Have you processed meat? At home/work: □ Yes □ No □ Don't know

How was offal handled? _____

Risk Factors – Water *During the 30 days before you became unwell did you…*

Drink water from any of the following sources (please specify):

Well/Bore	□ Yes	□ No	□ Don't know
Surface Stream/Lake/Spring	□ Yes	□ No	□ Don't know
Rainwater (roof collection)	□ Yes	□ No	□ Don't know

If 'Y' name location if possible and date: _____

Is there any potential for contamination with animal urine? □ Yes □ No □ Don't know

If 'Y', specify (type of animal, nature of contamination)_____

Have any direct contact with sewage/wastewater? □ Yes □ No □ Don't know

Go swimming i.e. beach, river, hot pools or local pool? □ Yes □ No □ Don't know

If yes location and date: _____ ___/____/____

Risk Factors - Activities *During the 30 days before you became unwell did you…*

Travel outside New Zealand? □ Yes □ No □ Don't know

What date did you depart New Zealand?__/__/__ Return to New Zealand? _/__/__

Travel within New Zealand? □ Yes □ No □ Don't know

If 'Y', list regions visited and dates: _____

Tramp or camp? If 'Y', where and for how long? _____

Have you been hunting? If 'Y' type of animal?_____

Source of water while tramping/camping/hunting: _____

Clear or help clear any bush areas on your or another property? □ Yes □ No

Done any other maintenance work on your on another property? □ Yes □ No

Worked on drainage on your or another property? □ Yes □ No

Worked near or in any wetlands river, stream or pool? □ Yes □ No

SUMMARY REPORT - what is the probable source of infection?

(NB there may be more than one)

- ❑ From contact with infected animal, specify _____
- ❑ From consumption of contaminated food, specify _____
- ❑ From consumption of contaminated water, specify _____
- ❑ Recreational water contact _____
- ❑ From at risk activities, specify _____
- ❑ Employment, specify (**NODS** Form offered)_____
- ❑ Other _____
- ❑ Unknown

COMMENTS

_____

_____

_____

_____

_____

_____

_____

Appendix 8 Questionnaire for Veterinary Student Study

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Study of Leptospirosis among Veterinary Students

Participant Questionnaire

The objectives of this study are to measure the prevalence of leptospirosis infection or exposure in veterinary students at Massey University, and to investigate risk factors for sero-prevalence during both scheduled study and other times. This study will quantify the risk of infection, identify sources of infection, and guide the faculty in developing control measures if appropriate.

In New Zealand, leptospirosis is the most common occupationally acquired zoonotic disease. Cattle, sheep and deer are the most important animal reservoirs. Human exposure to Leptospira is mainly through contacting infected animal urine via abrasions or cuts in the skin, or intact mucous membranes. The majority of notified leptospirosis cases in New Zealand are due to occupational risk factors.

Personal information included in the questionnaire will be treated in the strictest confidence and will not be published or disclosed to any third parties by the research team in a manner that would allow identification of participants.

The study has the approval of the Massey University Human Research Ethics Committee.

The research team appreciates your participation in this study of leptospirosis.

1. Participant identification

Note: please make sure you have read through and signed the consent and confidentiality form before taking part in this study.

Name	
Gender	Male ☐ Female ☐
Date of Birth	(day/month/year) _____/_____/_____
Which option best identifies your ethnic affiliation?	NZ-Maori ☐ Pacific Islander ☐ NZ-European ☐ Asian ☐ Other ☐ _____
Which year of the course are you currently in?	First ☐ Second ☐ Third ☐ Fourth ☐ Fifth ☐ * If you had repeated studying for your courses, please state _____ year
Track (Fifth year students only)	Companion Animal ☐ Production Animal ☐ Equine ☐ Wildlife Animal ☐ Avian Species ☐ Mixed ☐ Other ☐ (Please state)_____
What location/s have you lived in during the **past 18 months**? (please state the proportions if you have been living in more than one type of the locations)	Rural ☐ % Urban ☐ % Lifestyle* ☐ % * i.e. Small block with grazing animals

*2. Exposure within the veterinary curriculum **

(* Includes scheduled semester courses, vacation veterinary practical requirement, and farm practical as part of the veterinary curriculum only (other potential exposures are in section 3 below))

This section asks about your potential exposure to the risk of leptospirosis from each of the animal species you may have been in contact with during the veterinary curriculum.

2.1 Appliance of personal protective equipment during veterinary curriculum

During the veterinary curriculum, approximately what percentage of the time do you use the recommended personal protective equipment, such as gloves, overalls, face mask and/or goggles?

_____%

2.2 Contact with live animals during veterinary curriculum

In the <u>past 18 months</u>, have you experienced or been in a situation where you were at risk of exposure to animal urine (e.g. dealing with, handling or being around live animals in a manner that you could have come in contact with urine, dead animals in anatomy or post mortem, collection or test of urine samples, reproductive tract manipulations or treatment, urinary tract surgery, etc.) or exposure where you may have been in contact with urine but not directly hands-on the animal (e.g. farm animal mustering, milking cows etc.)?

YES ☐ Complete table below

NO ☐ Skip to **Section 3.**

Animal type	Exposure	Number of animals	How many times in past 18 months?
Beef cattle	Yes ☐ No ☐		_____ times
Dairy cattle	Yes ☐ No ☐		_____ times
Sheep	Yes ☐ No ☐		_____ times
Deer	Yes ☐ No ☐		_____ times

Horse	Yes ☐ No ☐		_____ times
Goat	Yes ☐ No ☐		_____ times
Dogs	Yes ☐ No ☐		_____ times
Cats	Yes ☐ No ☐		_____ times
Laboratory animals (i.e. rabbits, mice)	Yes ☐ No ☐		_____ times
Other animals (please state) _____	Yes ☐ No ☐		_____ times

3. Exposure outside the veterinary curriculum

This section asks about your potential exposure to the risk of leptospirosis from each of the animal species you may have been in contact with other than during the veterinary curriculum, i.e. during your personal life.

3.1 Own or have frequent contact with live animals

Do you own or have frequent contact (at least weekly) with live animals outside your veterinary curriculum?

YES ☐ Please specify the animal types

NO ☐ Skip to **Exposure during personal life table 3.2**

Animal type	**Own or have frequent contact**
Dogs, cats	Yes ☐ No ☐
Rabbits, guinea pigs or other small mammals	Yes ☐ No ☐
Horses	Yes ☐ No ☐
Cattle, sheep, goats, deer, pigs	Yes ☐ No ☐
Birds	Yes ☐ No ☐
Other_____	Yes ☐ No ☐

3.2 Contact with live animals at home, friend's or family's house

Over the **past 18 months**, have you had regular contact (at least weekly) with animals outside your veterinary curriculum (at home, friend's or family's house) that may lead to exposure or risk of exposure to animal urine, for example, emptying your cat's litter tray or assisting your ewe's with lambing?

YES ☐ Complete table below

NO ☐ Skip to **Wildlife table 3.3**

Animal type	**Exposure**	**Number of animals**	**How many times in past 18 months?**
Beef cattle	Yes ☐ No ☐		_____ times
Dairy cattle	Yes ☐ No ☐		_____ times
Sheep	Yes ☐ No ☐		_____ times
Goats	Yes ☐ No ☐		_____ times
Deer	Yes ☐ No ☐		_____ times
Pigs	Yes ☐ No ☐		_____ times
Dogs	Yes ☐ No ☐		_____ times
Cats	Yes ☐ No ☐		_____ times
Other_____	Yes ☐ No ☐		_____ times
Did you have contact with any of these animals overseas?	Yes ☐ No ☐ If yes, specify country(ies) _____		

3.3 Contact with wildlife animals

Over the **past 18 months**, have you seen rats, mice, possums, rabbits or hedge hogs at where you were living (house, garden, surrounding fields)?

YES ☐ Complete table below

NO ☐ Skip to **Home slaughter table 3.4**

How many times in past 18 months?	_____ times
Have you set trapped or poisoned for these animals at home?	Yes ☐ No ☐ Don't know ☐
Have you had contact with wildlife animals overseas?	No ☐ Yes ☐ If yes, specify country(ies) _____

3.4 Home slaughter

Did you home-slaughter or have you helped with home slaughtering any animals in the **past 18 months**?

YES ☐ Complete table below

NO ☐ Skip to **Outdoor exposures section 3.5**

Animal type	How many times in past 18 months?	Number of animals slaughtered?	When was the last time?
Cattle	_____ times		
Sheep	_____ times		
Goats	_____ times		
Deer	_____ times		
Pigs	_____ times		

3.5 Outdoor exposures

3.5.1 Hunting/Trapping exposures

Have you been hunting in the **past 18 months**?

YES ☐ Complete table below

NO ☐ Skip to **Other Outdoors exposure table 3.5.2**

Animals hunted	How many shot or trapped	When was the last time?	Animal butchered (by yourself)?
Deer			
Wild pig			
Small game *			
Goats			
Other_____			

* e.g. ducks, other birds, possums, rabbits, hares…

3.5.2 Other Outdoor exposure

Over the **past 18 months**, have you done outdoor activities where you were exposed to fresh water?

YES ☐ Complete table below

NO ☐ **Skip to Flooding section 3.6**

Outdoor activities fresh water	How many times in past 18 months?	When was the last time?	Region?
Camping beside lakes/rivers	_____ times		
Water sports in lakes/rivers e.g. swimming, boating, windsurfing, endurance events	_____ times		
Fresh water fishing	_____ times		
Did you do any of these activities overseas?	No ☐ Yes ☐ If yes, specify country(ies) _____		

3.6 Flooding

Over the **past 18 months** has where you were living been flooded?

YES ☐ When the last time? (please state) _____

NO ☐ **Skip to Previous illness.**

4. Previous illness

Have you ever been diagnosed with Leptospirosis by a medical practitioner?

YES ☐ Complete **Leptospirosis table 4.1**

NO / Don't know ☐ Skip to **Other illness table 4.2**

4.1 Leptospirosis (confirmed or suspected)

Approximate date:	_____	
How was it diagnosed (multi-choice)?	Self-diagnosed ☐ GP ☐ Blood test ☐	
Do you know the serovar and/or titre?	Serovar: _____ Titre:	No/ Don't know ☐
How many days were you away from work and/or study?	_____ days	
Please describe your symptoms	Fever ☐ Headache ☐ Sore muscles/bones ☐ Sore eyes ☐ Sweating ☐ Severe debility ☐ Yellow eyes ☐ Other (please state) _____	
Treatment?	Yes ☐ No ☐ Don't know/remember ☐ Antibiotic treatment ☐ If yes, how many days: _____	

4.2 Other illness

Have you had influenza-like symptoms in the past 18 months?	Yes ☐ No ☐ Approx.date of latest episode _____	
Have you been off study/work due to this illness?	Approx. date _____ # days_____	No ☐
Did you ask for professional help?	GP ☐ Nurse ☐ Other_____	No ☐
Were any blood tests done or samples collected?	Yes ☐ No ☐ NA ☐	
Was a diagnosis made?	Yes ☐, diagnosis of _____	No ☐ Do not remember ☐
Which of the following symptoms did you experience?	Fever ☐ Headache ☐ Sore muscles ☐ Sore eyes ☐ Sweating ☐ Severe debility ☐ Coughing ☐ Sore throat ☐	

	Other (please state) _____
Treatment?	Yes ☐ No ☐ Don't know/remember ☐ Antibiotic treatment ☐ If yes, how many days: _____

- **Comments**

Thank you so much for your participating in this survey. Please feel free to comment on your experience with leptospirosis or about this questionnaire.
