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Leptospirosis prevalence and vaccination practices in South-West Victorian dairy herds.

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1. Abstract

While previous studies of Leptospira Hardjo in Victoria demonstrated a relatively high prevalence of exposure in both cattle (40%; Milner et al. 1980) and humans (22%; Sutherland 1988) these estimates need to be revised since they were made more than 30 years ago and leptospirosis vaccination programmes are now commonplace on Victorian dairy farms.

This was a cross-sectional study to estimate the prevalence of *Leptospira borgpetersenii* sv *Hardjo* and *Leptospira interrogans* sv *Pomona* in dairy herds in South-Western Victoria. Fifty-three herds were enrolled into the study. Herd managers were asked to present 15 late-lactation cows that had fertility issues (cows that had not conceived or had delayed calving to conception intervals). Furosemide 500 mg was injected into the tail vein of eligible cows and a mid-stream urine sample of the second voiding collected to increase the likelihood of sampling leptospira .At the time of each herd visit a questionnaire was administered to herd managers asking them to provide details of methods used for controlling leptospirosis, including vaccination. Urine samples were pooled at the herd level and tested for leptospira spp. using qPCR. Pooled samples were then tested individually and samples that were positive, were tested for *Leptospira Hardjo* and *Leptospira Pomona* using qPCR.

Three of the 53 pooled urine samples returned a positive result. The leptospira positive pools returned a minimum of three positive individual cow urine samples. Testing of individual cow urine samples identified an additional positive herd (with one weak positive and one inconclusive result), giving an apparent prevalence of approximately 8 (95% CI 1 to 13) leptospira-positive herds per 100 herds at risk.

Based on the 53 completed questionnaires, leptospirosis vaccination programs were non-compliant with label directions in 35 out of 52 vaccinated herds: 67 (95% CI 54 to 78) out of 100 herds that routinely vaccinate for leptospirosis were doing so incorrectly. Of the 53 herds that took part in this study, only one herd was completely unvaccinated.

Based on the findings from this study, and assuming the herds that took part in this study were an unbiased sample of the dairy herd population at risk, we estimate that close to one out of 10 dairy farms in South-Western Victoria are leptospirosis positive. While most herds are vaccinating for leptospirosis, most are doing so incorrectly. We conclude that herd managers need to be better educated regarding leptospirosis vaccination programs.

2. Declaration

This thesis comprises only the author's original work towards the Master of Veterinary Science (Clinical) except where indicated in the preface.

Due acknowledgment has been made in the text to all other material used.

The thesis is fewer than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signed,

Elke Erregger
11/06/2020

3. Preface

In each instance, all fieldwork in this thesis was conducted by the author, with some assistance from farmers, veterinary clinic staff and veterinary students. Statistical analysis was conducted by the author, with assistance from Prof. Mark Stevenson and Dr. David Beggs. All transcripts were drafted by the Author, with some modifications made on the suggestion of supervisors. The Author contributed more than 85% of the work in all cases. No work herein was carried out prior to enrolling the degree. The original work in this thesis is currently unpublished material, with Chapter 3 being accepted with minor changes by the AVJ, and Chapter 5 being prepared for submission to a suitable journal.

Chapter 3 is in minor revision following peer review to the Australian Veterinary Journal:

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- This project was also supported through an Australian Government Research Training Program Scholarship

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5. Table of Contents

6. List of Tables, Figures and Illustrations

CHAPTER 1

7. Introduction

Leptospirosis is a 'well known' disease, which is almost dismissed as a disease from the past in modern Australian farming life. It is known to still exist in third world countries, especially where humans live in close proximity to animals. Leptospirosis is a notifiable disease in Australia, in both human and veterinary medicine. The disease is often reported in humans, as it can cause serious disease. In cattle the main time of testing is, when abortions occur and are investigated, or on less common occasions, when haemorrhagic urine, "odd" mastitis or serious calf disease/deaths occur. Anecdotally, leptospirosis-associated mastitis was a common occurrence some years ago but has declined significantly within the last years.

The difficulty with leptospirosis is, that it can resemble other diseases in both humans and animals and testing for the disease proves to be still very challenging. To make it even harder, cattle are most commonly infected subclinically.

Vaccination programs for leptospirosis have become a standard procedure on most dairy farms. With the rise of combination products and an increased focus on workplace health and safety, cattle leptospirosis vaccination is even more common these days, especially on dairy farms. In Australia, cattle vaccines are often a combination of Clostridia and Leptospira vaccination. With farmers being accustomed to vaccinating for clostridial diseases, this made the adoption of vaccinating for leptospirosis easier. The cattle vaccines available in Australia contain both serovars: *L. borgpetersenii* serovar *Hardjo* and *L. interrogans* sv *Pomona*.

In Australia Leptospirosis is still present, and human cases are mainly reported in Queensland, where the majority is due to banana pickers getting infected through rodents.
In South West Victoria human cases were reported frequently in the past, but numbers have decreased significantly within the last years.

Previously most infections occurred in people working on farms or being exposed to farm animals (e.g. slaughter house). The reason for the decline of cases could lie within the rise of dairy farms using vaccination. Nowadays a higher percentage of infections can be traced back to people coming back from overseas travels. Due to the non-specific clinical symptoms cases still might be overlooked and the number of infections might still be higher than indicated.
A study (Sutherland 1988) published in 1988 showed that 22.4% of Western Victoria dairy farm residents had positive leptospirosis serum samples when being tested without showing clinical symptoms, suggesting that in humans not every infection has to become clinical.

Within this thesis, chapter two looks at the relevant literature for this study.
Chapter three, four and five investigate different aspects of the study. Chapter three looks at the prevalence of leptospirosis in South -West Victorian dairy herds and how vaccination programs have been taken up by farmers. It also attempts to investigate different risk factors.
Chapter four has a close look at laboratory methods for leptospirosis testing, especially trying to evaluate pooled urine samples for leptospirosis PCR testing.
Chapter five looks at the environmental implications for leptospirosis survival within the study area, especially after identifying a correlation between previous human cases and leptospirosis positive herds.

CHAPTER 2

8. Literature Review

8.1. Leptospirosis – the disease

Leptospirosis is caused by the bacterium Leptospira, which causes disease in animals and humans, it is a zoonosis (Adler, 2014). Leptospira are slender, helical shaped, motile spirochetes. They are 0.2-0.3µm in diameter and 20-30µm long. Leptospira are devided into pathogenic species and free-living nonpathogenic species. Pathogenic leptospira are aerobes that use long chain fatty acids or fatty alcohols as their energy and carbon source.

Leptospira were first isolated from freshwater and reported in 1914 by Wolbach and Binger, and only a year later in Japan by Inada and Ido isolated from the blood of miners (Doerfler & Böhm, 1995). Over subsequent years more and more isolates from different locations were named.
The family leptospiracea contains three genera of spirochaetes: Leptospira, Leptonema and Turneriella. Species Leptospira itself clusters into pathogenic, non-pathogenic and intermediate group.

There are two classification systems for leptospira, the antigenic classification, based on the microscopic agglutination test and dividing leptospira into large groups of serovars and the genetic classfication (Doerfler & Böhm, 1995).

The nomenclature for leptospira has changed over time, and this is especially important when reading older articles. The initial nomenclature talked about *L. interrogans* serovar Hardjo subtype hardjobovis but differentiate between hardjobovis and hardjoprajitno.

Prior to 1989 there were only two species of Leptospira reported: L. *interrogans*, containing all pathogenic leptospira strains, and L. *biflexa*, containing all saprophytic strains(Levett, 2001). Since then the phenotypic classification has been replaced by the genotypic classification, and both pathogenic and saprophytic strains now can be found within a species.

The nomenclature contains more genomspecies, and some serovars are found in more than one species, like sv Hardjo. Serovar Hardjo can be found in species L. *borgpetersenii*, which would be the only serovar found worldwide including Australia, and species L. *interrogans*, which mainly is found in the United Kingdom.

8.2. Cattle

In cattle leptospira can cause both acute and chronic disease.

Acute disease is seen as septicaemia with evidence of endotoxemia such as haemorrhages, hepatitis, nephritis, meningitis and/or agalactia. It is caused by damage to the endothelium of small vessels, leading to bleeding and migration of leptospira into tissue.

Chronic disease is characterised by iridocyclitis, abortions, stillbirth, infertility, birth of weak calves. Leptospirosis can also manifest itself in a subclinical form, recognised only by development of antibodies. Severe clinical signs of leptospirosis include fever, muscle pain, haemoglobinuria, jaundice, anaemia, anuria or other signs of acute nephritis, signs of meningeal impairment, skin rashes, photophobia and gastro-intestinal signs.

In maintenance hosts, leptospira serovars tend to cause chronic rather than acute disease, the bacteria can persist in the kidneys and sometimes genital tract for months or years(Prescott & Zuerner, 1993). Serovar Hardjo most commonly causes chronic disease in cattle, which can manifest in abortion, stillbirths or delivery of weak and premature calves. Infected but apparently healthy calves may also be born (Prescott & Zuerner, 1993).

Ellis et al showed the presence of the bacterium in the genital tract back in 1986, which has been confirmed in a recent study in Brazil, which also used PCR to detect the pathogen (Cabral Pires et al., 2018). Persistence of serovar *Hardjo* in the reproductive tract could be the reason for leptospira to cause subclinical fertility issues (G. Dhaliwal, Musser, & Anderson, 1996).
Serovar *Pomona* is a well-known cause of bovine abortion and can cause fatal haemolytic disease in calves (Chappel et al., 1989).

Studies comparing groups of vaccinated cattle against unvaccinated cattle showed a connection between leptospirosis and fertility (G. Dhaliwal et al., 1996). Vaccinated cattle showed a higher conception rate and a lower culling rate. A different article compared fertility parameters in naturally infected herds with serological levels for *Leptospira Hardjo* (G. . Dhaliwal, Murray, Dobson, Montgomery, & Ellis, 1996). Results of this and another study, which analysed fertility data of 40 years from herds (G. S. Dhaliwal, Murray, & Ellis, 1996), suggested that herds were less fertile during the year after diagnosis of infection, but that optimum fertility slowly improved thereafter. It showed significant reductions in the pregnancy rate of dairy cows with seropositive titres to *L. Hardjo* (genotype hardjobovis was isolated from 2 of the cows that had titres≥ 100), in herds known to be infected with leptospirosis.

It is important to note that on the basis of antibody titres alone it is not possible to assess chronologically when an animal was exposed to the infection, as the rate of titre decline can alter from cow to cow, but in general terms It is believed that the higher the titre the more recent exposure has been.

Dhaliwal et al. also highlighted that serological prevalence of Hardjo varied between different ages of milking cows: 1st lactation dairy heifers usually have a higher sero-prevalence than older cattle (G. . Dhaliwal et al., 1996).

Dhaliwal and colleagues showed that poor fertility was significantly higher among sero-positive milking cows in their 2nd-4th lactation, or in their 5th or later lactation.

8.3. Humans

Humans with leptospirosis can show with variable symptoms, from a mild flu like illness, Weil's disease, meningitis/meningoencephalitis, pulmonary haemorrhage with respiratory failure, and on rare occasions death (World Health Organization, 2003). It is recognised by WHO that human leptospirosis might be underdiagnosed, as it is difficult to confirm, might be mistaken for other diseases, or when mild enough might not be investigated further.

Infection with the pathogen occurs most commonly from exposure to the maintenance host or infected environment. Due to the damage of the endothelial lining of small blood vessels, which can affect all internal organs, the wide range of clinical manifestation can be observed. Infection occurs when the bacterium enters either abraded skin, wounds, or mucus membranes. In pregnant woman and only in a few cases congenital infection has been reported.

Risk groups (World Health Organization, 2003):
For humans to be infected depends on where and how they are exposed to the bacterium. This can happen either through direct contact with infected animals or indirect through contaminated environment.
In some cases it can be a work-related risk, in other cases due to outdoors activities and water leisure activities, or simple due to keeping livestock and pets.
Risk groups for leptospirosis are as follows:

- Cattle farmers: especially dairy farmers during milking time, but also cattle farmers when handling aborted materials;
- Pig farmers;
- Vegetable farmers and gardeners, that might get exposed to the bacterium through infected rodents, e.g. banana pickers in Queensland;
- Farmers might be exposed to contaminated water when working or irrigating fields;
- Veterinarians and pet or production animal holders;
- Butchers and abattoir workers;
- People involved in food preparations could be exposed to rodent contaminated surroundings, especially where hygiene is not of high standards;
- Sewer workers, due to contaminated waters;
- Miners, when being exposed to contaminated waters;
- People when herding animals like cattle;

- People living in close contact with animals that could be infected (e.g. rodent infested housing);
- Inland fisherman, fish and prawn farmers;
- children when playing in yards, mud, stagnant waters, puddles;
- People involved in leisure or recreational activities, e.g. swimming, hiking;
- Contamination of drinking water sources; and
- Laboratory staff involved in diagnosis or research activities involving leptospira

Several articles have been written about the occupational risk of leptospirosis (Waitkins, 1986, Dillingham et al., 1971, Sci 1975).
Especially Watkins showed that back in 1986 in the United Kingdom the disease became more of an issue within the farming community, and with that that serovar Hardjo was on the rise (Waitkins, 2008).

8.4. Leptospira and cattle vaccination

Circulating maternal antibodies for Leptospira may be detected in calves up to 13 weeks (Palit, Middleton, Sheers, & Basilone, 1991).

A study on the influence of maternal antibodies and age of calves on effective vaccination against *L. Hardjo* suggested that early vaccination would reduce the chance of calf-hood infection and that a booster at 6-8 months of age would ensure continued good protection (Palit et al., 1991).

Several vaccine studies showed that vaccination either prevented leptospiruria to a significant degree or, if disease developed, vaccination greatly shortened the duration of clinical signs (Alexander et al., 1989; Mackintosh, Marshall, & Broughton, 1980; J M Sanhueza, Wilson, Benschop, Collins-emerson, & Heuer, 2018). It is noted that this is based on animals who were not infected at the time of vaccination. Infected animals don't seem to show a significant reduction in leptospiruria after vaccination. (Hancock, Wilks, Kotiw, & Allen, 1984)

Studies using Leptavoid-H vaccine (a vaccine containing *L. interrogans* serovar *Hardjo*) showed that vaccination induced good cell mediated immunity and differences in the humoral response, which together provided solid protection when animals were challenged with recent field isolates of L. Hardjo, whereas natural infection appeared to produce little, if any, cell mediated immunity, hence animals succumbing to disease if rechallenged (SSAB, 1996).

Further studies supporting and considering a higher importance for cell mediated immunity were published in 2002 and 2003 (Brown et al., 2003; Naiman et al., 2002).

Though a study presented at the 10th Leptospirosis meeting in New Zealand reported that serum antibodies alone would provide full protection in calves/ cattle (Klaasen & Van Der Veen, 2017).

Interestingly vaccination titres (MAT) generally appear to be short lived, whereas titres following natural infection persist longer and at higher values (G. Dhaliwal et al., 1996). This could be used to differentiate between vaccinated and infected animals (Marshall, Broughton, & Hellstrom, 1979). However, some animals develop high vaccination titres, and despite them decreasing over time they might still persist for 6 months or longer after vaccination (Bolin & Alt, 1998).

Despite the MAT titres disappearing a short period after vaccination, animals appear to be protected against natural Hardjo challenge for some months (Mackintosh et al., 1980).

The recovery of genotypes hardjobovis and hardjoprajitno from same animals and same organs indicates that natural infection by one genotype may not always confer immunity to infection by the heterologous genotype, which suggests that dual genotype vaccine should be used (Ellis et al., 1988).

8.5. Leptospirosis in Australia

In Australia, two serovars of *Leptospira interrogans* are responsible for most bovine leptospirosis. Firstly, *L. interrogans* serovar *Pomona*, with pigs as the reservoir host and cattle a secondary host, and secondly *L borgpetersenii* serovar Hardjo for which cattle are the primary host (Chappel et al., 1989). Pomona infection was the more common until the early 1970s but since then Hardjo infections have dominated (Alexander et al., 1989).

Leptospirosis has been reported in cattle in different states in Australia (Carroll & Campbell, 1987; Peterson, 1951). Chappel et al. (1989) investigated abortions in Victoria over a period of three years and found that leptospira were not a major cause of abortions. Chappel and co-workers only identified *Leptospira* serovar Hardjobovis, whereas in Ireland there was also genotype *Leptospira* serovar Hardjoprajitno (more frequently associated with abortions and mastitis), which appears to be more pathogenic (Chappel et al., 1989).

In Australia, Leptospirosis is a notifiable disease not only in animals but also humans. In the past, especially in Victoria, a strong association between occupations in contact with livestock and leptospirosis was recorded (Swart, Wilks, Jackson, & Hayman, 1983). In 2010, 14 human cases of leptospirosis were diagnosed in Victoria, Australia. Eight of these were due to *Leptospira Hardjo* (McBryde, 2010). Ten of the 14 cases were reported as occupationally acquired; six worked as dairy farmers, two as farm-hands and two as cattle farmers.

Since the early days numbers of leptospirosis infections in humans in Victoria have decreased, whereas numbers in Queensland and New South Wales have increased (Katelaris et al., 2020; J. K. G. Smith, Young, Wilson, & Craig, 2013; S. Smith et al., 2019; http://www9.health.gov.au/cda/source/rpt_4.cfm , accessed 02/20) .

8.6. Testing for leptospirosis

Leptospiral infection are sometimes hard to diagnose, because infections are mainly subclinical and serological titres vary greatly in their peak and duration. Leptospires can be excreted in the urine for up to 18 months, often intermittently. Higher rates of renal carriage have been observed in younger cattle than older cows, but nowadays this can depend on vaccination regimes (age of animals being vaccinated, continuity of vaccination).

Blood, urine, cerebrospinal fluids and tissues samples can be used to test for leptospira. The stage of disease can be critical when it comes to deciding on a test (Picardeau, 2013). There are two stages an infected animal or human will go through, the first stage is the acute stage or septicaemia and the second stage is the immune stage. During the first stage the bacterium may be detected in the blood until 15 days after onset of clinical signs. The second stage usually occurs during the second week but can be from 4 to 30 days.

Tests that can identify the bacterium should be aimed within the first week of symptoms whereas serological tests should be used at the later stage. Choosing the right test at the right time can prove difficult and could be one of the reasons why leptospirosis is hard to diagnose.
Once the bacterium infects its host, it can persistently colonise the proximal renal tubules of the kidney (Vinetz et al., 2003), or other compartments of the body, such as the reproductive tract (Cabral Pires et al., 2018) in cattle or the aqueous humor in horses (Faber et al., 2000).

Indirect methods

The Microscopic Agglutinations Test (MAT) is the most widely used laboratory test for diagnosis, its specificity and sensitivity are high, but sensitivity declines when animals are tested a considerable time after infection (C. R. Smith, Ketterer, McGowan, & Corney, 1994). It is an indirect testing method, using live organisms as antigen, and without paired serum samples one cannot distinguish between acute active infections from a previous infection (Hamond et al., 2014). The MAT identifies serovars or serogroups, but as shown in a study performed in Thailand, might not always identify the actual serovar that is causing the concurrent disease (Smythe et al., 2009). This could be due to the fact that the MAT only can identify serogroups and serovars that are used within the test, but also might depend on the time of testing. If a single MAT is performed before antibodies are present within the blood system, older infections, if present, might be picked up and their serovar erroneously identified.

Other methods relying on antibodies include enzyme-linked immunosorbent assay (ELISA) and complement fixation test.

The ELISA, (World Health Organization, 2003) cannot differentiate between serogroups/serovars but has the benefit of being specific for either IgM and IgG antibodies (C. R. Smith et al., 1994). Several studies identified the ELISA, depending on the ELISA type, to be useful when compared with the MAT (B Adler, Cousins, Faine, & Robertson, 1982; Bajani et al., 2003; Bercovich, Taaijke, & Bokhout, 1990; Goddard & Thornton, 1991).

Direct methods

Direct methods used are dark field microscopy (not serovar specific), histological staining techniques (silver impregnation techniques, which can only detect intact organisms), microbiological culture and polymerase chain reaction (PCR) (C. R. Smith et al., 1994). Leptospira are fastidious and culture procedures are laborious and require selective media which makes them difficult to manage in a laboratory setting, thus reducing the sensitivity of the test.

PCR is easier and faster and will identify animals shedding the bacterium at the time, but requires sophisticated equipment and laboratory facilities. However it has been reported that inhibitors of PCR may be found in clinical samples like urine and have been reported as participating on the decreasing of sensitivity and specificity on this method (Hamond et al., 2014).

As little as 5-10 leptospira cells per mL of urine can be identified with PCR (Çetİnkaya et al., 2000; Gerritsen, Olyhoek, Smits, & Bokhout, 1991; Merien, Amouriaux, Perolat, Baranton, & Girons, 1992). Gerritsen et al. described several sample preparation methods to improve detection of leptospira in urine by PCR (Gerritsen et al., 1991). He discussed using transport media, larger sample sizes, preventing loss of leptospira cells during pelleting and minimizing the presence of PCR- inhibiting factors.

Loss of leptospira during pelleting can happen when leptospira die during transport and lyse (Lucchesi, Arroyo, Etcheverría, Parma, & Seijo, 2004). The smaller DNA particles might be lost with the supernatant, leading to false negative results.

8.7. Leptospirosis and the environment

Leptospira interrogans serovar Hardjo do not tend to survive long in urine, as shown in a study in Malaysia which identified that leptospira in undiluted urine only survived 0-2 hours in direct sunlight, 2-6 hours in shaded areas and 2-48 hours refrigerated at 4 degrees (Khairani-Bejo, 2004b). Survival of leptospira is also pH dependent, they prefer a neutral urinary pH between 6.2-8.0 (F. Leonard, Quinn, & Ellis, 1992).

Each serovar tends to be maintained in a specific maintenance hosts, within which the bacterium may remain chronically infected but only have mild, if any clinical effects (Doerfler & Böhm, 1995). It is still not very well understood how pathogenic leptospira survive outside their hosts, but a study published in 2004 show how *Leptospira interrogans sv.* Canicola could survive outside the host in semi-solid media for up to 347 days due to cell aggregation (Trueba, Zapata, Madrid, Cullen, & Haake, 2004).

Wildlife might also play a role in maintaining leptospira in certain regions.
Leptospira Pomona was found in Australian wombats and infected animals were found in areas where leptospirosis was diagnosed in cattle previously. This suggested that wombats may have become infected from cattle, but it was not investigated if wombats could transfer the infection to cattle (Munday & Corbould, 2013). In New Zealand a connection was found between possums being seen in dairy herds and cows shedding the bacterium, suggesting that possums might transmit the bacterium to cows or vice versa (Yupiana et al., 2017). Another study in New Zealand found that, in the same environment, the same serovars in wildlife species were found in livestock, suggesting a transmission pathway (Moinet et al., 2017).

Despite a lot of literature on leptospirosis there are still questions open that require further investigation. For once there has been no recent study on the prevalence of leptospirosis in Australia and especially Victoria, but also testing urine samples and using serovar specific PCR is relatively new.
The environmental factor still has a lot of uncertainties, regards survival of leptospira in soil, water and possible intermediate hosts.
The next chapters will investigate leptospirosis prevalence in South-West Victoria, testing methods and environmental factors that might help identify circumstances for leptospira survival within herds and areas, in more details.

CHAPTER 3

9. A cross sectional pilot study to determine the prevalence of and risk factors for Leptospirosis in South-West Victorian dairy herds

9.1. Abstract

Leptospirosis is a zoonosis, found worldwide, affecting many species of animals. We conducted a cross-sectional study to estimate the prevalence of *Leptospira borgpetersenii* sv Hardjo and *Leptospira interrogans* sv Pomona in cattle in dairy herds in South-Western Victoria, Australia. Fifty-three herds were enrolled into the study, with urine samples retrieved from 15 late-lactation cows in each herd. A questionnaire was administered to herd managers at the time of each herd visit, asking them to provide details of methods used for controlling leptospirosis, including vaccination. Urine samples were pooled at the herd level and tested for leptospira spp. using real time PCR. Urine samples from individual cows within the positive pooled samples were then tested for *Leptospira* Hardjo and *Leptospira* Pomona using qPCR.

Four of the 53 herds showed positive leptospirosis results giving an apparent prevalence of 8 (95% CI 1 to 13) leptospira-positive herds per 100 herds at risk. Based on the 53 completed questionnaires, leptospirosis vaccination programs were not compliant with label directions in 35 out of 52 vaccinated herds: 67 (95% CI 54 to 78) out of 100 herds that routinely vaccinated for leptospirosis were doing so not accordingly to label directions. One herd was completely unvaccinated.

Based on our findings, we estimate that close to one in 10 dairy farms in South-Western Victoria are likely leptospirosis positive. While most herds are vaccinating for leptospirosis, most are doing so not accordingly to label directions. We conclude that herd managers need to be better educated regarding leptospirosis vaccination programs.

9.2. Introduction

Leptospirosis has been found in almost all domestic and wild mammalian species(Ben Adler, 2014). Studies of *Leptospira borgpetersenii* sv Hardjo (L. Hardjo) and *L. interrogans* sv Pomona (L. Pomona) carried out in the 1980s in Victoria, Australia, demonstrated a relatively high prevalence of exposure in both cattle (40% for L. Hardjo and 2% for L. Pomona(Milner, Wilks, & Calvert, 1980)) and humans (22%(Sutherland, 1988)). These estimates need to be revisited since leptospirosis vaccination programs are now commonplace in Victorian dairy herds. If vaccination programs are working well, a decline in herds with cows shedding the bacterium would be expected, as would the risk for humans of contracting leptospirosis.

Dairy herd sizes in Australia have been continuously increasing and there is a trend away from family-owned farms toward corporate style farms(Beggs, Fisher, Jongman, & Hemsworth, 2015; Dibden & Cocklin, 2010). In 2017-2018, approximately 37% of farms were expecting to increase their herd sizes in the following year(Dairy Australia, 2018). With these trends, the diversity of farming staff has increased. In Australia the use of overseas workers, backpackers and holidaymakers is common, which brings people on farms that might have not been exposed to leptospira before(Jarvis & Peel, 2013).

Australian law requires farm staff to be provided a safe work environment. The reason we chose to investigate dairy cows was because people working in the dairy shed are at risk of exposure to leptospirosis and subsequent zoonotic disease, as identified in a study conducted in the UK (Waitkins, 2008), especially if cows are unvaccinated or vaccinated ineffectively. It is common for cows to urinate during the milking process, especially when being stressed and/or exposed to new people. A small drop of infected urine on mucus membranes (e.g. eyes, oral mucus membranes) or wounds/ lacerated skin could be enough to cause infection in humans. Due to cows standing at an elevation above the milker, the risk is not just via being in the direct path of expelled urine but also via urine being splashed back from the floor or other surfaces. Thus, exposure to aerosol and splashed urine cannot always be avoided.

Leptospira in cattle:

Despite leptospirosis being a notifiable disease in all Australian states, relatively little data has been published about the disease in Australia and in cattle. A possible reason for a lack of reporting the disease is the relatively low pathogenicity of L. Hardjo in cattle in Australia.

In the early 1970s mastitis cases associated with leptospirosis were recorded in Australia. The first Victorian case study(Gordon & Sci, 1977) reported udders that appeared flaccid, had no swelling or heat, but abnormal milk in all quarters. The infections could be traced back to L. Hardjo(Gordon & Sci, 1977). A study investigating causes of abortion in cattle in Victoria from the late 1980s(Chappel et al., 1989) identified a low association between aborted foetuses and leptospira, despite still showing a fairly high seroprevalence in cattle (28% for Hardjo and 12% for Pomona), suggesting a high proportion of cattle having been exposed to the bacteria with no apparent effect.

The disease was not only seen in Victorian cattle. Serovar Pomona was reported in calf leptospirosis back in 1951 in Western Australia (Peterson, 1951), and an investigation into the reproductive performance of Central Queensland beef cattle looked at the seroprevalence of L. Hardjo and Pomona(Carroll & Campbell, 1987) on high or low water containing soils.
Most of these data are outdated and more recent research can be found in New Zealand, where the pathogen is still causing a serious zoonosis risk to people working within the livestock industry(Juan M Sanhueza, Baker, Benschop, Peter, & Cord, 2020). It also has to be added that in New Zealand another strain, sv Tarassovi, has emerged(Yupiana et al., 2019), which is not covered by vaccines on the market.

In comparison to the low pathogenicity in Australia, overseas studies, especially in the United Kingdom, have shown a correlation between leptospira seropositive cows and reproductive issues (G. . Dhaliwal et al., 1996)(G. S. Dhaliwal et al., 1996).
In the UK, besides *L. borgpetersenii* sv Hardjo, a different type of Hardjo is present: *L. interrogans* serovar Hardjo subtype Hardjoprajitno(Grooms, 2006). Studies of Australian isolates have only found *L. borgpetersenii* serovar Hardjo to date("Restriction endonuclease analysis of Australian isolates of Leptospira interrogans serovar hardjo.," 1989).

The nomenclature for leptospira has changed over time and older articles often use the nomenclature of *L. interrogans* serovar Hardjo subtype hardjobovis but differentiate between hardjobovis and hardjoprajitno.
Prior to 1989 there were only two species of Leptospira reported: L. *interrogans*, containing all pathogenic leptospira strains, and L. *biflexa*, containing all saprophytic strains(Levett, 2001). Since then the phenotypic classification has been replaced by the genotypic classification, and both pathogenic and saprophytic strains now can be found within a species.
The nomenclature contains more genomspecies, and some serovars are found in more than one species, like sv Hardjo. Serovar Hardjo can be found in species L. *borgpetersenii*, which would be the

only serovar found worldwide including Australia, and species L. *interrogans*, which mainly is found in the United Kingdom.

Leptospirosis in humans:

In humans leptospirosis may be misdiagnosed because the clinical presentation can be non-specific and may overlap with other causes of acute febrile illnesses(Lau, Townell, Stephenson, & Berg, 2018). In patients with risk factors for leptospirosis, a high index of clinical suspicion is important to ensure prompt diagnosis. Given the non-specific signs of disease it is likely that the true incidence of leptospirosis in human populations in Australia is under-reported. Whilst vaccines for leptospirosis in humans do exist in other countries, they have a serovar specific antigen and are designed to protect only against homologous or closely related, but not heterologous serovars(Doerfler & Böhm, 1995). Globally a vaccine called SPIROLEPT is available, based on L. *interrogans* ss Icterohemorrhagiae(Rodriguez-gonzalez, Fillonneau, Blanchet, & Suard, 2004) , but there are other vaccines available like one in Cuba, called vax-SPIRAL, containing L.*interrogans* serogroup Canicola sv Canicola, Icterohemorrhagiae sv Copenhageni and Pomona sv Mozdok(Verma, Khanna, & Chawla, 2013). Leptospirosis vaccines are not available for use in humans in Australia.

Studies from New Zealand have shown that leptospirosis is still a high risk to occupants in the livestock industry, showing that the disease is still very present, despite widespread vaccination programs in dairy cattle against leptospirosis(Juan M Sanhueza et al., 2020),(Yupiana et al., 2019). In Victoria between 1979 and 1981, of 2516 possible cases of leptospirosis in humans, 208 cases were confirmed. The occupations of individuals affected were: 101 farmers, 44 meat workers, and 11 meat inspectors(Swart et al., 1983). Occupations were not stated for 35 individuals, and of the remaining 17, eight had obvious occupational contact with animals, but unfortunately the kind of animals was not listed. In 2010, 14 cases of leptospirosis were officially notified in Victoria, 10 of which were work associated. Six dairy farmers, two farm hands and two beef cattle farmers were affected(McBryde, 2010). It is not clear whether the decrease in the number of occupational leptospirosis diagnoses in humans in Victoria(Australian Government Department of Health, 2018) is due to a decrease in the incidence of leptospirosis in cattle (possibly associated with an increase in the number of cattle vaccinated for disease), or failure of the medical community to diagnose cases of disease (due to the similarity of the symptoms of leptospirosis with those of other diseases).

Over the recent years more human cases have been reported in both Queensland and NSW. A NSW study investigated an outbreak in raspberry workers in 2018, whereas a retrospective study of the

ICU in tropical Queensland discussed microbiological confirmed cases between 1998 and 2018. Raspberry workers were most likely being infected by rodents, whereas no information on infection was reported by the ICU. In both studies L. Hardjo was not the predominant pathogen, making it unclear how common L. Hardjo is within their cattle populations. Despite the smaller amount of human cases in Victoria, data obtained from the Victorian Government Health Department at the end of 2018, showed that 45% of all confirmed human leptospirosis cases from January 2016 until September 2018 were still likely associated with farming or farm animals (Dept Health and Human Services, Victoria pers comm, 2019). Out of all cases *L. Hardjo* was still the predominant pathogen, and the majority of *L. Hardjo* positive cases were associated with farming and farm animals.

With this background, the aim of this study was to estimate the prevalence of leptospirosis in dairy herds in South-Western Victoria, Australia. A secondary aim was to estimate the proportion of dairy herds using leptospirosis vaccination as a routine management tool and the proportion of herds that were correctly using vaccination according to all the manufacturer's recommendations.

9.3. Material and methods

Ethics approval for this study was provided by the University of Melbourne Human Research Ethics Committee (Ethics ID: 1647214.1) and the Faculty of Veterinary and Agriculture Sciences animal ethics committee (Ethics ID: 1613950.1).

The source population for this study comprised all dairy herds in South-Western Victoria, Australia that sold milk for human consumption in 2016. The eligible population comprised 220 members of the source population that were clients of a large veterinary practice based in Warrnambool, located in the centre of the study area (Figure 9.1) and which had their herd tested to confirm the presence of pregnancy following the most recent mating period. The study group comprised those members of the eligible population whose herd managers agreed to take part in the study following an invitation distributed via a practice newsletter in October 2016. Initial attempts to identifying a similar amount of vaccinated and unvaccinated herds for sampling was unsuccessful, due to the majority of clients using vaccine.

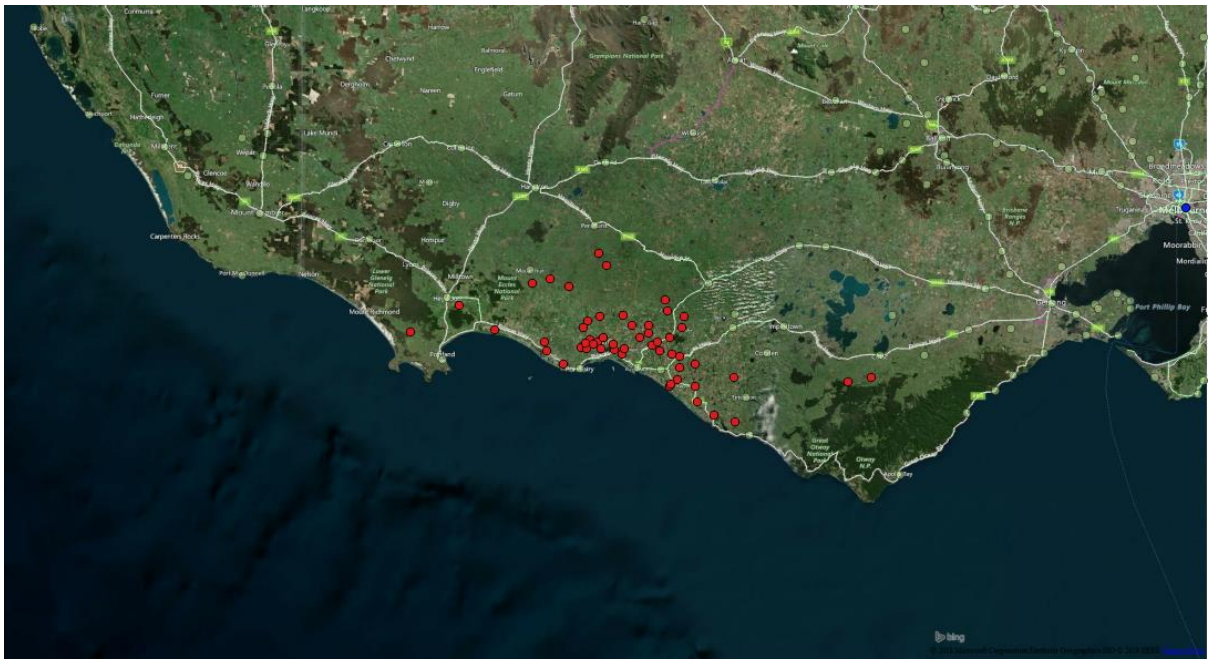

FIGURE 9.1: MAP OF SOUTH-WESTERN VICTORIA, AUSTRALIA SHOWING THE LOCATION OF STUDY FARMS. LOCATIONS HAVE BEEN JITTERED BY 1 KILOMETRE TO PRESERVE ANONYMITY.

Between November 2016 and August 2017 each of the herds enrolled into the study was visited by the first author. At the time of each herd visit a questionnaire was administered to the herd owner or herd manager. A focus of the questionnaire was to determine: (1) whether leptospirosis vaccination was routinely used; and (2) if vaccination was used, whether it was used in a way that was consistent with all of the manufacturer's recommendations. Additional details collected included the average size of the milking herd, the numbers of animals in each age class (calves, heifers and adult milking cows), the predominant breed of animals in the milking herd, the size of the effective milking area, details of the presence of other domestic animals resident on the property, presence or absence of biosecurity practices and whether or not cases of leptospirosis had been diagnosed in workers or owner-managers of the property in the past. A copy of the questionnaire is provided as supplementary material for this paper.

Sample size calculations were carried out to determine the appropriate number of cows to sample per herd to detect at least one cow with leptospirosis. We calculated required sample sizes assuming (from clinic records) that the typical size of dairy herds in South-Western Victoria ranged from 100 to 1500 cows. We designed the study to detect herds that had a within-herd prevalence of leptospirosis of 20% as it was thought this level would likely be biologically important in terms of the

possibility of zoonotic infection risk. Using a test with a diagnostic sensitivity of 95% and a diagnostic specificity of 95%, to be 95% confident that at least one cow would return a positive test if the within-herd prevalence of leptospirosis was 20%, at least 15 cows were needed to be sampled and tested from each herd.

Studies in the United Kingdom(G. . Dhaliwal et al., 1996) demonstrated a correlation between delayed calving to conception intervals and the presence of leptospirosis infection. Reduced conception rates were correlated with leptospira titres and impaired fertility was observed in the year in which infection was identified. Thus, in an effort to increase the probability of detecting leptospira-positive cows if they were present, we asked herd managers to present for sampling 15 cows that had either failed to become pregnant during the mating period or had taken longer to conceive, compared with the rest of the herd.

At the time of each herd visit cows selected for sampling were injected with furosemide (Frusemide Injection, Ilium Veterinary Products, New South Wales, Australia). This has been shown to change the osmolarity of the urine and to increase the likelihood of harvesting leptospira if they are present in the renal tubular lumen(Doerfler & Böhm, 1995). Urine from the first and second voiding post administration of furosemide was collected, but only the second voiding was used for analysis, based on findings of Nervig and Garrett (1979)(Nervig & Garrett, 1979b). Following collection, urine samples were transported in a cool box with ice blocks to the laboratory within 24 hours. Once received at the laboratory one ml of urine per sample was centrifuged at 14,000g to pellet any cellular tissue. For the pooled samples one ml urine from each cow tested per herd was filter centrifuged using a Pall Macrosep 0.2µm centrifugal filter. 60µl of PBS was added to the pellet before the sample was either run for DNA extraction or frozen to be run at a later stage for DNA extraction. Freezing and thawing of urine samples without preparation was less successful, thus cell pellets from samples arriving at the lab had to be prepared on the day of arrival.
For extraction of the DNA, Qiagen DNeasy extraction was used. With this method the DNA was much more stable, reliable and stronger.

Leptospira PCR

DNA extraction and PCR tests were undertaken at a commercial laboratory (Ace Laboratory Services, East Bendigo, 12 Gildea Lane, VIC 3550) using primers and probes described in Table 9.1, which were provided by Zoetis.

Oligo	Sequence	Dye
Pomona specific		
P7	5'- TTACAGACGGGAGAGATACAC-3'	
P8	5'- AAGGAATACGATCAGGAGTTTG-3'	
Pomona Probe	5'- TGCATCGAGATTATTTCCTTCTTGG -3'	FAM-IBFQ
Hardjo specific		
H11	5'- CCATTGTTTCCTGAAGTTGATG-3'	
H12	5'- CGATTCCTGTGGCAAAGC-3'	
Hardjo Probe	5'- TTCGAACACAAGGATTTACGCTTT -3'	FAM-IBFQ
Lepto all spp		
LipL32-45F	5'-AAG CAT TAC CGC TTG TGG TG-3'	
LipL32-286R	5'-GAA CTC CCA TTT CAG CGA TT-3'	
LipL32-Pr	5'-AA AGC CAG GAC AAG CGC CG-3'	FAM-IBFQ

TABLE 9.1: PRIMERS AND PROBES USED IN LEPTOSPIRA PCR

During DNA extraction it was noted by the lab that some Leptospira were caught in the Macrosep filter, hence the filter was swabbed firmly with a PBS wetted swab. DNA on the swab was also extracted via DNeasy and tested as a replicate to the concentrated urines for result confirmation. A positive control and a negative control were added to each PCR run to confirm specific and efficient amplification. Samples were considered positive if their Ct value was ≤ 36 . Above Ct 36 samples were called either weak positive or inconclusive. Samples were negative if no leptospira were detected after 40 cycles.

Samples were first tested at both the pooled (1 ml from each individual sample) and individual cow level for *Leptospira spp*. Positive herds were then tested with serovar specific PCR for *L. borgpetersenii* sv. Hardjo and *L. interrogans* sv. Pomona.

Statistical methods

We report the frequency of leptospira-positive herds as prevalence, expressed as the number of leptospira-positive herds per 100 herds at risk. The frequency of herd managers not vaccinating their herds according to label instructions is reported as a prevalence in the same manner. Exact confidence intervals for each prevalence estimate were calculated using the methods described by

Collett (1979)(Collett, 2003). Unconditional associations between each of the binary herd-level characteristics from the questionnaire and herd leptospirosis status were quantified using the prevalence ratio computed using the epiR package(R Core Team, 2018) in R(Collett, 2003).

9.4. Results

A total of 60 herd managers initially agreed to take part in the study. Seven of the 60 herd managers subsequently withdrew their consent to participate due to time restrictions or an inability to agree on a convenient time for the herd visit.

Survey:

Of the 53 herds that took part in the study only, one was identified as fully unvaccinated against leptospirosis. The vaccine used by the majority of farmers within the study area was Ultravac® 7 in 1 (Zoetis, Australia), others used similar vaccines such as Websters® 7 in 1 vaccine for cattle (Virbac, Australia). No use of monovalent vaccines was identified. Only 16 herd managers vaccinated their herds in complete accordance with the manufacturer's label instructions (i.e. calves were vaccinated within the first 3 months of life; the time between the initial two vaccines was 4-6 weeks; and calves that received their second injection at less than 12 weeks of age received a third vaccination up to 6 months later before going on to receive yearly boosters).

A total of 19 herds vaccinated all their stock (all age groups) but, for at least one part of the program, were not vaccinating at time intervals that were in accordance with the product label. Examples of non-compliance with label directions included: the interval between the first two vaccinations being too long; the 6-month booster not being given where calves received their initial two vaccinations at less than 12 weeks of age; calves being over 3 months of age at their initial vaccination; and not all cows receiving their 12-monthly booster vaccination. Herd managers of 17 herds stated that they vaccinated part of their herds, such as calves or calves and youngstock only.

Thirty-five of the 52 herd managers that vaccinated their herds were non-compliant in at least one aspect of the manufacturer's label instructions. There were 67 (95% CI 54 to 78) non-compliant vaccinated herds per 100 vaccinated herds at risk.

Unconditional associations between each of the binary herd-level characteristics and herd leptospirosis status are shown in Table 9.2. Of the 11 herd-level characteristics assessed, only the presence of a previous diagnosis of leptospirosis in an individual human resident on the farm was statistically significantly associated with the probability of a herd being leptospirosis positive at the time of testing. For herds where a case of leptospirosis had been diagnosed in humans, the risk of being leptospirosis positive was 12.9 (95% CI 1.5 to 111) times more than herds were no cases of human leptospirosis had been diagnosed.

PCR Assay:

Three leptospira-positive herds were identified out of the 53 herds that took part in the study. One herd had three positive samples, the other two each had five positive and one inconclusive sample (samples with a cycle threshold [Ct] value above 36 were considered weak positive or inconclusive – we chose to keep this as a separate category so as to avoid overstating or understating our results). Individual-sample testing of the remaining 50 herd samples identified an additional positive herd with one positive and one inconclusive sample. This gave a total of four positive herds out of the 53 herds that participated in the study, a prevalence of 8 (95% CI 1 to 13) leptospirosis-positive herds per 100 herds at risk.

A total of 794 individual urine samples were tested for leptospira spp, with one sample being lost during transit to the laboratory due to spillage within the esky.
Once individual-cow positive samples were identified, serovar specific PCRs were run for *Leptospira borgpetersenii* serovar Hardjo and *Leptospira interrogans* serovar Pomona. The number of cows that were positive, inconclusive and negative to Leptospirosis serovars based on PCR is shown in Table 9.3. Out of 17 positive or inconclusive individual urine samples, eight samples were positive and four samples were weak positive or inconclusive for *Leptospira borgpetersenii* serovar Hardjo. No positive samples were identified as positive for *Leptospira interrogans* serovar Pomona. Five samples were negative for both serovars.
71% of the leptospira positive individual urine samples were associated with *L. borgpetersenii* serovar *Hardjo*, containing both positive and inconclusive results.

Herd-level characteristic	Herd leptospirosis status		PR (95% CI) [a]
	Positive	Negative	
Vaccination of calves:			
Incorrect	0	4	1.7 (0.23, 12)
Correct	4	44	Reference
Vaccination of young stock:			
Incorrect	2	11	3.0 (0.47, 19)
Correct	2	37	Reference
Vaccination of adults:			
Incorrect	2	15	2.1 (0.32, 13)
Correct	2	33	Reference
Vaccination of entire herd:			
Incorrect	4	32	2.4 (0.30, 19)
Correct	0	16	Reference
Timing of vaccination:			
Incorrect	3	22	3.2 (0.36, 29)
Correct	1	26	Reference
Heifers adjusted:			
Yes	3	34	1.3 (0.15, 11)
No	1	15	Reference
Other animals present on farm:			
Yes	2	31	0.6 (0.09, 4.0)
No	2	18	Reference
Pigs present on farm:			
Yes	0	7	1.1 (0.14, 8.1)
No	4	42	Reference
Open herd:			
Yes	2	16	1.9 (0.30, 13)
No	2	33	Reference
Cattle quarantined:			
Yes	0	7	1.0 (0.13, 7.6)
No	4	39	Reference
Human cases of leptospirosis			
Yes	3	7	13 (1.5, 110)
No	1	42	Reference

TABLE 9.2: PREVALENCE OF AND RISK FACTORS FOR LEPTOSPIROSIS IN SOUTH-WESTERN VICTORIAN DAIRY HERDS, 2017. UNCONDITIONAL ASSOCIATIONS BETWEEN HERD LEPTOSPIROSIS STATUS AND ELEVEN EXPLANATORY VARIABLES.

[a] PR: prevalence ratio; CI: confidence interval.

Isolate	Cow status			Prevalence (95% CI) [a]
	Positive	Inconclusive	Negative	
L. hardjo	8	4	5	71 (44 to 90) [b]
L. pomona	0	0	17	0 (0 to 20)

TABLE 9.3: THE PREVALENCE OF AND RISK FACTORS FOR LEPTOSPIROSIS IN SOUTH-WESTERN VICTORIAN DAIRY HERDS, 2017. COUNTS OF COWS POSITIVE, INCONCLUSIVE AND NEGATIVE TO LEPTOSPIROSIS SEROVARS BASED ON PCR.

[a] CI: confidence interval.

[b]: Includes Leptospira positive and inconclusive isolates.

9.5. Discussion

Despite our attempts at the start to enrol similar numbers of vaccinated and unvaccinated herds, we found that most dairy clients in the study area used leptospirosis vaccination and thus finding unvaccinated herds proved to be difficult. Identifying partly vaccinated herds that were able to commit to testing was another restricting factor. Some of these herds were identified to be smaller and family run, with no extra help. Time limitations for selecting and collecting empty cows was another restricting factor, which lead to a smaller pool of participants. It is possible that our study population was biased toward farmers that vaccinate for leptospirosis because farmers that do not might be less likely to respond to the study invitation. If this is the case, our study would likely underestimate the true prevalence of leptospirosis positive dairy herds.

Survey

The survey focused on trying to elicit the correct information regarding vaccination regimes by asking the same question in 3 different ways.

Initially the clients were asked if they vaccinated their herds. This was followed in more detail by asking if they vaccinated calves, young-stock, heifers, cows and bulls. Thereafter, questions went into still more detail by asking when and at what age each group was vaccinated. In this way we attempted to identify issues with the vaccination program whilst attempting to confirm whether

answers had been given accurately.

Interestingly, taking out the single unvaccinated herd, we succeeded in enrolling 3 similar sized groups: correct/compliant to label-use vaccinated herds (all age groups), non-compliant vaccinated herds (all age groups) and partly-vaccinated herds. As the partly-vaccinated herds were also non-compliant vaccinated herds, these groups were merged together for analysis.

During the survey it was noticed that many farmers and their workers believed that they were vaccinating correctly, even when they did not comply fully with label directions.
We hypothesised that this may be partly because most farmers vaccinate against several different diseases using vaccines with different regimes and it is easy to make mistakes or get confused. Some clients were aware of the incorrect timing of their vaccination regime and were curious to see whether this would be reflected within their sample results. An example of a reason that some cattle were missed was on some farms non-pregnant cows missed the annual booster as farmer would only vaccinate at dry-off time and the empty cows were carried over to the next season and not dried off.

The number of farmers that identified as having had leptospirosis either themselves or in their family members in the past 25 years was not insignificant. The majority of farmers in this group vaccinated for leptospirosis due to experiencing the devastating impact the disease had on humans. We hypothesised that leptospirosis control programs would be more effective on farms where there had been previous human cases of leptospirosis, but this was not the case. It was of concern that, despite the low numbers in our study, there was still found a significant positive association between farms where there had been previous human cases and leptospiruria in the herd.

Urine Testing

Where vaccinating for leptospirosis is not common practice, serological studies could be used as being reflective of exposure to the bacterium. Serological studies nowadays might be inappropriate since vaccination of dairy herds has become a reasonably common practice. The development of 7-in-1 vaccines (clostridial diseases and 2 leptospira serovars) in Australia has led to leptospirosis vaccination being a common routine management procedure, because farmers were used to vaccinating against clostridial diseases and there is no extra work involved in also vaccinating for leptospirosis.

Previous work suggests that microscopic agglutination test (MAT) results after vaccination are rather weak and of shorter duration, whereas titres to natural infection are stronger and present over

longer periods of time(Marshall et al., 1979). It is difficult to use a MAT result to distinguish between the two because there is significant cross-over and vaccinated cattle that are subsequently exposed to the live organism can have very strong antibody responses despite being protected. Testing urine for the presence of leptospires is a much more reliable way to identify an infection(Hamond et al., 2014) although shedding of leptospirosis in cows can be intermittent, continuous for a period, or a combination of both, meaning that this will likely underestimate the true proportion of infected cows.

A study investigating the efficacy of the Australian bivalent leptospira vaccine for cattle showed that animals that are vaccinated correctly and at a young age, before being exposed to the bacterium, did not shed the pathogen(Alexander et al., 1989). However, this has not been demonstrated for all leptospirosis vaccines on the market. Preventing shedding of the bacterium should be the main aim of vaccination, as this helps to minimize the distribution of the disease to both animals and humans.

Vaccination label directions

Young animals are more typically naïve to the bacteria as they have not had as much time in which to encounter an infection through transfer of bacteria from infected older stock. In addition, the organism can be shed over a period of up to 60 weeks[20]. Thus the risk of exposure of calves is ongoing. It is important when implementing a vaccination program that cattle are protected over their lifetime to ensure that there are no 'windows of opportunity' for infection, hence the importance of compliance with vaccine label directions.

In this study we identified animals of any age with suboptimal reproductive performance going through the milking shed. This meant both first time calving cows and older animals were tested. Older animals could be at risk of infection especially on farms where only calves and young-stock were vaccinated, considering that a long-term efficacy study of commercial leptospira vaccine showed good protection of up to 55 weeks (Hancock et al., 1984). At 55 weeks only 2.7% of vaccinated heifers were found with leptospiruria compared with 58.5 % of unvaccinated heifers (although it should be noted that this study used a leptospira only vaccine, not the product in common use in our study).

Previous studies identified a correlation between reproductive problems and seropositive cows, although these studies also identified a higher fertility drop within the year of diagnosis, and that fertility recovered slowly after the introduction of leptospira (G. . Dhaliwal et al., 1996; G. S. Dhaliwal et al., 1996). Further studies have identified leptospira in the reproductive tract of cattle, which

might be another reason why fertility in cows with leptospirosis could be decreased(Bielanski, Surujballi, Thomas, & Tanaka, 1998; Cabral Pires et al., 2018; Ellis, Songer, Montgomery, & Cassells, 1986). Despite the majority of these studies being from overseas, especially the United Kingdom, we preferentially selected cows with fertility issues for sampling.

As mentioned previously, L. *borgpetersenii* sv *Hardjo* has not shown to be a major cause of abortions(Chappel et al., 1989) and not much information on reduced fertility has been reported in Australia neither. It appears that *L. borgpetersenii* sv Hardjo is less pathogenic than *L. interrogans* sv Hardjo (subtype Hardjoprajitno), which could be the reason abortions and fertility issues are more often described in UK dairies than in Australia(C. R. Smith et al., 1994).

Furosemide was used to induce urination but also to decrease the osmolarity of the urine which made the urine more favourable for survival of leptospira(Nervig & Garrett, 1979a). Initial voiding took around 10-15 minutes in this trial (from as early as 5 minutes to very rare cases of up to 30 minutes), and the time between first and second voiding was an average of 17 minutes. First voided urine was light to dark yellow depending on the cow's hydration status and temperatures on the day of collection. Second voiding recovered clear urine in all cases regardless of the colour of the urine from the first voiding.

Leptospira have a low survival time in urine, especially in more acidic urine as the bacterium prefers neutral to alkaline urine which would be the case in healthy cattle. The survival of leptospira in urine samples can be affected by sunlight, temperature and dilution of the sample. A study performed in Malaysia identified leptospirosis survival of 2-48hours in undiluted urine at 4 degrees Celcius(Khairani-Bejo, 2004a). Samples in our study were cooled and transported to the lab within 24 hours whenever possible. It is possible that leptospira might have died and lysed during the transport to the lab, and DNA could therefore be lost with the supernatant when being centrifuged (Lucchesi et al., 2004).

The testing method chosen for this project was qPCR, as PCRs are very sensitive and specific and easy to perform. PCR will not distinguish between alive or dead bacteria, which might be helpful for the case of leptospira dying during the transport and preparation process of the samples, as it will still identify leptospira positive samples.

Whilst our study has some limitations associated with sample size and the sensitivity of the tests used, these limitations are likely to mean our results are conservative when we estimate the prevalence to be10 infected herds per 100 at risk. Leptospirosis is an important zoonotic disease and further work is warranted to understand why such ahigh prevalence of leptospirosis dairy farms should exist, despite the high prevalence of leptospirosis vaccination over many years. Possible reasons include not complying with vaccine label directions as reported here but also incorrect storage of vaccine (temperature, duration) and incorrect administration/injection method/site of vaccine by farmers, which have been reported as reasons for failure of veterinary vaccines but which were not studied here.

9.6. Conclusions

Leptospira are present in South-Western Victorian dairy herds and remain a risk to farmers, their families and workers. This study suggests that almost one out of ten dairy herds may have cows shedding the bacterium, despite most farms implementing vaccination programs. Lack of compliance with vaccine label directions is one reason why prevalence remains relatively high. This suggests that further education of farmers and their workers is needed to outline the risks and deliver the message around the importance of good vaccine label compliance.

CHAPTER 4

10. PCR testing of pooled cattle urine samples as a possible method of screening dairy herds for the presence of Leptospirosis

10.1. Abstract

Leptospirosis is a bacterial infection that can be found in dairy cattle herds in Australia, even though vaccination programs have become common practice. In most cases infection is subclinical, posing a risk to humans, as testing is required to detect the presence of the bacterium in cattle herds. There are several different types of tests for leptospirosis in cattle. The MAT is regarded as the international reference test, but PCR methods become a more frequent and useful tool.

The value and efficacy of pooled PCR urine samples was investigated, for the possibility of a more cost efficient and faster laboratory testing method.
Urine of 15 cows per herd was collected, a pooled sample created and tested by real time PCR. Thereafter the test results were compared to the results of each herd after running each single sample by the same PCR method. Furthermore, positive herds were run with a serovar specific PCR, to identify L. *borgpetersenii* sv Hardjo and L. *interrogans* sv Pomona within the leptospirosis spp. positive animals.

Three herds were found to be leptospirosis positive on the pooled urine samples, and single animal samples identified one more herd. Positive pools had a minimum of three positive single animal samples, whereas the missed herd had one positive and one inconclusive animal sample. This showed that in herds with a high prevalence of leptospirosis pooled urine samples can be an easy screening tool, but with fewer positive animals or with inconclusive animals the sensitivity of the test is decreased, and leptospirosis in a herd could be missed.

10.2. Introduction

Diagnosis of leptospirosis can be undertaken by different diagnostic tests, using either blood, cerebrospinal fluids, urine or tissue samples. The stage of disease can be critical when it comes to deciding on a test (Picardeau, 2013). There are two stages an infected animal or human will go through, the first stage is the acute stage or septicaemia and the second stage is the immune stage. During the first stage the bacterium may be detected in the blood until 15 days after onset of symptoms. The second stage usually occurs during the second week but can be from 4 to 30 days. Tests that can identify the bacterium should be aimed within the first week of symptoms whereas serological tests should be used at the later stage. Choosing the right test at the right time can prove difficult and could be one of the reasons why leptospirosis is hard to diagnose.
Once a bacterium infect its host, it can persistently colonise the proximal renal tubules of the kidney (Vinetz et al., 2003), or other compartments of the body, like the reproductive tract (Cabral Pires et al., 2018) in cattle or the aqueous humor in horses (Faber et al., 2000).

Methods of testing for leptospirosis include both direct methods, demonstrating the presence of the bacterium, or indirect methods, demonstrating an immune system response.
Growing the bacterium in culture could still be seen as the gold standard, but for this live Leptospira have to get to the laboratory, and transport of Leptospira itself can be challenging depending on the medium used (e.g. urine samples (Gerritsen et al., 1991)), and culture requires a special culture medium and can take several weeks to grow (Won, 2013).
The most commonly cited reference test is the microscopic agglutination test (MAT)(World Health Organization, 2003). The MAT is based on the agglutination-lysis test developed by Martin & Pettit in 1918 and is used to determine antibody titres. Generally, the MAT is positive from10-12 days after first clinical signs, but can be as early as 5-7days, though antibiotic treatment has been shown to delay a response. It consists of two stages, one to determine the serogroup and the second to determine the serum titre. It requires a panel of live cell suspensions for the first stage.

Another test in common use, the ELISA, (World Health Organization, 2003) cannot differentiate between serogroups/serovars but has the benefit of being specific for either IgM and IgG antibodies (C. R. Smith et al., 1994). Several studies identified the ELISA, depending on the ELISA type, to be useful when compared with the MAT (B Adler et al., 1982; Bajani et al., 2003; Bercovich et al., 1990; Goddard & Thornton, 1991).

Both these tests are indirect methods, dependent on serology, and cannot easily differentiate between infection or vaccination titres, which can make interpretation in vaccinated herds difficult. A hardjo-pomona vaccination trial study done in New Zealand on 3-4 month old, serologically negative, female Friesian calves showed that the MAT response in vaccinated animals wasn't as strong or lasted as long as in naturally infected calves, which could help differentiate between the two, but would require the knowledge of when vaccinations were done (Marshall et al., 1979). The ELISA, due to the different immunoglobulins IgM and IgG, can be of use when differentiating fresh from older infections. Despite being very useful tests neither of them indicate where animals are actually shedding the bacterium.

A test involving a urine sample would seem to be sensible because it is an easy non-invasive sample to collect, and leptospires in urine are the most likely way for zoonotic spread. Thus, for our study we used urine samples. Direct methods include culturing the bacterium and dark field microscopy and PCR. Culturing/microscopy techniques need an experienced eye and silver impregnation staining methods. A limitation of this test is that it only detects intact leptospira.

PCR, on the other hand, can detect both dead or live leptospira as it detects bacterial DNA. Of all the direct methods PCR appears to be the easiest and fastest method to perform. Several studies showed that PCR is a useful tool in testing urine for leptospira and can be very accurate (Hamond et al., 2014; Hernández-Rodríguez, Díaz, Dalmau, & Quintero, 2011; Merien et al., 1992; Van Eys et al., 1989). A study looking into the effectiveness of long-term vaccination on dairy herds in New Zealand used real time PCR as one of the testing methods (Parramore, Wilson, & Hooijer, 2011). A more recent study, based on human samples, again showed the beneficial use of real time PCR of pathogenic leptospires, using the lipL32 sequence, which appears to be restricted to pathogenic leptospira (Levett et al., 2018).

In cattle herds leptospirosis does not always present clinically. Several studies showed that leptospirosis can reduce conceptions rate and herd fertility, but only rarely are signs like mastitis, sick animals and abortions seen, especially in countries like Australia, where only serovar hardjobovis has been diagnosed so far, which appears to be less virulent than hardjoprajitno (G. . Dhaliwal et al., 1996; Ellis et al., 1986).

Hence the main disease risk is not within the natural host but within accidental hosts like the human, where it can cause serious disease. Screening tests to identify the pathogen in a herd can be beneficial, as farmers can implement extra preventive measurements in herds with demonstrated infection.

Bulk milk tests for leptospirosis antibodies do exist, and this method was described in a study from Ireland where an ELISA test was used to show the exposure of dairy cattle to the pathogen (N. Leonard, Mee, Snijders, Mackie, & Elisa, 2004).

This method is only based on previous exposure to the pathogen, but again would not show which herds have cows actively shedding. This method works well in herds that are tested frequently for changes in their titres, to identify more recent infections.

As described in Chapter 3, only one out of the 53 investigated herds was completely unvaccinated. Despite the high number of farmers using leptospirosis vaccine, it was noted that most farmers vaccinated not compliant to label directions, and cows were still identified shedding the pathogen in their urine and being a risk to dairy workers. This highlights why screening of herds is of value.

For this study we aimed to determine whether real time PCR on pooled samples can be a useful tool to screen herds for leptospirosis when used on cattle urine by testing individual samples that had been combined as pools in a previous study to investigate the sensitivity of the pooled test.

10.3. Material and methods

Following on from Chapter 3 and the investigation into leptospirosis prevalence, urine was collected from 53 dairy herds, all within South-West Victoria. Herds for the project were recruited through Warrnambool Veterinary clinic and ethics approval was given by the University of Melbourne's animal ethics committee (ID 1613950.1).

Each of the 15 animals per herd were injected with 10mL of Furosemide, and midstream urine of the second voiding was collected in a 50mL pot (Nervig & Garrett, 1979a). Samples were transported in cool eskies and sent by express post to the laboratory (ACE laboratories, Bendigo) for further processing and testing.

The laboratory test used for this project was q-PCR (real time PCR). Pooled samples were tested initially, with each pool represented a single farm, using the urine from 15 individual cows. Thereafter each herd had their single animals tested with q-PCR and serovar specific PCR.

Pooled samples:

One mL each of the 15 urine samples per farm was pooled and concentrated using Pall Macrosep

0.2µm centrifugal filter. The concentrate was removed into a new 1.5mL tube.

500µL PBS was added to another Eppendorf tube. A swab was moistened in the PBS and used to gently rub both sides of the Macrosep filter to remove any lepto if present on the filter. The swab was rinsed off back into the PBS to wash off cells. This was repeated, rubbing the filter more firmly the second time around for possible semi-caught leptospira. Due to their structure spirochetes can get trapped in the filter. The swab was thereafter returned to the PBS and vortexed to release cells. Both tubes, pure concentrate and cells collected via swab, were spun and the supernatant discarded. The pellet from the swabs were tested as a replicate to the concentrated urines for result confirmation
60µL of PBS was added to both tubes and cells extracted with DNeasy either immediately or they were frozen for later extraction.

Individual samples:

One mL of each sample was suspended into a 1.5mL tube and centrifuged at 14,000g to pellet any cellular material. The supernatant was discarded and 60µL PBS added to the cell pellet. Thereafter the tubes were placed in the freezer for later analysis.

DNeasy nucleic acid extraction methods:
Optimisation trials comparing Qiagen DNeasy, Magnetic beads and lysis protocols were performed and the DNeasy extraction kit produced the best quality DNA for detecting Leptospira. The Qiagen DNeasy Blood & Tissue kit is designed to enable specific adsorption of DNA to a silica membrane (spin column) and optimal removal of contaminants and enzyme inhibitors.

To lyse the cells and release DNA:
The prepared cell pellets had a quick spin, to avoid loss of any sample. To each cell pellet 180µL ATL buffer and 20µL Proteinase K were added. This solution was incubated at 56 degrees for at least 10 minutes. For lysis 200µL of AL buffer was added and vortexed immediately. Again, the solution was incubated at 56 degrees for at least 10 minutes.

To bind the sample to the silica spin column:
200µL of 95% ethanol was added and immediately vortexed. Thereafter the entire lysate was transferred to a silica spin column. The column was spun for one minute to allow the nucleic acid to bind to the column. Following this, the column was transferred to a new collection tube and the old

tube discarded.

To wash away contaminants and inhibitors:
500µL of AW1 was added to the column and centrifuged for one minute. The column then was transferred to a new tube and the old discarded. 500µL AW2 was added and the column centrifuged at full speed for three minutes. It was made sure that no fluids stayed in the column, and the spin step was repeated if there was. The column was then transferred to a new tube.

To elute the purified DNA:
200µL of AE buffer was added to the column and incubated at room temperature for one minute. The column was placed into the centrifuge without touching the open lid and spun for one minute. The column was removed and discarded after this step and the eluate of purified nucleic acid used for PCR.

Leptospira PCR:

The initial PCR was to identify leptospira spp.. After identifying leptospira positive herds, only these would be tested with serovar specific PCR, for both L. Hardjobovis and L. Pomona.
Initially the primers and probes (Chapter 3, table 1) were trialled with a range of mastermixes available at the lab, like PathID, Agilent Brilliant II, Oasig, Universal mastermix, Bioneer, Quantitect, and AgPath.

Optimising trials compared Lepto spp PCR via Taqman (PathID) and SYBR methodologies using a range of DNA preparations and it was observed that Taqman using PathID mastermix was more sensitive than SYBR PCR. Thus, optimisation was continued for Taqman PCRs. Quanta Perfecta Multiplex qPCR ToughMix was purchased and all three amplifications were optimised with this mastermix.

Once the mastermix was optimised, samples were amplified with probes, primers and ROX as internal reference dye. A positive control and a negative control were added to each PCR run to confirm specific and efficient amplification.

Before running the research samples, serovar specific primers were run with positive samples for sv Hardjo, sv Pomona and sv. Copenhagenii, with no cross reactions being observed, and primers responding specifically to their corresponding serovars. Probes and primer sequences used for this

project can be found in the table below.

Samples were called positive if their Ct value was ≤ 36. Above Ct 36 samples were called either weak positive or inconclusive.

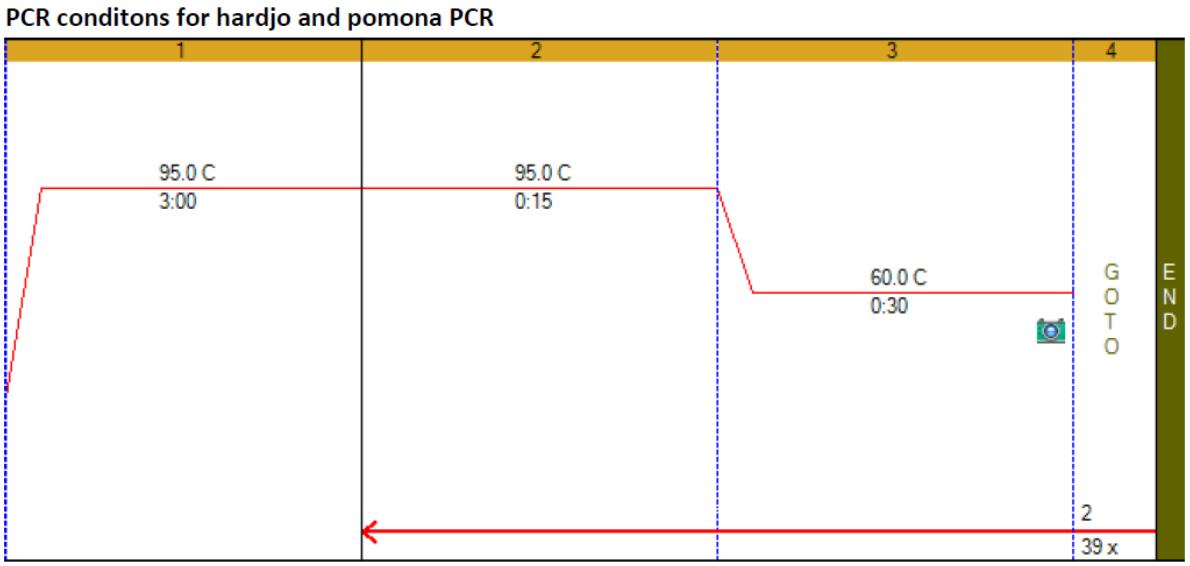

FIGURE 10.1: PCR CYCLE

10.4. Results

As already discussed in Chapter 3, of the 53 pooled samples, three samples came back positive for Leptospira spp. Of those pools a minimum of three individual animal samples came back positive, which showed that *Lepto* spp PCR was relatively sensitive, and suggests being able to detect one positive in 5 pooled urine samples.

This would mean a minimum of 20% of individual positive urine samples, so to speak herds with a moderate to high leptospirosis prevalence, would give a positive pooled urine sample.

	Lepto SPP			Lepto Pomona			Lepto Hardjo	
PCR	Run	Result	PCR	Run	Result	PCR	Run	Result
Tough	170731	ND						
Tough	170731	ND						
Tough	170731	ND						
Tough	170731	33.48	Tough	170901	ND	Tough	170901	30.6
Tough	170731	30.41	Tough	170901	ND	Tough	170901	31.0
Tough	170731	ND						
Tough	170731	27.06	Tough	170901	ND	Tough	170901	26.2
Tough	170731	ND						
Tough	170731	ND						
Tough	170731	ND						
Tough	170731	ND						
Tough	170731	ND						
Tough	170731	36.96	Tough	170901	ND	Tough	170901	36.7
Tough	170731	32.22	Tough	170901	ND	Tough	170901	31.7
Tough	170731	31.66	Tough	170901	ND	Tough	170901	30.7

TABLE 10.1: SINGLE RESULTS OF ONE POSITIVE POOL

After testing all 794 (one sample was lost during transit) single animal samples, one more herd was identified as leptospira positive. In this herd only one positive (Ct value 35.1) and one inconclusive sample (Ct value 37.1) were identified, which suggests that two weak positive urines in a sample of 15 does not contain enough target DNA.

A total of 17 single animal samples gave results to *Lepto* spp (both positive and weak positive/inconclusive), out of which 12 identified as Leptospira Hardjo. No positive samples were detected to L. Pomona.

Five samples were of unidentified serovar.

Ct values between L. spp and L. sv *Hardjo* were similar, with values for sv *Hardjo* ranging from 2.88 lower to 2.8 higher compared to the L.spp.

L. spp	L. Hardjo
33.48	30.6
30.41	31.0
27.06	26.2
36.96	36.7
32.22	31.7
31.66	30.7
37.7	38.1
35.1	37.5
25.4	27.2
29.7	ND
34	36.8
23.9	25.3
23.1	25.1
36.7	ND
33	ND
30.9	ND
24.6	ND

TABLE 10.2: CT VALUES OF LEPTOSPIROSIS SPP AND HARDJO POSITIVE AND INCONCLUSIVE ANIMALS

10.5. Discussion

Despite leptospirosis being a disease identified over 100 years ago, there appears to be no ideal testing method, but when compared with the MAT, PCR is being used more frequently.

Pooled samples are used as screening tools in many different diseases, as it is more cost efficient, cheaper to run, and less time consuming. In bulk milk samples PCR has shown to be a useful tool to identify certain mastitis pathogens. For leptospirosis only antibody tests on bulk milk exist, which will identify herds that have been exposed to the pathogen but will not identify if the bacterium is still being shed in the urine. It also does not differentiate between vaccinated and unvaccinated herds, and with more herds being vaccinated the test might not be as much of a value. Bulk milk testing is also not applicable for beef herds.

PCR on urine fits several of the criteria that are sought after for leptospirosis investigations.

Urine collection is minimal invasive, and PCR can be used to identify the bacterium as it is less time consuming compared to culture. PCR can show the presence of the bacterium, compared to serology, which is based on the immune reaction to the pathogen, but does not confirm concurrent shedding of the bacterium. It also is easy to create a pool with urine samples.

With no literature on testing pooled urine samples in cattle for leptospirosis, we attempted to see how sensitive the pooled PCR test could be, and if this could be a useful screening tool for the future.

One pooled sample out of four leptospirosis positive herds was not detected in this study, suggesting that pooling at this level might not be sufficiently sensitive. Pooling 15 samples together detected all farms where more than 3/15 individual samples were positive but failed to detect one farm where there were only two weak positive results. Thus, whilst pooling samples appears to be a useful strategy in herds with a moderate to high prevalence of leptospirosis, we have demonstrated that it is likely to miss herds with lower prevalence such as might be found in herds where vaccination is providing a limited level of protection.

A certain amount of Leptospira has to be present to be detected in a sample, and what could have happened is that by pooling samples the amount of Leptospira might have been diluted to the extent where the test is unable to pick up the DNA .

It appears that one positive per five samples will be detected, which indicates that doing smaller pools might be of a better advantage, in which case possibly the weak positives on the missed herd could have been detected too. This would still bring down costs on testing urine samples, as instead of testing 15 individuals there would be 3 pools. This way possibly the one or two weak samples might not be missed.

As discussed, the herds with positive pooled samples were strongly positive, having at least three positive single samples. Thus, pooled samples may well be worth doing, with the majority of positive herds in this study having a high prevalence of cows shedding leptospira in their urine, especially when focusing on cows with conception problems. A leptospira Hardjo vaccination trial (Ellis et al., 1986) on dairy cows showed that cows that were vaccinated had a higher conception rate and a lower culling rate. With the bacterium being identified in the uterus (Ellis et al., 1986), it is possible that the presence of the bacterium in the uterus causes the lower conception, and that cows that are carriers might be found within the empty or reduced fertility cows group.

The higher number of positive individual animals in our positive pools could suggest that herds that have a leptospirosis problem, have a higher herd prevalence than we expected. We assumed an intra-herd prevalence of 20%. If the disease is present in a herd, and young stock become infected every year either due to being vaccinated after infection or due to non-compliant vaccination intervals, there is a possible built up of cows shedding the bacterium, especially with cows shedding over prolonged periods of times. A prevalence study conducted in Spain did use a similar intra-herd prevalence with 25% (“Herd-level risk factors associated with Leptospira spp. seroprevalence in dairy and beef cattle in Spain,” 2001).

In a different study conducted in Ontario a high percentage of herds had intra-herd levels of either 0-10% or 10-20% (Prescott, Miller, Nicholson, Martin, & Lesnick, 1988). However, this was done by testing for each single serovar and not like in our study where we simply tested for Leptospira spp. Still this could mean that we should have aimed for higher sample sizes, instead of the 15 cows. In saying that, our positive herds did show a high number of individual leptospira positive urine samples. With three out of four herds having three to six positive single samples it supports the assumption that infected herds have a high intra-herd prevalence.

Within this study it was not attempted to calculate how many leptospira were in each sample, but it could be assumed that the more leptospira are within a sample the faster a reaction would be expected.

The fact that Ct values between L. spp and L. sv Hardjo varied is hard to explain. Within one herd (Table 10.1) the majority of samples had a lower Ct value for L. sv *Hardjo*., suggesting a possible better analytical sensitivity. This cannot be said for the other farms, where the sv *Hardjo* Ct value was higher, suggesting the more likely situation of lysis of Lepto post re-occurring thawing.

PCR has an advantage in that it does detect both live and dead leptospira, which can be of importance when testing samples that were in transit for up to 24 hours without any transport media. However, if the bacteria die and undergo lysis, it is possible that DNA would not be captured when centrifuged but lost in the supernatant. This effect requires further investigation. Gerritsen et al used a carrier medium to facilitate the collection of leptospiral cells, and EDTA was added to the urine sample to prevent DNAse activity during transport and storage, which was removed during the washing process. As a carrier medium L biflexa serovar patoc was used and therefore cultured in the lab. This was not an option for our project and would not be useful when considering sampling herds on a routine basis.

A study in Malaysia (Khairani-Bejo, 2004b) identified that leptospira in undiluted urine only survived 0-2 hours in direct sunlight, 2-6 hours in shaded areas and 2-48 hours refrigerated at 4 degrees. This indicates that some leptospira might have been lost during transport, as only cooling devices were used. Temperatures of 4 degrees would not have been consistent during the transport. Some of the sampling occurred late spring, summer and early autumn when higher temperatures were recorded. Despite some studies showing that as few as 5-10 leptospira per mL of urine can be detected by PCR (Çetİnkaya et al., 2000; Gerritsen et al., 1991; Merien et al., 1992), the fact that some leptospira might have degraded during transport and were lost during sample preparation might mean that less leptospira cells were present at the time of running the samples. Some cows, that are shedding the bacterium, might have gone undetected. This shows that we cannot expect PCR to pick up on single

bacteria in samples.
Another risk factor that might have caused lower positive samples could be the fact that only 1mL per sample was used. Gerritsen et al did show in his study that sample sizes of 10mL improved PCR results. The larger sample amount might mean more leptospira cells within the pellet. This indicated some leptospira positive urine could have been missed during our study, and it could be useful in both single and pooled samples.

Identifying serovars by PCR is still a recent development and has not yet been described on cattle urine in Australia. Leptospira interrogans sv Pomona and *L. borgpetersenii* sv Hardjo are currently the only two serovars present in Australian cattle vaccine, and therefore identifying the serovar could be of importance to confirm the status of the historically most common serovars within Australian cattle but also to identify if a shift away from these serovars occurs (Chappel et al., 1989; Milner et al., 1980). If different serovars became predominant, new vaccinations might need to be developed.

As mentioned, the MAT is regarded the test of choice to identify serogroups/ serovars.
A prospective study on humans in Thailand showed, that the MAT might not perform as well as generally expected (Smythe et al., 2009). Out of 106 culture positive samples only 78 were identified by the MAT, which could be due to the strains used within the test. More importantly this study showed that only in 33 percent of the cases the MAT identified the correct serovar of the infecting isolate and that in 53 percent of the cases the highest MAT titres was not the same as the infecting serovar identified by culture. This highlights that PCR could be more useful in identifying the correct serovar that is being shed during infection.

For our study it might have been of benefit to run more than just three serovars (sv Pomona, sv Hardjo, sv Copenhageni), to further qualify the specificity of the test, but as Reitstetter showed, only a few cross reactions were noticed in his study (Reitstetter, 2006). He did show that the interrogans group was split in two different groups, and sv Pomona and sv Copenhageni were using the same primer in his study, but as mentioned there was no cross reaction within our testing, when running Pomona positive samples.

There are limitations to our study. The PCR was repeated until we had confidence it would identify culture positive samples, but we still have not calculated sensitivity or specificity. Although we have some confidence that leptospirosis is present in the samples that tested positive, we are unable to know whether there were truly infected samples that were not picked up by the test, or cows which

were infected with leptospirosis but not shedding at the time of sample collection. Thus, whilst a negative pooled or individual test does not categorically rule out the presence of leptospirosis on a farm, a positive finding may be useful in alerting producers that the risk of leptospirosis zoonosis is one that needs managing carefully on their farms.

10.6. Conclusion

Despite leptospirosis being one of the most common zoonosis worldwide, laboratory testing methods to screen herds for leptospirosis are not in common use. Pooled urine PCR sampling detected all samples where more than 20% of the individual samples were positive but did not detect one sample where there were 2 weak positive samples. Pooled PCR testing may be a useful screening test for herds with a moderate to high prevalence of leptospirosis, but more work is required to understand how pooling might be most efficiently undertaken.

CHAPTER 5

11. Correlation between a wetness index and leptospirosis on dairy farms in South-West Victoria

11.1. Abstract

The aim of developing a wetness index was to investigate the correlation between the environment and the survival of leptospira in certain herds.

Vaccination programs are common practice these days on dairy herds in the South West of Victoria, as leptospirosis was a serious problem in the 80s up to the start into the new millennium. The disease was wide spread on dairy herds, highlighted by a strong seroprevalence within the cow population but also within farmers and farmworkers. Findings of a concurrent prevalence study indicate that leptospirosis is still present in an estimated 0.8 out of 10 herds, which suggests that despite vaccination programs leptospirosis is still surviving in this area of Australia.

During the analysis of possible risk factors, for which a survey was developed to collect required information, a prevalence ratio of 12.9 (95% CI 1.49-111.41; p-value: 0.003) between previous human cases and the continuous existence of leptospirosis on farms was identified. This high association appears to be suggestive of a possible environmental reservoir. Due to favour of water and moist areas for environmental leptospiral survival we developed a wetness index for this study, which provides a measure of water accumulation as a function of slope and catchment. Further we compared leptospirosis positive herds or herds with previous human cases with the wetness index of each farm and found some positive association on both, but more so on herds with previous human cases. Finally, a map was created to show areas of higher or lower wetness.

11.2. Introduction:

Leptospirosis is one of the world's most widespread zoonosis. In humans it can cause serious disease and even lead to death. Leptospirosis occurs in almost all species of mammals and in its main host does not always show any signs of disease. In such cases, animals are silent carriers and humans are unaware of the risk they are exposed to.

There are a large number of different leptospira serovars described, pathogenic, non-pathogenic and

intermediate ones (Doerfler & Böhm, 1995). Some need an animal host, others live in the environment as free-living saprophytes found in freshwater. Within animal hosts, the pathogen tends to live in the kidneys, in the renal tubules. A typical lifecycle tends to be: shedding in urine from host, persistence in ambient environment, infection of a new main or accidental host.
In cattle, Leptospira Hardjo may be present in the upper and lower reproductive tract, making this another reservoir and source of infection (Bielanski et al., 1998; Cabral Pires et al., 2018; Ellis et al., 1986).

Human leptospirosis most commonly occurs in underdeveloped countries, where humans live in close proximity to livestock, but the disease also occurs in well developed countries like New Zealand and Australia.
A recent study in New Zealand found that Leptospirosis is still a risk to farmers, their workers and workers of other livestock industries (Parramore et al., 2011). More recent studies identified a change in the type of leptospira predominantly being associated with shedding on New Zealand dairy farms (Yupiana et al., 2017).
In Australia it appears that *L. borgpetersenii* sv Hardjo is still one of, if not the main serovar found in cattle. Our concurrent study (Chapter 3) in South-West Victoria identified that 71% of the leptospirosis positive, incl. weak positive, samples were serovar Hardjo. This is a significant outcome, as cattle vaccines within Australia only cover serovar Hardjo and Pomona.

One significant finding in this recent study was that previous human cases would increase the risk of leptospirosis found within the dairy herd. Interestingly most of the human cases in this study were up to 27 years ago, with none happening in the last 5 years. This brought up the question how leptospira might sustain and survive within these areas.
There are studies showing how floods can cause leptospira outbreaks, suggesting the higher risk with excessive water amounts, already suggesting how contaminated water could be playing a role (Barcellos & Sabroza, 2005; Lau et al., 2012).
Leptospira have been proven to survive in different media for different lengths of time. They prefer an alkaline environment. A study looking at survival of pathogenic leptospira outside the host, showed that Leptospira canicola were culturable in semi-solid media for up to 347 days (Trueba et al., 2004). This was caused by cell aggregation, which was noticed in semi-solid media and could became visible within 48 hours. In distilled water, pH 7.2, leptospira showed motility for up to 98 days and was culturable for up to 110 days, whereas in 0.85% NaCl leptospira survived only two weeks. This study suggested that survival of leptospira also depends on viscosity and salt concentration of media they get into.

Another study (Andre-Fontaine, Aviat, & Thorin, 2015)could show that pathogenic leptospira, in this case L. Icterohaemorrhagiae, could survive and stay virulent in still, natural waters for up to 20 months. Interestingly in this study the bacterium still maintained virulence even with pH lower than 7.

We attempted to show how we might be able to identify risk areas for leptospirosis and show that there is a correlation between environment and the survival of leptospira.
Previous studies, like the one in Brazil investigating the correlation between floods and human leptospirosis, created maps with areas of flood risk, waste accumulation and human population compared to human leptospirosis cases. Based on this background information and the data found in the concurrent prevalence study we attempted to prove that the wetter an area is the more likely leptospira might be found in dairy cattle.
For this we created a wetness index which is measured by water catchment.

11.3. Material and Methods

For this investigation, we used data from the study described in Chapter 3.
53 dairy herds in South West Victoria, Australia, participated with both having their cow's urine tested for Leptospira and answer a survey. Clients were identified by Warrnambool Veterinary clinic and approached by both the main researcher and parts of the large animal team. Samples were collected between spring 2016 and summer 2017.
Qualifying criteria were pregnancy testing of the animals, to identify empty or late pregnant cows.

For this part of the study, a survey was answered by either farm owners or managers (Appendix D). Farm size was collected during the survey. Through urine PCR dairy farms that had cows actively shedding leptospira were identified.

Based on the development of a wetness index for Food and Mouth disease in Zambia (Hamoonga, Stevenson, Allepuz, Carpenter, & Sinkala, 2014), we created a wetness index of South West Victoria, to see whether positive herds were more common in wetter areas.

The Victorian state government has geospatial data sets online available on request. For this project the Vicmap Elevation DTM (digital terrain model) 10m and 20m was used, which is a raster

representation of Victoria's elevation with a resolution of either 10m (DTM 10m) or 20m (DTM 20m). It is based on source data of various resolution, accuracies and ages to produce an improved DTM containing increased detail in localised areas. Those DTM are hydrologically enforced and define the natural drainage and hydrological flow, suggesting how areas fill, hold and store water.
The area of interest, South West Victoria, was ordered.
Vicmap_elev_nonfloat_TIFF.zip file was extracted and, with the projection raster EPSG 3111 being used, imported into R (statistics program). Raster values that were less than 0 were set to NA. The dimension used in the end was raster cells of 20m.

A map identifying how the farms were spread across the clinic area and the local regions was created using the computer program R. Thereafter the survey was analysed, to attempt and identify risk factors, e.g. open vs closed herds, vaccination practices, other animals like pigs on the farm, and previous human cases.

To calculate the wetness index the DEM (digital elevation model) was imported into the geographic information system GRASS and r.topidx used to return a raster of TWI for the study area.
The dairy farm data were all geocoded and the wetness index of each farm was calculated by using the point location of the farm as the centroid. Thereafter around each point location a buffer was defined to reflect the farm area and summary statistics for the wetness cells that lie within that buffer calculated.
Box and whisker plots were used to compare the wetness index calculated for leptospirosis positive and negative herds, for both the animals and human side.

A wetness raster map with leptospirosis positive and negative dairy cattle herds was created by exporting the previous data into KML

11.4. Results

Farm sizes ranged from only 105 acres up to 3954 acres.

Mean	803.9623
Standard Error	94.37377
Median	620
Mode	550
Standard Deviation	687.0514
Sample Variance	472039.6
Kurtosis	8.870898
Skewness	2.726861
Range	3849
Minimum	105
Maximum	3954
Sum	42610
Count	53

TABLE 11.1 SUMMARY STATISTICS OF FARM ACREAGE

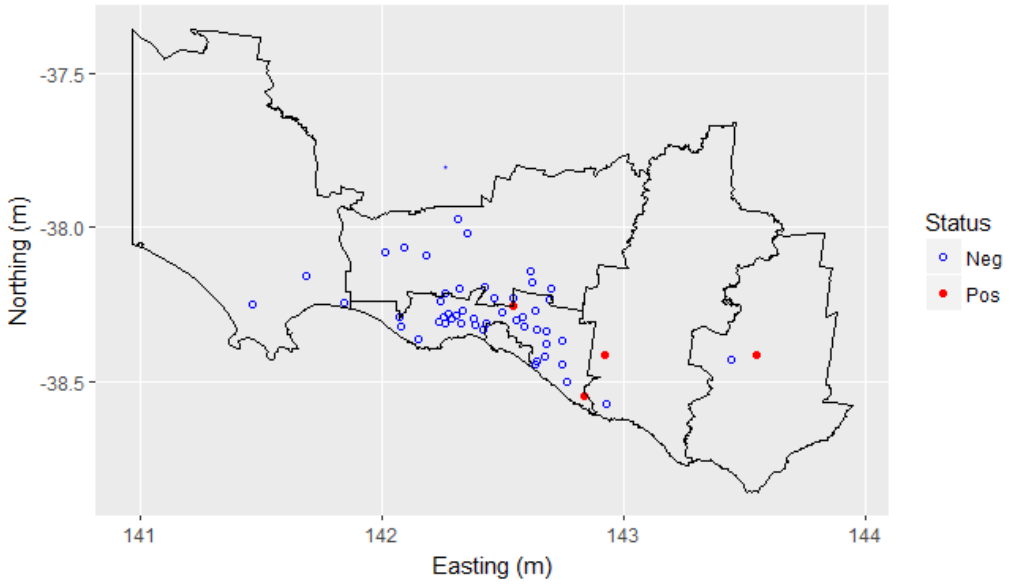

ILLUSTRATION 11.1 LEPTOSPIRA POSITIVE AND NEGATIVE FARMS BY REGIONS (SW VICTORIA)

The concurrent study identified four leptospira positive herds out of 53 dairy herds by urine PCR. Analysing the survey showed: 35 herds were run closed, whereas 18 herds were run open, only one herd was unvaccinated, 16 herds were vaccinated to label directions and 36 herds vaccinated not in accordance to label directions, only 7 herds also homed pigs on the properties.

In the survey farmers and farm managers were asked if there had been any human cases of leptospirosis in anyone associated with the farm within the past 5 years. Interestingly 10 out of 53 herds reported human cases in either the farmer or farm manager themselves or their relatives, but not within the past 5 years. Cases were reported to happen between 1983 and 2007. These cases were reported to display serious clinical disease that had to be seen by doctors and in some cases led to extended hospital stays or health implications.

Three of the four leptospira positive herds were out of the 10 herds that identified human cases, shown in the two by two table (table 2).

	Lepto+	lepto-	Total
Human cases+	3	7	10
Human cases-	1	42	43
Total	4	49	53

TABLE 11.2: THE RELATIONSHIP BETWEEN HUMAN CASES AND LEPTOSPIROSIS SHEDDING COWS/ HERDS

The relation between human cases and leptospirosis positive herds identified a prevalence ratio of leptospirosis of 12.9 in herds that experienced human cases, which was a significant finding (p = 0.003).

None of the other risk factors investigated showed significant findings.

Calculating the wetness index of all farms showed that the wettest farm identified was a leptospira positive herd.

On herd level the median wetness index of herds appears similar between leptospira positive and negative herds, but the upper and lower limits on the box and whiskers plot (Figure 10.1) show a positive correlation to the wetness level.

Farms with human leptospirosis cases have an even stronger correlation to the wetness index, as can be seen in Figure 10.1 .

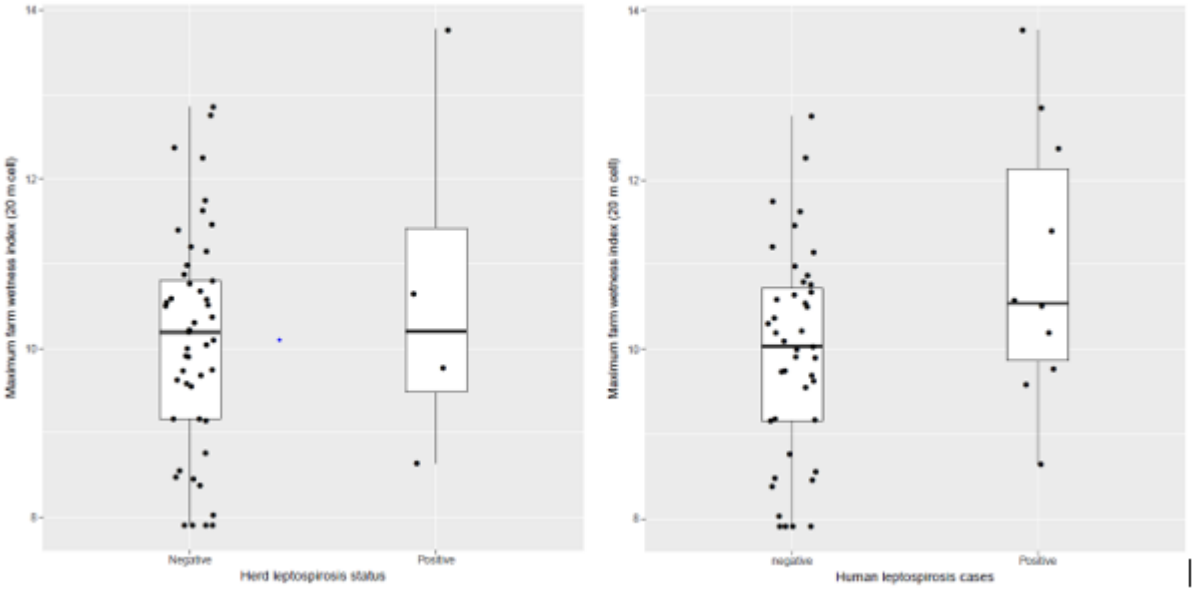

FIGURE 11.1: BOX AND WHISKER PLOTS FOR HERD LEPTOSPIROSIS CASES (L) AND HUMAN LEPTOSPIROSIS CASES (R); VERTICAL LINE SHOWS THE MAXIMUM FARM WETNESS INDEX OF EACH HERD

Results of the wetness index can finally also be visualized on the created wetness index map. The deeper the blue the wetter the area was calculated. The deepest blue is shown towards the right. Three of the four leptospirosis identified farms were situated towards the bluer areas as can be visualized on both the map and within the box and whisker plot.

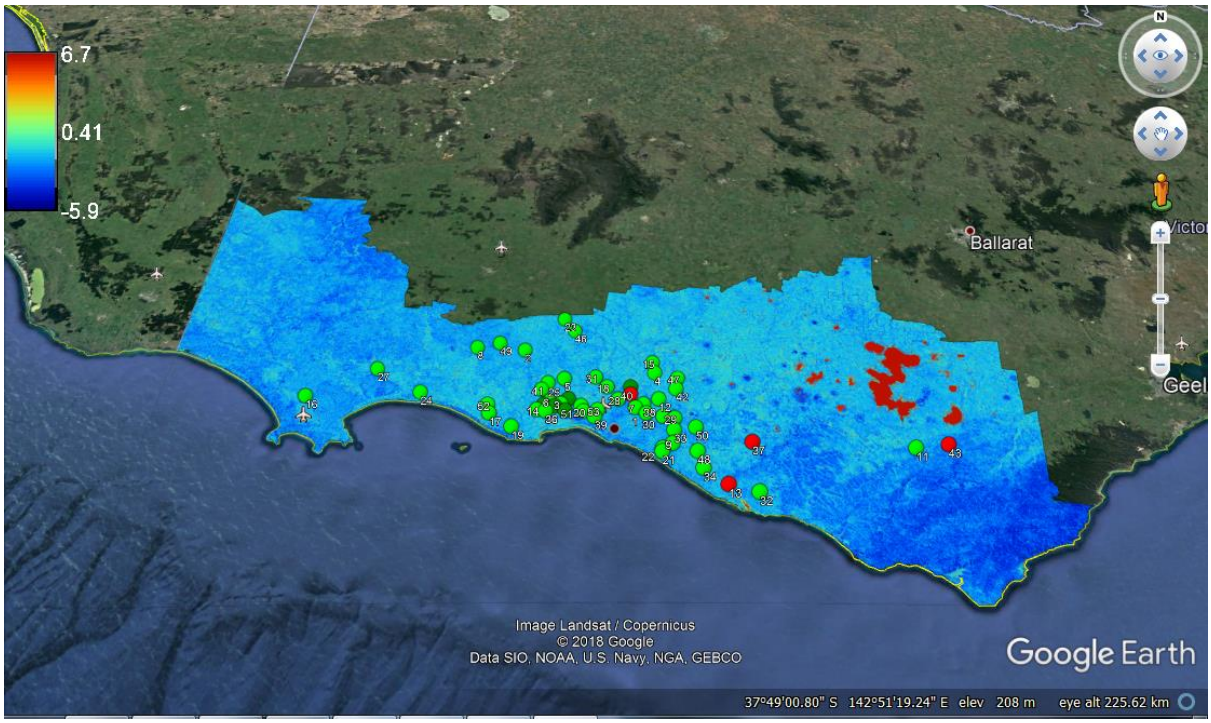

ILLUSTRATION 11.2: WETNESS INDEX MAP FOR STUDY AREA WITH LEPTOSPIROSIS POSITIVE (RED) AND NEGATIVE (GREEN) HERDS

11.5. Discussion

Data used for this investigation were of a pilot study, with a lower number of participants. This most likely is part of the reason why most suspected risk factors, like vaccinating only parts of herds or running herds open, showed no significant findings. Interestingly despite the low numbers one finding was significant, the higher risk of leptospirosis in cattle herds if human cases have been present within the farm over an extended period of time (up to 27 years ago).

The majority of farms investigated were family run herds, with only a few corporate herds. A majority of farmers have been on the same property for years, but this was unfortunately not investigated in detail, and could mean some error within the data.

Similarly, no medical certificates or other measures of proof of leptospirosis diagnosis were investigated. Though it should be said that this cases were seen by doctors and most likely affected people were given the diagnosis, as reported by some of the clients.

The fact that human cases could backdate up to 27 years and still have an effect on the farm and animals would suggest that there has to be a reservoir, keeping leptospira within this area.

Within this study we assume that the surviving factor is partly down to the environment, and

possible other intermediate hosts, keeping the pathogen within the environment. A study in Tasmania could show that wombats got infected and carried L. Pomona infections (Munday & Corbould, 2013). The most likely route of infection was from the cattle, that in this case had been shown have L. Pomona infection, but it cannot be ruled out that the infection might have been progressed in the other direction, with wombats re-infecting cattle. The study did not investigate whether wombats were shedding the bacterium with urine, and experimentally infected animals died within 14 days.

In New Zealand a study by Yupiana et al (Yupiana et al., 2017) suggested that possums may transmit the bacterium to dairy cows, shown by an increased risk of cows shedding the pathogen when possums were seen on farm.

Cattle are the main host for leptospira Hardjo and another main host, secondary to pigs, for leptospira Pomona. We still have to consider the factor that despite vaccinations cattle might still be the main reason, why the bacterium is kept within those areas. In human studies it was found that the bacterium might survive within patients, despite antibiotic treatment, and that it might be shed longer than previously assumed (years instead of months)("Detection of leptospires in urine by PCR for early diagnosis of leptospirosis," 1994). Treatments with antibiotics also have shown to not stop persistence of the bacterium in cattle kidneys back in 1985, though at the time only single injection or two injections 14 days apart were used as a treatment (Ellis, Montgomery, & Cassells, 2018).

The presence of positive human cases on each farm was based on the survey, answered by farmers and their managers. A limitation of this study is that the cases could not be officially confirmed, and we had to rely on information was given by farmers and their managers.

Additionally, it should be mentioned that leptospirosis in humans can range from a light infection to serious illness (World Health Organization, 2003). It resembles influenza virus, which can make it difficult to distinguish from other viral infections. With the drop of human cases in Victoria over the last 20 years, the disease is most likely not being tested on a frequent level by doctors. Without the right history it could easily be overlooked and remain undiagnosed. This it is likely that human cases are underestimated.

Leptospirosis in Australia is a notifiable disease. Government data showed that most human leptospirosis cases within the study area and the study period appeared to be associated with farming. In 2016 three human cases were identified within the study area, of which one patient was

a dairy farmer, another lived on a farm and the third person was a farmer/ farm manager.
2017 confirmed five human cases within the study area, of which two were dairy farmers, one worked as a dairy factory hand, another worked on a cattle farm and did earthmoving and the final patient lived and worked on a beef cattle farm.

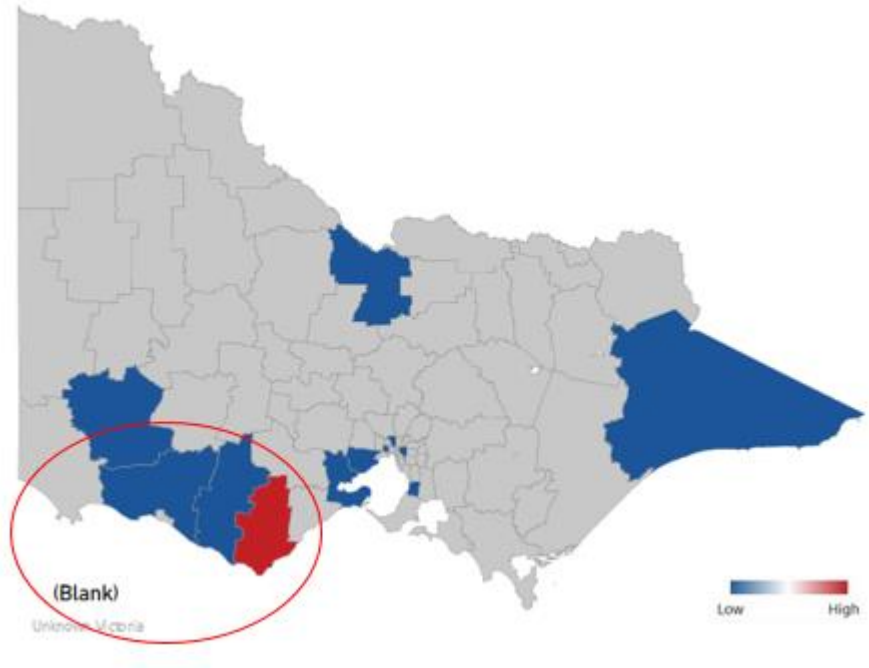

ILLUSTRATION 11.3: HUMAN CASES WITHIN THE STUDY AREA (CIRCLED RED) 2017

The above map, available to view on the Victorian government webpage, shows human cases in 2017 by regional areas. The study area is circled within this map and as can be seen more cases were found towards the wetter area (red coloured region), again supporting that wetter areas might be more susceptible to leptospirosis. Though those maps provided by the government only show where leptospirosis affected humans are located and might not be where they actually became infected. However, as noted previously, two cases were dairy farmers, suggesting that they would be living and working most likely within the same area and another case was a person living on a farm. Interestingly 2016 and 2017 were wetter years compared to the previous couple of years, as can be seen when looking at the rainfall maps provided by the Bureau of Meteorology, although going by their data the rainfall was 6% below the overall average, suggesting that some years have an even higher wetness risk.

Interestingly the areas which identified with a higher wetness index appear to also be areas where higher rainfall is measured. Rainfall, as defined by the Bureau of Meteorology is the amount of

precipitation of any type, measured in a fixed rain gauge, and therefore is different to the wetness index map, which is based on water accumulation as a function of slope and catchment.

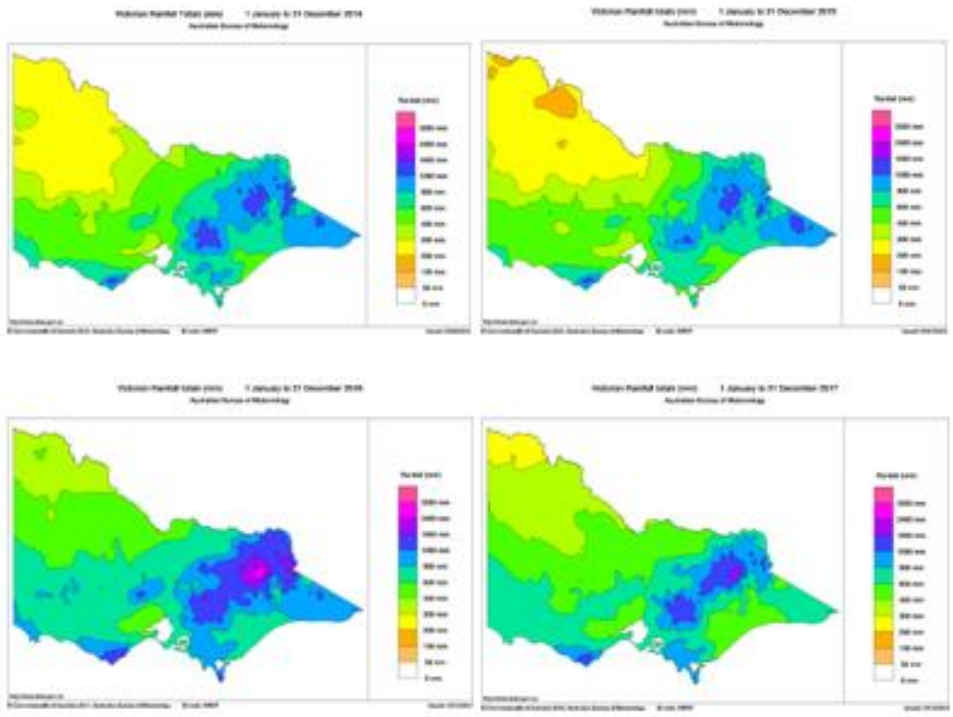

ILLUSTRATION 11.4: RAINFALL MAPS OF VICTORIA FOR YEARS 2014 TO 2017

http://www.bom.gov.au/climate/current/annual/vic/summary.shtml (accessed 20/12/2018)

The box and whisker plot shows a positive correlation between leptospirosis positive herds and the wetness index of the farm. With only a low number of herds the correlation could be going either way with a higher number of farms.

On the other hand the human cases showed a stronger positive correlation to the wetness index, with more human cases within the study herds. This could suggest that it might be the same with the dairy cattle, as wetter farms are more habitable for leptospira.

Another factor suggesting that the correlation between leptospira positive cattle and the wetness index might be heading the same direction as the one between human cases and the wetness index is the fact that most human cases between 2016/17 that were associated with farm animals or farming were two thirds due to L Hardjo.

Out of 30 confirmed human cases throughout 2016 and 2017 in all Victoria, fifteen were either travel associated or of unknown origin, whereas the other 15 were farming and farm animal associated. Out of those 15 cases 10 had titres to L. Hardjo.

It has to be mentioned again that the human cases within this study were spread over 25 years, and

most of the farms are using vaccination by now, even if not always correct to label directions. Not correct to label direction includes herds that only vaccinate parts of their herds or don't follow the recommended periods between vaccinations.
This might be the reason why less herds with cattle shedding leptospira were found within the cattle group.

The calculation of the wetness index of each farm was by using the farm address as the centre of the area and thereafter applying a buffer zone around each centroid depending on the farming acres. It should be mentioned that this obviously will not be a true measurement, but more of an approximate and suggestive measurement.
To be precise farm points would have to be measured to get a more accurate idea of the farming area. Some farming land might even be separated from the actual farm holding.

11.6. Conclusion

There appears to be a correlation between the farm wetness index and the possibility for finding leptospira in dairy herds. This study was only based on a pilot study and more data would need to be collected to make findings more significant.
The map of the study area and its wetness shows that most herds tested were in drier areas. Three of the positive herds (red spots) are towards the bluer/wetter area, as already shown.
This suggests that if further testing could be conducted, it should be concentrated on wetter areas identified by the map produced for this study.
If this shows to be a significant finding, similar maps could be used for future research into leptospirosis and to identify possible risk areas.

CHAPTER 6

12. Summary

Leptospirosis is a challenging bacterium to investigate, and not much recent literature has been published about leptospirosis in cattle, especially dairy cattle, in Australia. The infection in cows is especially important due to its zoonotic nature.

This study investigated the prevalence of leptospirosis in dairy cattle in South-West Victoria, how well vaccination regimes have been taken up, and also whether risk factors for infection of cows could be identified.

During the study it became clear that testing of leptospira still has its limits, and that there is no published information about hoe PCR of pooled urine samples in cattle might be used and interpreted.

As final part of this study a farm wetness index was created, which provides a measure of water accumulation as a function of slope and catchment, and leptospirosis positive and negative herds were compared with their wetness index.

The key findings of this project are:

Leptospira are present in South-Western Victorian dairy herds and remain a risk to farmers, their families and workers. Our work suggests that almost one out of ten dairy herds may have cows shedding the bacterium, despite most farms implementing vaccination programs.

A high lack of compliance with vaccine label directions was identified. This might be one reason why prevalence remains relatively high. This suggests that further education of farmers and their workers is needed to outline the risks and deliver the message around the importance of good vaccine label compliance.

Despite leptospirosis being one of the most common zoonosis worldwide, laboratory testing methods to screen herds for leptospirosis are not in common use. Pooled urine PCR sampling was able to detect samples where a minimum of 20% of the individual samples were positive but did not detect one sample where there were 2 weak positive samples. Pooled PCR testing may be a useful screening test for herds with a moderate to high prevalence of leptospirosis, but more work is required to understand how pooling might be most efficiently undertaken.

There appears to be a correlation between the farm wetness index and the possibility for finding leptospira in dairy herds. A limitation of our study was the small number of participating herds - further work is needed to confirm our findings. The map of the study area and its wetness shows that most herds tested were in drier areas (Illustration 11.2). Three of the positive herds (red spots) are towards the bluer/wetter area, as already shown. This suggests that if further testing could be conducted, it should be concentrated on wetter areas identified by the map produced for this study. If this shows to be a significant finding, a wetness index could be used for future research into leptospirosis and to identify possible risk areas.

Conclusion:

Despite leptospirosis being an ancient disease, further prevalence studies within Victoria and other states would be of benefit. This could be done especially using the wetness index, concentrating research on areas of higher interest/ wetter environment.

CHAPTER 7

13. References

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APPENDIX A

14. Appendix A - consent form

Project: A survey on the use of leptospirosis vaccine on SW Victorian dairy farms

Primary Researcher: Dr. Michael Pyman (Principal researcher) email: m.pyman@unimelb.edu.au

Additional Researchers:

Dr Elke Erregger (student researcher) email: e.erregger@student.unimelb.edu.au

Dr David Beggs (co-researcher)

Dr Stephen Jagoe (co-researcher)

Dr Jon Kelly (co-researcher)

Name of Participant: _____

I consent to participate in this project, the details of which have been explained to me, and I have been provided with a written plain language statement to keep.

I understand that the purpose of this research is to investigate the prevalence of leptospirosis on farms and the uptake of protective vaccination programs.

I understand that my participation in this project is for research purposes only.

I acknowledge that the possible effects of participating in this research project have been explained to my satisfaction.

In this project I will be required to answer project specific questions with a member of the research team (survey). Urine samples of cows will be collected at a later stage of the project.

I understand that my participation is voluntary and that I am free to withdraw from this project anytime without explanation or prejudice and to withdraw any unprocessed data that I have provided.

1. I understand that the data from this research will be stored at the University of Melbourne and will be destroyed after 5 years.

2. I have been informed that the confidentiality of the information I provide will be safeguarded subject to any legal requirements; my data will be password protected and accessible only by the named researchers.

3. The sample size for the study will be small. This may have implications for protecting the identity of participants, but no personal identifying information will be published.

4. I understand that after I sign and return this consent form, it will be retained by the researcher.

Participant Signature: _____ **Date:** _____

HREC Number: 1647214.1 Project Start Date: August 2016

APPENDIX B

15. Appendix B – Plain Language Statement

A survey on the use of leptospirosis vaccine on SW Victorian dairy farms

Plain Language Statement

PROJECT TEAM

A/Prof Michael Pyman (Principal Researcher) email: m.pyman@unimelb.edu.au

Dr Elke Erregger (student researcher, dairy cattle resident) email: e.erregger@student.unimelb.edu.au
Dr David Beggs (co-researcher) email: dbeggs@unimelb.edu.au

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Dr Jon Kelly (co-researcher) email: jon.kelly@wvc.com.au

With Support of:

PROJECT SUMMARY

Leptospirosis is a disease of cows that can potentially be transmitted to humans. Despite the existence of vaccines for cattle, cases still occur in dairy farmers and people handling cattle.

The aim of the project is to gather more information about the prevalence of leptospirosis in SW Victoria and the use of leptospirosis vaccine by Australian dairy farmers. We are conducting a survey to collect information on how people use leptospirosis vaccines.

The survey results will be compared with results from urine samples collected from cows, which have been pregnancy tested empty or have aborted and tested for leptospirosis. We aim to look at how the use of vaccine reduces the prevalence of leptospirosis on farm and thereby reduces the risk of infection for farmers and farmworkers. The study will seek to compare different vaccines, age of animals being vaccinated and booster intervals to see if there are important differences.

project SCOPE

The results of the project will be used to investigate the uptake, use and effectiveness of vaccines against leptospirosis.

The results will be published in the Warrnambool Veterinary Clinic newsletter, as an article in dairy industry publications and in a peer reviewed journal. We will also send a summary of the results and conclusions to people who are involved in the trial.

This survey should take about 30 minutes to complete

The project and questions have been given clearance by the University of Melbourne Human Ethics Committee.

The information collected is for the purpose of this research project only and involvement in this project is entirely voluntary.

All personal information (in the survey under general information) will be held in confidence and destroyed after the required time period.

The minimum retention period for research data and records is five years after publication, or public release, of the work of the research as stated in the University of Melbourne *Code of Conduct for Research* The Publication date for this study is February 2017.

If participants have any concerns about the conduct of the research then they can contact the Executive Officer, Human Research Ethics, The University of Melbourne, ph: 8344 2073; fax 9347 6739. They can quote the Ethics number of the project, which is: 1647214.1

APPENDIX C

16. Appendix C - Intervention Criteria Sheet

Steps:

1. *Determine the severity of abnormalities seen, using the Severity Table below.*
2. *Contact the AFM or AWO for advice if appropriate or required.*
3. *Take action in accordance with the Intervention Criteria below.*

Intervention Criteria	
Criteria are based on the severity of signs, as classified by the table below:	
Observations (based on the Severity Table below)	**Action required**
Normal (√ on monitoring sheet)	None
"mild to moderate or severe" signs (based on below)	Contact your Veterinary clinic/ vet immediately to get the animal examined. Vet will make decision on treatment or if euthanasia is required.

Severity Table	
	Mild to Moderate/ severe
Milk production	decreased
Appetite	Decreased appetite, rumen appears empty, not eating "grain" in parlour
Behaviour, Activity	Depressed, staying on her own (away from herd)
Altered respiratory pattern/depth	Showing respiratory distress (higher respiratory rate)

Other signs	Seek vet advice from the farms regular veterinarian (vet practice)

The AWO must be contacted in the case of unexpected adverse events. Necropsies must be performed on any animals that die or are humanely killed as a result of an unexpected adverse event.

The AFM must be notified of all deaths due to a cause other than humane killing.

APPENDIX D

17. Appendix E - Questionnaire

Leptospirosis Questionnaire for pilot surveillance project

Questionnaire for enrolled farms

This pilot surveillance project is designed to gather information regarding the effectiveness of different leptospirosis vaccination programs in dairy herds. While your personal details will be used in order for your veterinarian to provide you with your results, the data reported will not include any personal details apart from the general area where your farm is located. Thank-you for taking part in this survey.

1. General Information

1.1 Property

Name:_____

1.2 Contact

Person:_____

1.3 Postal Address:

_____

_____

1.4 Farm Address (if

different):_____

_____

1.5 District:_____

1.6 Phone:_____

1.7 Fax:_____

1.8 Mobile:_____

1.9

Email:_____

____

1.10 Farm Area:

(Ha)_____

1.11 Date of

Visit:_____

2. About your herd

2.1: Breed_____

2.2: When does your herd calve?_____

2.3 Numbers of cattle – Please complete the table below

Age Group	Numbers
2.3.1 Calves (0-12mths) on this farm	
2.3.2 Calves (0-12mths) on other farms	
2.3.3 Heifers (12mths -1st calf) on this farm	
2.3.4 Heifers (12mths -1st calf) on other farms	
2.3.5 Cows (24+ mths)	
2.3.6 Bulls (24+ mths)	

3. Vaccination

3.1 Are the animals vaccinated for leptospirosis? Yes ☐ No☐

If yes, please answer the following questions, if not, please go to question 3.3

3.1.1 What groups of cattle are being vaccinated? ☐ Calves

☐ **Heifers**

☐ **1st calvers**

☐ **Adult cows**

☐ **Bulls**

3.1.1 What year did the vaccination strategy for lepto start on this farm?

☐ **0-2 years**

☐ **3-5 years**

☐ **6-10 years**

☐ **10-15 years**

☐ **> 15 years**

☐ **unsure**

3.1.2 What vaccine is used for the prevention of leptospirosis?_____

3.1.3 If the adult cows are getting vaccinated, what time of year (months) are the adult cows getting vaccinated?

_____

_____

3.2 Calves/heifers

3.2.1 What age do your calves get their first vaccination?_____

3.2.2 What age do your calves get their second vaccination?_____

3.2.3 What age are they typically boosted after this?_____

3.3 Management

3.3.1 Do you send the heifers away for rearing?_____

3.3.2 Do you have other animals on your farm? Yes/No

3.3.3 If yes, what other animals?_____

3.3.4 Do you run a closed herd? Yes/No

3.3.5 If no, what animals have been introduced in the last 3 years?

2016_____

2015_____

2014_____

3.3.6 Are new animals treated with antibiotics? Yes/No

3.3.7 Are new animals held in quarantine? Yes/No

3.3.8 If so, for how long?_____

3.4 Leptospirosis history

3.4.1 Are you aware of any cases of leptospirosis on your farm in the past 5 years? Yes/No

3.4.2 Were these cases confirmed by your vet? Yes/No

3.4.3 Were these cases confirmed by laboratory tests? Yes/No

3.4.4 Have there been any cases of leptospirosis in humans in anyone associated with your farm in the last 5 years? Yes/No