Viability of Leptospira spp. in deer kidney from a New Zealand abattoir, as a model for New Zealand' wildlife

Photo: Deer farm, South Island New Zealand

M.M.Ratering Arntz#

Supervisors: Prof. P.R. Wilson*, Dr. J. Benschop*, Dr. J.M. Collins-Emerson* and Prof. P.A.M. Vos!

#Veterinary Medicine Student at University of Utrecht, The Netherlands

* Institute of Veterinary, Animal and Biomedical Sciences, Molecular Epidemiology and Veterinary Public Health Laboratory (mEpiLab) and Infectious Disease Research Centre (IDReC), Hopkirk Research Institute, Massey University, Palmerston North 4442, New Zealand

! University of Utrecht (The Netherlands), Faculty of Veterinary Medicine, Department Gezondheidszorg Landbouwhuisdieren

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Abstract

Leptospirosis is a spirochetal zoonosis and can affect all domestic animals and humans, ranging in severity from mild infections to serious systemic diseases. Leptospirosis is an important zoonosis and can have a major economic impact on livestock industries. The goal of this research is to have a more robust estimate of how long leptospirosis survives in dead animal kidney tissue and how long after death leptospiral DNA can be extracted for PCR determination and till what time leptospiral culture can be isolated.

Therefore, a cross-sectional study was carried out on slaughtered farmed red deer at a New Zealand abattoir from November 2015 to February 2016 to investigate the renal carriage rate, the viability of leptospires in dead kidney tissue, the seroprevalence and the serovar causing infection. Kidney that showed possible sign of leptospiral infection were target and blood samples were collected. Samples of 98 deer from 9 different farms were collected. The kidney samples were tested by quantitative real-time PCR (qPCR) and culture while serum samples (from coagulated blood samples) from positive animals were tested by microscopic agglutination test (MAT). The kidneys were left at ambient temperature and culture and PCR were repeated at intervals thereafter.

In total, 2 out of 98 kidney samples (2%) tested positive by qPCR. The 2 kidneys that were PCR positive on day one, were found PCR negative at day 2 (after 48h). All kidneys were found culture negative. There are no MAT results from blood samples available. The number of leptospirosis positive tested animals is too low to state firm conclusions. Either the prevalence of leptospirosis in deer at the abattoir was low or the methods of testing that were used were not suitable.

Chapter 1: Leptospirosis, a general introduction

1.1 Leptospira species

Leptospirosis is a worldwide distributed zoonosis and has emerged as a globally important infectious disease over the past decade. All mammalian species, including human, can be affected by this disease. Leptospirosis is caused by obligate aerobic spirochetes of the genus *Leptospira*. The spirochetes are Gram-negative motile helical bacteria with hook-shaped ends, about 0,1-µm in diameter and 6-20 µm in length and have an optimum growth temperature at 28-30 °C (fig. 1). The bacterium causing leptospirosis was first known as *Spirochaeta icterohaemorhagiae* but has been renamed *Leptospira* in 1971. Typically, leptospires were divided into two serological species, with most known or suspected pathogenic leptospires grouped within the "interrogans" complex. All other were placed in the "biflexa" complex. Both complexes have been divided into several serovars. This serovar classification is based on the expression of the surface-exposed epitopes in a mosaic of the lipopolysaccharide (LPS) antigens on the outer membrane [1–3]. Nowadays, a new classification of the genus Leptospira, based on DNA relatedness, is known. However, since epidemiologists and clinicians are more familiar with the serovar-classification, this new classification is not often used [4]. Leptospira is subdivided in 20 species and 300 different serovars are known nowadays. Although leptospires are found worldwide, some serovars appear to have a limited geographical distribution and therefore most serovars are associated with a particular host species since leptospirosis is a disease that shows a natural nidality [5].

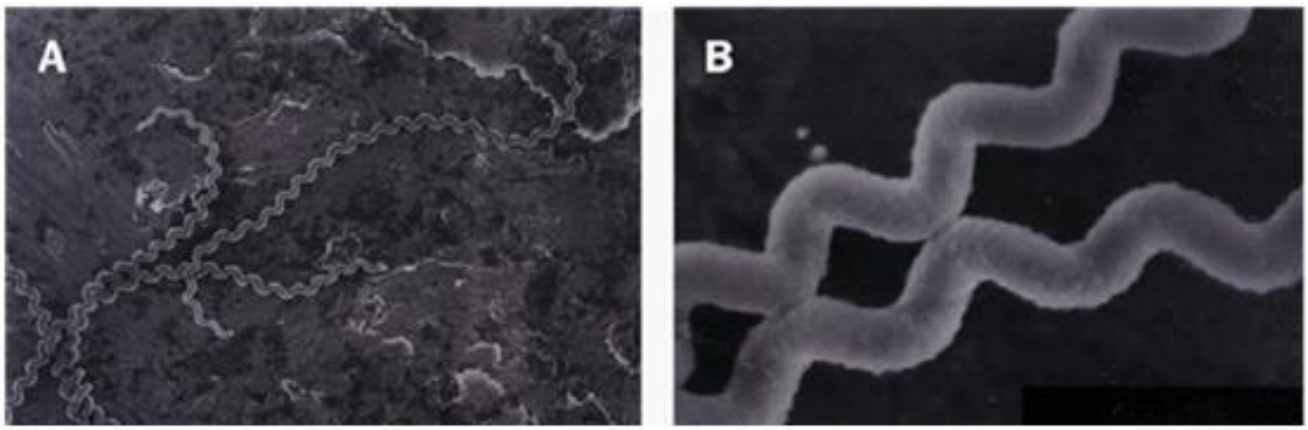

Figure 1: High-resolution scanning electron micrograph of *Leptospira interrogans* [4].

1.2 Epidemiology

The source of a Leptospirosis infection is direct contact with either urine, blood or animal tissue of infected animals, or most often, indirect by exposure to contaminated environment like water or soil. Leptospires penetrate the human body through skin lesions or mucosa of the eyes, mouth or nose or via the genital tract after contact with contaminated material [2]. Some state that it can also pass through aerosols [1]. After entry, leptospires spread rapidly and circulate in blood and lymphatics to target tissue and cause a bacteremia, which may last for a week and begins 1 or 2 days after infection. During this period, leptospires can be isolated from blood, most organs of the body and from the cerebrospinal fluid. This primary bacteremic phase ends with the appearance of circulating antibodies, which are detectable after 10–14 days. A secondary bacteremic period (after 15–26 days) has rarely been reported [5]. Figure 2 shows a schematic presentation of leptospirosis [1].

When the numbers of leptospires reach a certain level, leptospiral toxins and toxic cell components cause lesions and consequent symptoms. Once circulating antibodies appear, leptospires are removed from the circulation and tissue by opsonophagocytosis, a process by which a pathogen is marked for ingestion and eliminated by a phagocyte. The severity of a leptospirosis infection depends on each individual and the mechanisms by which leptospires cause host tissue damage, which are not yet well defined [3].

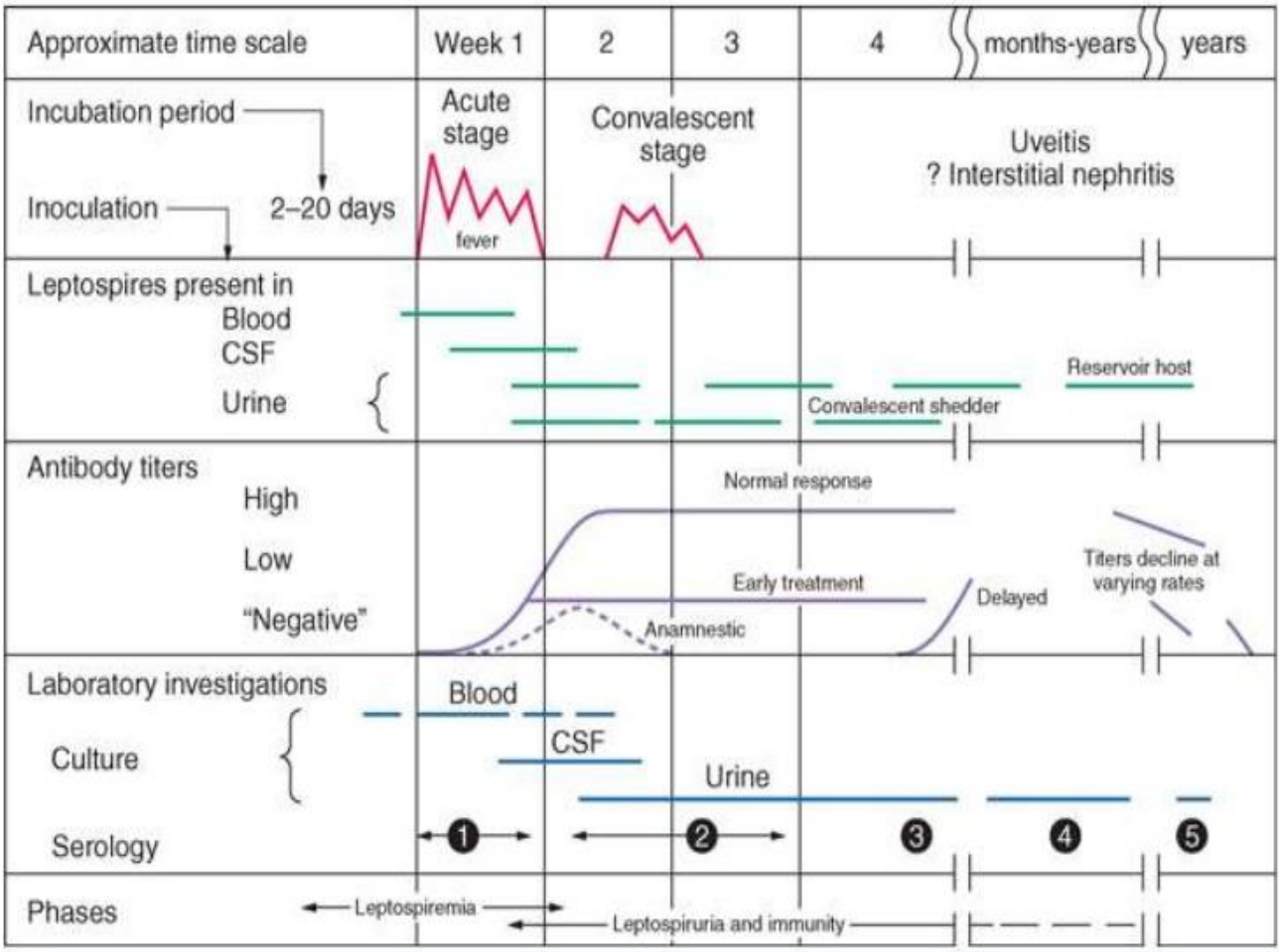

Figure 2: Schematic representation of the biphasic nature of leptospirosis and relevant diagnostic investigations at different stages of disease [1].

1.2.1 Hosts

Leptospires are found in humans and a variety of animal species, including domestic pets, livestock and a diversity of farm animals and wildlife. These mammalian hosts can be divided in two groups: accidental (incidental) hosts and maintenance hosts (carriers). Animal species can be maintenance host species of some serovars and accidental host species of others, some examples are listed in Table 1 [1,6]. Leptospirosis is maintained in nature by the persistent colonization (chronic infection) of the proximal renal tubules of carrier animals, where they are protected from antibody and other host defences [1]. The renal carrier state is a key component which is central to the persistence and epidemiology of leptospirosis. The molecular basis for this bacterial-cell association to the surfaces of renal proximal tubular epithelial cells is, however, unknown. The pathogenic urinary excretion can be intermittent or continuous and the urinary concentration of bacteria may be as high as 10^8/ml. Leptospires do not survive well in acid urine but remain viable in alkaline urine. Consequently, herbivores and animals whose diet

produces alkaline urine are relatively more important as shedders than animals that produce acid urine [3].

Maintenance hosts are highly susceptible for infection, but do not have a clinical disease. Different rodent species, mostly mice and rat, serve as the main reservoir for serovars; without showing clinical signs, they can harbor leptospires in their kidneys clinical and shed infectious organisms into their environment by urine. They will be infectious for months or even for a lifetime [7]. Wildlife plays an important role as a source of infection for humans, domestic animals and livestock. Because wildlife cannot be regulated it makes leptospirosis a disease that is hard to control [4,8]. In contrast to carriers, accidental hosts are less susceptible, but if they get infected they are more likely to develop an acute clinical disease and suffer from a serious clinical disease, which is elaborated in chapter 1.4. The renal leptospiral excretion of incidental hosts is usually of limited duration and thus does not play a key role in transmission of pathogen amongst other animals.

Serovar	Maintenance hosts	Incidental hosts
Bratislava	Pigs, hedgehogs, horses	Dogs
Canicola	Dogs	Pigs, cattle
Grippotyphosa	Rodents	Cattle, pigs, horses, dogs
Hardjo	Cattle, (sheep occasionally), deer	Humans
Icterohaemorrhagiae	Rats	Domestic animals, humans
Pomona	Pigs, cattle	Sheep, horses, dogs

Table 1: Maintenance and incidental hosts for important serovars of *Leptospira interrogans* [6]

1.2.2 Environment

Leptospires are dependent on several factors in the environment for survival; pH, temperature and the presence of inhibitory compounds. They are sensitive to heat, dryness, acids and common disinfectants. When environmental temperatures are moderate, leptospires can survive in ponds, rivers, surface waters, moist soil, mud and organ and tissue of live or dead animals for quite a long time (fig. 4)[6]. Leptospires survive longest in warm, humid conditions, the incidence is significantly higher during summer or fall [1]. Under favorable conditions, excreted leptospires remain infective in the environment up to 74 days [9]. Previous research showed that leptospiral serovar *Pomona* survived for 42 days in soil under stimulated winter conditions, 183 days in wet soil and 94 days in river water [10]. Leptospira can survive for several hours at extreme temperatures of less than -18°C and above 40°C. Besides extreme temperatures, leptospires are highly susceptible to dehydration, UV light, and UV sunlight; they survive for less than 0.2 hour when exposed to UV light, and less than 1.0 hours when exposed to UV sunlight [11].

Figure 2: Infected water road sign in Hawaii

1.3 Prevalence worldwide

Human leptospirosis occurs in diverse epidemiological settings and affects vulnerable populations. Worldwide, leptospirosis causes around 1.03 million clinical cases and 58,900 deaths each year. Mortality and morbidity are greatest in the poorest regions of the world, often observed in regions of East Africa, South/East Asia, Oceania, Caribbean, South, Central and Latin America (fig 5.) In those areas is surveillance not routinely performed. Most people infected with leptospirosis will only develop mild flu-like symptoms, therefore they usually do not seek medical attention or may be misdiagnosed resulting in under-reporting of the true leptospirosis incidence [12]. Occupations with frequent intensive contact with animals like farmers, meat workers, meat inspectors, veterinarians and rodent control workers are most at risk to develop leptospirosis[1].

Animal leptospirosis is also widespread. It has been found almost all around the world and has been detected in practically every animal species. Within the domestic species appears to be a range of susceptibility to infection. For example, horses being susceptible to a wide range of leptospiral serovars, while infection in cats is rare. Each species can develop a different set of symptoms, which are elaborated in the next chapter.

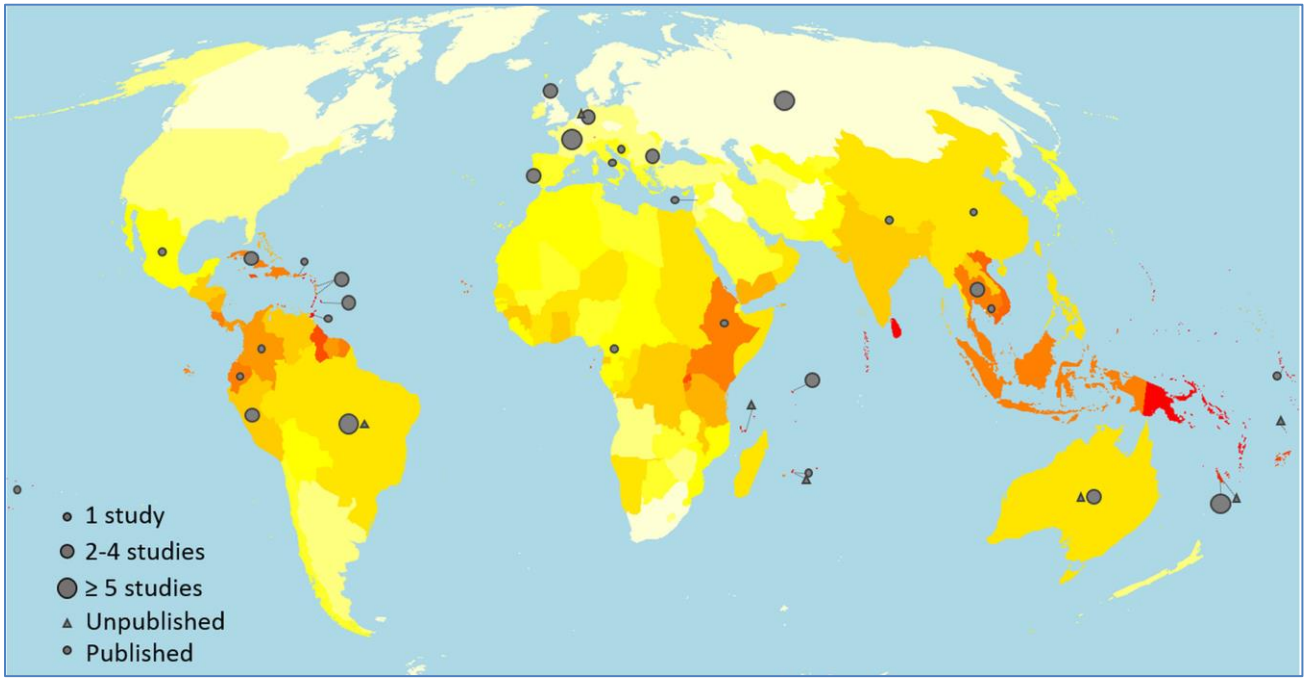

Figure 3: Estimated annual morbidity of leptospirosis by country or territory. Annual disease incidence is represented as a color gradient, from white (0-3 cases)), yellow (7–10), orange (20–25) to red (over 100 cases), per 100,000 population. Circles and triangles indicate the countries of origin for published and grey literature quality-assured studies, respectively (PLOS Neglected Tropical Disease [12])

1.4 Clinical Signs

Clinical presentation of the pathogenic Leptospira can vary strongly between species and can vary in severity according to the infecting leptospiral serovar and the age, health and immunological competence of the patient (table 2). Signs of Leptospirosis frequently include symptoms like fever, renal and hepatic failure, pulmonary hemorrhage, reproduction disorders and abortion [3]. This chapter will discuss the most often seen clinical signs per species.

Serovar	Hosts	Clinical conditions
Leptospira borgpetersenii serovar Hardjo	Cattle, sheep	Abortions, stillbirths, agalactia
L. interrogans serovar Hardjo	Humans	Influenza-like illness; occasionally liver or kidney disease
L. borgpetersenii serovar Tarassovi	Pigs	Reproductive failure, abortions, stillbirths
L. interrogans serovar Bratislava	Pigs, horses, dogs	Reproductive failure, abortions, stillbirths
L. interrogans serovar Canicola	Dogs	Acute nephritis in pups. Chronic renal disease in adult animals
	Pigs	Abortions and stillbirths. Renal disease in young pigs
L. interrogans serovar Grippotyphosa	Cattle, pigs, dogs	Septicaemic disease in young animals; abortion
L. interrogans serovar Icterohaemorrhagiae	Cattle, sheep, pigs	Acute septicaemic disease in calves, piglets and lambs; abortions
	Dogs, humans	Peracute haemorrhagic disease; acute hepatitis with jaundice
L. interrogans serovar Copenhageni	Domestic animals and humans	Peracute and acute disease; abortion in animals
L. interrogans serovar Pomona	Cattle, sheep	Acute haemolytic disease in calves and lambs; abortions
	Pigs	Reproductive failure; septicaemia in piglets
	Horses	Abortions, periodic ophthalmia

Table 2: Serovars of *Leptospira* which can cause leptospirosis in domestic animals [6].

1.4.1 Humans

In contrast to mammals, human suffer more often from acute infection and almost never become chronic carriers [3]. The clinical presentation ranges from a mild influenza-like biphasic illness to a very severe infection with renal and hepatic failure, pulmonary distress and can even lead to death. The type of serovar depends the severity of disease; *Hardjo* causes usually mild disease; whereas *Copenhageni* or *Australis* are more commonly associated with severe disease. The incubation period in humans is usually between 5–14 days but can range from 2–30 days. The most often seen predominant early clinical features are sudden emergence of headache, muscle pain and tenderness, fever, rigors, nausea, conjunctival suffusion, transient skin and mucosal rash, photophobia and other signs of meningism. However, most people infected by Leptospira will only develop mild fever and flu-like symptoms. In more severe cases, the disease will start with moderate fever where after symptoms such as general malaise, chills, headache, muscle pain, weakness, pneumonia, anemia, conjunctival suffusion and photophobia develop. Young patients may develop aseptic meningitis. The most severe form of leptospirosis is called Weil's syndrome. Symptoms include jaundice and acute renal failure[1]. The disease usually lasts for two to three weeks, however a long recovery is followed afterwards. Around 90% of the cases is self-cure after a period of 3 or more months. However, currently research shows that there might be a recurrent effect of Leptospirosis in human [13,14].

1.4.2 Animals

Mammals can be both acute and chronic carriers of leptospirosis, and there are great variations in symptoms between species.

1.4.2.1 Wildlife

Feral and wild animals, which roam on and near farms, play often the most important key role in infecting other individuals, acting as the most substantial transfer host of leptospirosis. They are the most important maintenance host for different serovars and, once infected, they do not show clinical signs. They are chronic carriers of Leptospirosis that do not suffer of any clinical symptoms themselves, but harbour virulent leptospires in their renal tubules meaning they will excrete infectious leptospires in the urine in enormous numbers for a long period of time, in that way infecting others in a direct or indirect way via the environment.

1.4.2.2 Livestock

In livestock, leptospirosis is a serious problem. Leptospirosis is more often severe in younger than in older animals. It is a highly invasive, virulent and is often a fatal disease in young animals [5]. Signs of leptospirosis can include mastitis, reproductive failure, abortion, stillbirth, foetal mummification, weak newborns and agalactia [3]. Lower growth rates and macroscopic renal lesions can be seen, even when animals clinically recovered from Leptospirosis [11]. Also, clinically recovered animals may become asymptomatic chronic carriers and will excrete infectious leptospires in their urine. In cattle, shedding periods average about thirty days, but can exceed to one hundred days. Sheep may void leptospires for at least nine months. On a dense populated farm, they can easily infect other animals on farm or contaminate water and soil during this period.

1.4.2.4 Horses

Although prevalence of a leptospiral infection is common in horses, clinical disease is not frequent. However rarely, signs include abortion in mares and renal disease in young horses can been seen. Also, equine recurrent uveitis (ERU) (periodic ophthalmia, moon blindness) can be seen as a chronic manifestation of leptospirosis in horses [3,6].

1.4.2.3 Domestic pets

Figure 6: Direct contact between animals

Four syndromes have been identified in dogs; icteric (Weil's syndrome), haemorrhagic, uremic (Stuttgart disease) and reproductive (abortion and premature or weak pups) disease. In dogs, typical leptospirosis may present with fever, jaundice, vomiting and diarrhea. There can also develop intravascular disseminated coagulation, haemorrhages and uraemia caused by renal failure. Leptospirosis can occasionally lead to death [3]. Infection is most common in male dogs aged 4 to 7 years with hunting dogs more at risk [6]. They can get infected by direct or indirect contact with leptospiral contaminated material (fig 6).

1.5 Pathology and histology

Pathologic lesions of leptospirosis are similar in animals and humans, commonly marked in the lung, liver, heart and kidney, but can differentiate between species and state of infection. The differences in pathology findings are dependent on the type of serovar, the immune state of the host and the chronic-carrier state [15]. Damage of the small blood vessel-endothelium lead to

localized ischemia in organs and is believed to be caused by the toxins produced by leptospires. At histology, this localized ischemia is defined by the occurrence of vasculitis (fig. 7) and inflammatory infiltrates composed of plasma-cells, histiocytes, monocytes and neutrophils. The vascular changes lead to ischemic damage and consequent necrosis of target organs, mostly seen in liver, lung, kidney, muscle, brain and placenta [11]. Tissue damage may be reversible and followed by complete repair (e.g. kidney, liver), although long lasting damage (e.g. myocarditis) may be a complication and leads to scarring, well recognized in kidneys, where it may be observed macroscopically as "white spots" (fig. 8). Those histopathological lesions in the kidney are typical of leptospirosis. Cortical cellular necrosis, petechiae and ecchymotic hemorrhages particularly in the glomeruli and the proximal tubuli are often seen, along with infiltration of mononuclear cells (neutrophils and monocytes), interstitial nephritis and fibrosis [15]. At immunohistopathology of infected individuals, leptospires stained with specific antiserum (arrow) are seen lining the proximal renal tubules [3] (fig. 9). The brush borders of the tubular cells are affected, the basement membrane is thickened and there is less mitochondrial activity [1]. In the liver, vacuolation, centrilobular necrosis and retention of bile occurs. This will damage hepatic tissue[15]. Besides nephritis and hepatitis, the most common pathologic findings of leptospirosis are jaundice, edema and pulmonary hemorrhage (fig. 10), meningitis and encephalopathy. Also, placentitis, stillbirth, abortion, interstitial myocarditis and acute muscle tenderness are seen [1,15]. There is usually a mild granulocytosis and splenomegaly [3] .

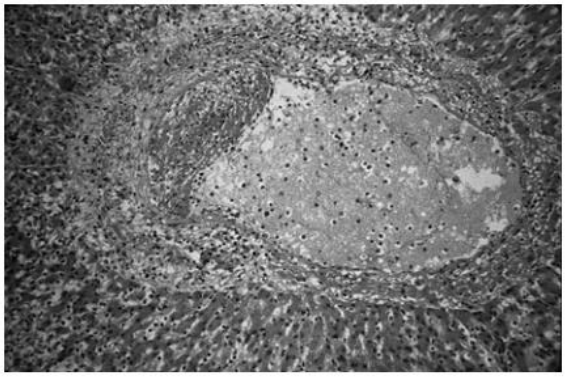

Figure 4: Vasculitis in a horse which died of acute leptospirosis [5].

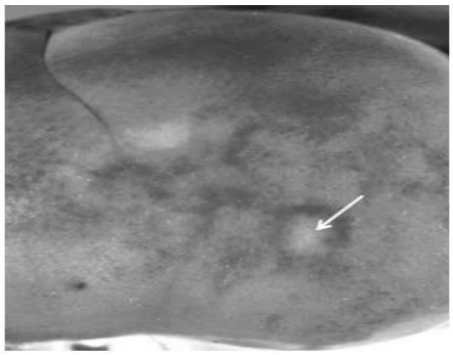

Figure 5: Focal pale lesion surrounded by hyperaemia on a serovar Hardjo infected Bovine kidney [5]

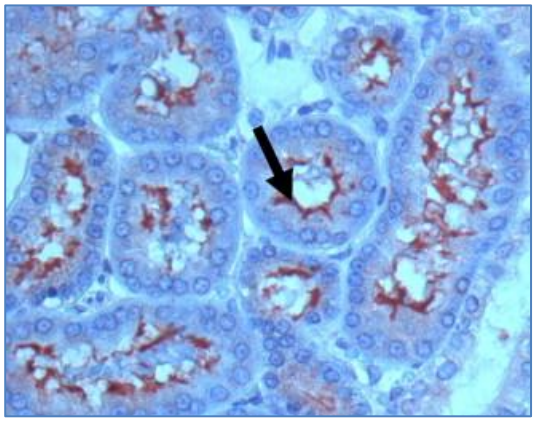

Figure 9: Immunohistopathology of infected hamster kidney. Leptospires stained with specific antiserum (arrow) are seen lining the proximal renal tubules [3].

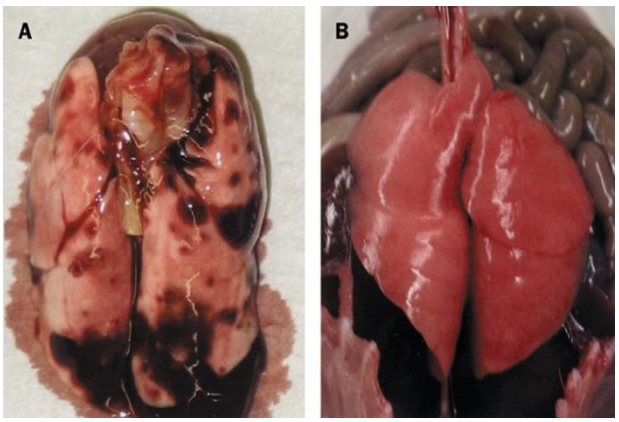

Figure 10: (A) Pulmonary haemorrhage in a guineapig infected with a strain of L interrogans serovar copenhageni obtained from a Brazilian patient with pulmonary haemorrhage.(B) Lungs from a normal guineapig are shown at right for comparison [4].

1.6 Diagnosis

Diagnosis of leptospirosis based on symptoms is difficult because the disease has a wide diversity of clinical signs. Leptospirosis diagnosis depends therefore upon a variety of laboratory assays. The most often used laboratory diagnosing tests are culture, PCR and detection of anti-leptospiral antibodies in the patient's blood (serology). Figure 11 lists the most appropriate diagnostic procedures to the stage of infection [5]. The golden standard for serological diagnosis is the microscopic agglutination test (MAT), because this test has a high specificity and can test serovar-specific antibodies. Other techniques, like indirect hemagglutination assay (IHA) or immuno-enzymatic assays (ELISA) can also be used. As explained earlier, leptospires are presented in the blood after infection until they are cleared after 5-10 days after onset of the disease, following the production of anti-Leptospira antibodies. Initially, it is mainly the IgM class that will increase, thereafter the IgG class can be detected. However, in week 1 an early diagnostic serology gap occurs and the antibody titers will test negative (fig 2), since leptospires circulate in the blood notably 4-7 days after onset of the disease. Several other techniques like PCR on blood can fill this gap. PCR is a successful method to detect Leptospiral DNA in blood in the first week of infection. After the first week, leptospires in blood cannot longer be detected by PCR anymore. Leptospires cannot only be detected in blood, their components can also be detected in urine or tissue by techniques like culture, dark field microscopy, immuno-staining or PCR, these techniques will be discussed later on [1,3,4].

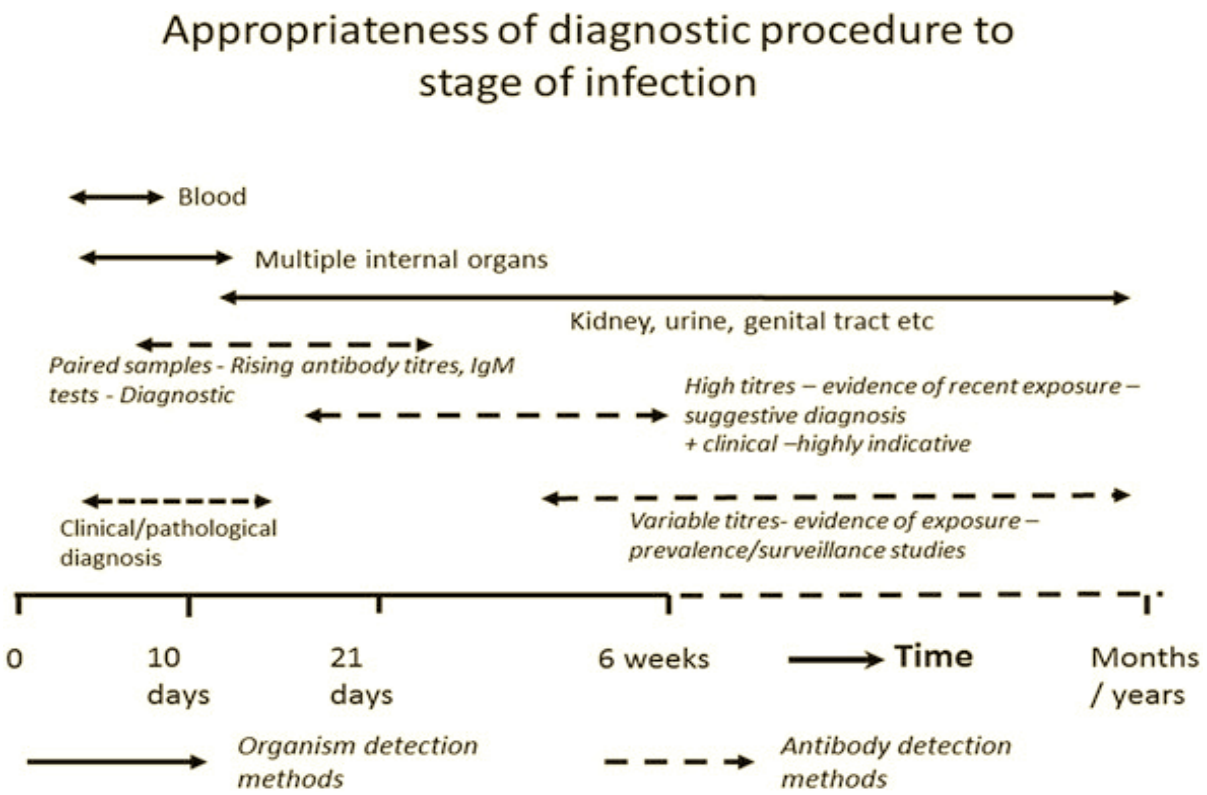

Figure 11: The appropriateness of serological and organism based tests at various stages of infection [5].

1.7 Treatment

The preferred antibiotic drugs for treatment of human leptospirosis are currently penicillin and doxycycline [15]. During acute illness, antibiotics should be given as quickly as possible. Additionally supportive treatment (fluid therapy and dialysis) is necessary for patients who developed acute renal failure, jaundice and pulmonary symptoms [1,4]. The treatment of acute leptospirosis in individual animals or herds is generally similar to human. It often dependents on the use of effective antibiotics plus supportive symptomatic treatment [16]. While principles are the same for all species, the antibiotics used may vary according to their safety, their availability, the cost and the route of administration. Animals that suffer from acute or chronic Leptospirosis are usually treated with a combination of penicillin, doxycycline and streptomycin, but ampicillin, amoxycillin, tetracyclines, tulathromycin and third generation cephalosporins have also been used[5,15]. In food producing animals, withdrawal times are an important consideration.

Vaccination in risk herds can help to prevent and treat leptospirosis. Vaccines induce the production of antibodies against the lipopolysaccharides found on the leptospiral surface[17]. Commercial Leptospira vaccines are globally available for cattle, pigs and dogs [3]. Vaccines to prevent human leptospirosis are available in some countries and however the use is still debatable due to possible unacceptable side effects, its short-term effect and the induction of autoimmune diseases [4]. Clinical and subclinical disease are significantly reduced by a leptospiral vaccination, since it reduces bacterial shedding. However, vaccination alone may not stop symptoms if infection has already taken place [5,18]. Continued epidemiological studies are required for a successful vaccination program to measure the incidence of different *Leptospira* serovars present in the geographical region in question, since each vaccine effects only a specific serovar [3]. Besides vaccination and adequate antibiotic treatment, fluid and supportive therapy is almost always indicated. If needed, blood transfusions and dialysis, if possible, is suitable.

1.8 Control

The key factor of controlling leptospirosis is to restrict the transmission (direct and indirect) of leptospires between contaminated environment, hosts and carriers. Leptospirosis carriers in the maintenance host populations should be eliminated. Antibiotic treatment, preventive vaccination and reducing transmission risk factors by management are important control factors. Management is needed to control leptospiral infections in animals and to reduce the zoonotic risk [5]. Since many human leptospirosis cases are occupational related, methods to increase hygiene such as protective clothing and avoidance of splash from urine or water are often useful but difficult to implement since they often interfere and complicate work[3]. Wildlife control, including rodents, is very important control factor for leptospirosis, since they play a large role in the transmission of leptospires across countries, via direct and indirect contact. If a combinations of the factors described above (preventive vaccination in animals, biosecurity, good animal husbandry and rodent control) is established, immunity against leptospirosis will be provided in most cases [19].

Chapter 2: Viability of Leptospira ssp. in deer kidney from a New Zealand abattoir, as a model for New Zealand' wildlife

2.1 Introduction

The first outbreaks of leptospirosis in New Zealand' livestock were reported during the 1950s. There are six endemic serovars in New Zealand: *Hardjo(bovis), Pomona, Ballum, Tarassovi, Copenhageni* and *Balcanica*. Tabel 3 shows the different serovars occurring in New Zealand and their carriers[20]. The epidemiology of leptospirosis in New Zealand is unique with ruminant livestock species as maintenance hosts and large numbers of transfer hosts such as possum and rats being key factors of Leptospiral spreading. However, vaccination is still not yet mandatory from the 1980s to the present, there has been widespread uptake of vaccination of farm animals. This increase of vaccinations has been highly successful in reducing the incidence of Leptospirosis in New Zealand and consequently the numbers of infected cases decreased rapidly over the years. Still, leptospirosis infects a significant number of humans and animals each year in New Zealand [21–23].

Genospecies	Serogroup	Serovar	Maintenance host (s)	Accidental host(s)
L. borgpetersenii	Sejroe	*Hardjo(bovis)*	Cattle, deer, sheep	Cattle, humans
		Balcanica	Brushtail possum *(Trichosurus vulpecula)*	Cattle, humans
	Ballum	*Ballum*	Black/ship rat (*Rattus rattus*), House mouse *(mus musculis)*, Hedgehog *(Erinaceous europaeus)*	Cattle, humans
	Tarassovi	*Tarassovi*	Pig	Cattle, dogs, humans
L. interrogans	Pomona	*Pomona*	Pig, deer, cattle, sheep	Cattle, sheep, humans
	Copenhageni	*Copenhageni*	Brown (Norway) rat *(Rattus norvegicus)*	Cattle, dogs, horses

Table 3: Classification of Leptospira species in New Zealand and maintenance host species to which the serovars are adapted to [20].

2.2 Prevalence of leptospirosis in New Zealand

2.2.1 New Zealanders

Serovars *Hardjo, Pomona* and *Ballum* are responsible for most of the human cases in New Zealand [8]. In the late 70s, the New Zealand's reported annual incidence of leptospirosis in humans was one of the highest in the world. An annual peak of 875 human cases was reported in 1974. After herd vaccination became widespread, the incidence of human cases declined dramatically to approximately 100 cases per year. This number seems to keep constant over the last past years (fig. 12) [18,19,24]. However, those numbers are likely to be underestimated because many individuals with leptospirosis may not seek medical attention since the symptoms are like a mild influenza or the disease is misdiagnosed. Like discussed in the previous chapter, occupation is a risk factor of developing leptospirosis. New Zealand' veterinarians, meat workers, farmers and rodent control workers are most at risk. A study to detect leptospirosis in New Zealand' veterinarians showed that 5,1% was seropositive [25]. More at risk are meat workers. A survey on sheep abattoir showed an estimated median daily exposures for meat workers ranged from 11 to 54 kidney culture-positive carcasses per day during high risk season (May–November) and 3–18 during low-risk periods [26]. Also, another serological survey showed

a 11% prevalence of leptospirosis in meat workers. These findings suggest significant exposure to leptospirosis for meat workers in New Zealand [19,27].

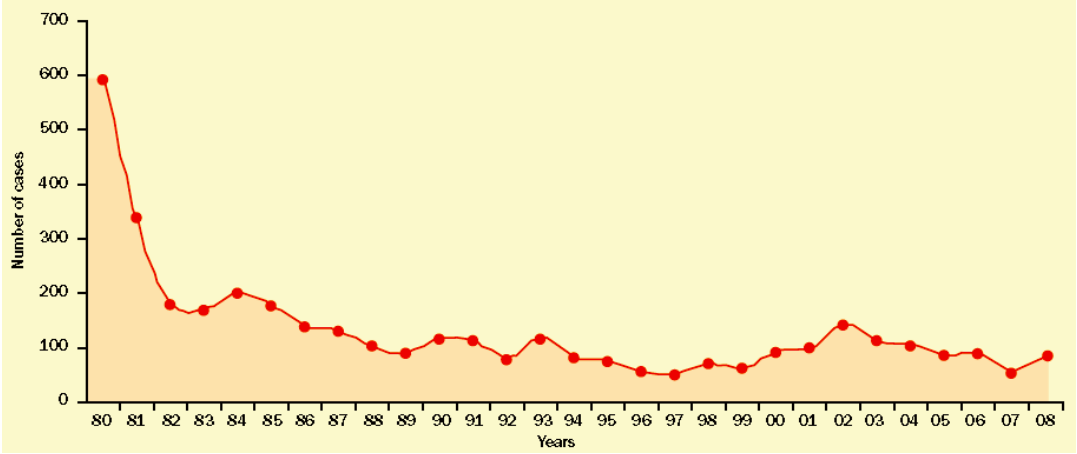

Figure 12: New Zealand leptospirosis notifications and laboratory reported cases by year 1997- 2014 [28].

2.2.2 New Zealand' wildlife

The most common maintenance hosts of Leptospirosis in wildlife in New Zealand are the black rat (*Rattus rattus*) for serovar *Ballum*, the house mouse (*Mus musculus*) for serovar *Ballum*, the Norway rat (*Rattus norvegicus*) for serovar *Copenhageni* and the brushtail possum (*Trichosurus vulpecula*) for serovar *Balcanica* [7].

2.2.3 New Zealand' complain animals

In 2013, a study on prevalence of serovars *Hardjo, Pomona, Copenhageni and Ballum* in 655 New Zealand' dogs showed a 15.2% Leptospira prevalence. *Copenhageni* was most common found (10.3 %). The prevalence of *Hardjo, Pomona* and *Ballum* was 3,5%, 1,1% and 0,8% respectively [29].

2.2.4 New Zealand' livestock

Serovar *Hardjo* and *Pomona* are the two most commonly seen serovars in New Zealand cattle and sheep. Pigs are conventionally regarded as the maintenance hosts for serovar *Tarassovi* and *Pomona*. Different surveys are done towards the exposure of different serovars. In 2010, seroprevalence for leptospirosis was 50% in sheep and 58% in cattle [8]. Approximately, the same numbers were found in 2015, 57% of sheep was seropositive and 73% of cattle. From those seropositive, 29% of sheep an 21% of cattle were found PCR positive for leptospirosis. Among those seropositive animals 40% had renal carriage and leptospirosis shedding through urine [19].

2.2.5 New Zealand' deer

Leptospirosis is an important clinical disease in New Zealand farmed deer, previous literature shows [22,21]. Serovar *Hardjobovis* and *Pomona* are most common detected. However, *Tarassovi* and *Copenhageni* were also reported in farmed deer [21]. Deer are maintenance hosts for *Hardjobovis* and an accidental host for *Pomona*, since clinical cases are observed with *Pomona*. Young animals are generally more at risk to develop leptospirosis disease than adults [22]. Animals of 9-30 months have the highest

Fig 13: Deer farm, Palmerston North, New Zealand

seroprevalence and there are no differences between sexes [23]. A survey of 110 New Zealand farms, not using leptospiral vaccines, showed a seroprevalence of 81% [23]. Also, a research of Dreyfus et al. towards non-vaccinated animals showed an in between-herd prevalence of 59% for *Hardjobovis*, 47% for *Pomona* and 75% for either serovar[20]. It can be concluded that the seroprevalence of leptospirosis in deer is high in New Zealand and that deer play an essential role in transmission of leptospirosis [23].

2.3 Aim of research

This is a pilot study to support a larger scale research-project to address what the role is of New Zealand's abundant wildlife species (e.g. possum, deer, pig, hedgehog, rabbit, rat, mouse: fig. 14) in leptospiral transmission to domestic animals. The traditional reservoir and spill-over host model for leptospiral transmission is timely for reconsideration given the rising importance of serovar *Ballum* in humans and the role of leptospiral animal vaccination programs to protect human and animal health in New Zealand. Massey University wants to embark on a wildlife screening project of Leptospirosis involving animal trapping and opportunistic sampling of wildlife species. However, the optimum and maximum time to sample from dead animals for leptospirosis culture and PCR from kidney tissue is unknown. It is believed that the organism does not survive long in tissue after death, but this has not been tested adequately according to the lack of published literature. DNA may persist for some time, yet unknown and unpublished. The goal of this research is to have a more robust estimate of how long leptospires survive in dead animal kidney tissue and can be cultured and how long after death DNA can be extracted for PCR. However, collection of wildlife specimens for this pilot study would be problematic given the sporadic and unpredictable nature of infection in wildlife and the logistics of sample collection and timing of collection. Therefore, it is planned to use kidneys from deer as a model, since large numbers of potentially infected samples can be collected relatively simple and in a controlled manner. Also, it allows good quality serum samples to be collected simultaneously for testing for Leptospira serovar. Deer kidneys that show possible sign of leptospiral infection are target and left at ambient temperature, culture and PCR are repeated at intervals thereafter. All the kidneys were tested by PCR, thereafter the positive kidneys were tested by culture and paired blood samples were tested with MAT-serology.

2.4 Hypothese

In studies with wildlife, animals are usually captured overnight and collected the next morning, or even after a few days. This means that the animals can be dead for several hours or days before they will be dissected and analyzed. During this period of time, there will be a considerable DNA degradation in animal tissue, mainly due to humidity and oxygen[30]. These postmortem changes influence the survival and detection of leptospires. This study is to investigate the viability of leptospires in kidney tissue over time, detected by PCR and culture, when the kidneys are stored at room temperature and tested by PCR and culture at intervals after collection. The kidneys will be kept at room temperature and will be exposed to humidity and oxygen, to simulate a natural environment. Previous research shows that samples which cultured positive for serovar *Pomona* on the first day, were culture negative by 24 hours or less after inoculation regardless of the temperature they were held by. It is thought that autolysis causes an anaerobic environment in which leptospires cannot live, since they are aerobic organisms. A decline in pH could also contribute to the decrease in leptospiral numbers. These changes thought to be caused by the putrefactive, rotting bacteria's and hydrolytic enzymes released from lysozymes of damaged tissue cells [30]. When the leptospires are not viable anymore, it is unable to get a culture. It is

known that Leptospira could be found in kidney-tissue when tested by PCR, however no data is available about the viability of leptospires over time, when tested by PCR or culture.

Hypotheses

"Leptospira can be cultured up to 24 hours after death in kidney tissue"
"Leptospira DNA can be detected in kidney for up to seven days"

Figure 14: New Zealand' wildlife (l.t.r possum, rat, deer, hedgehog, mouse. rabbit, pig)

2.5 Material and Methods

2.5.1 Sample collection and testing

A random cross-sectional study was conducted from November 2015 to February 2016 on slaughtered farmed red deer from different suppliers. Collection of kidney-samples was mostly planned from October through summer, since the deer will be 12 months, and lambs about 5-6 months old, the likelihood of being positive on Leptospirosis is highest[20,27,22,23,31]. Also in summer, the prevalence of leptospirosis is higher, since the survival of leptospira is enhanced by higher temperatures and a humid environment [1]. Samples were collected at the deer abattoir Venison Packers in Fielding, New Zealand by the author and an assistant, both wearing personal protective equipment (fig. 15). For each selected carcass, blood (coagulated) samples and kidney samples were taken. Collection and processing of samples had been kept as sterile as possible to avoid cross-contamination. Several lines per supplier of origin were tried to sample, to increase the changes for infected leptospirosis kidneys. Since the deer abattoir is a relatively slow line, paired blood and kidney samples could be taken. On 5 days collecting days, 15-25 samples were collected each time (table 4). In total, 98 blood samples and 98 kidneys were collected from deer of 9 different farms. The opportunistic sample size depended on the slaughterhouse 'offer at that moment. Specified total sample size for adequate power of detection could not be calculated in this study since the samples size was too small. All samples were under fresh condition stored in a chilly-bin and transported to the Hopkirk Leptospirosis Research Laboratory (HLRL, Massey University, Palmerston North, New Zealand) on the day of collection.

2.5.1.1 Collection of kidney samples

At the evisceration and inspection area (fig. 16), kidneys were initially target on possible signs of leptospiral infection and one whole kidney of each animal were collected. However, mostly all the kidneys were collected randomly. They were put into a zip-lock bag and kept at ambient temperature and cooling packs were put in the chilly-bin if necessary on a hot day.

2.5.1.2 Collection of blood samples

Blood samples of all the animals were collected by free flow into a new 60-ml container, after the skinning at the middle of the slaughter line (fig. 16).

Species	No. Collected	Abattoir collected	Date collected	How provided
Deer	18	Venison Packers	26th of November, 2015	Fresh
Deer	15	Venison Packers	7th of January, 2016	Fresh
Deer	15	Venison Packers	18th of January, 2016	Fresh
Deer	25	Venison Packers	1th of February, 2016	Fresh
Deer	25	Venison Packers	9th of February, 2016	Fresh

Tabel 4: Sample data

Fig 15: Left: Venison Packers Deer abattoir. **Right:** protective clothing.

Figure 16: Left: Evisceration and inspection area. **Right**: collecting a blood sample

2.5.3 Analysis of samples

2.5.3.1 Analysis of the blood

To prevent lysis of the red blood cells, immediately after arriving at the HLRL, the collected blood samples were transferred into Greiner© centrifuge tubes in the biohazard cabinet (after 10 min of UV-light) and spun down at 3000g for 10 minutes. Sera were collected and put into a 1.5ml Eppendorf tube and stored in the cooling room (2°C). 2 ml of sera of each sample was put into Cryovial© containers and stored in a -80°C freezer, as a backup-sample. In the afternoon, the sera were taken out of the cooling room and 30µl of each serum sample was put together with 150µl of Saline into a sterile Masterplate©, to get a 1:6 dilution. The Masterplate© covered with parafilm and stored in a freezer (-20°C). Serum samples were tested by the microscopic agglutination test (MAT) for antibodies against serovars *Hardjobovis* and *Pomona* (HLRL laboratory strains), based on the standard procedure previous described by Faine [32]. These two serovars were chosen because serological evidence of leptospirosis in domestic livestock, deer in this case, is most commonly found with serovars *Hardjobovis* and *Pomona* in New Zealand [22,21,23]. 16 serial dilutions of serum in standard saline were prepared in a 96-well plate together with a positive and negative control. The plates were incubated at 30°C for 1.5–4 hr. The analysis and degree of agglutination was determined by examination under a dark-field microscope with magnification of 100x. A MAT titer of ≥1 : 48 was considered positive for both serovars, based on previous studies of leptospirosis in sheep and cattle [19]. The end-point titer is the lowest dilution at which approximately 50% of the organisms were agglutinated or lysed. Positives samples were further diluted to determine their titer. The exact protocol can be found in appendix E.3 and E.4.

2.5.3.2 Analysis of the kidneys

Kidneys were transported to the HLRL in a chillybin and were left at ambient temperature, at all times.

2.5.3.2.1 Classification

The kidney surface was assessed based on the macroscopic criteria, namely the presence of focal pale (necrosis) or red (petechia) discoloration scattered throughout the cortical surface, white or red mottling, ecchymotic hemorrhages, fibrotic scarring, the aspect of the cortical surface and possible cysts. Findings were classified and written down and photos of suspicious looking kidneys were taken. This classification is made by the author based on previous research [7,21,33]. The results and classification-scale can be found in Appendix A and B.

2.5.3.2.2 Preparation for PCR and culture

A 10gr section of each kidney, extending from the renal cortex to the medulla, was removed aseptically. The remainder of the kidneys were stored at ambient temperature in a sealed container overnight. The removed sections were washed with 70% ethyl alcohol using funnel and flask, followed by flaming with a Bunsen burner and put into a stomacher bag. The sections of kidney were then individually homogenized in 10ml of 0,01M Phosphate Buffer Saline (PBS) using a Colworth© stomacher 400 (AJ Seward Ltd, London, United Kingdom) for 2 minutes per sample. 1.5ml of fluid was captured from the stomacher bag and put into a sterile Eppendorf tube [34]. This was used to for PCR, the rest was used for culture. Details of the protocol can be found in appendix E.1.

2.5.3.2.2. Culturing

A 100µl aliquot of the suspension was used for culture in an Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. This culture was checked under a dark-field microscope weekly after four weeks post inoculation until thirteen weeks post inoculation. The remainder of the aliquot was left at ambient temperature (appendix E.1). Although most serovars need 1 to 1,5-week incubation time. L. *Hardjobovis* can take a longer time.

2.5.3.2.3 PCR

DNA extraction*:* A 160µl aliquot of PBS-kidney suspension was used to extract DNA, using a High Pure PCR template preparation kit (Roche©, Mannheim, Germany) [34]. 40µl of (cooled) Proteinase K, 200µl of Tissue Lysis buffer and 160µl were put into a sterile Eppendorf, mixed and incubated at 55°C for one hour. 200µl of Binding buffer was added and the samples were incubated again at 70°C for ten minutes. 100µl of Isopropanol was added and the suspension was vortexed and centrifuged. The fluid was put into a High Filter Tube (HFT) of a HFT-Collection Tube (CT) assembly and spun down at 8000g for 1 minute. 500µl of Inhibitor Removal Buffer was added to the HFT assembled to a new CT and was spun down at 8000g for 1 minute. The fluid was washed and centrifuged (8000g for 1 minute) twice with 500µl of Washing buffer. At last, 200µl of prewarmed (70°C) was added to the HFT-CT assembly and was spun down at 13000g for 1 minute. The remaining liquid, the extracted DNA, was put into a sterile Eppendorf.

DNA amplification: The qPCR reaction solution was made of 5 pM of primers targeting the gyrB gene [35], of sequence 5'-TGAGCCAAGAAGAAACAAGCTACA-3' (2For) and 5'-MATGGTTCCRCTTTCCGAAGA-3' (504Rev), 1.2 µM of SYTO9 (Invitrogen Corp., Carlsbad, CA, USA), 12.5 µL of a commercial mastermix (Roche LightCycler 480 Probes Master 04707494001, Roche Diagnostics GmBH, Mannheim, Germany), 2 µL of the DNA preparation (sample) and 7.3 µL double distilled water for a total volume of 25 µL. The qPCR was run on a Rotor-Gene Q (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand). An initial denaturation of 10 minutes at 95°C was followed by 40 cycles, consisting of 10 seconds of denaturation at 95°C, 20 seconds of annealing at 63°C and 10 seconds of elongation at 72°C. The melting temperature was measured by monitoring the fluorescence on the green channel, every 0.2°C from 78–90°C. *L.interrogans*

serovar *Pomona* (ESR laboratory strain), which can detect *Harjobovis* and *Pomona* DNA fragments, was used as a positive control and double distilled, DNAse and RNase free water was used as a negative control, for each PCR run. The qPCR used was adapted from Subharat et al. [36], Fang et al. [34] and Vallee et al. [37] and was shown to have a detection limit of 10^3 cells/mL for a leptospiral serovar, since it is not possible to distinguish concentrations below 10^3 cells/mL from the negative control. Confirmation of positive samples was determined by comparing the melting temperature with the positive control[38]. Positive samples were defined as having a cycle-to-threshold (Ct) value less or equal to 38 cycles. The remaining DNA extracted fluid was kept at -20°C and was kept as a backup sample. The exact PCR protocol can be found in appendix E.2.

2.6 Results

Kidney and blood samples were collected from 98 red deer from 9 different farms at a New Zealand' abattoir, Venison Packers Fielding (appendix A). From the dataset available, the average age of the animals is 2 years. The sex of the animals is mostly unknown.

PCR

In total, 2 out of 98 kidneys (2%) were found PCR positive for leptospirosis on day one (within 24 hours). The same 2 kidneys were found PCR negative on day 2 (after 48h) when stored overnight at ambient temperature. The animals were both 2 years old, originating from different farms. One positive tested animal (#39) had multiple white foci (>15) on her kidney and was classified C4. Remarkably, the kidney of the other leptospirosis positive animal (#48) did not show macroscopically signs of leptospirosis (table 5). The full range of PCR test runs are listed in appendix C.

Nr	Sample #	Date	Farm	Age	Sex	description	C	1th PCR result	2th PCR result	Culture result
39	21	18-1-16	Farm D	2	H	>15 white foci	C4	+ve	-ve	-ve
48	30	18-1-16	Farm E	2	unk	-	C0	+ve	-ve	-ve

Table 5: Results of all the samples from animals that tested PCR positive.

Cultures

All 98 kidneys samples were found culture negative when checked after 2 and 4 weeks of incubation. Little contamination or overgrowth of bacteria occurred during the culturing process.

MAT

Blood samples of the leptospirosis positive animals have not been tested yet, due to occupied facilities at the Hopkirk Laboratory. Therefore, it remains unknown which leptospiral serovar causes leptospirosis in the two PCR positive samples. Also, no information is available about the seroprevalence of leptospirosis.

2.7 Discussion

Previous research shows leptospires could be found in kidney-tissue, however no exact data is available about the viability of leptospires over time when tested by PCR or culture.
The hypothesis *"Leptospira DNA can be detected in kidney for up to seven days"* was formulated and seems to be incorrect. Only 2 out of 98 kidney samples were PCR positive on leptospirosis.

When these two samples were stored at ambient temperature and tested 48 hours later, the PCR results turned negative. It seems that leptospiral DNA is only detectable within 24 hours after dead, thereafter a numerous DNA degeneration takes place [30]. Even though this study found that DNA was only detectable within 24 hours, the amount of positive PCR results of this study is so little, no firm conclusions can be stated about the survival time of leptospiral DNA for PCR detection. Conjointly, it cannot be established whether these are the correct numbers, or the method of testing was unsuccessful. Possibly, the way of storage might not simulate a natural environment in which leptospires could have survived. Earlier literature demonstrate that leptospires are dependent on several important factors for survival; pH, UV-light, temperature, humidity and oxygen [1,6,10,11]. Autolysis of kidney tissue causes an anaerobic environment and a decline in pH in which leptospires cannot live, since they are aerobic organisms. These postmortem changes influence the survival and detection of leptospires, since a considerable DNA degradation takes place [30]. During this research, the kidneys were placed in a plastic zip-lock bag, without oxygen supply, a factor they need for survival. The depository could also have negatively influenced the pH and the temperature of kidney tissue. The kidneys have not been exposed to UV-light, a factor that decreases survival rate. Also, it must be considered that there is no accurate result of 24 kidney samples (#74 – 98) available, because an error in the 5th PCR test occurred on day 1 and 2. For that reason, the PCR result that could be considered valid took place three days after collection, consequently DNA degeneration could have influenced their results.
Considering the optimal culturing time of leptospirosis, it is demonstrated that only viable leptospires can be cultured. Previous research shows that samples which cultured positive for leptospirosis on the first day, were culture negative by 24 hours or less after inoculation regardless of the temperature they were held by[30]. Looking at this study, the hypothesis formulated earlier; *"Leptospira can be cultured up to 24 hours after death in kidney tissue",* turned out to be incorrect. Even the 2 kidneys that were PCR positive gave negative cultures. This can be either due to errors in the precise culturing techniques, the specific serovars require a longer incubation time or the fact that the leptospirosis infection of the 2 PCR positive infections was not acute consequently leptospires were not viable anymore and could not be cultured.

Deer kidneys from the abattoir were used as a model for wildlife, since previous research showed a high prevalence of leptospirosis in New Zealand deer [22,21,23]. Studies illustrate a leptospirosis seroprevalence of 81% in non-vaccinated New Zealand farmed deer [22,21,23]. It was assumed that large numbers of potentially infected samples could be collected relatively simple and in a controlled manner in the deer abattoir. Nevertheless, this research shows a low prevalence of leptospiral field infection (2%: 2 out of 98) in deer, which does not match earlier literature. It is important to consider and investigate whether the design of this research is incorrect or the factually prevalence of leptospirosis in the deer abattoir was low. Considering the design of this research a few assumptions can explain the low outcome:
First, the sample size was very small therefore the power of study was too low to state firm conclusions. The slow transport line in the deer abattoir limited the sampling size. Moreover, sampling could only occur in the mornings, due to the time that must be considered for processing of kidneys and blood samples in the laboratory afterwards.
Second, collection of kidneys was at random. At first, data was collected from opportunistic sampling, with the sample size depended on the slaughterhouses' offer of kidneys at that moment. Initially, only deer kidneys that showed possible sign of leptospiral infection were targeted since this increases the chances of positive results, however this plan was changed

during research since the occurrence of macroscopically signs was minimal. Also, blood samples were collected before the kidney surface could be examined in the abattoir. For that reason, not only leptospirosis suspicious looking kidneys were collected. The plan of an opportunistic sample size was therefore changed into a plan of random sample collection to see whether kidneys that did not show the classical signs of leptospirosis were leptospirosis positive. Remarkably, not all the deer' kidneys with a high score (macroscopically signs of leptospirosis) were suffering from leptospirosis. This supports Wilson et al, although they found a high prevalence of renal lesions caused by leptospirosis some kidneys also did not have lesions while suffering from leptospirosis [22,21]. However, it cannot be ruled out whether the animals were suffering from another kidney disease or the PCR and culture results for leptospirosis were false or truly negative.

Third, there was no distinction of age while previous research points out that young animals are more susceptible to leptospirosis than adults. Collection of samples was mostly planned from October through summer, the deer would be 12 months and lambs 5-6 months old, when the likelihood of positive leptospirosis results is highest [20,27,22,23,31]. With the dataset that is available, the average age of the tested slaughtered animals is 2 years, which may have caused a low number of positive results. We could however not confirm this since there is no complete dataset available with all ages.

Fourth, there was no distinction in vaccinated and non-vaccinated animals since no data was available. Kidneys from non-vaccinated animals are preferable considering they are most likely to incur a leptospirosis field infection. Also, herd size was not included in the study. Larger herds are more susceptible for leptospirosis, since they are likely to retain shedders and are more frequently engaged in stock trading [20,27,31]. Moreover, it was not evaluated whether the farmer managed leptospirosis risk factors e.g. rodent control, grazing on wetlands or if co-farming occurred. For example, studies proved animals were more likely to have increased antibody titers against multiple leptospiral serovars when co-grazing occured[15,20,27,31].

Conjointly, samples were collected from only one abattoir in the Palmerston North area for this study, from October to February 2016. Therefore, results reflect only this spatial and temporal window and cannot be directly extrapolated for the rest of New Zealand or worldwide.

2.8 Conclusion

This research was performed to support a large-scale research-project to address the role of New Zealand's wildlife species in leptospiral transmission to domestic animals. The goal of this research was to have a more robust estimate of how long leptospires survive in dead animal kidney tissue, how long after death DNA can be extracted for PCR and leptospires can be cultured. To investigate the viability of leptospires in dead kidneys tissue over time, kidneys were stored at room temperature to stimulate a natural environment and tested by PCR and culture at intervals thereafter. Also paired blood samples were tested with MAT-serology to detect the type of leptospiral serovar.

A low prevalence of leptospirosis was found; in total, 2 out of 98 kidney samples (2%) tested positive by PCR. The 2 kidneys that were PCR positive on day one, were found PCR negative after 48 hours. It can be concluded that Leptospira ssp. is detectable by PCR in kidney tissue stored at ambient temperature within 24h after dead, thereafter, it is not detectable anymore. However, the number of leptospirosis positive tested animals is too low to state firm conclusions. Either the prevalence of leptospirosis in deer at the abattoir was low or the methods of testing were not suitable. All kidneys were found culture negative. The low outcome of positive cultures can be due to either errors in culturing techniques or the absence of viable leptospires in kidney

tissue. There are no serology MAT results from blood samples available, which would have provided useful information about seroprevalence and type of leptospiral serovar.
This research set-up was not elaborated and reliable enough to extrapolate and to draw conclusions. Concluding, further investigation of the current prevalence in non-vaccinated animals with large, carefully selected, sample sizes should be undertaken to see what the true prevalence numbers of leptospirosis in New Zealand' deer are. More advanced pilot studies should be carried out to discover the optimal method of sampling for the Massey University' wildlife screening project involving animal trapping and opportunistic sampling to address what the role is of New Zealand's wildlife species in leptospiral transmission to domestic animals.

2.9 Recommendations

To achieve improved research results in future projects, set-up of research should be modified. Stated below are a few recommendations.

The way of transporting and storing the kidney tissue should be further evaluated, since this can be a factor which decreased the viability of leptospires and not synchronize the open air of the natural environment. Due to hygienic and zoonotic reasons it is not possible to store the kidneys in the open air. Perhaps the zip-lock bays can be changed for vacutainers with air.

Kidneys from animals that have a higher prevalence of Leptospirosis should be chosen. Perhaps sample collection at a sheep abattoir is more suitable, since the likeliness of lambs being infected by leptospirosis is higher than deer[19]. Furthermore, slaughter house workers mentioned a higher rate of suspicious looking kidneys in the sheep plan, compared to the deer plan. For example, sampling can be done at Oviation, 61 Kawakawa Rd, Feilding 4775, New Zealand. Contact has already been established by the author at the introduction phase of this research. The line frequency (number of animals per minute) is a lot quicker at the sheep abattoir than at the deer plan and therefore more kidneys can be collected in the same amount of time. Also, the kidneys are put together in a bin, so suspicious looking kidneys can be targeted easily. However, a paired blood sample is harder to collect, since collection of blood samples and kidneys take place at a different place. Pairing them up will be difficult, unless you have a big sampling team. Furthermore, it is recommended that the kidneys are target for macroscopically leptospirosis signs and sampling is done with a greater sample size to increase the chances for positive results. Animals should also be a maximum of 1 year old and vaccinated animals should be excluded from research. Information about herd size and management of leptospirosis risk factors should be available for research. Moreover, to increase the reliability and the degree of extrapolation, sampling should be done at different deer abattoir across New Zealand.

The most perfect set up for this research is to follow a leptospirosis infected animal to infected kidney, which can be done in several ways. This can mean including a local veterinary practice. Therefore, the author contacted a few local veterinary clinics to find out whether it was possible to get information about some leptospirosis positive farms. At that time however, no data was available. It can be further assessed, when a next research towards leptospirosis is set up. Another option is to induce leptospirosis in animals, for example, in experiment-hamsters. However, artificial infection will probably result in a slightly different reaction of Leptospira in kidney tissue as compared to a naturally infected animal.

For practical reasons, it must be reconsidered which test is used to detect leptospirosis. This research planned to do three different tests; PCR, culture and MAT. However, it is more efficient to focus on one type of test. The standard serology test to diagnose leptospirosis is MAT[11,19]. Nonetheless, this test is based on the infected host's response but does not show whether there are viable leptospires and cannot differentiate between actual infection or increased antibodies acquired by vaccination[19]. Also, MAT serology is very time-consuming and requires expertise from a specialist to perform and interpret the results[1].
Techniques to detect leptospires directly are PCR and culture. Both methods require precise manual work, consequently mistakes can be made quickly. Culturing has a relatively lower sensitivity since it requires viable leptospires, it is very time-consuming and often complications e.g. overgrowth of other bacteria's or fungal contamination occurs[11,19,32]. PCR, on the other hand, is a relatively rapid method for detecting leptospiral DNA and does not require viable leptospires[19]. Several different leptospiral genes have been developed for qPCR to detect leptospirosis [2]. A study was performed by Dorjee et al. to compare the prevalence of leptospirosis in sheep kidney tissue by PCR and culture. The PCR technique detected leptospires in 50% of the seropositive sheep, whereas the culture method detected leptospires in only 22% of the sheep[39]. This indicates that PCR has a higher sensitivity compared to culture[36,39].
In a future research project, it is preferable to focus on the PCR method. However, MAT should be continued to be used when a result is PCR positive since the association between the results of MAT serology and kidney PCR will provide useful information for perceiving shedding/carrier status in deer that tested positive by MAT. Also, this will give valuable information about the type of leptospiral serovar.

Finally, exact protocols should be defined before starting. The research should not be a trial and error. Before the actual sampling, it must be established how many samples can be done in one day. Additionally, a margin of error must be built in. There must be considered that proceedings are slower at the beginning, since learning lab-work and setting up the sampling protocols takes time. All the research materials need to be ordered in front to avoid delay. Practicalities in abattoir (e.g. lunchbreak) must be considered for the sampling collection to be the most effective. Conjointly, a longer research period and an assistance during sampling collection and laboratory work is essential for follow up research.

Epilogue

During this research, I learned to work independently and strengthen my perseverance since my results were not as expected. I learned how research must be planned wisely and requires strict time management. I gained experience in complex, precise and punctual laboratory-procedures; PCR, culture and MAT-serology. Working hard until late hours is part of the research. Also, I noticed communication is the key for every good research. Many specialists were involved in this project and each had different opinions about it. I set up several meetings with all the staff-members to discuss the main research points. In this way, all the team members were on the same page again. I would like to thank the Massey University leptospirosis research unit, for having me as a part of their team and all the support they gave me. I enjoyed doing research and learned a lot from my stay at Massey University.

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- Peter Vos (Department of Large animals, Veterinary Medicine, Utrecht University, Utrecht, The Netherlands)

Co-supervisors

- Dr Julie Collins-Emerson, Senior Research Officer, IVABS
- Dr Jackie Benschop, Senior Lecturer, Molecular Epidemiology & Veterinary Public Health, IVABS

Lab- and Fieldwork

- Mr Neville Haack, Technician, Hopkirk Leptospirosis Research laboratory, Hopkirk Institute, Massey University, Palmerston North, New Zealand
- Yuni Yupiana, PhD, Hopkirk Leptospirosis Research laboratory, Hopkirk Institute, Massey University, Palmerston North, New Zealand

Collection of samples

- Chris Beattie, Deer plan manager, Venison Packers Fielding, New Zealand
- Geoff Purchas, Technician, Institute of Vet, Animal & Biomedical science, Massey University, Palmerston North, New Zealand
- Catriona Jenkinson, Research Technician, Institute of Vet, Animal & Biomedical science, Massey University, Palmerston North, New Zealand
- Aron Meijer, student Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

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Appendix A: Raw data

Nr	Sample #	Date	Farm	Age	Sex	Kidney description	C	1th PCR result	2th PCR result	Culture result
1	1	26-11-15	Farm A	unk	unk	Hyperemic, red vasculation	C0	-ve	-	-ve
2	2	26-11-15	Farm A	unk	unk	Red mottling, 2 small nodules	C2	-ve	-	-ve
3	3	26-11-15	Farm A	unk	unk	Red mottling	C1	-ve	-	-ve
4	4	26-11-15	Farm A	unk	unk	-	C0	-ve	-	-ve
5	5	26-11-15	Farm A	unk	unk	-	C0	-ve	-	-ve
6	6	26-11-15	Farm A	unk	unk	-	C0	-ve	-	
7	7	26-11-15	Farm A	unk	unk	White mottling	C1	-ve	-	-ve
8	8	26-11-15	Farm A	unk	unk	White + red mottling	C1	-ve	-	-ve
9	9	26-11-15	Farm A	unk	unk	Weird shaped, white and red mottling	C1	-ve	-	-ve
10	10	26-11-15	Farm A	unk	unk	White mottling	C1	-ve	-	-ve
11	11	26-11-15	Farm A	unk	unk	>15 small foci, white mottling	C4	-ve	-	-ve
12	12	26-11-15	Farm A	unk	unk	1-5 small concave nodules	C2	-ve	-	-ve
13	13	26-11-15	Farm A	unk	unk	White mottling	C1	-ve	-	-ve
14	14	26-11-15	Farm A	unk	unk	-	C0	-ve	-	-ve
15	15	26-11-15	Farm A	unk	unk	-	C0	-ve	-	-ve
16	16	26-11-15	Farm B	unk	unk	White+red mottling	C1	-ve	-	-ve
17	17	26-11-15	Farm B	unk	unk	-	C0	-ve	-	-ve
18	18	26-11-15	Farm B	unk	unk	White mottling	C1	-ve	-	-ve
19	1	7-1-16	Farm C	2	unk	Red vasculation, red mottling	C1	-ve	-	-ve
20	2	7-1-16	Farm C	2	unk	-	C0	-ve	-	-ve
21	3	7-1-16	Farm C	2	unk	Red mottling	C1	-ve	-	-ve
22	4	7-1-16	Farm C	2	unk	White mottling/pale	C1	-ve	-	-ve
23	5	7-1-16	Farm C	2	unk	1 concave nodule	C2	-ve	-	-ve
24	6	7-1-16	Farm C	2	unk	-	C0	-ve	-	-ve
25	7	7-1-16	Farm C	2	unk	-	C0	-ve	-	-ve
26	8	7-1-16	Farm C	2	unk	Swollen, red mottling	C1	-ve	-	-ve
27	9	7-1-16	Farm C	2	unk	Swollen, red mottling	C1	-ve	-	-ve
28	10	7-1-16	Farm C	2	unk	-	C0	-ve	-	-ve
29	11	7-1-16	Farm C	2	unk	Pale, mottling, red vasculation, >15 white foci	C4	-ve	-	-ve
30	12	7-1-16	Farm C	2	unk	-	C0	-ve	-	-ve

31	13	7-1-16	Farm C	2	unk	Vasculation	C1	-ve	-	-ve
32	14	7-1-16	Farm C	2	unk	Red mottling	C1	-ve	-	-ve
33	15	7-1-16	Farm C	2	unk	10-15 white foci	C3	-ve	-	-ve
34	16	18-1-16	Farm D	2	H	-	C0	-ve	-	-ve
35	17	18-1-16	Farm D	2	H	-	C0	-ve	-	-ve
36	18	18-1-16	Farm D	2	H	Pale, mottling	C1	-ve	-	-ve
37	19	18-1-16	Farm D	2	H	Darkened, mottling	C1	-ve	-	-ve
38	20	18-1-16	Farm D	2	H	-	C0	-ve	-	-ve
39	21	18-1-16	Farm D	2	H	>15 white foci N4	C4	+ve	-ve	-ve
40	22	18-1-16	Farm D	2	H	-	C0	-ve	-	-ve
41	23	18-1-16	Farm D	2	H	-	C0	-ve	-	-ve
42	24	18-1-16	Farm D	2	H	Very pale	C1	-ve	-	-ve
43	25	18-1-16	Farm E	2	unk	Pale, smelly	C1	-ve	-	-ve
44	26	18-1-16	Farm E	2	unk	-	C0	-ve	-	-ve
45	27	18-1-16	Farm E	2	unk	Very pale	C1	-ve	-	-ve
46	28	18-1-16	Farm E	2	unk	-	C0	-ve	-	-ve
47	29	18-1-16	Farm E	2	unk	pale	C1	-ve	-	-ve
48	30	18-1-16	Farm E	2	unk	-	C0	+ve	-ve	-ve
49	31	1-2-16	Farm F	3	S	-	C0	-ve	-	-ve
50	32	1-2-16	Farm F	3	S	Dark, red mottling	C1	-ve	-	-ve
51	33	1-2-16	Farm F	3	S	-	C0	-ve	-	-ve
52	34	1-2-16	Farm F	3	S	-	C0	-ve	-	-ve
53	35	1-2-16	Farm F	3	S	Very dark, red mottling	C1	-ve	-	-ve
54	36	1-2-16	Farm F	3	S	Dark, 1 big black spot	C2	-ve	-	-ve
55	37	1-2-16	Farm G	2	unk	3 big white spots	C2	-ve	-	-ve
56	38	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
57	39	1-2-16	Farm G	2	unk	Dark	C0	-ve	-	-ve
58		1-2-16	Farm G	2	unk	Red mottling, 2 black spots	C2	-ve	-	-ve
59	41	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
60	42	1-2-16	Farm G	2	unk	3 red/black spots, red cortex	C2	-ve	-	-ve
61	43	1-2-16	Farm G	2	unk	Red mottling, vasculation, smelly	C1	-ve	-	-ve
62	44	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
63	45	1-2-16	Farm G	2	unk	Red mottling, vasculation	C1	-ve	-	-ve
64	46	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
65	47	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
66	48	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
67	49	1-2-16	Farm G	2	unk	Swollen, pale, 1 big red mottling spot	C2	-ve	-	-ve
68	50	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
69	51	1-2-16	Farm G	2	unk	Pale, red mottling	C1	-ve	-	-ve
70	52	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
71	53	1-2-16	Farm G	2	unk	-	C0	-ve	-	-ve
72	54	1-2-16	Farm G	2	unk	Red + white mottling, red cortex	C1	-ve	-	-ve

73	55	1-2-16	Farm H	unk	S	Big	C0	-ve	-	-ve
74	56	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
75	57	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
76	58	9-2-16	Farm I	unk	unk	Red mottling	C1	-ve	-	-ve
77	59	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
78	60	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
79	61	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
80	62	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
81	63	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
82	64	9-2-16	Farm I	unk	unk	Pale, red and white mottling	C1	-ve	-	-ve
83	65	9-2-16	Farm I	unk	unk	Red mottling	C1	-ve	-	-ve
84	66	9-2-16	Farm I	unk	unk	hydropyelum	C0	-ve	-	-ve
85	67	9-2-16	Farm I	unk	unk	Pale, red cortex	C1	-ve	-	-ve
86	68	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
87	69	9-2-16	Farm I	unk	unk	Dark	C1	-ve	-	-ve
88	70	9-2-16	Farm I	unk	unk	Pale, 3 black spots	C2	-ve	-	-ve
89	71	9-2-16	Farm I	unk	unk	Pale	C1	-ve	-	-ve
90	72	9-2-16	Farm I	unk	unk	Pale, >15 small white foci	C4	-ve	-	-ve
91	73	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
92	74	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
93	75	9-2-16	Farm I	unk	unk	Red mottling	C1	-ve	-	-ve
94	76	9-2-16	Farm I	unk	unk	Dark	C1	-ve	-	-ve
95	77	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
96	78	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve
97	79	9-2-16	Farm I	unk	unk	Dark, 3 white foci, vasculation	C2	-ve	-	-ve
98	80	9-2-16	Farm I	unk	unk	-	C0	-ve	-	-ve

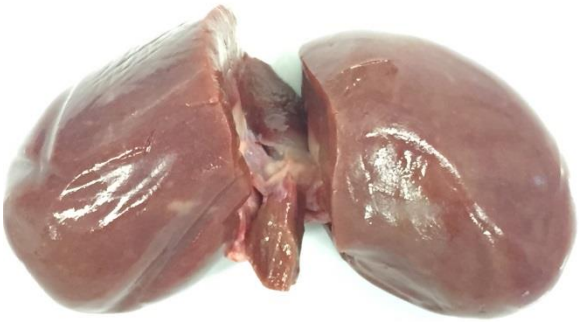
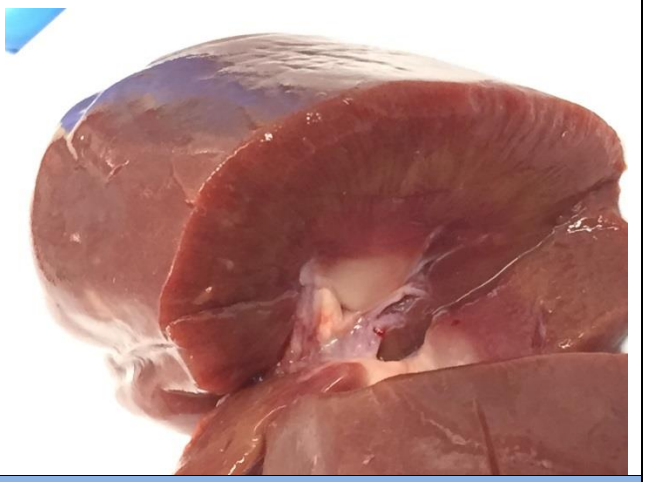
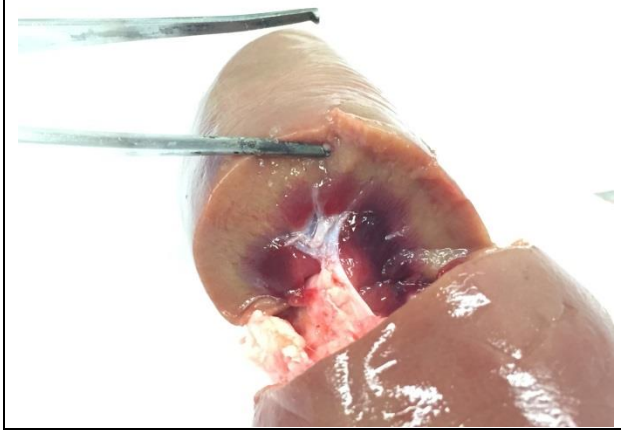
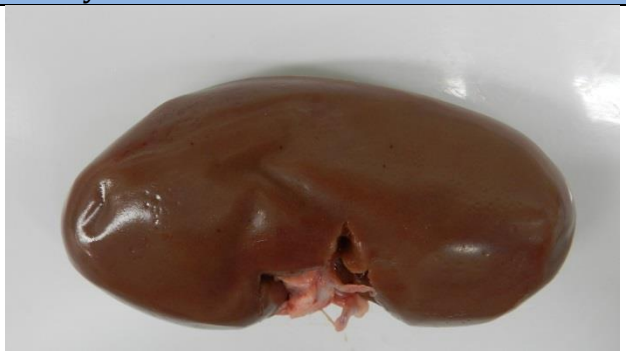
*S = stag = male
*H = hind = female
*C = kidney classification
*unk = unknown
* information about suppliers, not for public (!):
*Farm A = unknown
*Farm B = unknown
*Farm C = Greenhills Partnership
*Farm D = 1028 Forest Gate (2012) Ltd, c/- D Holden 1730 State Highway 50 RD 1 Onga Onga
*Farm E = 0524 G Nicol, Rawhiti Farm 178 Lindsay Road LEVIN 5510
*Farm F = 0978 Puhanga Trust CI- LF & RA alcock RD 3 Te Kuiti 3983
*Farm G = 1014 Beauly Trust, RD 4 Taumarunui 3994
*Farm H = 0155 CE&CM Satherley PO boc 62 Mangaweka 4746
*Farm I = 1048 G&R Partnership, 347 Ohurakura Road RD 2 Napier 4182

Appendix B: Assessment of kidneys[7,21,33]

Description
Cortical cellular necrosis - 1-5 small white spots - 5-10 small white spots - 10-15 small white spots - >15 small spots
Petechia: - 1-5 small red spots - 5-10 small red spots - 10-15 small red spots - >15 small red spots
White mottling
Red mottling
Ecchymotic haemorrhages / vascualisation - Glomerulair - Proximal tubulus
Fibrotic scarring
Cyst(s) (and amount)
Cortical surface - Smooth - Rough

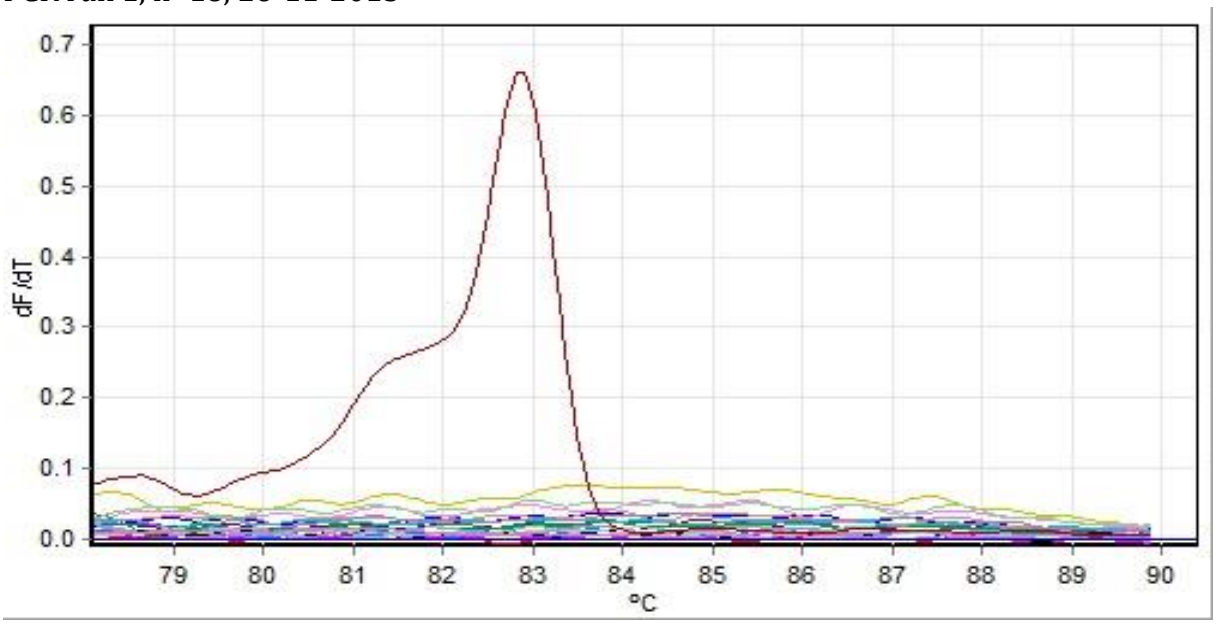
Classification	Description
Stage 0	no laesions, smooth cortical surface.
Stage 1	Smooth cortical surface, white or red mottling, ecchymotic haemorrhage
Stage 2	Smooth cortical surface, 1-5 white spots or petechia, white or red mottling, ecchymotic haemorrhage
Stage 3	Smooth cortical surface, 5-15 white spots or petechia, white or red mottling, ecchymotic haemorrhage
Stage 4	Smooth cortical surface, > 15 white spots or petechia, white or red mottling, ecchymotic haemorrhage
Stage 5	Rough cortical surface, >15 white spots or petechia, white or red mottling, ecchymotic haemorrhage
Stage 6	Rough cortical surface, >15 white spots or petechia, white or red mottling, fibrotic scarring, cysts, ecchymotic haemorrhage

A.1 photos of kidneys with macroscopically remarkable aspect

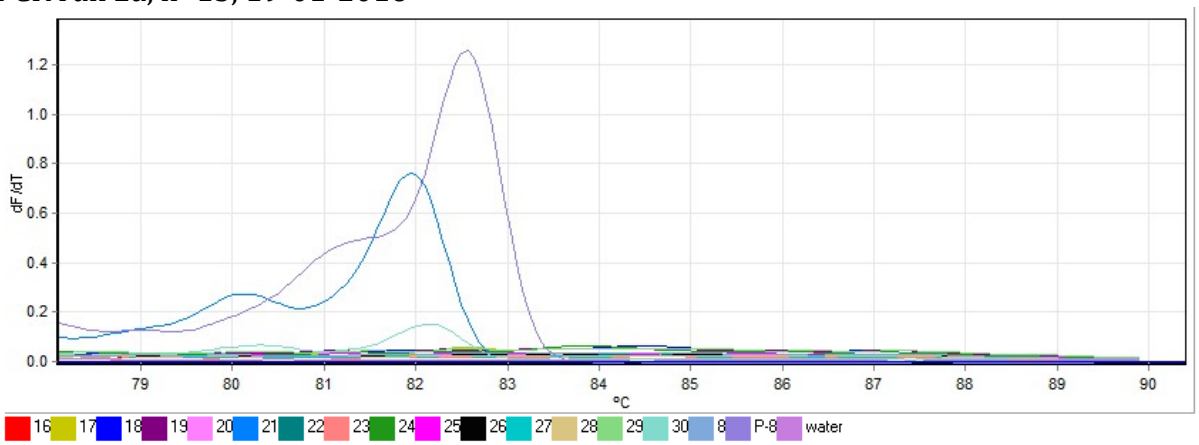
Kidney 11	Kidney 21
	 
Kidney 30	**Kidney 42**
	

Appendix C: PCR Results

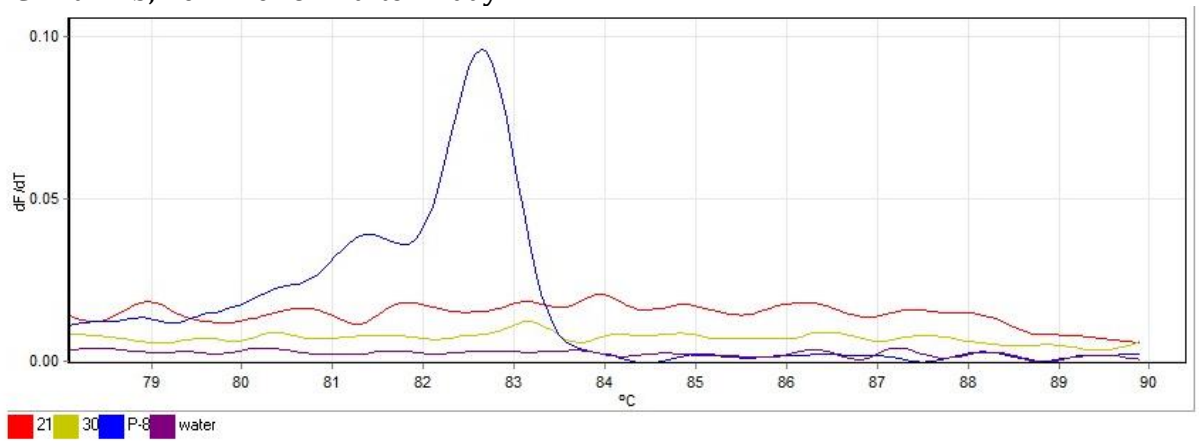
PCR run 1, n=18, 26-11-2015



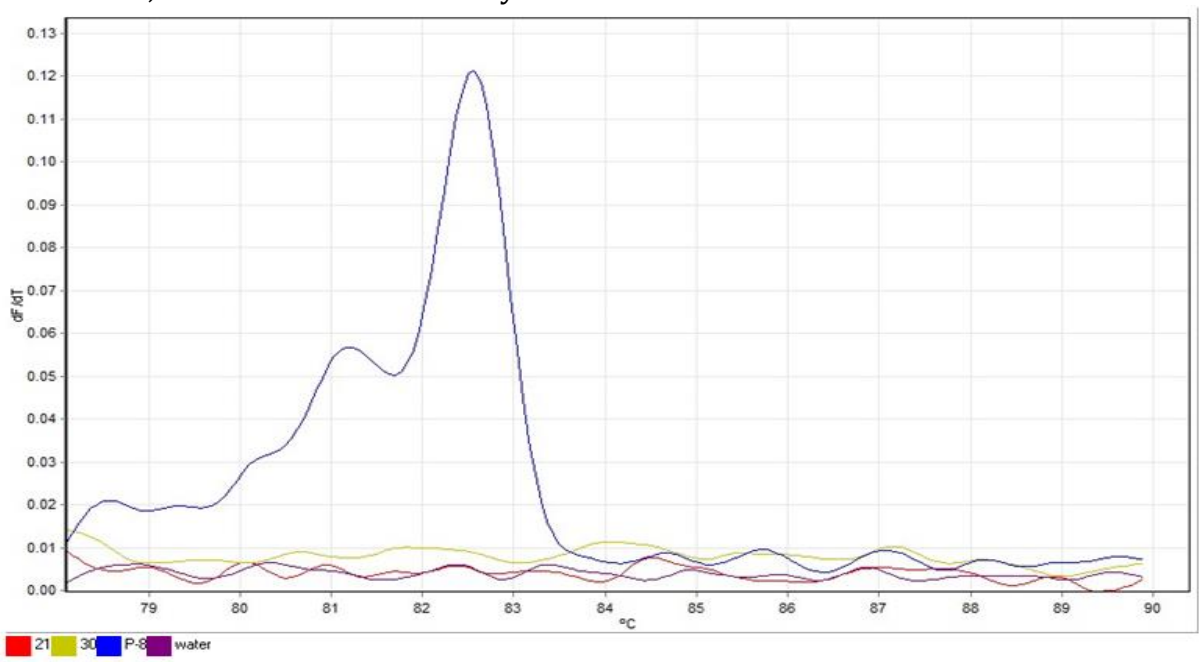
PCR run 2a, n=15, 19-01-2016



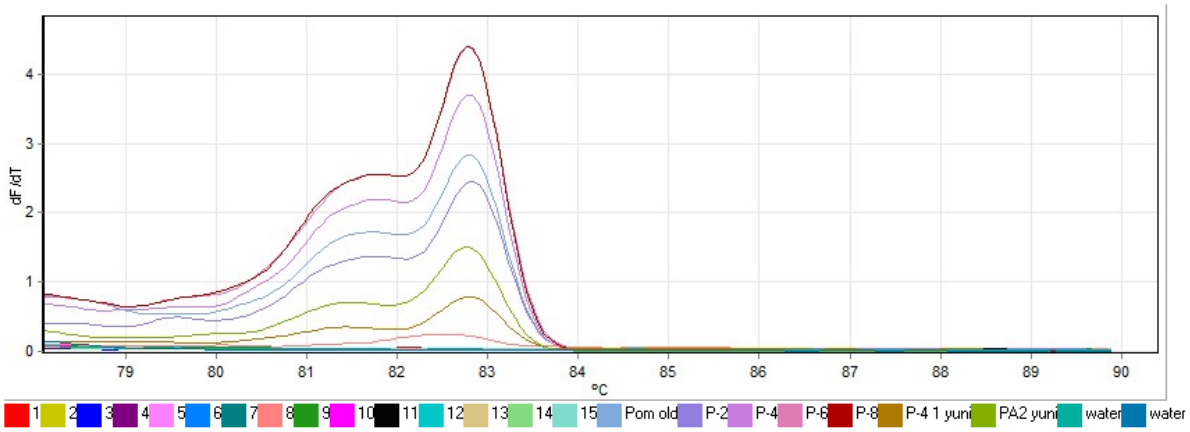
PCR run 2b, 20-1-2016 → after 1 day



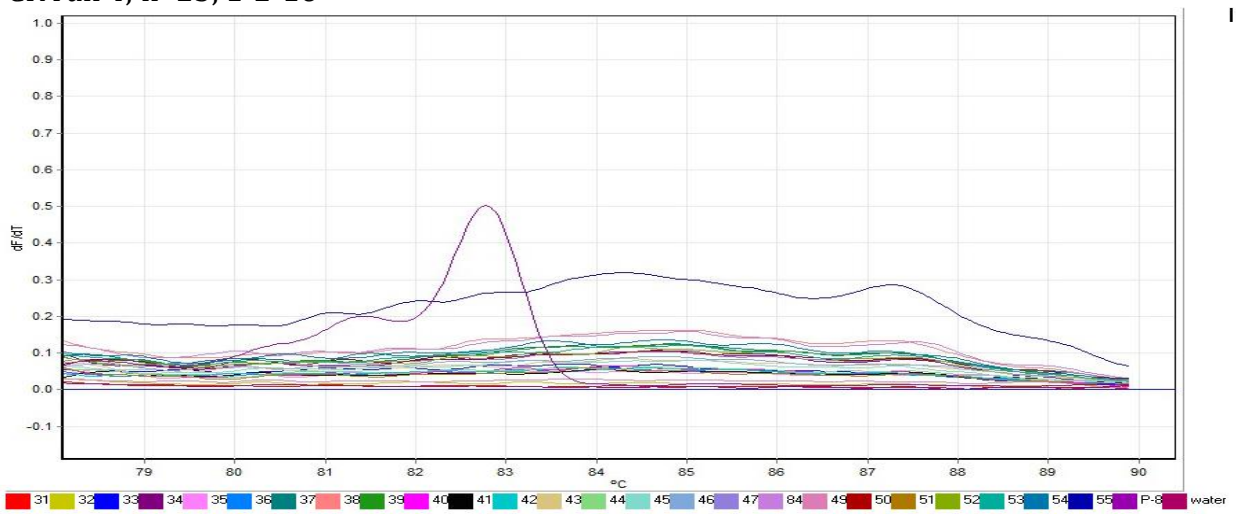
PCR run 2c, 22-01-2016 → after 3 days



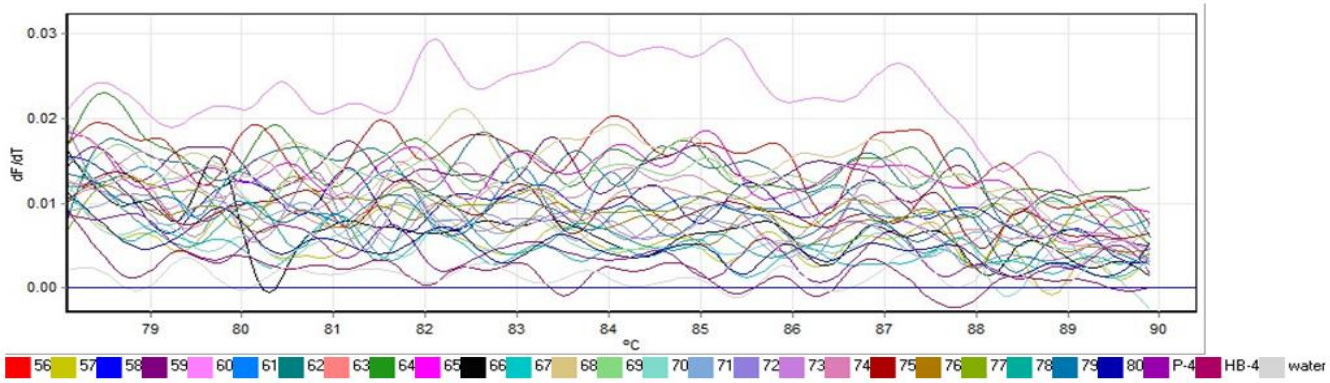
PCR run 3, n=15, 11-01-2016



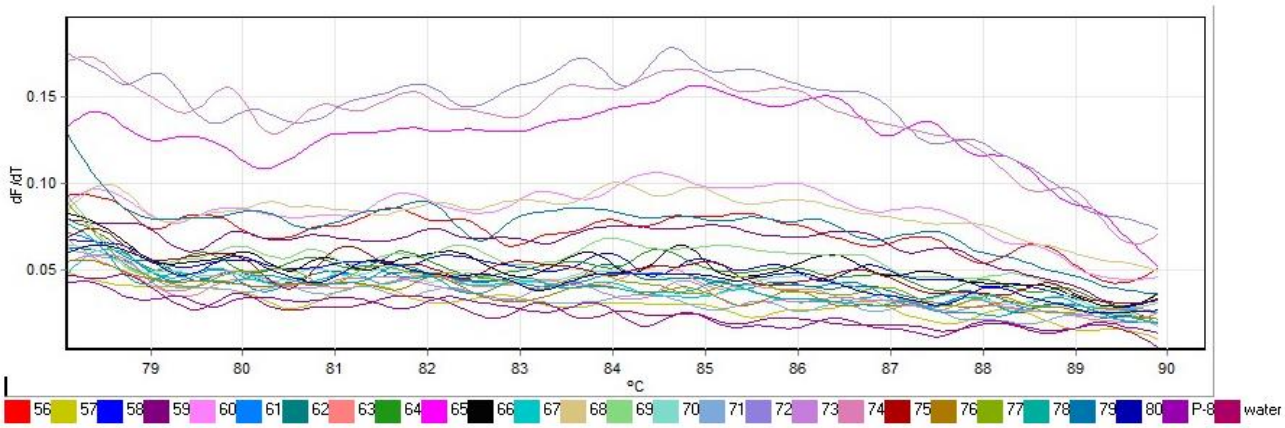
PCR run 4; n=25, 1-2-16



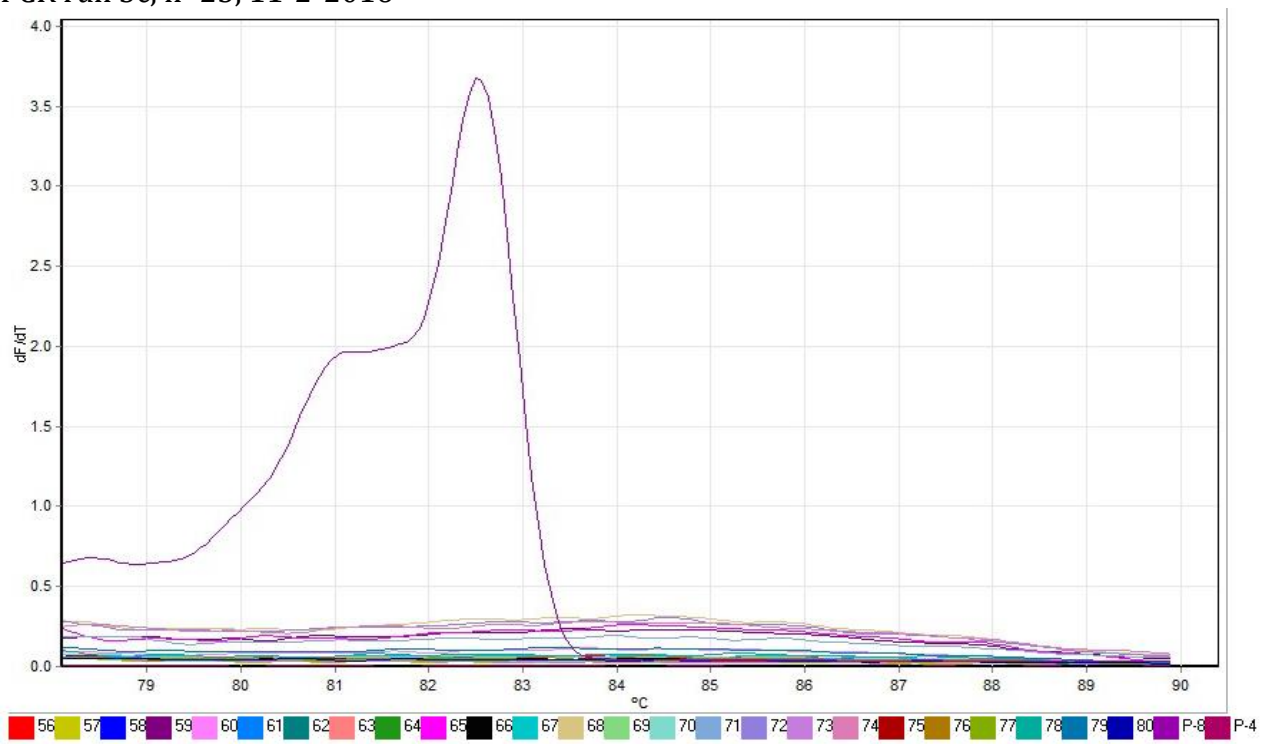
PCR run 5a, n=25, 9-2-2016



PCR run 5b, n=25, 10-2-2016



PCR run 5c, n=25, 11-2-2016



Appendix D: Protocol for sample collection at abattoir

Sample
Kidney and blood

Time
January & February

Location
Deer (Venison Packers Feilding, Kawakawa Rd, Feilding 4775, New Zealand)

Materials
Personal protective equipment (white overal, gumboots, gloves, goggles, hair-net, hat), zip-lock bags with stickers, permanent markers, 2 chillybins, blood collection containers (KJ520 60ml container)

Method
Collecting blood samples of all the animals by free flow into a new 60-ml container, after the skinning at the middle of the slaughting line. Write down the number of each animal on the container.
At the evisceration and inspection area, look for possibly infected kidneys and collect one whole kidney of each animal*. (*The kidneys are assessed based on the macroscopic criteria, namely the presence of foci of pale (necrosis) or red (petechiën) discoloration scattered throughout the cortical surface, white or red mottling, ecchymotic haemorrhages, fibrotic scarring, the aspect of the cortical surface and possble cysts.).* Put the kidneys in a Zip-lock bag and write down the number (matching with the blood sample) on the outside of the bag.

Transport all the samples under fresh condition and stored in a chilly-bin to the Hopkirk Leptospirosis Research Laboratory on the day of collection. Rap cooling elements in newspaper and put them together with the kidneys in the chilly-box. Centrifuge the coagulated blood samples shortly after collection at 3000 g for 10 min at the Hopkirk Laboratory and collect the sera. Leave the kidneys at ambient temperature in the chilly-bin at the laboratory.

Appendix E: Protocols for labwork[40]

E.1. Preparation of the kidneys for PCR and/or culture on day 1 (Repeat at 12u/24u/48u/etc)

Materials
lab coat, latex gloves, 1.5ml Eppendorf cups, plastic tray, paper towels, sterile yellow tips, a pipette bucket, Bunsen burner, sterile standard saline solution (PBS), scalpel blades, 70% alcohol, funnel, sterile stomacher bags, stomacher machine, chiller or freezer, culture bottles containing 5ml EMJH[40](yellow cap Vacutainer©), a 28-30gr incubator, sterile glass pipette.

Protocol
1. Take a piece of 10gr from each kidney.
2. Put the rest of the kidney back into the Zip-lock bag and put it back into the chillybin. Store the chillybin at ambient temperature.

3. Sterilize the (piece of) kidney:
    a. A sample of tissue (1-10gm) is placed in the funnel, which is perched on the flask
    b. Sterilise the surface by dousing in alcohol (70% ethanol)
    c. Flame it with a Bunsen burner
4. Put the piece of kidneys separately in a stomacher bag and put 0,01M PBS into the bag (for every gram of kidney you must put 1ml of PBS in the bag) = 10ml PBS
5. Stomacher the bag with (pieces of) kidney for 2 minutes in a Colworth stomacher 400 (AJ Seward Ltd, London, United Kingdom), to let it pulverise until the tissue has been broken down to a pulp.
6. Leave the bag to sit for 5 minutes.
7. Make up a line with new sterile 1,5 ml Eppendorf tubes.

If you perform only PCR on the kidney go to step 8. If you perform culture, go to step 11.

8. Take 800µl from the stomacher bag and put it in a 1,5ml Eppendorf tube.
9. Close the lid and write the number of the sample on the lid of the Eppendorf
10. Put the Eppendorf tube in the chiller (8°C) or freezer (-20°C) or at ambient temperature (depending on when the extraction will be). Due to time management, the extraction will be held the next day, so we leave it at ambient temperature.

If you perform only PCR, go to point 17. If you perform culture, go to step 11.

11. Make up a series of 3 yellow cap Vacutainer© with each 5ml of EMJH medium and write on the bottles: A/B/C, own name, sample number, the date.
12. Take 30µl from the stomacher bag with a sterile pipette and put in in bottle A
13. Take 30µl from bottle A and put in in bottle B.
14. Take 30µl from bottle B and put it in bottle C.
15. Put the bottles in the incubator (28-30°C) on a shaker.
16. Check the culture bottle routinely (approximately every 1-2 weeks) for the presence of *Leptospira.*
    a. Take the culture bottle out of the incubator
    b. Take up a sample of the medium with a flamed platinum loop
    c. Place it on a microscope slide
    d. Put the rest of the culture back in the incubator*
    e. Examinate the microscope slide under dark field microscopy.

* the cultures are held for up to 3 months after which time they are discarded, after first putting them through the autoclave for decontamination purposes.

17. Throw away the rest of the content of the stomacher bag

PCR on day 2/3/4/5/etc.: Repeat protocol.

E.2. Quantitative real-time PCR (qPCR) on kidney samples on day 2

Materials

Roche kit, Ethanol (96-100%), 1.5 ml microcentrifuge tubes, pipet tips with aeorosol barrier, Microcentrifuge (with roto for 2 ml tubes), Vortexer, Water bath or heating block at 55°C and 70°C, PBS, pipettes and pipette tips.

Protocol

DNA extraction

(using the Roche High Pure Template Preparation Kit)
Hands-on time: approx. 30 min, total time approx. 2h

1. Preparation of working solutions: (only needs to be done the first time)
    a. Dissolve **Proteinase K** (3 pink) in 4.5ml **double distilled water**, aliquot solution and store at -15 to -25°C
    b. Add 20ml **absolute ethanol** to **Inhibitor Removal buffer** (4a black) and store at +15 to +25°C.
    c. Add 80ml **absolute ethanol** to **Wash buffer** (4 blue) and store at +15 to +25°C.
2. Turn on the 55°C and 70°C heater.
3. Warm up the **Elution Buffer** to 70°C by placing it on the 70°C heater.
4. Prepare 1.5ml sterile Eppendorf tube for each sample in a tray. Label with number of sample on the cap of the tube.
5. To each labelled 1.5ml Eppendorf tube add:
    *use 200 pipette and filter pipette tips
    a. First, 40µl **Proteinase K**
    b. Second, 200 µl **Tissue Lysis Buffer** (1 white)
    c. Third, 160 µl of **kidney sample** after stomacher procedure. Vortex the Eppendorfs with sample before using them.
6. Vortex the Eppendorf tubes with its content
7. Incubate (=warm) all the Eppendorf tubes at 55˚C for 1 hour.
8. Get the tubes out of the heater and put them back in the tray.
9. Add 200µl if **Binding buffer** (2 green) in the tubes
10. Vortex the Eppendorf tubes with its content.
11. Put clips on the tubes to prevent "popping"
12. Incubate all tubes for 10 minutes at 70˚C
13. Get the tubes out of the heater and put them back in the tray.
14. (centrifuge them shortly at smart-start, to remove condense)
15. Add 100µl of **isopropanol** (not in kit) in the tubes.
16. Vortex the Eppendorf tubes with its content.
17. If necessary, draw big chunks of tissue out with a 1ml disposable pipette tip. Withdraw and discard pipette tip.
18. Centrifuge all tube shortly at 8000 for 15 s = quick start
19. In a tray, put one Collection Tube for each sample and insert one High Filter Tube into the Collection Tube. Label the cap of the tube with the number of the sample.
20. (Put 3x empty Collection Tube in a row in the same tray)
21. Pipet the remainder of the liquid sample into the upper buffer reservoir of the corresponding High Filter Tube.
22. Insert the entire High Pure Filter Tube assembly into the centrifuge and run at 8000g for 1 min.
23. Put the tubes back in the tray.
24. Put new collection tubes for each sample in the tray.
25. Remove the Filter Tube from the collection tube and put them in a new Collection tube.
26. Discard old collection tubes and discard the flow through liquid.

27. Add 500 µl of **Inhibitor Removal Buffer** (reconstituted) into the upper reservoir of the filter tubes, then close cap
28. Centrifuge filter tube and collection tube assemblies at 8000g for 1 min.
29. Put the tubes back in the tray.
30. Put new collection tubes for each sample in the tray.
31. Remove the Filter Tube from the collection tube and put them in a new Collection tube.
32. Discard old collection tubes and discard the flow through liquid.
33. Add 500 µl of **Wash Buffer** (reconstituted) into the upper reservoir of the filter tubes, then close cap.
34. Centrifuge filter tube and collection tube assemblies at 8000 g for 1 min.
35. Put the tubes back in the tray.
36. Put new collection tubes for each sample in the tray.
37. Remove the Filter Tube from the collection tube and put them in a new Collection tube.
38. Discard old collection tubes and discard the flow through liquid.
39. Add 500 µl of **Wash Buffer** (reconstituted) into the upper reservoir of the filter tubes, then close cap.
40. Centrifuge filter tube and collection tube assemblies at full speed (13000g) for 10 seconds to remove residual Wash Buffer.
41. Put new 1.5ml sterile Eppendorf tubes for each sample in a tray and label them. Cut the cap off with a scissors
42. Discard each collection tube and insert filter tubes into new 1.5ml microcentrifuge tubes.
43. Add 200 µl prewarmed **Elution Buffer** (5 colorless, from the 70°C heater) to the upper reservoir of each filter tube and put the cap back on.
44. Centrifuge the filter and microcentrifuge tube assemblies at 8000g for 1 min.
45. Put new Eppendorf tubes for each sample in a tray and label them.
46. Discard the filter tubes and pipette the result content from the Eppendorf tube into the new Eppendorf tubes. Put the cap on.
47. These microcentrifuge tubes contained the eluted DNA, stable nucleic acids for PCR analysis or frozen storage for later use. Store at +2 to +8°C or -15 to -25°C.

DNA amplification

1. Defrost your kidney samples from the day before if they were stored in the freezer (keep them on the bench in the DNA room). Here, the samples were kept in the cooling room.
2. Defrost the positive control
3. Go to the DNA room
4. Each qPCR reaction solution contains:

PCR mix	**25 µL (1 sample)**	**Bulk mix 23x# µL(# sample)**
Forward primer	1 µL	1 µL x# +3
Reverse primer	1 µL	1 µL x#+3
H20 (double distilled water = nr 2 top)	7.3 µL	7.3 µL x#+3
Master mix (red top)	12.5 µL	12.5 µL x#+3
Syto 9 (brown top)	1.2 µL	1.2 µL x#+3
Sample	2 µL	

a. Defrost mastermix and double distilled water

b. Make up a **bulk mix** (yellow tip, on the left bench at the DNA room)
   * use 40 µL pipette tips
   i. 7.3 µL (x #+3) of double distilled water
   ii. 1.2 µL (x #+3) of a commercial mastermix (Roche LightCycler 480 Probes Master 04707494001, Roche Diagnostics GmBH, Mannheim, Germany)

iii. 1.2 µL (x #+3) of SYTO9 (Invitrogen Corp., Carlsbad, CA, USA)
    1. Use 1.2 µL from stock (contains 100µL, keep in fridge and in the dark)
    2. Make a 1:100 working solution: take 1 µL of Syto stock solution and add 99 µL of distilled water
iv. 1 µL (x #+3) of each forward and backward primers targeting the gyrB gene [35], of sequence 5'-TGAGCCAAGAAGAAACAAGCTACA-3' (2For) and 5'-MATGGTTCCRCTTTCCGAAGA-3' (504Rev)
    1. Prepare new primers:
        a. Rehydrate them (primers are freeze dried)
            i. Spin the dehydrated primer at 13000rpm for 3 min before starting
            ii. Look at the number of nmoles in the tube and add the same number of uL of water to it.
            iii. This gives you a 1ng/ul stock solution
        b. OR dilute them from the primer stock/making up a working solution from stock (stock contains 500 µL and you use 1 µL each time):
            i. Do a 1:500 dilution i.e. 1uL stock solution:499uL water

c. Vortex the bulkmix
d. Centrifuge the bulkmix
e. Go to the other side of the DNA room and bring the samples, tray, rotogene pipettes and the bulkmix through the window
f. Turn of the light and turn on the LED-light
g. Put 23 µL of the bulkmix in order (#) into each well (roto-gene tips)
h. Put 2 µL of sample in each well, in the right order
i. Use for the 1th and 2th well a resp. positive and negative control.
j. Sterilise the metal tray when finished.

5. Run the qPCR on a Rotor-Gene Q (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand) *
    a. Put cups with lids on in the Rotor.
    b. Fill the circle up with empty ones
    c. Put top rotor on it
    d. Login on the computer and open the Rotorgene program
    e. Click at edit sample (lower right) and give each sample the right name:
        i. 1. +ve control
        ii. 2. –ve control
        iii. 3. sample 1
        iv. 4. sample 2
        v. Etc
    f. Run the Rotogene
        i. 10 minutes of denaturation at 95°C
        ii. 10 seconds of denaturation at 95°C (40 cycles)
        iii. 20 seconds of annealing at 63°C
        iv. 10 seconds of elongation at 72°C.

** Measure the melting temperature by monitoring the fluorescence on the green channel, every 0.2°C from 78–90°C.*
** For each PCR run a positive control consisting of either DNA extracted from a live culture of a strain of L. interrogans serovar Copenhageni isolated from sheep in New Zealand or DNA extracted from sheep urine inoculated with a live culture of a New Zealand strain of L. borgpetersenii serovar Hardjo,*
** For each PCR run a negative control of double distilled water.*

6. Confirm the positive samples by comparing the melting temperature with the positive control.

E.3. Preparation of blood samples for serology (MAT) on day 1

Materials
Bloodsample, labcoat, gloves, centrifuge, sterile Eppendorf tubes, pipette.

Protocol
1. Transfer 10ml of blood out of the KJ520 60ml container into Greiner © Eppendorf tube.
2. centrifuged at 3000 g for 10 min
3. take the sera out of the tube and put 1.5ml in a sterile Eppendorf tube and label it. Store it in the cooling room (2°C)
4. Put 2µl of each serum sample in a Cryovial container and label it on the white top.
5. Store the Cryovial in the freezer at -80°C, as a backup sample.

E.4 Serology (MAT) of the serum on day x

E.4.1 Master plate preparation

Materials
Labcoat, PBS, serum samples, a 96 well flatt-bottomed serology plate (12x8), sterile yellow tips, P100 pipette, P200 pipette, or a multichannel dispenser, Parafilm or sealing film, re-sealable plastic bag, disposal container for used yellow tips.

Protocol
1. Use a pipetteman to dispens a 30µl sample of serum into a well
2. Dilute 1:6 by adding 150µl of PBS
3. Cover the plate with Parafilm or sealing tape and seal carefully so that serum will not leak between wells.
4. Replace the lid of the plate and write down the number of the master plate on the side of the plate.
5. Store the plate in the -20°C freezer.

E.4.2 MAT serology

Materials
Labcoat, sterile standard saline solution (PBS), serum samples, a 96 well flat-bottomed serology plate, sterile yellow tips, P100 pipette, P200 pipette, or a multi-channel dispenser, Parafilm or sealing film, re-sealable plastic bag, multi-diluter machine, tweezers, Bunsen burner, serology serovar standards, sterile petrie dishes and/or V-shaped reservoirs, serology plates filled with dist. H20 to be used as wash plates, paper towels, antigen culture*, McFarland nephelometer standards, Virkon or equivalent, buckets, glass slides, dark field microscope, “dropper” for placing an aliquot from the wells onto the slide, serology result sheets for recording, disposal container for glass slides and pipete tips, 70-100% Ethanol (EtOH).

Protocol
1. Take the master plates out of the freezer and allow them to thaw.
2. Pour PBS into a clean petri dish/V-shaped reservoir
3. Set the multipipetter at 25µl and fill the serology plates with 25µl of PBS with the multipipetter.
4. Turn on the Bunsen Burner

5. Remove the combs of the multidiluter (which hold 25µl of liquid) and held with the tweezer dipped in 70% ethanol and touched into a flame to let the ethanol burn off. This sterilises the combs and removes any grease.
6. Replace in their mounts in the machine.
7. Check the combs for their carrying capacity by eye.
8. Agitate them in a row of the wash plate, containing distilled H20, and blot the combs on absorbent paper.
9. Check the circumference of the circles to see that all the combs are carrying similar amounts.
10. Dilute a row of sera from the master plate by placing the combs in the multidiluter in the wells and mix them.
11. Take up a 25µl sample of serum.
12. Place the combs in the top row of a prepared serology plate and mix it.
13. Repeat this process through successive rows in the serology plate to make a serial dilution.
14. Wash the combs by agitating them in distilled H20 in a row of wells in a serology wash plate, at the end of the serology plate.
15. Blot the combs with a paper towel.
16. Ad 25µl of antigen to each well using the multipipetter. (This process results in eight, two-fold dilutions covering the range 1:24 – 1:3072)
17. Check the culture for density with the Mc Farland nepholometer scale. It should be close in density to that of standard no.3 (approx. equivalent to 1-2x10^8 organisms/ml).
18. Look at the culture at under the microscope to see whether any auto-agglutination has occurred. If more than a few clumps are observed, then the culture is not suitable for use.)
19. Replace the lid on the plate.
20. Place the plates in a re-sealable plastic bag and keep at room-temperature (20-30 degrees) for 1.5-4 hours.
21. Use the dropper (= pipet) to place a sample from each of the eight dilutions from each well into a microscope slide.
22. Look for the result
    a. *The end-point of an agglutination reaction is deemed to be the dilution at which approximately 50% of the organisms have been agglutinated.
    b. *Read the standard plate first to ensure that the antigen reacts appropriately with its antiserum standard.
    c. *A record of all standard plates is kept in the laboratory so that it can be checked that the end-points for standard agglutinations fall within the acceptable titration range. (That is, up to 2 dilutions either side of the average titration for the particular antiserum. 1 dilution factor if the antigen is at approximately #3 on the nephelometer scale or, up to two dilutions higher or lower if the concentration of the antigen is respectively a little higher or lower than #3)
23. Place the serology plates in a bucket of Virkon solution (for a minimum of half an hour) after use, to decontaminate them.
24. Wash the serology plates with tap water and rinse them with distilled H20 and leave to air dry.