Detection and localization of Leptospira spp. by real-time PCR and culture in the genital tract and fetal tissues in the beginning and late pregnancy of slaughtered deer

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Abstract

Leptospirosis is an economically important bacterial infection of livestock that causes reproductive losses due to abortions, stillbirths and infertility and non-reproductive losses due to septicaemia and nephritis. In addition, leptospirosis is presumed to be the most widespread zoonotic disease in the world.
Earlier studies in red deer in New Zealand show reproductive effects, mainly lower weaning rates. No effect has been proved yet on calving rate *per se.* Abortion due to leptospirosis has been demonstrated only experimentally in (white tailed) deer. The economic consequence of reproductive effects in rising-two-year-old deer in NZ averaged 15.5$ per hind.
This study attempts to find evidence for detection and localization of *Leptospira* spp. by PCR and culture in the genital tract and fetal tissues in the beginning and late pregnancy of slaughtered deer.
From July to October 2011, 57 mixed age hinds, phenotypically Cervus elaphus, but possibly containing some Wapiti genes, from 7 commercial deer farms were sampled at a deer slaughter premises in Feilding, New Zealand. From each deer farm 3 to 23 hinds were randomly sampled at the slaughterhouse. In total, 57 kidneys, 57 blood samples, 57 uteri and 23 fetuses were collected at a deer slaughter premises.
Serum reactivity was tested for serovars Hardjo-bovis, Pomona and Ballum. A titre of ≥1:48 was considered as positive. Bacterial culture and real-time PCR were performed on the kidney, uteri and fetus (kidneys) samples.
For the 57 blood samples, 29 (50.1%) were positive for serovar Hardjo-bovis, 41 (71.9%) were positive for serovar Pomona and 2 (3.5%) were positive for serovar Ballum. Eight of 43 (18.6%) kidneys from 2 farms were culture positive. . Three of 48 kidney samples were PCR positive all of which were culture positive. One of the culture positive kidney samples was not positive by real-time PCR. From 4 culture positive kidney samples it was not possible to perform real-time PCR.
This research failed to isolate *Leptospira* spp. from the uterus or fetus of deer by bacterial culture and real time PCR.

1. Introduction

Deer Industry in New Zealand

The deer industry in New Zealand is a relatively young industry compared to other livestock industries in the country. It has grown rapidly in a small amount of time. (Archer JA 2003, 233-236)
Before 1969 deer farming was illegal in New Zealand. Deer were characterized as noxious animals and were seen as a pest. However, a small number of individuals, who kept deer in captivity revolted against the authorities. In 1969 Deer Farming Regulations were invoked and they strictly controlled deer farming, stock, fences and site. For farming, transport and capture of deer licenses were needed, because the authorities had a big fear that farmed deer would escape and become a plaque again.

Since the first license was issued in 1970, deer farming steadily increased. In 1980 deer were classified by law as game instead of stock. The advantage of this classification is that venison incurs only a 5% tariff in the EEC instead of a 20% tariff on meat.
To expand the deer farming industry Deer Slaughter premises and more deer farms were built and at the end of 1986 there were approximately 3000 deer farms in the country. Most of them now farm in conjunction with sheep and beef. Farming beef, sheep and deer together is a good protection against wild swings in income experienced with single enterprise farming. Currently 85% of deer farms also farm cattle, goats or sheep.(Wilson 2010)

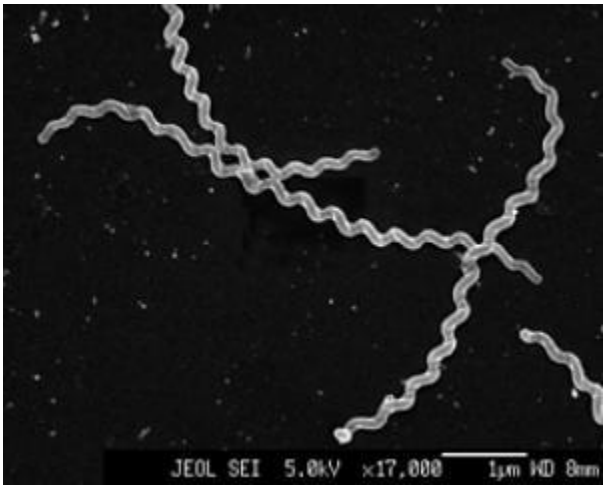
At present there are approximately 3500 of deer farmers in New Zealand. There have been ups and downs in the development of the deer industry, but the prices for velvet and venison have firmed and there is confidence in the industry. New Zealand nowadays has one of the biggest populations of farmed deer in the world. Approximately 85% of the farmed deer population are red deer, 10% are wapiti/red deer crossbreeds and the remaining 5% comprising fallow deer.
The red and wapiti deer are becoming more and more popular, because these are larger breeds and the industry steers towards the use of larger deer for use as terminal sires.
The red deer in New Zealand originate from the UK. Because deer of UK origin are genetically the smallest, New Zealand imported various strains of larger red deer from Eastern and Central Europe. There are currently 28 sub-species of red deer. They are farmed for velvet, venison and co-products. Red deer are known to be adaptable to farming, easy to handle, easily managed and easy to transport. In captivity they reproduce well and they are cross-bred with the larger wapiti deer. (Wilson 2010; Shadbolt et al. 2008, 1-54)

The major product of the deer industry is venison. All but a very small amount of venison is exported. Currently about 300 tonnes were consumed locally (<2.0% of total production), but the local market is expanding slowly. Around 40% of venison is exported to Germany and another 40% is goes to other European and Scandinavian countries. Approximately 12% is exported to the USA.
To obtain the venison, deer are slaughtered in special slaughterhouses called deer slaughter premises (DSP's). DSP's are separate entities from abattoirs, because of the game classification of deer. (Shadbolt et al. 2008, 1-54)

Leptospirosis
Bacteria properties

Leptospirosis is a disease caused by spirochetes of the genus *Leptospira*. Leptospires are ideal zoonotic bacteria causing infection and disease in humans and animals. They have a broad host spectrum and produce non life-threatening infections in most maintenance or carrier hosts.

Leptospires are long, slender, filamentous and motile rods measuring 0.2 to 0.3 micrometers in diameter and 10 to 30 micrometer in length. They have a double membrane wherein the peptidoglycan cell and cytoplasmic membrane are closely linked and are covered by an outer membrane.(Cullen, Haake, and Adler 2004, 291-318) LPS, which is located within the outer membrane, is the primary antigen for leptospires. This LPS is similar to the structure and function of LPS from gram negative organisms. However, leptopires LPS is less toxic to cells or animals.(Faine 1993)



Electron micrograph of L. interrogans.(Cornell University)

The genome of leptospires consists of two circular chromosomes. The leptospiral genome is relatively large compared to other spirochetes, which may explain why leptospires can live well in different environments: animals and freely in the environment. Usually each end of the spirochaete is hooked and the characteristic motility is caused by two periplasmic flaggelae. (Bharti et al. 2003, 757-771) The motility and appearance of the spirocheates depends on the medium in which they grow. Leptospires cells appear bent or hooked at one or both sides when they are grown in liquid media, but straight mutants can also be found.
In some other cultures, the leptospires can appear as little granules filled with coiled remnants of the cell. The leptospires can move in different ways. The first type of movement is rotation around a central axis, the second is a circular motion and the last type is progressive movement in the direction of the straight end.(Ellis et al. 1983, 323-335)

The growth of leptospires is very slow on primary isolation. They are obligate aerobeic bacteria with an optimum growth temperature between 28 and 30 degrees Celsius. To grow leptospires a simple medium with vitamins B12 and B1, ammonium salts and long-chain fatty acids is needed. At present, the most used medium is the Ellinghausen- McCullough-Johnson- Harris medium, containing oleic acid, bovine serum albumin and Tween. To protect the medium against contamination with other bacteria additions with 5-fluorouracil, gentamicin, rifampicin or nalidixic acid are used.(Adler and de la Pena Moctezuma 2010, 287-296)

Traditionally *Leptospira* were divided into the pathogenic species L. interrogans and L. borgpetersenii and the free-living, non pathogenic species, L. biflexa and L. parva. L interrogans can now be subdivided into serovars corresponding to stable antigenic differences and serogroups on the basis of common antigens. Presently, 17 genomospecies, more than 200 serovars and 23 serogroups have been identified.(Guerra 2009, 472-478)

Wilson et al. reported serological evidence of serovars Hardjo-bovis, Pomona Copenhageni, Tarassovi, Ballum, Balcanica and Australis in farmed deer in New Zealand. They also reported bacterial cultural evidence of serovars Hardjo-bovis, Copenhageni and Pomona.(Wilson et al. 1989, 131-139)

Pathogenesis

Leptospira can enter the body through small abrasions or cuts in the skin and through mucous membranes. After entering the body, leptospires circulate in the blood stream with a bacteremic phase that can last up to seven days. After the numbers of spirochaetes reach a critical level in blood and tissues, lesions caused by the action of undefined toxins and symptoms appear. At first, local ischemia in organs due to the damage of the endothelium of small blood vessels appears. This leads to hepatocellular and pulmonary damage, renal tubular necrosis, meningitis, placentitis and myositis. Once antibodies appear, the leptospires are killed by opsonophagocytosis. The affected tissues can be completely repaired after an infection, although long lasting tissue damage also has been described.(Bharti et al. 2003, 757-771)
It is still not clear by which mechanisms leptospires cause host tissue damage and disease. There are reports of leptospiral mechanisms, but in all reports the specific leptospiral component responsible for the damage was not identified. A couple of leptospiral proteins involved in adhesion, survival *in vivo* and renal colonisation have been studied, but these studies indicate a high degree of redundancy in the proteins. Adler et al. 2010 suggest that it will be hard to identify and specify the virulence factors with single gene inactivation.(Adler and de la Pena Moctezuma 2010, 287-296)

Leptospirosis in red deer

The seroprevalence of *Leptospira* spp. serovars in deer in New Zealand is high and distributed in farmed deer herds throughout the whole country. An earlier Massey prevalence research showed that 61% of deer herds have been exposed to serovar Hardjo-bovis alone, 4% to serovar Pomona alone and 16% to both serovars (overall 81%). Thus, serovar Hardjo-bovis is endemic in most herds whereas serovar Pomona occurs sporadically (Ayanegui-Alcerreca et al. 2010, 184-189). However, other serological surveys show variation in the prevalence of various *Leptospira* spp. serovars. Age and sex of deer and serological interpretation may contribute to the variation in prevalence between the different surveys.(Ayanegui-Alcerreca et al. 2007, 102-108)
Farmed deer appear to be maintenance hosts and a maintenance population for serovar Hardjo-bovis, since there is evidence of widespread infection and persistent infections in herds over 21 months.(Ayanegui-Alcerreca et al. 2004, 34-38) For serovar Pomona deer are incidental or accidental hosts and probably a maintenance population, because research showed that some infections can persist for several months.(Ayanegui-Alcerreca et al. 2007, 102-108)
Most reports of clinical leptospirosis in deer are associated with culture evidence and serological evidence of *Leptospira* spp. serovar Pomona. However, there are also reports of clinical cases caused by serovar Hardjo-bovis.(Wilson and McGhie 1993, 19-21) Based on laboratory results on serology alone it is difficult to make conclusions about the cause of disease, because a lot apparently healthy deer are seropositive to some serovars. Most of the clinical disease reports are from deer in their first year of life. It appears to be that young deer get infected during autumn, when the animals are moved from farm to farm and mixed with other animals.(Ayanegui-Alcerreca et al. 2007, 102-108; Wilson and McGhie 1993, 19-21) Serovar Pomona can be

transmitted to and/or from newly introduced cattle and sheep to young deer and it is likely that serovar Copenhageni can be transmitted by rats. (Dean et al. 2005, 121-123; Flint, Marshall, and Winter 1986, 70-71)

Clinical disease in deer is often manifested by haemolysis, renal lesions, jaundice, haemoglobinurie, and sudden dead. There is only one case report of confirmed leptospirosis in live deer, describing lethargy and red urine observed on the floor of the yard.
An earlier Massey University survey showed reproductive effects, mainly lower weaning rates. No effect has been proved yet on calving rate *per se*. Abortion due to leptospirosis has been demonstrated only experimentally in (white tailed) deer.(Trainer, Karstad, and Hanson 1961, 278-286)

Reproductive Cycle

The uterus of cervids is bicornuate, it has a small body and two long horns. Each of the horns contains four or five caruncles to which trophoblast cotyledons attach to form placentomes. In contrast to placentation in other ruminants, placententation in red deer is oligocotyledonary with eight to ten placentomes formed and synepitheliochorial. (Hamilton, Harrison, and Young 1961, 1-33)
The ovalutory cycle of cervid species, including red deer, is polyoestrous. Non pregnant females show continuous oestrus/luteal cycles or alternating periods of oestrous cyclicity and anoestrum. In red deer, the first oestrus starts in autumn and will end in spring. Between autumn and spring up to 5-8 oestrus cycles will be expressed.(Asher GW et al. 1994, 257-277) The anoestrum can persist for 4 to 6 months from spring to early autumn.(Asher and Fisher 1991, 474-484)
The length of the oestrus cycle is approximately 18 to 20 days at the beginning of the breeding season. As the breeding season continues, the length of the oestrus cycle progressively increases. This phenomenon was only observed in red deer, black-tailed deer, fallow deer and white tailed deer.(Guinness, Lincoln, and Short 1971, 427-438)
During every oestrus cycle, a couple of growing follicles will be selected and these follicles will grow and develop quickly. Only one or two of the selected follicles will eventually ovulate. These follicle growth patterns have been studied in other domestic ruminants, but little is known about it in red deer.(Asher GW et al. 1994, 257-277)
McLeod et al. have monitored the growth and development of ovarian follicles in red deer. The conclusion of their study was that during the oestrus cycle and anoestrus one or two antral follicles are present. Between the breeding season and anoestrus the morphology of follicle populations differ little, with the exception of preovulatory development. This suggests that the seasonal anoestrus in deer is probably not due to a gonadal block.
The luteal phase during the oestrus cycle in cervids is similar to other domestic ruminants. During luteinization, the progesterone secretion increases. The highest progesterone levels are achieved between day 10 and day 16 of the oestrus cycle(Asher GW et al. 1994, 257-277). The absolute and relative levels of progesterone measured in peripheral blood or urine may vary between individuals of the same deer species. Concentrations of progesterone are measured in blood plasma and urine by direct radioimmunoassay as described for red deer by Asher et al. (1992). The variety of progesterone levels between blood and urine is probably caused by the induction of superovulation, which results in higher ovulation rates and thus a higher level of plasma progesterone (Asher et al. 1992, 261-273).

Leptospires in the genital tract

Infection with leptospires can affect the fertility in several domestic animals. It has been shown to affect the fertility in cattle, sheep and goats.(Subharat et al. 2010, 281) Besides affecting fertility, leptospiral infection can cause early embryonic death, abortion, stillbirth and weak newborns in pigs and cattle.(Ellis et al. 1986, 294-295; Ellis et al. 1982, 192-194; Ellis et al. 1982, 147-150)
Leptospires primarily localise and persist in the proximal renal tubules of kidneys. Several attempts have been

made to investigate the localization and presence of leptospires in the genital tract in several domestic animals. Table 1. shows the results of different researches in cattle, pigs, deer and sheep. It shows that leptospirosis infection is identified in the genital tract and fetuses of these animals. A screening of deer uteri and early foetuses (up to early July) was done in 2010 by Massey University Deer Research Group in order to find evidence for vertical transmission of Leptospira spp and to attempt to explain the pathogenesis of

Table 1.

Species		Localisation of leptospira in animal	Localisation of leptospira in fetus	Aborted fetus	Age fetus	Strain	Author
Cattle	Pregnant heifers	Placentae, kidneys	Liver, kidneys, lungs	yes		L. interrogans serogroup Hepdomadis	(Ellis and Michna 1977, 229-236)
	Normal fetuses and uteri	Uteri	kidneys	no	5-7 months	L. hardjo boviis	(Ellis et al. 1982, 192-194)
	Pregnant heifers after calving or abortion	Vaginal discharges, uteri		Yes and no		L. hardjo boviis	(Ellis et al. 1985, 296-298)
	Aborted fetuses		Kidneys, eye	yes		L. hardjo bovis	(Ellis et al. 1982, 147-150)
	Beefcattle	Kidneys				L. hardjo boviis	(Prescott, Miller, and Nicholson 1987, 229-231)
Pigs	Swine	Kidneys, uteri, oviducts				L. Bratislava, L. hardjo	(Bolin and Cassells 1992, 87-89)
	Aborted sows	Kidney, upper genital tract				L. Bratislava, L. Muenchen	(Ellis et al. 1986, 294-295)
	Stillborn and weak pigs from sows	Placenta	Sera, kidneys, lungs			L. Bratislava	(Bolin and Cassells 1990, 1601-1604)
Deer	Pregnant and non-pregnant deer	Kidneys	kidney	1 positive fetus in slaughterhouse	3 months	L. Hardjo.	(Subharat et al. 2010, 281)
Goats	Females and bucks	Semen and vaginal fluid				L. Hardjo, L. Shermani, L. Gryppothyphosa	(Lilenbaum et al. 2008, 837-842)
Sheep	Naturally infected sheep	Kidneys				L. hardjo bovis	(Cerri et al. 1996, 175-178)
	Sheep only abattoir	Kidneys, blood				L. hardjo bovis L. Pomona	(Dorjee et al. 2008, 164-170)
	Aborted, stillborn lambs		kidneys	yes		L. Pomona, L. Hebdomadis, L. Australis	(Ellis et al. 1983, 291-293)

observed reproductive losses in farmed deer. Real-time PCR provided evidence of infection in one fetus that was approximately 3 months of gestation age, but not in uterin tissue. This tentatively indicates that that leptospiroses may pass from the dam to the fetus in dear in early pregnancy. (Subharat et al. 2010, 281)

Estimating fetal age in red deer

By day 27 the trophoblast is extended throughout both horns and the end of the chorion lies close to the utero-tubal junctions. Both yolk sac and amnion are present by day 27 and length of the allantois is about a fifth the length of the chorion.
By day 34 the allantois fills the entire chorion and the yolk sac is considerably smaller and is not evident thereafter. Around day 34 placentome formation begins and by day 55 the placentation is well developed in all but especially the most cranial caruncles. The limb buds appear by day 34 and the phalanges separate into hooves and dew claws by day 48. The most accurate age prediction in early pregnancy can be achieved by measuring the crown-rump length and amnion length.(McMahon et al. 1997, 723-730)
Table 2 shows the results.

Table 2.

Days of pregnancy	Embryo weight (g)	Crown-rump length (mm)
27	0.02	5.7
34	0.35	13.1
41	0.96	28.2
48	3.02	37.7
55	7.56	55.3

The most convenient way to estimate foetal age in the slaughterhouse is by weighing and measuring the crown – rump length. To estimate the age with known crown-rump length under mentioned equation can be used:

$$t = -5.7 + 15.75 \ln X$$

Where X is crown-rump length in mm and t is fetal age in days. (Revol and Wilson 1991, 241-253)

To estimate fetal age with known weight figure 1 can be used. Wenham et al. were able to plot fetal body weight(g) against fetal age (days) using under mentioned equation.

$$Ln\ W = 10.131 - 14.341 \exp(-0.01089t)$$

Where W = fetal body weight (kg) and t is fetal age in days.(Wenham, Adam, and Moir 1986, 336-349)

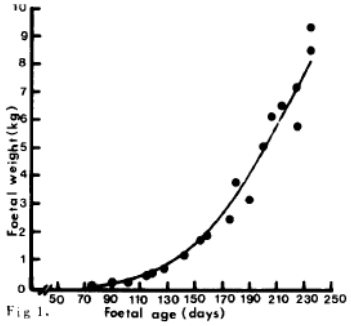


Fig 1.

Aim of the study

This study is a part of a four year during PhD research programme. The PhD includes examining the potential role of leptospirosis in late pregnancy and the perinatal period to identify at which stage of the reproductive cycle losses are occurring.

It has been shown that leptopiral infections in cattle and pigs cause reproductive losses. However, little is known about the role of leptospirosis on reproduction in farmed deer in New Zealand.

Two earlier Massey University surveys show reproductive effects in deer, mainly lower weaning rates. Both studies looked at the improvement of weaning rate in vaccinated deer against Leptospirosis on deer farms with a leptospiral infection history. One of the studies found a significant 5.7 percentage point increase of weaning rates, and the other study found a 9.0 percentage point increase in weaning rates due to vaccination on farms with evidence of serovar Hardjo-bovis and Pomona infection. (Ayanegui-Alcerreca et al. 2010, 184-189) (Subharat et al. 2011, 743-752) No effect has been proved yet on calving rate *per se*.

These results indicate that leptospirosis can cause reproductive losses in farmed deer in New Zealand. The economic consequence of reproductive effects in rising-two-year-old deer in New Zealand averaged 15.5$ per hind.

A screening of uteri and early foetuses (up to early July) was conducted recently by Massey University Deer Research Group in order to find evidence for vertical transmission, as a first approach for investigating the physiopathology of the reproduction effects: infection of foetuses in early pregnancy was demonstrated, but not at the uterus tissue. Real-time PCR provided evidence of infection in one fetus, which was approximately 3 months of gestation age. This tentatively indicates that leptospiroses may pass from the dam to the fetus in dear in early pregnancy. (Subharat et al. 2010)

This study attempts to find evidence for detection and localization of *Leptospira* spp. by PCR and culture in the genital tract and fetal tissues in the beginning and later stages of pregnancy of slaughtered deer.

2. Material and Methods

2.1 Animals

From July to October 2011, 57 mixed age hinds, phenotypically Cervus elaphus, but possibly containing some Wapiti genes, from 7 commercial deer farms were sampled at a deer slaughter premises in Feilding, New Zealand. From each deer farm 3 to 23 hinds were randomly sampled at the slaughterhouse. Nothing was known about the leptospiral infection history of the farms. In total, 57 kidneys, 57 blood samples, 57 uteri and 23 fetuses were collected. The number of samples required was estimated as 64 animals using FreeCalc Software v2.0 (AusVet Animal Health Services), to find at least one animal with evidence of Leptospira in the reproductive tissue. The calculation was based on the real-time PCR test, using DNA gyrase subunit B gene primers specificity of 99.2% and sensitivity of 85.0% and an assumed prevalence of disease of 10%.

2.2 Blood collection

Blood samples were collected after sticking the animals from the jugular vein, into 10-ml plain vacutainers. The samples were held at 4ºC for transport to the laboratory. In the laboratory the samples were centrifuged at 3000 rpm for 10 minutes and after that the serum was aliquoted into 1.5 ml microcentrifuge tubes. These tubes were stored at -20ºC for Micro Agglutination Test.

2.3 Kidney collection

The kidneys from the hinds were collected at the evisceration and inspection area in the slaughterhouse. They were put aseptically in a sterile plastic bag, with identification. The samples were transported at 4 ºC. In the laboratory, the kidneys were transported to the biohazard cabinet and the surface of the kidneys was swapped with 70 % of alcohol. The kidneys tissue was randomly excised with a sterile 16- G needle into a 5 ml sterile syringe. 50 mg of tissue was used for DNA extraction and 50 mg for culture preparation.

2.4 Uterus and fetus collection

Uteri were collected at the slaughterhouse with or without fetus at the same time as the kidney collection. They were put in a sterile plastic bag, with identification and transported to the laboratory at 4ºC. The uteri were transported to the post mortem room for further processing. For non pregnant and pregnant hinds, the uteri were cut open and the mucosal layer of the uteri was randomly excised with a sterile scalpel blade in the horns and bodies to take approximately 50 mg of tissue. For pregnant uteri, the fetal membranes were cut open and the whole fetus was taken out aseptically. The fetuses were incised and the kidneys were removed and further processed in the biohazard cabinet.

2.5 Microscopic Agglutination Test

The Microscopic Agglutination Test was used to test the serum samples for reactivity to Leptospira serovars Pomona, Hardjo-bovis and Ballum. MAT is considered to be the golden standard assay for the diagnosis of leptospirosis. The used method is developed by the Leptospirosis Research Unit, Institute of Veterinary, Animal and Biomedical Sciences, Massey University based on the guidelines for the control of leptospirosis.(Faine 1982, 125-134) To consider a sample as positive, a titre of ≥1:48 was required.

2.5.1. Materials

Lab coat
Sterile standard saline solution
Serum samples
96 well flat-bottomed serology plate
Sterile yellow tips

P100 pipette
P200 pipette
Parafilm
Re-sealable plastic bag
Disposal container
Multidiluter machine
Bunsen burner
Serology serovar standards
Sterile petrie dishes
Serology plates filled with distilled H2O to be used as wash plates
Antigen culture
Virkon
Buckets
Glass slides
Dark field microscope
"Dropper"
Serology result sheets for recording

2.5.2 Masterplate preparation

Using a pipetteman, a 30µl sample of serum was dispensed into each well of a 96 well flat-bottemed serology plate and then diluted 1:6 by adding 150µl of standard saline dilution. The plate was then covered with Parafilm and carefully sealed so that the serum would not leak between wells. After that the lid of the plate was replaced and the masterplate was stored at -20 ºC. The number of the masterplate was written down the side of the plate bearing the letters A-H, and the ID's of sera were recorded on a masterplate record sheet which was stored in the masterplates and standerds file.

2.5.3 MAT

Masterplates were taken out of the freezer to thaw. Sterile standard saline was poured into a clean petri dish and the multipipetter was set at 25 µl. The wells of the 96 well plates were prepared by filling with 25 µl of sterile standard saline using the multipipetter.

The combs of the multidiluter machine (which hold 25µl of liquid) were removed and flamed to sterilize and remove any grease. After sterilizing they were replaced in their mounts in the multidiluter machine.

25 µl of serum from the masterplate was added into each well of the top row of the 96 well plate. Serial dilutions were made by placing the combs of the multidiluter in the wells, mixing and taking up 25 µl serum sample and adding it to the next rows.

At the end of the serology plate, the combs were washed by agitating them in distilled H2O in a row of wells in a serology wash plate.

After this, 25 µl of antigen was added into each well of the serology plate using the multipipetter. This process results in eight, two-fold dilutions covering the range 1:24 -1:3072. The culture used as an antigen was checked before adding to the serology plates, because the antigen must be living and generally between 4-14 days old.

The positive and negative control plates were prepared in the same manner as the serum sample serology plate. For the positive control antiserum against each serovar was used and standard saline for the negative control.

After this, the lids of the serology plates were replaced and the plates were placed in re-sealable plastic bags

and kept at room temperature (20-30 ºC) for 1.5-4 hours.
The 'dropper' was used to place a sample from each of the eight dilutions from each well onto a microscope slide. The end point of an agglutination reaction was deemed to be the dilution at which approximately 50% of the organisms have been agglutinated.
The standard plate was read first to ensure that the antigen reacts appropriately with its antiserum standard. After use the serology plates were placed in a bucket of Virkon solution for half an hour to decontaminate them.

2.6 Bacterial culture

2.6.1 Materials
Labcoat
Glass slides
Darkfield microscope
Sterile standard saline solution
Sterile stomacher bags
Stomacher
Latex gloves
Sterile plastic pipettes
Culture bottles containing 5ml EMJH+ 5-fluoroucil
Incubator
Biohazard cabinet
Disposal container

2.6.2. Culture preparation
50 mg of kidney, uterus and fetal kidney sample was taken out of the 5 ml sterile syringes, which were prepared after collection. For each sample, the tissue was then placed in a sterile stomacher bag and standard saline solution, equivalent to the approximate volume of tissue. After that the bag was placed in the stomacher machine and pulverized until the tissue had been broken down to a pulp. 100µl of the resulting supernatant was drawn off with a sterile plastic pipette and transferred into a labelled culture bottle containing 5 ml of EMJH with 5-fluouracil. 100µl of this bottle was then cultured into another labelled culture bottle and the process was repeated yet again to obtain three serial dilutions. The procedure was carried out in a biohazard cabinet and the culture bottles were placed in a 28-30 degrees C incubator.
The cultures were weekly checked for growth of Leptospires under the darkfield microscope for 4 months. After 4 months the cultures were discarded.

2.7 DNA extraction
The leptospiral DNA was extracted from the kidney, uteri and fetal samples. The Roche High Pure Template Preparation Kit was used for the extraction.

For each sample 1.5ml sterile eppendorf tubes were labeled with the number of the sample on the cap of the tube. After labeling, 40µl of Proteinase K, 200µl Tissue Lysis buffer and 160µl of kidney/uterus/fetal sample after stomacher procedure were added to each labeled tube, mixed by pulse- vortexing and incubated at 55 degree C for 1 Hour. 200µl of Binding Buffer was added to each tube and all tubes were incubated again for

10 minutes at 70 degrees C. 100µl of isopropanol was added to the tubes and mixed by pulse-vortexing. The tubes were centrifuged at 8,000g for 15 seconds. For each sample, one filter tube was inserted into one collection tube and the caps of the tubes were labeled with the number of the sample. The entire samples were transferred into the upper reservoir of the corresponding filter tubes and the caps were closed. Filter tubes and collection tubes were centrifuged at 8,000g for 1 minute. Each collection tube was discarded and filter tubes were inserted into new collection tubes. 500µl of Inhibitor Removal Buffer was added into the upper reservoirs of the filter tubes and the caps were closed. Filter tubes and collection tubes were centrifuged at 8,000g for 1 minute. Each collection tube was discarded and filter tubes were inserted into new collection tubes. 500µl of Wash buffer was added into the upper reservoirs of the filter tubes and the caps were closed. Filter tubes and collection tubes were centrifuged at 8,000g for 1 minute. Each collection tube was discarded and filter tubes were inserted into new collection tubes. Adding 500µl of Wash Buffer and centrifuging them at 8,000 for 1 minute was repeated. After that, the filter tube and collection tubes were centrifuged again at 13,000g for 10 seconds to remove residual Wash Buffer. Each collection tube was discarded and the filters were inserted into new labeled 1.5 ml microcentrifuge tubes. 200µl Elution buffer (heated up to 70 degree C) was added to the upper reservoir of each filter tube and filter tubes were recapped. The filter and microcentrifuge tube assemblies were centrifuged at 8,000g for 1 minute. The filter tubes were discarded and the microcentrifuge tubes were recapped. These microcentrifuge tubes contained the eluted, stable nucleic acids for PCR analysis. The tubes were stored in the freezer.

2.8 Real time PCR

Real time PCR was done on the kidney, uteri and fetus samples. The PCR technique was a modification of the method described by Subharat et al. (2011), with a sensitivity and specificity of 85% and 99.2%,.(Subharat et al. 2011, 743-752) As a double-stranded DNA-specific intercalating dye, SYTO9 (Invitrogen, Eugene OR, USA) was used. The forward primer 2 5'-tgagccaagaagaaacaagctaca-3' and reverse primer 504 5'-matggttccrctttccgaaga-3' were used.

For each sample plus a negative control and positive control microtubes were prepared. To each microtube 12.5 µl of Mastermix (LightCycler 480 Probes Master, Roche Diagnostics, Mannheim, Germany), 1 µl forward primer, 1 µl reverse primer, 7.3 µl double-distilled water, 1.2 µl SYT09 and 2 µl DNA extracted from the samples were added. For the positive control, instead of 2 µl DNA extracted from the samples, 2 µl of a field isolate of Leptospira serovar Hardjo-bovis was added. For the negative control, 2 µl of double-distilled water was added to a microtube.

After this, the microtubes were put in the Rotor-Gene 6000 machine (Corbett Research, Mortlake, Australia) and initially denaturized at 95ºC for 10 minutes, after that 40 cycles of denaturation at 95ºC for 10 seconds, annealing at 60ºC for 20 seconds and finally extension at 72ºC for 20 seconds.

Fluorescence change was monitored every 0.1ºC while heating the PCR product from 70ºC to 90º to determine the melting temperature of the PCR product. The positive samples were confirmed by comparing the melting temperatures with that of the positive control.

3. Results

Age Fetuses

The age of 5 fetuses was estimated by using the method described in Material and Methods. The results are summarized in Table 2. The age of the other 18 fetuses was not determined, because the weight and crown rump length were not measured.

Sample ID	Sampling date	Weight (g)	Crown rump length (mm)	Age by using weight (days)
53	05-10-2011	3005	440	175
54	05-10-2011	3940	470	187
55	05-10-2011	2390	400	166
56	05-10-2011	3520	460	182
57	05-10-2011	3520	460	182

Table 2. Age of fetuses from sample 53, 54, 55, 56 and 58.

Fetus from sample ID 55. Age is 166 days.

MAT

Serum reactivity was tested for serovars Hardjo-bovis, Pomona and Ballum. A titre of ≥1:48 was considered as positive. For the 57 blood samples, 29 (50.1%) were positive for serovar Hardjo-bovis, 41 (71.9%) were positive for serovar Pomona and 2 (3.5%) were positive for serovar Ballum.
Results are summarized in Table 3. Full data for each individual animal are presented in Appendix 1.

Line	n	Hardjo-bovis	Pomona	Ballum
A	1	1/1	0/1	0/1
B	22	10/22	16/22	0/22
C	10	7/10	5/10	1/10
D	12	4/12	9/12	0/12
E	9	4/9	8/9	0/9
F	3	3/3	3/3	1/3
Total	57	29/57	41/57	2/57

Table 3. Summarised of results from the MAT test for *Leptospira* serovars Hardjo-bovis, Pomona and Ballum (number positive/number tested)

Culture

Cultures were checked weekly for 4 months. Eight kidneys from 2 farms were culture positive (n=43, 18.6%). Five culture positive hinds were seropositive for both Hardjo-bovis and Pomona. One culture positive hind was seropositive for Hardjo-bovis, Pomona and Ballum. Two culture positive hinds were only seropositive for Hardjo-bovis. None of the 12 fetuses or 34 uteri were culture positive. The culture results are summarized in Table 4. Full date are in Appendix 1.

Line	n	Kidney	Uterus	Fetus kidney
A	1	0/1	0/1	n/a
B	22	4/22	0/22	0/6
C	10	4/10	0/10	n/a
D	12	0/10	0/1	0/12
E	9	n/a	n/a	n/a
F	3	n/a	n/a	n/a
Total	57	8/43	0/34	0/12

Table 4. Summary of results of the bacterial cultures for *Leptospira* spp. of kidneys, uteri of the dams and kidney fetuses (number positive/number tested).

Real-time PCR

Real-time PCR was done on 48 kidney samples, 57 uterus samples and 23 fetuses. Three of 48 kidney samples were PCR positive all of which were culture positive. One of the culture positive kidney samples was not positive by real-time PCR. None of the 57 uteri or 23 fetuses were PCR positive. Results are summarized in Table 5.

Line	n	Kidney	Uterus	Fetus kidney
A	1	0/1	0/1	n/a
B	22	3/22	0/22	0/6
C	10	0/1	0/10	n/a
D	12	0/12	0/12	0/12
E	9	0/9	0/9	0/2
F	3	0/3	0/3	0/3
Total	57	3/48	0/57	0/23

Table 5. Summary of results of the real-time PCR for *Leptospira* spp. of kidneys, uteri and fetuses (number of positive/number tested)

4. Discussion

The results of this study show that *Leptospira* spp. can only be detected and localized in the kidneys of slaughtered deer in both early and late gestation. No evidence of *Leptospira* spp. in uterus or fetal tissues, collected between July and October 2011 (second and third trimester) has been found. The lines in this study were sampled randomly, so nothing was known about the leptospiral infection history of the dams. That is why beside blood, uterus and fetus samples, kidney samples were taken to find more evidence of the infection status of the dam. Kidneys are known to be the predilection site for *Leptospira* spp. If lines with a history of leptospiral infections and abortion were sampled, the chance of finding *Leptospira* spp. in the genital tract could have been higher. Further, this study has aninsufficient number of samples to be highly confident that leptospires do not populate the reproductive tract or fetus. The context of this study is that it was preliminary to a larger study to continue in 2012, which will collect a greater number of samples for a range of potential infectious causes of abortion

The age of the sampled hinds could also influence the chance of detecting *Leptospira*. In this study mixed-age hinds were sampled. Yearlings are more likely to be recently infected and the change of detecting infection might be higher in them. Older deer can already be immune and may not be shedding or carrying the bacteria any more.(Subharat et al. 2010, 281)

Serology results show that each sampled farm is seropositive for one or more serovars. The only thing that could be said about the seropositive animals is that they have been infected with one or more serovars. Nothing can be said about the infection status of the seropositive deer. The Microscopic Agglutination Test was used to test the serum samples for reactivity to Leptospira serovars Pomona, Hardjo-bovis and Ballum. Serovar Hardjo-bovis is endemic in most herds whereas serovar Pomona occurs sporadically. Earlier Massey surveys show that these two serovars are apparently responsible for lowered weaning rates and thus responsible for economic losses. In this study serovar Ballum was also tested to investigate the percentage of seropositive deer herds in New Zealand. Only 2 hinds were seropositive for serovar Ballum with a titre of 1:48, which is a very low titre, considering that a titre of ≥1:48 was required to consider a sample positive.

The reason why one kidney sample was culture positive, but not PCR positive may have been due to the concentration of leptospires of the sample. The detection limit is 10^3 cells/ml and the concentration in the kidney sample may have been lower than the detection limit. Subharat et al. validated the PCR and they found a sensitivity of 99.2% and specificity of 85.0%. Thus, it is possible to find false-positive and false-negative results. In this research the PCR method was adapted and another Mastermix was used. More research has to be done to evaluate the validity of this new method. (Subharat et al. 2011, 743-752) Another reason for not detecting leptospires by PCR could be because the DNA extraction failed.

Bacterial culture results show that 8 of the 43 kidney samples were positive. Culture is known as the golden standard for the detection of leptospires. However, growing leptospires in a culture depends on the microbiological viability of the organism. If the leptospires are dead they will not grow in the medium and the test result will be negative. Another limitation of this method is the susceptibility to contamination. Many of the uteri and fetal cultures were contaminated in this study, which made these samples non valid. 5-fluouracil, was used as an antibiotic to prevent the culture against contamination. Other sorts of antibiotics can be used to prevent contamination. Further research has to be done to check whether other antibiotics prevent contamination or not.

The literature review describes the placenta type of deer compared to other ruminants. The placenta of deer hardly differs from that of other ruminants. It is very unlikely that the small difference in placenta type can be the reason for not finding leptospires in fetal tissue.

This research failed to isolate *Leptospira* spp. from the uterus or fetus of deer. It could be that *Leptospira* spp. don't localize in the uterus of deer. Another explanation can be that the seropositive deer developed immunity and cleared the infection. It could also be possible that some seropositive deer have been treated with antibiotics, which makes it less likely to detect *Leptospira* spp. in tissue.
(Anonymous2002, 381-384)

Further research has to be done on more samples to investigate whether leptospires can be isolated from the genital tract and fetal tissues from pregnant and non pregnant deer. During this study it was not possible to take samples from aborted fetuses. The chance of detecting leptospires will probably be higher in aborted fetuses compared to normal fetuses.
Another option for more research could be to test the vaginal fluid for the presence of *Leptospira* spp. in pregnant and non pregnant deer. Lilenbaum et al. tested vaginal fluid from goats and sheep and found evidence for three *Leptospira* serovars.

5. Conclusion

This research failed to isolate *Leptospira* spp. from the uterus or fetus of deer by bacterial culture and real time PCR. The results show that *Leptospira* spp. can only be detected in the kidneys of slaughtered deer in both early and late gestation. Reasons for not detecting Leptospira spp. can be: this study has an insufficient number of samples to be highly confident, mixed-age hinds were samples which are less likely to be recently infected and might not be shedding the bacteria any more, the concentration of bacteria in the uteri and fetuses was to low to be detected by PCR, the PCR method which was used needs to validated and it could also be possible that Leptospira don't localize in the uterus of deer.
However, an earlier Massey University research showed molecular evidence for a fetal infection in one case. Thus, further research has to be done on more samples to investigate whether leptospires can be isolated from the genital tract and fetal tissues from pregnant/ non pregnant deer and aborted fetuses.

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Appendix 1.

Sample ID	Line	MAT Hardjo-bovis	MAT Pomona	MAT Ballum	Culture Kidney	PCR Kidney	Culture Uterus	PCR Uterus	Culture Fetus	PCR Fetus
1	A	1:384	1:24	0	Neg	Neg	Neg	Neg		
2	B	1:768	1:48	0	**Pos**	**Pos**	Neg	Neg		
3	B	1:48	1:192	0	Neg	Neg	Neg	Neg	Neg	Neg
4	B	0	1:768	0	Neg	Neg	Neg	Neg	Neg	Neg
5	B	1:1536	1:384	0	**Pos**	**Pos**	Neg	Neg		
6	B	1:192	1:192	0	Neg	Neg	Neg	Neg		
7	B	1:48	1:192	0	Neg	Neg	Neg	Neg	Neg	Neg
8	B	1:768	1:192	0	**Pos**	Neg	Neg	Neg		
9	B	0	1:384	0	Neg	Neg	Neg	Neg		
10	B	1:48	0	0	Neg	Neg	Neg	Neg	Neg	Neg
11	B	1:768	1:3072	0	**Pos**	**Pos**	Neg	Neg		
12	B	1:48	1:24	0	Neg	Neg	Neg	Neg		
13	B	0	0	0	Neg	Neg	Neg	Neg		
14	B	0	1:48	0	Neg	Neg	n/a	Neg		
15	B	1:1536	1:96	0	Neg	Neg	Neg	Neg	Neg	Neg
16	B	0	0	0	Neg	Neg	Neg	Neg		
17	B	0	0	0	Neg	Neg	Neg	Neg		
18	B	0	1:96	0	Neg	Neg	Neg	Neg		
19	B	0	1:1536	0	Neg	Neg	Neg	Neg		
20	B	0	1:768	0	Neg	Neg	Neg	Neg		
21	B	0	1:48	0	Neg	Neg	Neg	Neg		
22	B	0	1:384	0	Neg	Neg	Neg	Neg		
23	B	0	1:24	0	Neg	Neg	Neg	Neg	Neg	Neg
24	C	0	0	0	Neg	Neg	Neg	Neg		
25	C	1:384	0	0	**Pos**	n/a	Neg	Neg		
26	C	0	1:24	0	Neg	n/a	Neg	Neg		
27	C	1:768	1:768	0	Neg	n/a	Neg	Neg		
28	C	1:48	0	0	Neg	n/a	Neg	Neg		
29	C	1:24	1:192	0	Neg	n/a	Neg	Neg		
30	C	1:3072	1:1536	1:48	**Pos**	n/a	Neg	Neg		
31	C	1:384	1:384	0	**Pos**	n/a	Neg	Neg		
32	C	1:192	0	0	**Pos**	n/a	Neg	Neg		
33	C	1:192	1:192	0	Neg	n/a	Neg	Neg		
34	D	1:48	1:24	0	Neg	Neg	n/a	Neg	Neg	Neg
35	D	1:96	1:96	0	Neg	Neg	Neg	Neg	Neg	Neg
36	D	1:24	1:192	0	Neg	Neg	n/a	Neg	Neg	Neg
37	D	0	1:192	0	Neg	Neg	n/a	Neg	Neg	Neg
38	D	1:24	1:24	0	Neg	Neg	n/a	Neg	Neg	Neg
39	D	1:24	1:192	0	Neg	Neg	n/a	Neg	Neg	Neg
40	D	1:24	1:192	0	Neg	Neg	n/a	Neg	Neg	Neg
41	D	0	1:3072	0	Neg	Neg	n/a	Neg	Neg	Neg
42	D	1:96	1:24	0	Neg	Neg	n/a	Neg	Neg	Neg
43	D	1:48	1:1536	0	Neg	Neg	n/a	Neg	Neg	Neg
44	D	0	1:768	0	Neg	Neg	n/a	Neg	Neg	Neg
45	D	1:24	1:48	0	Neg	Neg	n/a	Neg	Neg	Neg

46	E	0	1:384	0		Neg		Neg		
47	E	0	1:384	0		Neg		Neg		
48	E	1:192	1:192	0		Neg		Neg		
49	E	1:384	1:384	0		Neg		Neg		
50	E	1:384	1:384	0		Neg		Neg		
51	E	1:192	1:192	0		Neg		Neg		
52	E	0	1:24	0		Neg		Neg		
53	E	1:24	1:48	0		Neg		Neg	Neg	Neg
54	E	0	1:384	0		Neg		Neg	Neg	Neg
55	F	1:384	1:48	0		Neg		Neg	Neg	Neg
56	F	1:48	1:192	0		Neg		Neg	Neg	Neg
57	F	1:96	1:96	1:48		Neg		Neg	Neg	Neg