EPIDEMIOLOGY OF INFECTION WITH LEPTOSPIRA SPECIES IN LIVESTOCK IN PAPUA NEW GUINEA

Thesis submitted by

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Declaration

I declare this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

....................................

Peter Meiwan WAI’IN
Abstract

The role of infection with *Leptospira* as a cause of infertility in Papua New Guinea (PNG) has not been confirmed, mainly because of the lack of robust and simple diagnostic tests in PNG. The aims of this study were to determine the seroprevalence and distribution of infection in livestock in PNG and to develop and validate a diagnostic test for use in PNG that was sufficiently accurate and reliable for confident interpretation of the results. The nested and real-time PCRs were assessed for use as diagnostic tools.

The first survey was conducted on 3 commercial, 3 smallholder cattle farms and 4 abattoirs in March 2004 in PNG. Each herd was stratified into 3 age groups (< 2, 2-5 and >5 years), and sera from 1379 animals were sampled in Lae and Kimbe. In addition, 73 kidneys were collected from cattle at the abattoir and aseptically processed for culture. Two hundred and eighty three sera were collected from pigs killed at the abattoirs and 79 pig kidneys were collected and cultured. All sera were tested using the microscopic agglutination test (MAT). The dominant serovar infecting the cattle in PNG was Hardjo with a seroprevalence of 53.7%. The prevalence of serovar Hardjo in the six farms and the abattoir was significantly higher than serovars Tarassovi and Pomona (P < 0.05). All pig sera were negative for *Leptospira*. Leptospires were isolated by culture and the isolates were typed and identified as *L. borgpetersenii* serovar Hardjo. Cattle are a recognized reservoir for serovar Hardjo and may have a role in transmission to humans.

The second survey was conducted in June 2006 to determine if cattle from smallholder farmers, village pigs and dogs in the Markham Valley in Lae, PNG were infected with *Leptospira*. In addition, pigs from a commercial piggery and horses from commercial and smallholder farms were also sampled. A total of 69 pig sera, 22 dog sera, 15 horse sera and 111 cattle sera were collected. The results showed that 1 dog and 1 pig were seropositive with serovar Canicola. Of the 111 cattle sampled, 21 were seropositive for Hardjo. It was concluded that the seroprevalence with serovar Hardjo in these cattle was significantly lower than cattle from commercial properties. Smallholder cattle may therefore not be a major source of Hardjo infection for animals on commercial farms and pigs do not appear to be infected with *Leptospira*.
The Ab-ELISAs were constructed using one crude preparations of *L. interrogans* serovar Pomona and 2 different crude preparation of *L. biflexa* serovar Patoc. The three antigen preparations were evaluated using 21 MAT-positive and 96 MAT-negative pig sera to determine which antigen preparation was suitable for use in an Ab–ELISA. The selected antigen preparation (L1) was validated in the test using serum from 2 cattle and 1 pig population that were seropositive for *Leptospira*. A sub-population of seronegative cattle and pigs were also used. The Ab-ELISA was used to test 1,465 bovine sera from 8 cattle populations and the results were compared with the MAT using a Bayesian framework, to obtain an unbiased estimate of the accuracy of the tests. The ELISA had high sensitivity and specificity. Results from the Bayesian analysis showed that the sensitivity and specificity estimates for the Ab-ELISA were high compared to the MAT. Based on the test accuracy and its performance the Ab-ELISA using the L1 antigen described in this study is suitable for use in countries like PNG where the MAT is difficult to perform.

Samples of kidneys from livestock in PNG were tested using culture and a PCR-based assay to detect *Leptospira* species. A total of 72 samples of kidney were collected from cattle and a total of 74 samples were collected from pigs slaughtered in Lae and Port Moresby. A second study was designed to assess the use of a real-time PCR for detecting leptospiral DNA in urine from cattle. One hundred and ninety-three urine samples were collected from a beef cattle farm in WA. Whole genomic DNA from kidney samples was extracted from each kidney using the QIAamp DNA Mini kit (Qiagen). Heat lysis was used to extract genomic DNA from clear urine samples and the QIAamp Mini Kit was used for urine that was contaminated with faeces. The PCR-based test was able to detect a higher number of *Leptospira*-positive kidneys compared to culture in EMJH medium. Results of testing DNA extracted from urine using the real-time PCR showed that this test is sensitive and able to detect cattle infected with pathogenic leptospires.
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This thesis is dedicated to my dear dad, David Bomboman Wai’in who passed away in March 2006.

“Though you left me, you’re never gone”
Abreviations

<  
less than

>  
greater than

≥ 
greater than or equal to

%  
percent

µ (pref1x)  
micro (10^-6)

MAT  
microscopic agglutination test

EMJH  
Ellinghausen-McCullough-Johnson-Harris medium

°C  
degrees Celsius

Ab-ELISA  
antibody-detection enzyme-linked immunosorbent assay

ABTS  
2,2’-azino-di-(3-ethylbenzylhiazoline-6-sulfonate)

AUC  
area under the curve

CI  
confidence interval

C_T  
threshold cycle

d_(0)  
cut-off value calculated from the TGROC computer program that gives equal test sensitivity and specificity

DMSO  
dimethyl sulfoxide

EDTA  
ethylenediamine-tetraacetic acid, tri potassium salt

ELISA  
enzyme-linked-immunosorbent assay

FAO  
Food and Agriculture Organisation

et al.  
and others

g  
unit of gravitational field

HRP  
horseradish peroxidase

IgG  
immunoglobulin G

IgM  
immunoglobulin M

IR  
intermediate range for the cut-off value for the ELISA calculated using the TGROC computer program

kb  
kilo base pairs (a unit of measurement of DNA)

kHz  
kilohertz (10^3 cycles per second)

L  
litre

LDC  
Livestock Development Corporation

LR  
likelihood ratio

m  
metre
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>molar concentration</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NHSS</td>
<td>Numundo half stand system</td>
</tr>
<tr>
<td>NPOL</td>
<td>New Britain palm oil limited</td>
</tr>
<tr>
<td>NPV</td>
<td>negative predictive value</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>sq km</td>
<td>square kilometre</td>
</tr>
<tr>
<td>OIE</td>
<td>Office International Des Epizooties</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>P</td>
<td>probability of an event due to chance alone</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pH</td>
<td>minus log of the hydrogen ion concentration</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PNG</td>
<td>Papua New Guinea</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating curve</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TEN-T</td>
<td>Tris (hydroxymethyl) methylamine EDTA and NaCl with 0.05% (v/v) Tween 20</td>
</tr>
<tr>
<td>TEN-TC</td>
<td>TEN with 0.05% (v/v) Tween 20 and 0.2% (w/v) casein</td>
</tr>
<tr>
<td>TG-ROC</td>
<td>computer program for the calculation of a cut-off for the ELISA using two graph response-operating characteristic curves</td>
</tr>
<tr>
<td>WA</td>
<td>Western Australia</td>
</tr>
<tr>
<td>w/v</td>
<td>weight in volume (%)</td>
</tr>
<tr>
<td>v/v</td>
<td>volume in volume (%)</td>
</tr>
</tbody>
</table>
CHAPTER 1
GENERAL INTRODUCTION

The most common cause of bovine leptospirosis worldwide is infection with Leptospira serovar Hardjo. Two serologically indistinguishable but genetically distinct types of serovar Hardjo have been identified, Leptospira interrogans serovar Hardjo (type hardjoprajitno) and L. borgpetersenii serovar Hardjo (type hardjobovis) (LeFebvre et al., 1987). Both of these serovars are unusual among microbial pathogens in that they are specifically adapted to and maintained by cattle. They can be introduced to countries free of infection via infected cattle.

In Papua New Guinea there is no tradition of cattle grazing in village agriculture. Beef cattle production was introduced by the Australian Administration in the 1950s and 1960s. In the 1980s and 1990s several cattle herds from Queensland were shipped to PNG to increase the size of the national herd. Serovar Hardjo infection in cattle has been reported in Queensland (Amatredjo et al., 1976; Carroll and Campbell, 1987). Serological evidence suggests that serovar Hardjo infection is present in cattle in other Pacific island countries including the Solomon Islands, Fiji, Palau and Samoa (Angus, http://www.spc.int/rahs/Projects/zoonoses3E.htm, accessed 2003). However, despite anecdotal evidence that infection with Leptospira species is one of the causes of poor fertility in commercial cattle in PNG, the role of Leptospira as a cause of infertility has not been confirmed, mainly because of the lack of robust and simple diagnostic tests in PNG.

This study was undertaken in order to confirm the presence of infection with Leptospira species in the livestock population and to provide information on the extent of such
infections in PNG. There were two major aims to this study. Firstly, to determine the prevalence of infection with *Leptospira* species and which leptospiral serovars were present in livestock. Secondly to develop and validate a diagnostic test suitable for active surveillance for these infections in PNG.

Using the newly developed serological test and the traditional microscopic agglutination test two surveys were undertaken in Lae and Kimbe districts in the major commercial and smallholder farms and villages in PNG to detect the presence of infection with *Leptospira* species in cattle, pigs and dogs. In addition, culture was used for bacterial isolation and to identify the leptospiral serovars present in the animals. Finally, PCR tests were adopted for rapid detection of leptospires in clinical samples such as kidney and urine.
CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Leptospirosis is an infectious disease caused by pathogenic strains of the genus *Leptospira*, which are transmitted directly or between animals and from animals to humans. Leptospirosis occurs worldwide but is most common in tropical and subtropical areas with high rainfall (WHO and International Leptospiral Society, 2003). The disease occurs mainly in areas where humans or other animals come into contact with the urine of infected animals or a urine-polluted environment. Although leptospirosis has been recognised for many years it is considered an emerging or re-emerging disease of humans in many regions, exemplified by recent outbreaks around the world in Nicaragua (Trevejo *et al.*, 1998; Ashford *et al.*, 2000), Brazil (Romero *et al.*, 2003), India (Chaudhry *et al.*, 2002) and Malaysia (Sejvar *et al.*, 2003)s. In addition, leptospirosis is a major cause of economic losses to the livestock industry as a result of death and ill health of farmed animals and disease in people associated with livestock (Faine *et al.*, 1999).

Little information is available on the incidence and impact of leptospirosis in the Western Pacific Islands, including Papua New Guinea. A recent study in New Caledonia showed that the incidence of human leptospirosis is high, with 180 cases per 100,000 people per year (Mancel *et al.*, 1999). Serological surveys have been conducted in several Pacific Island countries including Solomon Island, Palau, Kiribati, Tonga and Micronesia and a number of potentially zoonotic leptospiral serovars have been detected.

2.2 History of the aspects of leptospirosis

The reported symptoms of jaundice associated with leptospirosis date back to the 1700’s (Faine et al., 1999). The acute form of leptospirosis, characterised by renal failure with accompanying jaundice and nephritis, was first reported by Adolf Weil in 1886 in Germany and was later named Weil’s disease (Faine et al., 1999). Clinical reports of “infectious jaundice and fever” in soldiers and sewer workers were documented but for a long time there was no knowledge of the causative agent (Faine et al., 1999). In 1914, Inada and colleagues isolated the causative agent of leptospirosis from the blood of Japanese miners with infectious jaundice and named it *Spirochaeta icterohaemorrhagiae* (Inada et al., 1916). A non-pathogenic form was also found in fresh water and named *Spirochaeta biflexa* (Wolbach and Binger, 1914).

The importance of occupation as a risk factor and the role of rats as a source of human infection were discovered in 1917 (Ido et al., 1917) and the occurrence of leptospirosis in livestock was recognised some years later (Alston and Broom, 1958). A number of leptospiral serovars affecting humans and animals were subsequently described (Table 2.1). The list of leptospiral serovars grew as scientists realised the zoonotic potential of leptospirosis and hence more research was carried out on the disease in most parts of the world.

Leptospirosis was first reported in Australia in 1933 and the diagnosis was made through histological examination of necropsy material (Morrisey, 1934; Johnson, 1951). Subsequently, several leptospiral serovars were isolated from human patients in
Australia including; *L. interrogans* serovars Australis, Zanoni, Kremastos, Robinsoni, Broomi, Pomona, Szwajizak; *L. kirschneri* serovar Valbuzzi and *L. weilli* serovar Celledoni (Morrisey, 1934; Johnson, 1951; Wellington *et al.*, 1951). *Leptospira interrogans* serovars Pomona and Hardjo were isolated from cattle in Australia in the early 1970s (Sullivan and Callan, 1970; Hoare and Claxton, 1972). Other serovars that have been isolated from cattle in Australia include serovar Australis (Campbell and Stallman, 1975), Zanoni (McClintock *et al.*, 1993), Celledoni and Grippotyphosa (Abdollahpour *et al.*, 1996). Leptospirosis is one of the most commonly reported zoonoses in Australia with farming occupations comprising the majority of cases (Slack *et al.*, 2006).

Table 2.1 Leptospiral serovars isolated from animals and humans

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Date</th>
<th>Place</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumnalis</td>
<td>1918</td>
<td>Japan</td>
<td>Humans</td>
<td>(Kitamura and Hara, 1918)</td>
</tr>
<tr>
<td>Bataviae</td>
<td>1923</td>
<td>Indonesia</td>
<td>Rodents</td>
<td>(cited by Faine <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>1928</td>
<td>Russia</td>
<td>Humans</td>
<td>(cited by Faine <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>Andaman A</td>
<td>1931</td>
<td>Andaman Is.</td>
<td>Humans</td>
<td>(Taylor and Goyle, 1931)</td>
</tr>
<tr>
<td>Canicola</td>
<td>1933</td>
<td>Netherlands</td>
<td>Dogs</td>
<td>(cited by Faine <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>Pomona</td>
<td>1937</td>
<td>Australia</td>
<td>Humans</td>
<td>(Lumley, 1937)</td>
</tr>
<tr>
<td>Australis</td>
<td>1937</td>
<td>Australia</td>
<td>Humans</td>
<td>(Clayton <em>et al.</em>, 1937)</td>
</tr>
<tr>
<td>Hardjo</td>
<td>1958</td>
<td>USA</td>
<td>Cattle</td>
<td>(Alston and Broom, 1958)</td>
</tr>
</tbody>
</table>

2.3 **History of the cattle industry in Papua New Guinea**

Significant numbers of beef cattle were introduced by the Australian Administration in the 1950s and 1960s for meat production in PNG. Cattle numbers peaked in 1976 at around 150,000 head with more than 1,000 smallholder projects running an estimated
total of 50,000 head. Since this time, numbers have declined sharply in both the large farming and smallholder sectors. By 1991 cattle numbers had fallen to around 75,000 head. The greatest decline has been in smallholder and customary land cattle projects. The current national cattle herd in PNG is around 12,000 head with 10,000 head being slaughtered domestically and 2,000 head being exported live per year. The industry has struggled over the last decade, during which economic conditions were generally adverse, however the industry has proven to be a long term survivor as it has a well established production base of large holders and a persistent core of smallholders.

Beef cattle production is now considered to be an important animal industry in the country and its future prospects look bright. Many agricultural companies like Trukai Industries, Ramu Sugar Limited and NBPOL have taken the lead in 2000 to increase the industry. This has led Trukai Industries to establish a market in the Philippines for live cattle while Ramu Sugar and NBPOL have purchased extra land for cattle grazing to increase the supply of locally produced beef to the local market.

Diseases are a major constraint to animal production and the recent low herd fertility of 50 and 60 percent has been raised as a matter of concern by the PNG Cattlemen Association. One agent responsible for some of this low reproductive performance is Leptospira and this has been identified as a research area of importance due to reduced cattle production (I. Puana, pers. comm.) Reliable estimates of the extent of infection have not been performed in PNG and a comprehensive study of leptospirosis in livestock in Papua New Guinea is required. The disease can also be a barrier to export of live cattle due to leptospirosis being classified by WHO/OIE as a re-emerging zoonotic disease.
2.4 Biology of leptospires

2.4.1 Morphology

Leptospires are tightly coiled spirochaetes, usually measuring 10 to 20 µm, but occasionally cultures may contain longer cells. The helical amplitude is approximately 0.1 to 0.15 µm, and the wavelength is approximately 0.5 µm (Faine et al., 1999). The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Figure 2.1). Two axial filaments with polar insertions are located in the periplasmic space (Swain, 1957). Leptospires exhibit two distinct forms of movement, translational and rotational (Faine et al., 1999). Morphologically all leptospires are indistinguishable, but the morphology of individual isolates may vary with subculture in vitro and can be restored by passage in hamsters (Ellis et al., 1983).

Figure 2.1   Electron micrograph of Leptospira interrogans (From Faine et al., 1999)

Leptospires have a distinctive double membrane structure in common with other spirochaetes, with the cytoplasmic membrane and peptidoglycan cell wall closely associated and overlain by an outer membrane (Figure 2.2) (Haake, 2000). The outer membrane appears to be fluid and contains porins that allow solute exchange between the periplasmic space and the environment. The envelope can be disorganised by salt water and desiccation. Leptospiral lipopolysaccharide has a composition similar to that
of other Gram-negative bacteria (Vinh et al., 1986b), but has lower endotoxic activity (Shimizu et al., 1987).

Members of the genus *Leptospira* are obligate aerobes with an optimum growth temperature of 28 to 30°C. They are unable to synthesize fatty acids and in nature only reproduce within animal hosts (Plank and Dean, 2000). They grow well in simple media enriched with vitamins (vitamins B2 and vitamins B12 are growth factors), long-chain fatty acids and ammonium (Levett, 2001). Long-chain fatty acids are utilized as the sole carbon source and are metabolized by beta oxidation salts (Levett, 2001).

![Figure 2.2 Schematic diagram of the *Leptospira* structure. A = prolipoprotein; B = subsurface lipoprotein in the cytoplasmic (inner) cell membrane; C = subsurface lipoprotein in the inner leaflet of the outer membrane; D = surface exposed lipoprotein (possible antigen determinant) in the outer leaflet of the outer membrane; Lsp = prolipoprotein signal peptidase (From Haake, 2000)](image-url)
2.4.2 Genomic organisation

Leptospires have a complex genome and its entire sequence of serovar Lai was recently established (Ren et al., 2003). The genome is large compared with the genomes of other spirochaetes such as Treponema and Borrelia. This gives leptospires the ability to live in a variety of habitats such as animals or freely in the environment (Bharti et al., 2003). The genome of both the pathogenic and saprophytic species of Leptospira is approximately 5,000 kb in size (Baril and Saint Girons, 1990) although smaller estimates of 2,000 kb have been reported (Taylor et al., 1991). The genome is composed of two sections: a 4,400 kb chromosome; and a smaller 350 kb chromosome. A physical map of the chromosome of serovars Pomona subtype kennewicki (Zuerner, 1991) and Icterohaemorrhagiae (Boursaux-Eude et al., 1998; Takahashi et al., 1998) have been constructed. Little is known about genetic exchange among the Leptospira, although lateral transfer has been suggested (de la Pena-Moctezuma et al., 1999).

Pathogenic leptospires have two sets of 16S and 23S ribosomal rRNA genes but only one 5S rRNA gene, and each rRNA gene is located far from the others on the genome (Fukunaga and Mifuchi, 1989; Baril et al., 1992). Copies of several insertion-sequence (IS)-like elements (IS1500 and IS1533) coding for transposases have been identified in pathogenic leptospiral serovars but not in saprophytic species (Boursaux-Eude et al., 1995; Kalambaheti et al., 1999). The IS1533 has a single open reading frame (ORF) and IS1500 has four ORFs (orfA-orfD) (Zuerner, 1994).

Advances in molecular techniques have improved our understanding of the genus Leptospira. Analysis of 16S rRNA gene sequences indicates that leptospires are
phylogenetically related to four other groups of spirochaetes which include *Treponema*, *Borrelia*, *Leptonema*, and *Brachyspira* (Paster and Dewhirst, 2000).

### 2.5 Taxonomy and classification

#### 2.5.1 Serological classification

Leptospires are spirochaetes in the order *Spirochaetales* and the family *Leptospiraceae* which includes two genera, *Leptospira* and *Leptonema* (Faine *et al.*, 1999). Based on serological classification, the genus *Leptospira* was divided into two species, *Leptospira interrogans*, comprising all pathogenic strains and *Leptospira biflexa*, containing the saprophytic strains isolated from the environment (Johnson and Faine, 1984).

Leptospires are classified into over 250 serovars according to the microscopic agglutination test (MAT) that uses specific antisera to identify the distinct serovars. Serovars that are antigenically related have traditionally been grouped into serogroups (Kmetry and Dikken, 1993). While serogroups have no taxonomic standing, they are useful in epidemiological studies. The serogroups of *L. interrogans* and their common serovars are shown in Table 2.2.

Within some serovars, further subgroups have been identified by genomic analysis. These subgroups are types of the serovar and are serologically indistinguishable from one another (e.g. serovar Hardjo, types hardjoprajitno and hardjobovis). It is generally considered not acceptable to refer to leptospires by the generic name followed by the serovar in italics, e.g. *Leptospira hardjo, Leptospira pomona* (Faine *et al.*, 1999) and these should be referred to as *Leptospira* Hardjo and *Leptospira* Pomona respectively.
Table 2.2 Serogroups and serovars of clinical importance in \textit{L. interrogans} sensu lato (Adapted from Levett, 2001)

<table>
<thead>
<tr>
<th>Serogroups</th>
<th>Serovars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Icterohaemorrhagiae</td>
<td>Icterohaemorrhagiae, Copenhageni, Lai</td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>Kremastos, Hebdomadis, Jules</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>Autumnalis, Fortbragg, Bim</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Pyrogenes, Zanoni</td>
</tr>
<tr>
<td>Bataviae</td>
<td>Bataviae</td>
</tr>
<tr>
<td>Sejroe</td>
<td>Hardjo, Sejroe, Saxkoebing</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>Grippotyphosa</td>
</tr>
<tr>
<td>Pomona</td>
<td>Pomona</td>
</tr>
<tr>
<td>Canicola</td>
<td>Canicola, Portlandvere</td>
</tr>
<tr>
<td>Tarassovi</td>
<td>Tarassovi</td>
</tr>
<tr>
<td>Australis</td>
<td>Australis, Bratislava</td>
</tr>
<tr>
<td>Javanica</td>
<td>Javanica</td>
</tr>
<tr>
<td>Ballum</td>
<td>Ballum, Arborea</td>
</tr>
<tr>
<td>Djasiman</td>
<td>Djasiman</td>
</tr>
</tbody>
</table>

2.5.2 Genotypic classification

The use of phenotypic characteristics to classify the species of \textit{Leptospira} has recently been replaced by the use of molecular methods based on the DNA-DNA homology of the leptospiral serovars. This has given rise to a number of genomo-species, which include serovars of both \textit{L. interrogans} (later \textit{L. interrogans} sensu lato) and \textit{L. biflexa} (later \textit{L. biflexa} sensu lato). Genetic heterogeneity was initially demonstrated by
(Brendle et al., 1974) and DNA hybridization studies led to the defined genomo-species of *Leptospira* (Ramadass et al., 1992). Unfortunately, genomo-species of *Leptospira* do not correspond to the previous two species (*L. interrogans* and *L. biflexa*) and pathogenic and non-pathogenic serovars can be classified within the same species (Table 2.3). However, serogroup and serovars reliably predict the species of *Leptospira* therefore a combination of methods are often used. A recent study demonstrated the genetic heterogeneity within serovars which resulted in the classification of certain serovars into more than one species (Table 2.4). In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans* from *L. biflexa* do not differentiate the genomo-species (Levett, 2001). Therefore, a reclassification of leptospirles on genotypic grounds is taxonomically correct and provides a strong foundation for future classification.

The molecular method of classification causes problems for clinical microbiologists because it is incompatible with the system of serogroups which has served clinicians and epidemiologists well for a long time. Until simpler DNA-based identification methods are developed and validated, it will be necessary for clinical laboratories to retain the serological classification of pathogenic leptospirles (Levett, 2001).
Table 2.3 Genomo-species of *Leptospira* and distribution of serogroups (Levett, 2001)

<table>
<thead>
<tr>
<th>Genomo-species</th>
<th>Serogroup&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. interrogans</em></td>
<td>Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisana, Mini, Sarmin</td>
</tr>
<tr>
<td><em>L. noguchii</em></td>
<td>Panama, Autumnalis, Pyogenes, Louisana, Bataviae, Tarassovi, Australis, Shermans, Djasiman, Pomona</td>
</tr>
<tr>
<td><em>L. santarosai</em></td>
<td>Shermans, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri</td>
</tr>
<tr>
<td><em>L. meyeri</em></td>
<td>Ranarum, Semaranga, Sejroe, Mini, Javanica</td>
</tr>
<tr>
<td><em>L. fainei</em></td>
<td>Hurstbridge</td>
</tr>
<tr>
<td><em>L. biflexa&lt;sup&gt;b&lt;/sup&gt;</em></td>
<td>Semaranga, Andamana</td>
</tr>
<tr>
<td><em>L. borgpetersenii</em></td>
<td>Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis</td>
</tr>
<tr>
<td><em>L. kirschneri</em></td>
<td>Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae</td>
</tr>
<tr>
<td><em>L. weilii</em></td>
<td>Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe</td>
</tr>
<tr>
<td><em>L. inadai</em></td>
<td>Lyme, Shermans, Icterohaemorrhagiae, Tarassovi, Manhao, Cynopteri, Panama, Javanica</td>
</tr>
<tr>
<td><em>L. alexanderi</em></td>
<td>Manhao, Hebdomadis, Javanica, Mini</td>
</tr>
</tbody>
</table>

<sup>a</sup> Serogroups Semaranga and Andamana contain non-pathogenic leptospires

<sup>b</sup> Non-pathogenic species
Table 2.4  Leptospiral serovars found in multiple genomo-species (Levett, 2001)

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Genomo-species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bataviae</td>
<td><em>L. interrogans, L. santarosai</em></td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td><em>L. kirschneri, L. interrogans</em></td>
</tr>
<tr>
<td>Hardjo</td>
<td><em>L. borgpetersenii, L. interrogans, L. meyeri</em></td>
</tr>
<tr>
<td>Pomona</td>
<td><em>L. interrogans, L. noguchii</em></td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td><em>L. interrogans, L. inadai</em></td>
</tr>
<tr>
<td>Kremastos</td>
<td><em>L. interrogans, L. santarosai</em></td>
</tr>
<tr>
<td>Szwajizak</td>
<td><em>L. interrogans, L. santarosai</em></td>
</tr>
<tr>
<td>Pyrogenes</td>
<td><em>L. interrogans, L. santarosai</em></td>
</tr>
</tbody>
</table>

2.6  Pathogenesis

The most severe clinical and pathological manifestations occur in young, weanling animals, infected when they are beginning to lose maternally-derived passive immunity. In domestic farm animals this frequently coincides with their attainment of reproductive age so that abortion and stillbirth, loss of milk production and failure to thrive are common features of the disease (Faine et al., 1999). Congenital infection and its sequelae are common in all species. The most important difference between animal and human leptospirosis is the chronic carrier state. In animals the leptospires survive, grow and are transmitted to other animals from reservoirs in the kidneys and genital tract.

The mechanism by which leptospires cause disease is not well understood, and a number of putative virulence factors have been suggested. The pathogenic mechanisms of leptospirosis may be divided into direct effects caused by *Leptospira* and those caused
by the host immune response to infection. One possible mechanism of virulence is the motility of leptospires in tissues (Ellinghausen and McCullough, 1965). Motility is probably important in initial infection and in the dissemination of organisms from the site of entry to sites of end-organ damage such as the lung, liver, kidney, eye and brain. Associated with motility are the twelve methyl-accepting chemotaxis proteins, which are likely to confer selective advantage in adapting to, and migrating through, host tissues (Ren et al., 2003). Virulent leptospiral strains have been shown to exhibit chemotaxis towards haemoglobin and can be lethal (Yuri et al., 1993). Differences in pathogenicity have been observed in the two genotypes of serovar Hardjo. Hardjoprajitno is more pathogenic and commonly associated with the development of clinical disease, while hardjobovis is more commonly associated with asymptomatic renal infections (Ellis et al., 1988).

### 2.6.1 Entry

The route and mode of entry of leptospires in natural infection is not clear. Leptospires are presumed to enter directly into the bloodstream or lymphatics via a number of sites. These include the conjunctivae, the genital tract in some animals, the nasopharyngeal mucosa and possibly through the cribiform plate or the lungs following inhalation of aerosolised organisms. There is also evidence of transplacental infection at any stage of pregnancy. It is unlikely that penetration of intact skin occurs (Faine et al., 1999).

### 2.6.2 Spread and growth

The ability of leptospires to survive and grow in tissues is a major contributor to their virulence. After entry through the open skin, leptospires are immediately exposed to the effects of non-specific factors such as pH, redox potential, electrolytes, fatty acids and other small organic molecules, some of which may be nutrients that will affect the
ability of the leptospires to survive and grow (Faine et al., 1999). Their survival in the tissues of animals is mediated by their resistance to innate immunoglobulins in tissue fluids or plasma. Leptospires do not cause an acute inflammatory response when present in tissues (Arimitsu et al., 1989).

Leptospires spread almost immediately from the site of entry via lymphatics to the bloodstream where they circulate to all tissues. The rapid penetration of the bloodstream following intraperitoneal inoculation is faster than other bacteria (Faine et al., 1999) and leptospires are found at first in the lungs and later in the liver and spleen (Faine, 1964). In the renal tubule leptospires migrate through the interstitial space and attach to the renal epithelial cells. Avirulent leptospires which reach the bloodstream are cleared rapidly, within several minutes of entry, by reticuloendothelial phagocytosis (McGrath et al., 1984).

The time taken to develop lesions is a function of the size of the inoculum (infecting dose), the rate of growth of the organisms in the host, their toxicity, and the rate of development of opsonic immunity. In natural infections the infecting dose is usually assumed to be relatively small and composed wholly of virulent organisms, which will grow uniformly without hindrance until immunity develops. Toxicity is mainly a function of the serovars of leptospires in a given host (Faine et al., 1999).

2.6.3 Persistence and carrier sites

Leptospires may persist and multiply in certain tissues in immunologically privileged sites following clearance from the bloodstream. These tissues include the proximal renal tubules, brain, anterior chamber of the eye and the genital tract (Faine et al., 1999). In
the kidney, growth continues exponentially, reaching a maximum concentration about 21 to 28 days after infection (Faine, 1962).

### 2.6.4 Toxin production

Endotoxin activity has been reported in several leptospiral serovars (Levett, 2001). Leptospiral lipopolysaccharide preparations exhibit activity in biological assays for endotoxin but at much lower potencies than in the host (Masuzawa et al., 1990). The haemolysin exotoxin produced by serovars Pomona, Hardjo, Tarassovi and Ballum can cause haemolytic disease in cattle (Bernheimer and Bey, 1986; del Real et al., 1989).

A protein cytotoxin has been demonstrated in strains of serovars Pomona and Copenhageni (Miller et al., 1970) and cytotoxic activity has been detected in the plasma of infected animals (Knight et al., 1973). \textit{In vivo}, studies have shown that this toxin induces a typical histopathological effect with infiltration of macrophages and polymorphonuclear cells (Yam et al., 1970). A glyco-lipoprotein fraction with cytotoxic activity has also been recovered from serovar Copenhageni (Vinh et al., 1986a).

### 2.7 Pathology

The primary histological lesion observed in clinical leptospirosis is damage to the endothelial membrane of small blood vessels, which is caused by leptospiral toxin. The immediate effect is to loosen the junctions between cells, allowing fluid and leptospires to migrate into extravascular spaces followed by erythrocytes wherever the damage is severe or prolonged. The secondary effects of ischaemic change, anoxia and increased pressure in the tissues reinforce damage resulting in cellular functional disintegration and death of the cell (Ellis, 1990).
Perhaps the most significant manifestation of infection with serovar Hardjo is the result of persistent infection in the reproductive tract, which can lead to infertility. The precise pathogenesis is not clearly understood but it is believed that the presence of leptospires in the epithelium of the uterus and oviducts of infected cows interferes with implantation of the embryo or other events in early pregnancy (Dhaliwal et al., 1996c). In the kidneys, interstitial nephritis is the major finding, accompanied by an intense cellular infiltration composed of neutrophils and monocytes (Penna et al., 1963), however renal disease is not commonly reported.

2.8 Epidemiology

2.8.1 Geographic distribution in Australia and the Pacific Islands

Leptospirosis was first recognised in Australia in 1934, among cane-workers in North Queensland with infections commonly resulting from contact with rodent urine (Emanuel et al., 1964). The agricultural workers in Queensland and other states of Australia are commonly infected with serovars Australis, Zanoni, Hardjo, Pomona, Tarassovi and Bratislava from cattle, pigs, sheep and rodents. Leptosomal serovars dominant in the tropics of Australia are Zanoni, Hardjo and Australis whilst Hardjo, Pomona, Tarassovi and Bratislava predominate in temperate regions (Smythe et al., 2000). Serological surveys conducted in selected Pacific Island countries showed that infections with *Leptospira* species are present in the region (Angus http://www.spc.int/rahs/Projects/zoonoses3E.htm, accessed 2003) (Table 2.5). In PNG no published reports on the extent of leptospiral infection in humans or animals have been made.
Table 2.5 The prevalence of leptospiral infection in animals in selected Pacific Island countries

<table>
<thead>
<tr>
<th>Country</th>
<th>Year reported</th>
<th>Seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
</tr>
<tr>
<td>Micronesia</td>
<td>1997</td>
<td>No data</td>
</tr>
<tr>
<td>Fiji</td>
<td>1994</td>
<td>No data</td>
</tr>
<tr>
<td>Kiribati</td>
<td>1996</td>
<td>No data</td>
</tr>
<tr>
<td>Palau</td>
<td>1999</td>
<td>50%</td>
</tr>
<tr>
<td>Samoa</td>
<td>1999</td>
<td>40%</td>
</tr>
<tr>
<td>Solomon Island</td>
<td>1999</td>
<td>83%</td>
</tr>
<tr>
<td>Tonga</td>
<td>1996</td>
<td>19-45%</td>
</tr>
<tr>
<td>Wallis &amp; Futuna</td>
<td>2000</td>
<td>No data</td>
</tr>
</tbody>
</table>

(From Angus http://www.spc.int/rahs/Projects/zoonoses3E.htm, accessed 2003)

2.8.2 Sources and modes of transmission of leptospires

Domestic and wild mammals, rodents, reptiles and amphibians are maintenance hosts for different leptospiral serovars. Rodents and cattle are considered the most important source of human infection (Smythe et al., 2000; Levett, 2001). Leptospires colonise the kidneys of carrier animals (Babudieri and D’Amore, 1958) and are shed in urine, which is the main source of environmental contamination. Estimates of the number of leptospires shed range from 10,000 to 1,000,000 organisms per millilitre of urine (Faine et al., 1999). Humans or other animals are usually infected by exposure to urine from infected animals. Other sources of transmission are contaminated surface water (includes rivers, lakes, ponds), mud and soil (Levett, 2001).
The modes of transmission can be either direct or indirect. Direct transmission occurs from chronically infected animals to other susceptible animals through animal’s urine (Ellis et al., 1986a; Ellis and Thiermann, 1986b). The kidneys are the site of leptospires localisation and urine is the medium for transmission (Ellis and Michna, 1976). In cattle and pigs there is evidence that leptospires can cross directly from the genital tract to the placenta and infect the foetus, which could have a primary or secondary role in abortion (Ellis et al., 1986b; Ellis and Thiermann, 1986b). Indirect transmission occurs when animals or humans acquire infection with *Leptospira* from the environment through the conjunctivae, the oral mucosa, respiratory tract mucous membrane or cuts in the skin (Levett, 2001).

### 2.8.3 Cycle of host infection

The epidemiology of human leptospirosis reflects the ecological relationship between humans and chronically infected mammalian reservoir hosts. Humans are considered an incidental end-host from which further transmission has not been demonstrated, although individuals can excrete leptospires in their urine for several weeks (Bharti et al., 2003).

There are two natural cycles of transmission of *Leptospira* in Australia. A sylvatic cycle exists between rodents and marsupials and a domestic cycle involves cattle, pigs, dogs and sheep (Figure 2.3) (Faine et al., 1999).

In the sylvatic cycle, leptospirosis is accidentally transmitted to farmed animals and humans from numerous species of rodents and marsupials. The principal means of spread and continuity of infection in rodents or marsupials is by direct transmission from the mother to the young. Humans can be infected through contact with an environment
contaminated with rodent’s urine. The most important sources for human infections are the various species of rodents with which humans live in domestic, agricultural or occupational association. Rodents closely associated with human habitation, such as the black and brown rats (Rattus rattus and R. norvegicus) and the common domestic mouse (Mus musculus) can act as sources of leptospires for humans, dogs and farm animals (Carter and Cordes, 1980).

Maintenance hosts are animals which do not generally show signs of clinical infection but which can shed leptospires for long periods of time. Urine contaminated with Leptospira from these animals can infect humans or other non-maintenance hosts resulting in disease. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serogroups Icterohaemorrhagiae and Ballum, and mice are the maintenance hosts for serogroup Ballum (Levett, 2001). Urban black rats (R. rattus) are a maintenance host for serovars Copenhageni or Ballum whereas R. norvegicus harbours only serovar Copenhageni (Hathaway and Blackmore, 1981).
Figure 2.3  Generalised scheme of the transmission of leptospires between rodents, farm animals and humans. Leptospires are excreted into the environment by various carrier animals (Modified from Faine et al., 1999)

The domestic cycle of leptospirosis involves of cattle, pigs, sheep, buffalo, goats and dogs. Domestic animals are maintenance hosts of specific serovars; cattle usually maintain serovars Hardjo, Pomona and Grippotyphosa; pigs harbour serovars Pomona, Tarassovi or Bratislava; sheep may harbour Hardjo and Pomona; and dogs may harbour Canicola (Levett, 2001).
2.8.4 Survival of leptospires in the environment

The extent to which infection with *Leptospira* is transmitted depends on the survival of leptospires in the environment and on many factors, including temperature, climate, soil pH and soil moisture (Okazaki and Ringen, 1956). Moisture of the soil is important and is dependent on rainfall and water holding capacity of the soil. The survival of leptospires in soil was shown to increase as soil moisture increased (Smith and Self, 1955). Serovar Pomona was found to retain viability, pathogenicity and antigenicity for up to 74 days when recovered from soil which had a moisture content of 15.2 to 31.4% and a pH of 6.7-7.2 (Zaitsev *et al.* , 1989). In an acidic soil environment, serovar Pomona was found to survive for up to 49 days (Hellstrom and Marshall, 1978).

*Leptospira*, like other spirochaetes, are well adapted to viscous environments, in which the organisms show greater translational motility than any other bacteria (Kaiser and Doetsch, 1975). Under laboratory conditions, leptospires can remain viable for several months in water at room temperature and a pH 7.2 to 8.0 (Crawford *et al.*, 1971). The presence of domestic sewage decreases the survival time to a matter of hours (Chan *et al.*, 1987) but in an oxidation ditch filled with cattle slurry, viable leptospires were detectable for several weeks (Diesch, 1971). When soil was contaminated with urine from infected rats or voles, leptospires survived for approximately 2 weeks (Karaseva *et al.*, 1977). A recent study showed that serovar Canicola could survive in water and remain motile for 110 days at a pH of 7.2, however little is known about the mechanisms by which pathogenic leptospires persist for long periods in aqueous environments (Trueba *et al.*, 2004).
2.8.5 Human infections

Humans are incidental hosts of *Leptospira* and are at a high risk of contracting the disease when they come into direct contact with infected animals or their products (Waitkins, 1986). Some examples of risky activities include managing domestic and farm animals; milking cattle; assisting in births, removing stillbirths or removal of abortion products; dressing carcases; cleaning urine spills from dogs; and practicing as veterinarians and meat inspectors (Blackmore and Schollum, 1982b; Faine *et al.*, 1999). Many cases are documented of infection in livestock farmers, veterinarians, abattoir workers (Blackmore and Schollum, 1982a; Campagnolo *et al.*, 2000), rodent control workers (Demers *et al.*, 1985), and zoologists or other occupations which involve direct or indirect contact with animals (Looke, 1986). In Australia, an investigation into the sources of infection with *Leptospira* in abattoir workers found that they were most commonly infected with serovars Pomona and Hardjo and that each affected individual was exposed to large volumes of animal urine during the course of their work (Terry *et al.*, 2000).

Indirect infections are also common in workers who are exposed to environments contaminated with urine from excretor animals. In Asian countries the practice of wet rice planting can lead to infection of rice planters via abraded skin, as their arms and legs are constantly being immersed in water contaminated by urine from rodents, buffalos and pigs (Padre *et al.*, 1988; Zamora *et al.*, 1990; Laras *et al.*, 2002). Other occupational risk groups are sewer workers (Chan *et al.*, 1987), miners (Onyemelukwe, 1993), septic tank sewer cleaners (De Serres *et al.*, 1995), canal workers (Andre-Fontaine *et al.*, 1992), banana farmers and workers (Smythe *et al.*, 2000) and sugar cane cutters (Ginebra Gonzalez, 1976).
There is also a significant risk associated with certain recreational activities associated with water sports (Sejvar et al., 2003), including swimming (de Lima et al., 1990; Jackson et al., 1993), canoeing (Shaw, 1992), white water rafting (van Crevel et al., 1994) and kayaking (Jevon et al., 1986). Many of these outbreaks have followed extended periods of hot, dry weather, when pathogenic leptospires have presumably multiplied in freshwater ponds or rivers (Levett, 2001).

Sporadic cases of leptospirosis in tropical regions are acquired following exposures that occur during daily activities, and are particularly associated with high seasonal rainfall (Everard et al., 1992; Perrocheau and Perolat, 1997). For example, infections have resulted from walking barefoot in damp conditions or gardening with bare hands (Douglin et al., 1997; Bharti et al., 2003), from swimming or bathing in contaminated water and from accidental ingestion of contaminated water (Bharti et al., 2003). Inhalation of contaminated aerosols of water may also result in infection via the mucous membranes of the respiratory tract (Levett, 2001). Dogs are a significant reservoir for human infection in many tropical countries and may be an important source of outbreaks (Weekes et al., 1997).

2.9 Clinical features of leptospirosis

2.9.1 Humans

The majority of infections caused by leptospires are either subclinical or of very mild severity and medical attention may not be sought (Levett, 2001). This mild (anicteric) syndrome usually lasts for about a week, and coincides with the appearance of antibodies. The early symptoms resemble those of many other common febrile illnesses including influenza, hepatitis and several acute illnesses of viral origin. Symptoms
include fever, headache, myalgia, abdominal pain, conjunctival suffusion and, less often, a skin rash (Martins et al., 1998; Faine et al., 1999). The rash is often transient, lasting less than 24 hours. The headache is often severe and resembles the typical presentation that occurs in dengue fever, with retro-orbital pain and photophobia (Levett, 2001). Mortality is rare in anicteric leptospirosis (Edwards and Domm, 1960). However, in a Chinese outbreak, death was reported in 2.4% of anicteric patients, associated with massive pulmonary haemorrhages (Wang et al., 1965).

Icteric (acute) leptospirosis is a more severe, progressive disease characterised by generalised pains in the neck, abdomen and limbs, severe muscle pains, especially in the calf muscles, thigh and back, and pain over the tibia, affecting the gait and ability to move (Faine et al., 1999). As the disease progresses, signs of renal and hepatic failure appear leading to varying degrees of uraemia and jaundice, accompanied with, or followed by, skin and mucosal haemorrhages, haemoptysis, myocarditis, progressing to death if left untreated (Faine et al., 1999). Leptospirosis is a common cause of acute renal failure, which occurs in 16 to 40% of cases (Edwards et al., 1990; Abdulkader, 1997).

Ocular manifestations of severe leptospirosis were identified in a large cluster of cases that occurred after flooding in India (Rathinam et al., 1997). Anterior uveitis, either unilateral or bilateral, occurs after recovery from the acute illness in a minority of cases (Barkay and Garzozi, 1984). Uveitis is a late complication that can cause reversible or irreversible blindness in people and in horses, and may present weeks, months, or occasionally years after the acute stage of the disease (Rathinam, 2002).
2.9.2 Cattle

The most common cause of bovine leptospirosis worldwide is infection with leptospires belonging to serovar Hardjo. Two serologically indistinguishable but genetically distinct types of serovar Hardjo have been identified, *Leptospira interrogans* serovar Hardjo (type hardjoprajitno) and *L. borgpetersenii* serovar Hardjo (type hardjobovis) (LeFebvre *et al.*, 1987). Serovar Hardjo type hardjobovis is common in cattle worldwide, whilst type hardjoprajitno is found primarily in cattle in Europe (Ellis *et al.*, 1986c).

The disease cycle of bovine leptospirosis is displayed in Figure 2.4. The bacteria gain entry via the eyes, mouth, nose, or through abraded skin, and enter the bloodstream. The organism multiplies for 4 to 20 days in the blood and spreads to the brain, liver, uterus, udder and kidneys, where infection is established (Ellis, 1990). Serovar Hardjo generally results in asymptomatic infections or relatively mild clinical signs with an associated decreased reproductive efficiency and milk production (Higgins *et al.*, 1980). Persistent infection of the uro-genital tract is also a prominent feature of infection with serovar Hardjo. Leptospires in the proximal renal tubules of the kidney, genital tract and mammary gland appear to be protected from circulating antibodies (Smith *et al.*, 1994). Urinary shedding of leptospires may infect other cattle in the herd and humans that come into contact with the urine.

Abortion usually occurs 6 to 12 weeks after infection in cows infected for the first time during pregnancy and most commonly in the last 4 months of gestation (Hathaway *et al.*, 1982; Faine *et al.*, 1999). Abortion is likely to be accompanied by placental retention and may lead to infertility. Abortions due to infection with serovar Hardjo tend to occur sporadically as opposed to an abortion “storm” which may occur as a result of infection.
with serovars Pomona or Grippotyphosa (Herr et al., 1982). Infection late in pregnancy may result in small, weakly viable calves. Diagnosis is complicated because the clinical signs are not pathognomonic for leptospirosis and the antibody titres of the dam may be low or falling at the time of abortion.

Figure 2.4 The disease cycle of bovine leptospirosis
(Modified from Pitman–Moore Ltd, Crewe Hall, Crewe, Cheshire CW1 1YR)

2.9.3 Pigs

Common serovars causing leptospirosis in pigs include Pomona, Tarassovi, Bratislava, and Grippotyphosa. A new serovar isolated from pigs in Australia which has been responsible for reproductive problems is *L. fainei* serovar Hurstbridge (Perolat et al., 1998). Infected pigs may shed large number of leptospires in their urine for as long as a year after infection (Faine et al., 1999). Pigs may also carry serovar Bratislava in their genital tracts for long periods of time after infection, although urinary shedding is less dramatic with this serovar (Ellis and Thiermann, 1986a). Young pigs suffering from acute leptospirosis rapidly become weak, lose appetite for food, and may have red eyes, jaundice and convulsions. New born piglets may develop haemorrhages in the kidneys and lungs, jaundice, signs of renal failure and may die. There may be also haemorrhages
and oedema in the foetal membranes of aborted sows (Faine et al., 1999). Survivors of acute leptospirosis may appear sickly and have the potential to shed leptospires in the urine.

Foetal infection results from maternal leptospiraemia and invasion through the placenta. Its consequences depend on the stage of pregnancy; in the first trimester foetuses may recover, but in the last trimester, abortion and stillbirth occur (Faine et al., 1999). Abortion, stillbirth, or birth of weak or ill piglets appearing 14 to 60 days after infection are often the first and only signs of leptospirosis (Bolin and Cassells, 1990).

2.10 Laboratory diagnosis

Diagnosis often depends on laboratory methods because clinical presentation can vary greatly. The diagnostic method selected varies depending on the samples available and the purpose of testing. Identification of the infecting serovar is of importance both epidemiologically and clinically, since this may assist in determining the source and outcome of infection. Different assays have been developed in an attempt to provide accurate diagnosis of leptospirosis, but the majority are not suitable for use in developing countries due to their requirement for maintenance of multiple strains or expensive equipment. The tests can be divided into those that detect bacteria, their antigens or genomic material and those that detect host antibody to the infecting serovars.

2.10.1 Microscopic demonstration

Leptospires may be visualized in clinical material by dark field microscopy, immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of body fluids such as blood, urine, cerebro-spinal fluid and
Dialysate fluid has been used to rapidly detect the presence of leptospires and is useful in situations where laboratory resources are limited, however the technique lacks sensitivity (Faine et al., 1999). The limit of detection of dark-field microscopy is approximately $10^4$ leptospires/ml (Turner, 1970). Microscopic examination of blood is of value only when leptospiraemia occurs during the first few days of acute illness (Levett, 2001). In addition, false positives can occur due to misinterpretation of fibrin or protein threads, which may show brownian motion. A high degree of operator skill is therefore required and no information on the infecting serovar can be gained (Smith et al., 1994).

Staining methods have been applied to increase the sensitivity of direct microscopic examination. Standard stains for *Leptospira* have been silver impregnation techniques, strong carbol fuchsin and methylene blue, or Gram stain using a carbol fuchsin counterstain, however they are tedious and difficult to perform (Faine et al., 1999). Immunofluorescent staining is also used to demonstrate leptospires in clinical and environmental specimens such as urine, other body fluids, frozen kidneys, water and soil, because it is easy to identify leptospires and the serovars can be determined presumptively (Ellis et al., 1982; Bolin et al., 1989; Faine et al., 1999). More recently, immuno-histochemical methods have been applied to demonstrate the expression of various specific leptospiral antigens in the tissues of experimentally infected animals and to improve the detection of leptospires in canine renal tissue (Barnett et al., 1999; Haake et al., 2000; Wild et al., 2002).

### 2.10.2 Cultural methods

Leptospires grow in culture media containing dilute animal serum or bovine serum albumin (BSA) (Faine et al., 1999). The most widely used medium commercially

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available today is the Ellinghausen-McCullough-Johnson-Harris formula, known as EMJH medium. It is based on a serum-free-oleic acid-albumin medium with derivatives containing Tween 80 as the source of fatty acids and BSA as the detoxifier (Ellinghausen and McCullough, 1965). The growth of contaminants from clinical specimens can be inhibited by the addition of 5-fluorouracil (Johnson and Rogers, 1964). The liquid media can be made into semi-solid and solidified media by adding agar at concentrations of 0.1 to 0.2% and 0.8 to 1.5% respectively (Faine et al., 1999).

Unfortunately, culture is slow, requires several weeks of incubation, and has low sensitivity (Bharti et al., 2003). Media should be inoculated within 24 hours of sample collection (Palmer and Zochowski, 2000). Even under optimal conditions, organisms grow slowly and cultures can be reported as negative only after a minimum of 6-8 weeks incubation, preferably as long as 4 months, before being discarded (Levett, 2003). Pure subcultures in liquid media however usually grow within 10 to 14 days. In semi-solid media, growth reaches maximum density zones beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. The pattern of growth is related to the optimum oxygen tension and is known as a Dinger’s ring or disk (Faine et al., 1999).

The visible growth of leptospiral cultures in liquid media can be seen when swirling the container against a dark background as the solution is cloudy. Fully grown cultures at cell concentrations of approximately 5x10^7-10^8 leptospires/ml are usually turbid to the naked eye (Faine et al., 1999). However, leptospiral cultures rarely achieve the densities obtained with “conventional” bacteria, and sometimes strains which grow poorly may not attain concentrations greater than 1-5 x 10^6 leptospires/ml (Faine et al., 1999).
Leptospiral cultures may be maintained by repeated subculture, or preferably by storage in semisolid agar containing haemoglobin (Faine et al., 1999). Long-term storage by lyophilization (Annear, 1974) or at -70°C in glycerol (Palit et al., 1986) are also used.

Isolation of leptospires is frequently attempted from a variety of clinical specimens during acute and chronic infections. Suitable specimens including whole or clotted blood, serum, urine, cerebro-spinal fluid and tissue samples, can be inoculated into EMJH medium containing 5-fluorouracil. Cultures are incubated at 28 to 30°C and are examined weekly by dark-field microscopy for up to 13 weeks or more (Faine et al., 1999; Levett, 2001). Contaminated cultures may be passed through a 0.2-µm or 0.45-µm filter before subculture into fresh medium (Rittenberg et al., 1958). Identification of isolates to the serovar level is usually carried out at reference laboratories and involves time consuming cross-absorption agglutination procedures with panels of monoclonal antibodies (Smith et al., 1994; Levett, 2001).

2.10.3 Microscopic agglutination test (MAT)

The MAT is the most widely used laboratory test for the diagnosis of leptospirosis and is the reference method for serological diagnosis of the disease (Cole et al., 1973; Levett, 2003). Antibodies are measured by incubating cultures of specific serovars of live leptospires with serial dilutions of the test serum. Antibodies in the serum bind to leptospires and cause agglutination, which can be detected using dark-field microscopy. The MAT detects both immunoglobulin-M (IgM) and immunoglobulin-G (IgG) classes of antibodies (Adler and Faine, 1978). The end point of dilution is the highest dilution of serum at which 50% agglutination occurs (Faine et al., 1999)
The MAT is a complex test to standardise, perform, and interpret, as live cultures are used and various factors, such as the age and density of the antigen culture, can influence the agglutination titre (Turner, 1968). In humans, the MAT cannot differentiate between agglutinating antibodies due to current or past infection and, in animals, MAT cannot differentiate between titres after vaccination and those of natural infection, since the titres may be of a similar magnitude (Adler et al., 1982). In the case of acute infections, a four-fold increase in titre between paired sera is essential to support the diagnosis of leptospirosis. There is no standard titre that is considered indicative of infection when single samples are collected from clinically normal animals. A titre of 1:100 is considered positive by some workers, whilst others accept 1:200, 1:400 or 1:800 as diagnostic for current or recent infection (Smith et al., 1994; WHO and International Leptospiral Society, 2003).

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between leptospires of the same serogroup or between closely related serovars as they possess the same agglutinating epitopes (Faine et al., 1999). For example, infection with serovars Szwajizak, Medanensis, Balcanica and Kremastos can cross-react with serovar Hardjo and produce false positive results (Mackintosh et al., 1981; Thiermann and Garrett, 1983; Black et al., 2001). Serovars Hardjo, Szwajizak and Medanensis were originally included in the serogroup Hebdomadis but are now in two new serogroups. Serovars Hardjo and Medanensis are in the Sejroe serogroup while Szwajizak belongs to the Mini serogroup (Faine et al., 1999).

There are no published reports of attempts to estimate the sensitivity and specificity of the MAT. Generally the specificity and sensitivity of the MAT test are considered to be
high, but sensitivity declines when animals are tested a considerable time after infection due to a reduction in their antibody levels (Blackmore et al., 1984).

The MAT has a number of disadvantages: it is laborious, hazardous, and requires the continuous weekly subculture of leptospires as the panel of serovars for testing needs to be maintained. The assay may be less sensitive for detecting infections in the maintenance host than in the non-maintenance host (Hartman et al., 1984). In reading the agglutination test result, considerable effort is required to reduce the subjective effect of the observer (Levett, 2001). In addition, chronically infected animals may be serologically negative, even though leptospires may be cultured from aseptically collected urine samples or from the cortex of the kidney (Faine et al., 1999).

2.10.4 Enzyme linked immuno-sorbent assay

Enzyme linked immuno-sorbent assays (ELISAs) were developed due to the deficiencies of the MAT and to produce a faster, safer and more precise assay for the detection of anti-leptospiral antibodies (Adler et al., 1982). Nevertheless, a antibody ELISA is only able to detect genus-specific antibodies and is not suitable for serogroup or serovar identification of the *Leptospira* (Ribotta et al., 2000). The major benefit of the ELISA is that it can be specific for the detection of IgM or IgG antibodies (Smith et al., 1994). The presence of IgM may indicate current or recent leptospirosis, but it should be noted that IgM-class antibodies can remain detectable for several years (WHO and International Leptospiral Society, 2003).

A study by Adler and co-workers (1982) using IgG and IgM ELISAs and MAT to investigate the immune response of cattle vaccinated or experimentally infected with
serovar Hardjo suggested that the IgM ELISA and MAT detect different IgM antibodies by virtue of the different antigen preparations used in the tests.

A variety of different antigens have been tested in the ELISA. These include a carbohydrate antigen produced by phenol extraction of whole cell preparations (Thiermann and Garrett, 1983), outer sheath protein (Cho et al., 1989), whole lysed bacteria (Bercovich et al., 1990), formalin-fixed whole culture extract (Ribotta et al., 2000) and proteinase-K-treated antigen (Ribeiro et al., 1995). Comparisons of protein and carbohydrate antigens in a indirect ELISA revealed similar sensitivities and specificities for the detection of antibodies in cattle (Dhaliwal et al., 1996b). Irrespective of the antigen used, the specificity of the ELISA was shown to be limited to the genus level and cross-reactivity between serovars was reported (Thiermann and Garrett, 1983; Bercovich et al., 1990).

The time post-infection that antibody may be detected varies depending on whether an IgM- or IgG-ELISA is used. Ribeiro et al. (1995) showed that anti-leptospiral IgM could be detected in the acute phase of human infection and that the ELISA was more sensitive than the MAT. In contrast, IgG-detecting ELISA may detect antibody later in the course of infection than the MAT (Bercovich et al., 1990; Gerritsen et al., 1993).

The principle advantages of the ELISA are that it can be standardised, is easy to perform and is less expensive than the MAT. The disadvantages are that some ELISA systems are less specific than the MAT (Cho et al., 1989). The genus-specific antigen used in an ELISA does not give an indication of the infecting serovar and doesn’t allow differentiation between vaccinated and infected cattle (Ribotta et al., 2000).
2.10.5 Polymerase chain reaction (PCR)

The PCR has been evaluated by several groups for its usefulness in the detection of leptospiral DNA from both human and animals. Although many PCRs for pathogenic \textit{Leptospira} are described in the literature, only a few have been used on clinical or veterinary samples such as urine, aqueous humor during ocular complications of the disease and tissues from aborted bovine foetuses (Van Eys \textit{et al.}, 1989; Bal \textit{et al.}, 1994; Merien \textit{et al.}, 1995; Richtzenhain \textit{et al.}, 2002). The PCR has also been used to investigate the efficacy of antibiotic treatment in stopping the shedding of \textit{Leptospira} by cattle (Gerritsen \textit{et al.}, 1994; Alt \textit{et al.}, 2001).

PCR-based strategies for detecting specific leptospiral DNA are more useful but they require selection of specific primers to allow for amplification of the DNA. A number of primer pairs have been described based on specific gene targets (Renesto \textit{et al.}, 2000), including the 16S or 23S ribosomal RNA genes found in all pathogenic leptospires (Merien \textit{et al.}, 1992) and others have been constructed from genomic libraries (Gravekamp \textit{et al.}, 1993).

There is evidence that PCR assays are more sensitive than conventional diagnostic methods such as culture and dark-field microscopy, although the sensitivity of culture may vary between laboratories (Merien \textit{et al.}, 1992; Brown \textit{et al.}, 1995; Heinemann \textit{et al.}, 2000). The PCR may be especially useful when the immune response of the host to the infecting serovar is poor, as with the response of cattle to serovar Hardjo, or where a poor sample quality may have rendered bacteria non-viable (O'Keefe, 2002). The ability of PCR assays to identify specific serovars is limited, and authors often describe genotypic groupings of serovars rather than serovar-specific groupings (O'Keefe, 2002).
A recent study examined five published PCR protocols and compared them with culture and the immunofluorescence test for the ability to detect serovar Hardjo in bovine urine. The PCR was as sensitive as immunofluorescence test (90% for genus-specific detection) and had a high specificity. None of the methods were 100% sensitive (Wagenaar et al., 2000). The PCR protocols could be readily applied to routine serovar typing of clinical samples from individuals, but they may be useful for screening herds or pooled samples (O'Keefe, 2002).

### 2.11 Measures for control and prevention of leptospirosis

#### 2.11.1 Treatment and vaccination in humans

Immediate treatment of acute leptospirosis is recommended. Treating patients with leptospirosis relies on supportive management and the use of penicillin and tetracycline antibiotics, even though their utility in severe disease remains unclear (Vinetz, 2001). Currently, doxycycline is recommended for both prophylaxis and the treatment of mild disease. Ampicillin and amoxicillin are also recommended for the treatment of mild disease, and penicillin G and ampicillin are indicated for the treatment of severe disease (Bharti et al., 2003).

An effective vaccine to prevent leptospirosis in humans has not yet been developed (Vinetz, 2001). Large scale vaccine trials have been reported in Cuba, Russia and China. However, only a few patients developed detectable antibody responses to the cocktail of serovars included in the vaccines (Bharti et al., 2003). Several problems have been faced during the development of a human vaccine. Firstly, unacceptable side-effects using killed bacterial vaccines have often been reported, such as fever and pain at or near the injection site (Philip and Tennent, 1966; Faine et al., 1999). Secondly, the killed
bacterial vaccines appeared to provide only short-term, and possibly incomplete, protection, similar to that reported in anti-leptospiral vaccines in animals (Faine et al., 1999). Thirdly, the locally variant serovars of Leptospira involved in infections may preclude the development of a suitable vaccine (Bharti et al., 2003). Fourthly, there is incomplete knowledge of the protective immunity against leptosomal infection (Bharti et al., 2003).

2.11.2 Control measures in animals

An optimal program to control leptospirosis in domestic livestock should be designed to prevent clinical disease and urinary shedding of leptospires. The most effective control programs in livestock are based on the prevention of exposure, which includes measures such as isolation, herd management, antibiotic prophylaxis and vaccination.

Isolation and herd management involve strategies to prevent direct and indirect transmission of leptospires from infected adults to susceptible young stock, because active infection often persists in older animals. For this programme to be successful, successive cohorts of animals have to remain isolated to remain free from infection, until all the infected cohorts have passed through the population. In addition, adult carriers in the herd should be culled and procedures implemented to vaccinate and prophylactically treat all animals introduced onto the property (Little et al., 1992a). If pigs are kept on the farm, their effluent should be contained separately and be inaccessible to cattle, and waterways should be fenced off so animals do not have direct access (Occupational Safety and Health Service, New Zealand, http://www.osh.dol.gov.nz, accessed, 2005).

Tetracycline and amoxycillin are the antibiotics recommended for the treatment of carrier animals (Faine et al., 1999). Antibiotic prophylaxis coupled with specific herd
management procedures have been suggested as a method to eliminate infection with serovar Pomona in pigs (Stalheim, 1969) however failures have been reported under field conditions (Hodges et al., 1979). Similar failures have been reported in attempts to eradicate infections with serovar Hardjo in cattle (Ellis et al., 1985a).

Vaccination is the most important method of preventing leptospirosis in livestock (Little et al., 1992b). Depending on the degree of exposure or the level of risk, vaccinating the herd one to two times a year may be warranted (Faine et al., 1999). Calves as young as four weeks or older should initially be vaccinated, followed by a second dose four to six weeks later (Little et al., 1992b). Annual revaccination maintains protective immunity but does not prevent infected animals from shedding leptospires (Faine et al., 1999).

Several field studies have shown that vaccination of cattle with infection with serovar Hardjo reduces reproductive losses and leptospiruria (Marshall et al., 1979; Little et al., 1992b). However, there have been reports that protection against infection with Hardjo in heifers has been suboptimal (Faine et al., 1999). A recently developed monovalent vaccine of Leptospira borgpetersenii serovar Hardjo has been shown to offer good protection against renal colonization and urinary shedding and has been shown to induce a cell-mediated response (Bolin and Alt, 2001). Variation in the efficacy of vaccines of serovar Hardjo is likely to be a result of a variation in vaccine composition, husbandry practices, and the pathogenicity of strains of serovar Hardjo prevalent in the region (Faine et al., 1999). Vaccines are also available for pigs and these have been shown to reduce abortion and stillbirth rates, and to reduce, but not eliminate, renal colonisation and leptospiruria (Faine et al., 1999).
CHAPTER 3
SURVEYS IN PAPUA NEW GUINEA TO DETECT THE PRESENCE OF LEPTOSPIRA SPECIES IN LIVESTOCK

3.1 Introduction

Leptospirosis is a disease with a world-wide distribution that is caused by over 200 different serovars of the Genus *Leptospira* (Faine *et al.*, 1999). The disease affects both animals and humans and, besides reducing animal production, is one of the commonest occupationally acquired zoonoses of agricultural workers in many countries (Faine *et al.*, 1999). It is an economically important disease in livestock that is characterized by reproductive failure such as abortions, stillbirths and birth of weak offspring, infertility and loss of milk production (Faine *et al.*, 1999). Different leptospiral serovars are prevalent in particular regions and are associated with one or more maintenance hosts, which serve as reservoirs of infection. The epidemiology of leptospirosis within an ecosystem is thus often complex and leptospires from several serogroups may be maintained by different animal species.

Leptospirosis is endemic throughout south-east Asia (Laras *et al.*, 2002) and the Pacific Island countries (Merien and Perolat, 1996). These areas present suitable environmental conditions for survival and transmission of leptospires, such as regular flooding as a result of high rainfall and the presence of suitable animal reservoirs. The results of serological surveys conducted in several Pacific Island countries showed that infection with a number of potentially zoonotic serovars, including Pomona, Hardjo, Tarassovi, Copenhageni and Icterohaemorrhagiae, were common in pigs, cattle and humans (Angus http://www.spc.int/rahs/Projects/zoonoses3E.htm, accessed 2003).
There is anecdotal evidence that leptospiral infection is one of the causes of poor fertility in commercial cattle in PNG. However, the role of *Leptospira* as a cause of infertility has not been confirmed, mainly because of the lack of robust and simple diagnostic tests available in PNG. This is the first systematic study of the prevalence and impact of infection with *Leptospira* in livestock in PNG.

### 3.1.1 Objectives

The objectives of this study were to:

1. Determine the prevalence and distribution of infections with *Leptospira* in livestock in PNG.
2. Determine the role of cattle as a reservoir for zoonotic serovars of *Leptospira*.
3. Identify risk factors for leptospiral infections in cattle.

### 3.2 Geographic location

Papua New Guinea occupies the eastern half of the island of New Guinea and has many outlying islands including Manus, New Britain, New Ireland and Bougainville. Papua New Guinea lies 6° South and 147° East and has a total area size of 462,840 sq km, with a land area of 451,710 sq km.

Field sites for the study were chosen in Lae in the Morobe Province and Kimbe in the Province of West New Britain because these are the most important areas for cattle production in PNG (Figure 3.1). Lae is the second largest city in PNG and is situated on the shores of Huon Gulf, 6° South on the northeast coast of PNG. The main livestock rearing areas in Lae are in the Markham-Ramu Valley plains where the grazing areas are dominated by soils that are poorly drained. Kimbe in West New Britain is 5° to the
South and 150° to the East of the equator. The surrounding area is covered with rainforest and the main revenue for the province is from oil palm and timber logging.

Figure 3.1 Map of Papua New Guinea with shaded areas showing the main livestock rearing areas in Lae and Kimbe

3.2.1 Climate of Papua New Guinea

The climate in PNG is tropical and is characterized by a northwest monsoon season from December to March and a southeast monsoon season from May to October. The interception of moisture-laden air with mountains creates areas of high rainfall and rain shadows. Rainfall does not necessarily increase with elevation and ranges from 980 mm in Port Moresby to 1,000 mm at Leron Plains in the Markham–Ramu valley. Most cattle are reared in lowland areas with 1,000 to 2,500 mm rainfall per year (Figure 3.2).

Most of PNG receives maximal rainfall during January-April whereas the Huon Peninsula in the Morobe Province and the southern portions of New Britain receive maximal rainfall from May-August. The seasonality and the quantity of rainfall produce
highly variable soil moisture. For example, Kiapit in the Markham Valley (average annual rainfall of 1,500 mm) experiences 20 weeks when soil water capacity is approximately 0.5-0.66 maximum. Several rivers and streams traverse areas where cattle are grazed. The two main rivers in the Markham valley are the Markham and the Leron, which overflow onto the pastures during rainy seasons.

Daily temperatures in PNG are strongly affected by altitude. Lowland areas below 600 m (maximum temperature 30-32°C, mean minimum 19-23°C) cover 66% of the country and supports 54% of the human population. Mid altitude regions from 600-1,200 m (maximum temperature 27-30°C, mean minimum 16-19°C) cover 14.2% of land and supports only 5.9% of the human population.

Figure 3.2 Map of Papua New Guinea showing the monthly rainfall distribution.
(Obtained from the PNG Bureau of Meteorology)
3.3 Materials and Methods

3.3.1 Study sites

The survey was undertaken from March to April of 2004 on cattle farms in Lae and Kimbe. Three large commercial farms (Trukai farm, Ramu beef and Rumion), and two smallholder enterprises (Prhalda and Rearaguntu) in Lae and one large commercial property in Kimbe (Numundo beef) were included in the survey (Figure 3.3). Collectively these farms represent approximately 90% of the total cattle population of PNG. In addition to samples from live cattle, samples of blood and kidneys were collected from cattle slaughtered at abattoirs in Lae, Kimbe and Port Moresby.

Figure 3.3 Sketch of Lae showing farms located in the Markham–Ramu Valley Plains. A: Trukai Industries; B1 and B2: Ramu Beef; C: Rumion; D: Prhalda and E: Rearaguntu
3.3.1.1 Trukai farm

Trukai industries operate a beef cattle production enterprise at Trukai farm at Erap in the Markham Valley. The farm has two grazing areas adjacent to each other at Erap (approximately 1,200 hectares) and Valley View (approximately 800 hectares), which is separated by the Markham river (Figure 3.4). The herd is predominantly Brahman with some cross breeds with Javanese Zebu, Santa Gertrudis, Droughtmaster, Africander, Belmont Red and Charolais.

The farm has approximately 6,000 cattle of which upto 3,000 are female. Trukai currently mate around 1,500 females in January/February of each year and calving begins in mid-September and is completed by mid-December. Heifers are mated earlier than adult cows to increase their reproductive efficiency for second and subsequent conceptions.

The conception rate on the farm is approximately 79% with a calving rate of around 63%. Anecdotal reports of abortions and/or stillbirths were related by farm workers. Calves are weaned at 7 months of age. The farm’s policy on the selection of cows is simple, each female must rear a live calf to weaning to hold their place in the herd.

Trukai farm buys cattle from local smallholder farmers in the Markham Valley, and Sialum District in the Huon Peninsula, to increase their annual turnoff and to provide a market for smallholders to sell their cattle. The recent establishment of a market for the export of live cattle to the Philippines has significantly increased the market for cattle in PNG.
Figure 3.4 Valley View farm showing cattle grazing on the hill side pastures. Most of the cattle on this farm are purchased from smallholders from the Markham Valley and in Sialum District

3.3.1.2 Ramu Beef farms (Gusap Downs and Leron Plains)

Ramu Beef is wholly owned by Ramu Sugar Industry, which is the biggest sugar producing company in PNG. Ramu Beef operate from two farms, at Gusap Downs in the Ramu Valley and at Leron Plains in the Markham Valley. The farm at Leron Plains has a total area of approximately 9,000 hectares and a total herd size of about 2,000 and the farm at Gusap Downs has up to 4,000 hectares and a herd size of approximately 1,000. Cattle mainly graze on open pastures. The herd is predominantly Brahman with some cross breeds with Javanese Zebu, Santa Gertrudis, Droughtmaster, Africander, Belmont Red and Charolais.
Ramu Beef mate their herds around January/February and calving begins in September to December. Historically, the farms have had very low calving percentages of around 45%-50%. This has improved more recently to approximately 75% in 2005. The conception rate on the farm is about 82%. A female must rear a live calf to weaning to hold a place in the herd. The farm workers reported seeing evidence of the abortions and/or stillbirths.
Pre-weaned calves and older cows are kept for breeding at Leron Plains (Figure 3.5) and after weaning heifers are transported to Gusap Downs farm in the Ramu Valley where they are kept with steers (Figure 3.6). Ramu farm buys cattle from local farmers in the Markham-Ramu Valley to provide a market for smallholders. Ramu Sugar also has an abattoir at Gusap Downs to slaughter cattle for the local market.

3.3.1.3 Rumion farm

Rumion farm is located between Trukai and Ramu beef farms on the Leron Plains. Rumion has a mixed farming system with cattle and intensively-reared pigs. It has approximately 2,100 cattle and about 7,500 pigs on an estimated total area of 4,000 hectares. It supplies beef and pork to the local market in Lae. The cattle herd is predominantly Brahman and Droughtmaster. There is a stream which runs through the farm that is commonly contaminated with effluent from the piggery (Figure 3.7). Cattle graze on adjacent pastures and drink from this stream.

Figure 3.7 Stream running through Rumion farm which receives effluent from the piggery
Mating commences around January/February each year and calving begins in mid-September. Conception rate is approximately 80%, with a calving rate around 60% and occasional evidence of abortions and/or stillbirths.

### 3.3.1.4 Prhalda farm

This farm is owned by a local businessman and is a mixed farming system with cattle and pigs. The farm is located approximately 3 kilometres from Rumion farm. This is a small farm with a herd size of approximately 400 cattle and 200 pigs. The cattle are mainly Brahman and Droughtmaster. The cattle are reared to supply beef for the local market and the piggery provides pork to several of the owner’s restaurants in Lae.

### 3.3.1.5 Rearaguntu farm

The farm is a co-operative business owned by the Reara and Guntu villagers in the Markham valley. The farm has about 250 cattle and is located next to several villages.

![Figure 3.8](image)

Figure 3.8 An example of a village adjacent to Rearaguntu farm. Dogs, pigs and humans regularly come into contact with cattle.
3.3.1.6 Numundo Beef

Numundo Beef in Kimbe, West New Britain, is a business arm of NBPOL. The integration of beef cattle and oil palm started in 1998 replacing the previously unprofitable grazing system of raising cattle under coconut palms. This beef production system is known as the “Numundo Half Stand System” (NHSS) and involves the rotational grazing of cattle in a half stand of oil palm and pasture areas (Figure 3.9). This system allows the sustainable growth of both oil palms and cattle on a constant area of land throughout the entire production cycle of the oil palm. The NHSS is a vertically integrated beef production operation from breeding through to retail.

Figure 3.9 Cattle grazing in pastures with oil palms in the background in Numundo farm

The farm currently has a total grazing area of 692 hectares which can support approximately 2,000 cattle. The current herd of 2,036 adult cattle is grazed on 96 hectares of sole pastures and 596 hectares of half stand oil palm and finished in a 1,200 head capacity feedlot. The present breeding herd consists of 1,600 cows supplying
replacement breeding females and cull stock. The breeding herds are run in five separate
groups that are control mated to provide a staggered calving over 9 months of each year,
ensuring continuous supply of cattle to the feedlot. For a cow to hold a place in the herd
it must rear a live calf to waening. The initial herd was predominantly Brahman with the
recent introduction of Droughtmaster and Charolais. Both these breeds have been cross-
bred with the initial herd to improve economically viable traits while maintaining the
adaptation to the environment.

Numundo Beef has a conception rate of around 80% and a calving rate of approximately
70%. In 2003, this farm experienced an unacceptably high level of abortions and/or
stillbirths in their first calf heifers in late gestation. For the year ending December 2003,
72% of the calf loss was the result of stillbirths and/or abortions. Reports from the farm
showed the greatest incidence of stillbirths and/or abortions occurred in maiden heifers.
In addition, white spots are regularly observed on the surface of the kidneys of feedlot
cattle at slaughter (approximately 10-15% of slaughtered cattle). White spots on the
kidney surface are considered to be indicative of leptospiral infection (Uzal et al., 2002;
Boqvist et al., 2003).

The cattle in the closed and open feedlots are fed with a supplementary feed which
consists mostly of palm kernel expeller. The supplementary feed is stored uncovered
where it may be contaminated with urine from rodents before being fed to the cattle
(Figure 3.10). A stream runs through the farm and is accessible to the cattle while
grazing (Figure 3.11).
3.3.1.7 Abattoirs

Five abattoirs were visited during this study and kidneys and serum were collected from cattle and pigs after slaughter. Two abattoirs that slaughter pigs and cattle in Lae and Port Moresby are owned by the Livestock Development Corporation (LDC). The facilities and equipment in these two abattoirs are old and poorly maintained. Pelgens
smallgoods abattoir in Lae only kills pigs from its own piggery farms. Ramu abattoir at Gusap Downs is owned by Ramu Sugar Ltd and slaughters only cattle. Numundo Beef abattoir in Kimbe is owned by NBPOL and only kills cattle reared from their farm.

The numbers of animals slaughtered in these abattoirs vary. The LDC central abattoir in Lae kills 50 to 80 cattle and 100 pigs per day while the abattoir in Port Moresby kills up to 50 cattle and 50 to 80 pigs per day. Pelgens abattoir kills 50 to 80 pigs per day while Ramu and Numundo Beef abattoirs have the capacity to kill up to 80 cattle per day.

![Figure 3.12 Killing floor at the Numundo Beef abattoir](image)

3.3.2 Collection of samples

Whole blood was collected from the coccygeal artery or vein of a total of 1,300 female cattle from Trukai, Ramu Beef, Rumion, Prhalda, Rearaguntu and Numundo Beef farms.
Blood was collected with a vacutainer and samples were carefully stored upright in an insulated box with ice bricks. Each herd was stratified into 3 age groups (<2, 2-5 and >5 years) which were systematically sampled to provide approximately equal numbers of samples from each age stratum. In Table 3.1 the number of blood samples collected from the female cattle in the three age groups from each farm is summarised. In addition, 79 blood samples were collected from cattle during slaughter at the Lae abattoir and 73 kidneys were collected from cattle at the abattoirs in Lae, Ramu beef, Numundo beef, and Port Moresby. Blood samples were left overnight to clot at 4°C before centrifugation. Serum was removed, labelled and stored at -20°C until required.

Blood was collected from pigs originating from commercial farms which were killed at the abattoirs in Lae and Port Moresby. The two commercial pig farms in Lae were Rumion piggery and Pelgens piggery and both are located outside Lae township. Hagen Planters piggery is located in Port Moresby. Ninety four blood samples from pigs from Rumion farm were collected at the central abattoir in Lae and 111 blood samples were collected from pigs killed at Pelgens piggery abattoir in Lae. Seventy eight blood samples and 79 kidneys from Hagen Planters piggery were collected at the abattoir in Port Moresby. Blood samples were left overnight to clot at 4°C before centrifugation. Serum was removed and was stored at -20°C until required.

Samples of kidneys collected from cattle and pigs were placed into sterile plastic bags and stored in an insulated box with ice bricks. The kidney samples were cultured within 3 to 4 hours of collection.
Table 3.1  Number of blood samples collected from cattle in each age group

<table>
<thead>
<tr>
<th>Farm</th>
<th>&lt;2 years</th>
<th>2-5 years</th>
<th>&gt;5 years</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trukai</td>
<td>126</td>
<td>122</td>
<td>121</td>
<td>369</td>
</tr>
<tr>
<td>Ramu Beef</td>
<td>71</td>
<td>71</td>
<td>82</td>
<td>224</td>
</tr>
<tr>
<td>Rumion</td>
<td>47</td>
<td>54</td>
<td>71</td>
<td>172</td>
</tr>
<tr>
<td>Prhalda</td>
<td>67</td>
<td>45</td>
<td>22</td>
<td>134</td>
</tr>
<tr>
<td>Rearaguntu</td>
<td>45</td>
<td>21</td>
<td>62</td>
<td>128</td>
</tr>
<tr>
<td>Numundo Beef</td>
<td>84</td>
<td>83</td>
<td>106</td>
<td>273</td>
</tr>
<tr>
<td>Abattoir</td>
<td>16</td>
<td>40</td>
<td>23</td>
<td>79</td>
</tr>
<tr>
<td>TOTAL</td>
<td>1,379</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.3  Culture and isolation

Kidney samples collected in Lae were transported to the Agriculture Laboratory at the University of Technology in Lae, samples collected in Numundo were transported to the Oil Palm Laboratory in Kimbe and samples collected in Port Moresby were transported to the National Veterinary Laboratory for further processing and culture.

Ellinghausen-McCullough-Johnson-Harris (EMJH) semi-solid medium containing 5-fluorouracil (5 mls) was prepared by the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis in Brisbane shipped to PNG prior to the study and stored at room temperature until required (Ellinghausen and McCullough, 1965).

The capsule of each kidney was removed and the surface was swabbed with 70% ethanol and allowed to dry. A sterile scalpel blade was used to cut each kidney into two pieces. A 1-2 mm³ piece was excised from the medullary region of the kidney and placed into
the EMJH semi-solid agar containing 5-fluorouracil and a similar sized portion was stored in 10% dimethyl sulfoxide (DMSO) and saturated sodium chloride (NaCl) solution for subsequent PCR analysis.

All EMJH cultures were incubated at 28°C for 3 to 4 days. After this time approximately 5 ml of the culture medium was sub-cultured aseptically into fresh EMJH semi-solid agar containing 5-fluorouracil and incubated at 28°C for a further 21 days. After that all cultures were sent to the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis in Brisbane for identification and serovar typing of any leptospires isolated.

3.3.4 Microscopic agglutination test

All sera were tested for the presence of anti-leptospiral antibodies using a reference panel of 21 leptospiral serovars: Pomona, Hardjo, Tarassovi, Grippotyphosa, Celledoni, Copenhageni, Australis, Zanoni, Robinsoni, Canicola, Kremastos, Szwajizak, Medanensis, Bulgarica, Cynopteri, Ballum, Bataviae, Djasiman, Javanica, Panama and Shermani using the MAT. The MAT was performed at the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis in Brisbane.

3.3.5 Interpretation of MAT titres

The MAT is not strictly serovar-specific and may give rise to a positive reaction to other serovars within the same serogroup, and even to serovars within other serogroups (Faine et al., 1999). Sera with titres to more than one serovar were regarded as seropositive for all those serovars unless the serovar was known to cross-react, in which case the sera were scored as positive to the serovar with the highest titre (Black et al., 2001).
Three of the commercial farms, Numundo beef, Trukai Industries and Ramu beef routinely vaccinate female cattle with a leptospiral vaccine (Ultravac 7:1 from CSL, Australia) that contains serovars Hardjo and Pomona. The vaccine contains a combination of leptospiral serovars Hardjo and Pomona and antigens of five clostridial species which commonly affect cattle. For this study a MAT titre of ≥ 400 was used as the cut-off to classify a vaccinated animal as infected (NerVig et al., 1980) while a titre of < 400 was considered to be due to vaccination (Trueba et al., 1990). The other three farms (Rumion, Prhalda and Rearaguntu) did not vaccinate cattle so a titre of ≥ 50 was used as the cut-off to classify animals as seropositive. To measure the rate of active infection a titre of ≥ 400 was used on the six farms (Faine et al., 1999).

3.3.6 Analysis

Apparent seroprevalences were calculated for all of the 21 leptospiral serovars. Age-specific seroprevalences of the most common serovars from the six farms and abattoirs in the study were calculated. Cattle in each age group from Trukai farm were further allocated into two groups according to their origin, determined by their brand (i.e. Trukai-born, non-Trukai born), and the seroprevalences within age groups calculated. The program SPSS Ver. 14.0 (SPSS Inc) was used for descriptive data analysis including chi-square tests and Excel 2000 (Microsoft) was used to plot graphs. The 95% confidence intervals were also calculated.

3.4 Results

3.4.1 Overall seroprevalence of leptospiral serovars in cattle in PNG

Antibodies to 13 leptospiral serovars were detected using the MAT (Table 3.2). The main serovars detected included Hardjo, Szwajizak, Tarassovi, Medanensis, Pomona, and Kremastos. Antibodies to other leptospiral serovars were detected but each at an
apparent seroprevalence of <2% with exception of Australis at 2.2%. The apparent seroprevalence of serovar Hardjo was significantly higher than all other serovars (P < 0.0001).

Table 3.2 Proportion of cattle (n=1379) from the study with antibodies to leptospiral serovars determined using the MAT

<table>
<thead>
<tr>
<th>Leptospira species</th>
<th>Serovar</th>
<th>No. positive</th>
<th>% positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptospira</td>
<td>Hardjo</td>
<td>742</td>
<td>53.7</td>
<td>51.1, 56.4</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Szwajizak</td>
<td>416</td>
<td>30.2</td>
<td>27.7, 32.6</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Tarassovi</td>
<td>214</td>
<td>15.5</td>
<td>13.6, 17.4</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Medanensis</td>
<td>176</td>
<td>12.8</td>
<td>11, 14.5</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Pomona</td>
<td>116</td>
<td>8.4</td>
<td>6.9, 9.9</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Kremastos</td>
<td>91</td>
<td>6.6</td>
<td>5.3, 7.9</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Australis</td>
<td>31</td>
<td>2.2</td>
<td>1.5, 3.0</td>
</tr>
<tr>
<td>L. noguchii</td>
<td>Panama</td>
<td>27</td>
<td>2.0</td>
<td>1.2, 2.7</td>
</tr>
<tr>
<td>L. santarosai</td>
<td>Sherman</td>
<td>21</td>
<td>1.5</td>
<td>0.9, 2.2</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Bataviae</td>
<td>17</td>
<td>1.2</td>
<td>0.7, 1.8</td>
</tr>
<tr>
<td>L. borgpetersenii</td>
<td>Ballum</td>
<td>13</td>
<td>0.9</td>
<td>0.4, 1.5</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Djasiman</td>
<td>11</td>
<td>0.8</td>
<td>0.3, 1.3</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Robinsoni</td>
<td>6</td>
<td>0.4</td>
<td>0.1, 0.4</td>
</tr>
</tbody>
</table>

3.4.2 Overall age-specific seroprevalence of antibodies to leptospiral serovars

Age-specific seroprevalences were calculated for each of the three age groups, <2 years, 2-5 years and >5 years (Figure 3.13). There was an increase in the seroprevalence of serovar Hardjo with increasing age and was significant. There was no age-specific difference in the seroprevalence of other serovars except with serovar Szwajizak. The
seroprevalence of Hardjo was found to be significantly higher in each age group compared to the other serovars (P < 0.0001).

Figure 3.13  Age–specific seroprevalence of the principal leptospiral serovars identified in cattle

3.4.3 The seroprevalence of leptospiral infection in cattle from 6 farms and an abattoir in PNG

The farm-specific seroprevalences of serovars Hardjo, Pomona and Tarassovi in cattle from six farms in PNG are summarised in Table 3.3. The seroprevalence of serovar Hardjo infection was significantly higher in unvaccinated cattle from Rumion compared to the Numundo, Trukai and Ramu farms that vaccinated (P < 0.05). There was a low seroprevalence of serovar Pomona infection from cattle in all farms.
Table 3.3 The prevalence of leptospiral serovars Hardjo, Tarassovi and Pomona from cattle on six farms and an abattoir (95% confidence interval in parentheses)

<table>
<thead>
<tr>
<th>Farm</th>
<th>Total sera</th>
<th>% Seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hardjo</td>
</tr>
<tr>
<td>Numundo</td>
<td>273</td>
<td>38.5 (32.7, 44.2)</td>
</tr>
<tr>
<td>Trukai</td>
<td>369</td>
<td>22.5 (18.2, 26.8)</td>
</tr>
<tr>
<td>Ramu</td>
<td>224</td>
<td>19.2 (14.0, 24.4)</td>
</tr>
<tr>
<td>Rumion</td>
<td>172</td>
<td>57.6 (50.2, 64.9)</td>
</tr>
<tr>
<td>Prhalda</td>
<td>134</td>
<td>32.8 (24.9, 40.8)</td>
</tr>
<tr>
<td>Rearaguntu</td>
<td>128</td>
<td>35.2 (26.9, 43.4)</td>
</tr>
<tr>
<td>Abattoir</td>
<td>79</td>
<td>19 (10.3, 27.6)</td>
</tr>
</tbody>
</table>

*MAT titre ≥ 400 was used as cut-off for positivity for serovars Hardjo and Pomona in these farms to discriminate infection from vaccination titres.

3.4.4 Seroprevalence of serovars Hardjo, Pomona and Tarassovi in cattle from the three age groups

3.4.4.1 Serovar Hardjo

The age-specific seroprevalence to serovar Hardjo is shown in Figure 3.14. The seroprevalence of Hardjo in older cows (>5 years) was significantly higher in cattle from Rumion farm than from older cattle on all other farms (P < 0.05). In the farms that vaccinate for *Leptospira* the seroprevalence to Hardjo from Numundo Beef was significantly higher in the three age groups compared to cattle from Trukai and Ramu beef (P < 0.05). The seroprevalence of Hardjo in maiden heifers was significantly lower in all farms compared to the older cattle (P < 0.05). In contrast the prevalence of serovar
Hardjo in cattle slaughtered at the Lae abattoir was significantly higher in cattle 2-5 years old compared to the cattle >5 years old.

Figure 3.14 Age-specific seroprevalence of serovar Hardjo in cattle from the six farms and from cattle slaughtered in Lae abattoir

* MAT titre of > 400 was used as the cut-off for infection with serovar Hardjo on Numundo, Trukai and Ramu farms that vaccinate cattle using a leptospiral vaccine.

3.4.4.2 Serovar Tarassovi

The age-specific distribution of antibodies to serovar Tarassovi in cattle from the six farms in PNG is shown in Figure 3.15. The seroprevalence of Tarassovi was significantly higher in 2-5 year old and >5 year old cattle from Rumion and Rearaguntu farms than in all other farms. The seroprevalence of Tarassovi in heifers <2 years old was significantly higher on Trukai and Numundo Beef farms compared to the other farms (P < 0.05). In Numundo Beef and Trukai farms the seroprevalence decreased as age increased while in Rumion the seroprevalence tended to increase with age.
3.4.4.3 Serovar Pomona

The age-specific seroprevalence of antibodies to serovar Pomona is displayed in Figure 3.16. The seroprevalence of serovar Pomona on the farms was less than 8% in all age groups.
3.4.5 Age-specific seroprevalence of serovars Hardjo and Tarassovi in cattle from Trukai farm

3.4.5.1 Serovar Hardjo

The seroprevalence of Hardjo infection was significantly lower in the maiden heifers (<2 years) born on Trukai farm (1.9%) compared to heifers not born on Trukai farm (17.6%) (P < 0.05) (Figure 3.17). The seroprevalence of Hardjo infection was significantly higher in cattle in the 2-5 years age group born on Trukai (47.4%) compared to cattle not born on Trukai (26.2%) (P < 0.05).

* A titre of ≥ 400 was used as the cut-off for infection with serovar Pomona in Numundo, Trukai and Ramu farms that vaccinate cattle using a leptospiral vaccine.
Figure 3.17  Age-specific seroprevalence of serovar Hardjo in cattle born on farm (Trukai) and those not born on Trukai (MAT titre of ≥ 400 was used as the cut-off in both groups)

3.4.5.2 Serovar Tarassovi

There is a decreasing prevalence of infection with serovar Tarassovi with age on Trukai farm (Figure 3.18). The seroprevalence for serovar Tarassovi in each age group was significantly higher in cattle born on Trukai farm compared to cattle not born on Trukai farm.
Figure 3.18  Age-specific seroprevalence of serovar Tarassovi in cattle born on farm (Trukai) and those that were not born on Trukai

![Graph showing age-specific seroprevalence of serovar Tarassovi in cattle born on farm (Trukai) and those that were not born on Trukai.]

3.4.6 Active infection with serovar Hardjo in cattle from six farms in PNG

Active infection of serovar Hardjo in maiden heifers (<2 years) and older cattle (≥2-5 years) was estimated using a MAT titre cut-off of ≥400 in cattle from the six farms and the results are displayed in Figure 3.19. The prevalence of active infection with serovar Hardjo in maiden heifers was significantly lower compared to the older female cattle on all the farms except Prhalda (P < 0.05). The prevalence of active infection in older cattle was significantly higher (P < 0.05) in Numundo Beef than in all other farms. The rate of active infection in maiden heifers from Numundo Beef was higher than Ramu farm.
Active infection of serovar Hardjo in animals on the six farms surveyed.

MAT titre of $\geq 400$ was used.

3.4.7 Geographic location of leptospiral infections

Cattle from Numundo farm in Kimbe had a significantly higher seroprevalence to Hardjo compared to cattle from Trukai, Ramu, Rumion, Prhalda and Rearaguntu farms in Lae ($P < 0.001$) (Table 3.4).

Table 3.4 Results of testing 1,300 cattle for the presence of antibodies to serovar Hardjo ($\text{titre} \geq 400$ for infection in vaccinated cattle)

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of sera</th>
<th>No. positive</th>
<th>% positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kimbe</td>
<td>273</td>
<td>105</td>
<td>38.5*</td>
<td>32.7, 44.2</td>
</tr>
<tr>
<td>Lae</td>
<td>1,027</td>
<td>165</td>
<td>16.0*</td>
<td>13.8, 18.3</td>
</tr>
<tr>
<td>All sera</td>
<td>1,300</td>
<td>270</td>
<td>20.8</td>
<td>18.6, 23.0</td>
</tr>
</tbody>
</table>

* Significant difference ($P < 0.001$)
Table 3.5  Age-specific seroprevalence of *Leptospira* Hardjo in cattle from Kimbe and Lae (titre $\geq$ 400 for infection in vaccinated cattle)

<table>
<thead>
<tr>
<th>Location</th>
<th>Age</th>
<th>No. sera tested</th>
<th>% Positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kimbe</td>
<td>&lt;2 years</td>
<td>84</td>
<td>13.1</td>
<td>5.9, 20.3</td>
</tr>
<tr>
<td></td>
<td>2-5 years</td>
<td>83</td>
<td>60.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.7, 70.8</td>
</tr>
<tr>
<td></td>
<td>&gt;5 years</td>
<td>106</td>
<td>41.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.1, 50.9</td>
</tr>
<tr>
<td>Lae</td>
<td>&lt;2 years</td>
<td>356</td>
<td>18.0</td>
<td>14.0, 22.0</td>
</tr>
<tr>
<td></td>
<td>2-5 years</td>
<td>313</td>
<td>18.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.2, 22.8</td>
</tr>
<tr>
<td></td>
<td>&gt;5 years</td>
<td>358</td>
<td>12.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.6, 15.4</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> significant difference (P < 0.001)

The seroprevalence of *Leptospira* Hardjo was significantly higher in cattle from Kimbe in the age groups of 2-5 and >5 years compared to those in the same age groups from Lae (P <0.001) (Table 3.5). There was no significant difference in the seroprevalence for heifers (<2 years) from either location.

### 3.4.8 Seroprevalence of leptospiral serovar in pigs

No antibodies to the 21 leptospiral serovars were detected in any of the 283 pig sera tested using the MAT.

### 3.4.9 Leptospiral culture

Leptospires cultured from 2 of the 73 kidney samples collected from cattle slaughtered at the central abattoir in Lae. Both isolates were typed and identified as *L. borgpetersenii* serovar Hardjo. All the other cultures of bovine kidneys were either negative or
contaminated. All the 79 pig kidneys cultured in EMJH medium were negative for leptospires.

3.5 Discussion

The main aim of this study was to determine the seroprevalence of *Leptospira* infection in cattle and pigs in PNG. Cattle are generally accepted as a major reservoir host for *Leptospira* serovar Hardjo (Ellis *et al.*, 1981; Faine *et al.*, 1999) and infection with this serovar usually leads to infertility and reproductive failure in chronic infections (Faine *et al.*, 1999). Environmental factors such as rainfall, soil water holding capacity, temperature and acidic content have extensive effects on the survival and prevalence of leptospirosis (Hellstrom and Marshall, 1978; Carroll and Campbell, 1987; Trueba *et al.*, 2004). The wet season in the Markham–Ramu valley in Lae is from January to April and in Kimbe from January to August. The soils in these two regions have a high water holding capacity of about 50-70% during and after the wet seasons making it viable for the leptospires to survive. Temperature could be another factor that affects survival and leptospires survive and grow best in a temperature range of 28 to 30°C (Faine *et al.*, 1999). Lae and Kimbe have daily minimum and maximum temperatures from 19 to 32°C which could be suitable for the survival of leptospires.

The MAT was used in this study because it is considered the reference test for antibody detection (WHO and International Leptospiral Society, 2003). The detection of leptospiral antibodies however does not confirm the presence of clinical disease and failure to detect antibodies does not guarantee lack of exposure to leptospiral infection. The sensitivity and specificity of the MAT have not been described due to the difficulty in establishing a suitable gold standard for comparison with the test. Culture is often difficult and therefore is unsuitable for use as a ‘gold standard’ for the MAT (Faine *et
al., 1999). Despite these drawbacks the MAT has been the test of choice to detect exposure to *Leptospira* for many years.

A MAT titre of $\geq 400$ was used in this study to differentiate between the antibody response induced by active leptospirosis and the antibody titre response induced by vaccination. Anti-*Leptospira* antibody titres usually reach their peak 2 weeks after a two-dose vaccination course (Stringfellow *et al.*, 1983) and vaccination titres vary greatly from 0 to about 800 (Smith *et al.*, 1994). NerVig *et al.*, (1980) observed that a titre of $\geq 400$ was indicative of past or present leptospiral infection, regardless of vaccination status.

A total of thirteen leptospiral serovars were identified in cattle originating from the main livestock rearing regions in PNG. Serovar Hardjo was found to be the most prevalent serovar infecting cattle, which is not surprising as cattle are considered the principal reservoir of this serovar. Other results showed that the seroprevalence of serovar Tarassovi was higher than that of serovar Pomona. The significant seroprevalence of serovars Szwajizak and Medanensis was noted but as there is evidence that they both cross-react with serovar Hardjo the results may be misleading (Thiermann and Garrett, 1983; Black *et al.*, 2001).

This first age-stratified study of leptospirosis in cattle in PNG showed that the overall seroprevalence to serovar Hardjo in the three age groups was significantly high compared to all other serovars, in agreement with a study in central Queensland (Black *et al.*, 2001). There was a strong association between age and seroprevalence of Hardjo infection, but no association with serovars Pomona and Tarassovi. The seroprevalence of
Hardjo infection was found to increase linearly with age and it was significantly higher in older cattle (P < 0.05). In PNG the majority of infection with Hardjo occurred in the first-calf heifers (2-5 years) and older cows (>5 years) and this is consistent with studies in Queensland (Black et al., 2001). The increase in serologic prevalence with age does not necessarily indicate that a greater risk of infection occurs among the older cattle. This increase may reflect the long duration of antibodies circulating in cattle naturally infected with *Leptospira*.

The farm-specific seroprevalences showed that the dominant serovar in the six farms is Hardjo and that the infection is endemic on all farms. The vaccinated farms Numundo Beef, Trukai and Ramu had an average seroprevalence of 27% and the unvaccinated farms had an average seroprevalence of 42%. Preventive measures such as culling the older cattle that are chronic carriers, and vaccination, should be taken to control the spread in the farms.

The age-specific seroprevalence of serovar Hardjo within the age groups in the farms showed that the seroprevalence in maiden heifers was significantly lower than in the older age groups (P < 0.05). Despite this low seroprevalence the maiden heifers are a high risk group because when infected for the first time during pregnancy, abortion, stillbirth or infertility may result (Hathaway et al., 1982). In Numundo Beef farm the greatest occurrence of abortions and/or stillbirths occurred in maiden heifers with 14% of calves born lost post-natally compared to the herd average of 5%. Serovar Hardjo is most likely transmitted to the maiden heifers from older carrier cattle and this seems to be a problem with Numundo Beef farm.
Within farms, the level of active infection with serovar Hardjo is significantly higher in older cattle on Numundo Beef farm than on Trukai and Ramu farms. The significance of this result suggests two things: firstly, in Numundo Beef, vaccination is not effective in controlling leptospiral infection; secondly, in Trukai and Ramu farms the practice of buying cattle from smallholders in the Markham and Ramu Valleys and the Sialum District in Huon Peninsula may result in the introduction of infected animals into their herds.

Cattle farms from two geographic locations were compared. Numundo farm on the island of Kimbe east of the mainland and the rest of the farms were in the Markham-Ramu Valleys in Lae on the mainland. The seroprevalence of serovar Hardjo in Kimbe was significantly higher than in Lae (P < 0.0001) (Table 3.4) and cattle in Kimbe were 2.4 times more likely to be infected than the Lae cattle. In all age groups the seroprevalence to Hardjo was generally higher in cattle from Kimbe (1.8%) than in Lae (0.9%). Infection with serovar Pomona may be a concern in Kimbe but not in Lae.

A decrease in the seroprevalence of Hardjo was noted in older cattle (>5 years) in Numundo, Trukai and Ramu farms because of culling for infertility, which may suggests that leptospiral infection is the cause of the infertility (Figure 3.14).

Despite the high seroprevalence of infection with serovar Hardjo, there is evidence that farms that vaccinated against leptospirosis had a lower seroprevalence of serovar Hardjo in all age groups than to farms that do not vaccinate. For example, Trukai and Ramu farms showed a significantly lower seroprevalence of Hardjo in cattle in farm-bred
maiden heifers (<2 years) which suggests a good response to herd vaccination, while Numundo Beef farm showed evidence of vaccination failure (Figure 3.14).

The apparent vaccination failure on Numundo Beef farm could be caused by several factors, with one possibility being the vaccine potency. The potency of the vaccine can be affected during shipment if the vaccine is not stored and maintained at an optimum temperature of 2-7°C during transportation from the manufacturer in Australia to Port Moresby and then onto Numundo Beef farm in Kimbe. In Port Moresby, the ambient temperature ranges from approximately 25 to 33°C. This is important because there is a possibility that the vaccine could be subjected to high temperatures when it is transferred between international and domestic flights. If the optimum temperature is not maintained, the potency will be reduced and the required level of protection may not be induced in the cattle. The vaccine at the farm should also be stored at the required temperature after receival and during transport to the cattle yard. The other factor for the vaccine to be effective is that the correct dose should be given to all the cattle on the farm at the required 4-6 weeks intervals, with an annual booster revaccination recommended.

Antibodies to serovar Tarassovi are relatively common in all ages of cattle from all farms (Figure 3.15). This is likely to be a result of natural infection with serovar Tarassovi because it is not included in the vaccine used in PNG and the serovar doesn’t cross-react with other serovars. The results of this study suggest that the infection is actively present in the cattle population although it is not possible to identify the source of infection. Serovar Tarassovi was not isolated from the kidney samples collected during this study however, as already noted, culture is difficult and insensitive.
A serological study in beef cattle in Queensland also identified antibodies to serovar Tarassovi (Black et al., 2001). The significance of this serovar is uncertain as the organism has not been isolated from Australian cattle (Durham and Paine, 1997; Black et al., 2001). Pigs are the preferred host for serovar Tarassovi and are known to excrete the organism in their urine (Davos, 1977). This raises the possibility that cattle are an incidental host for this serovar and suggests a reservoir host within the vicinity of the farms.

The high seroprevalence of Tarassovi in young and old cows in Rumion farm suggests evidence that the piggery on that farm may be the source of infection. However, serological results from the piggery did not show any evidence of infection with Tarassovi in the pigs. Rodents and small marsupials could also be a possible reservoir for this serovar on Rumion farm. In another farm in Rearaguntu, a high seroprevalence of serovar Tarassovi was detected in older cattle and this may result from daily contact with pigs, dogs or rodents, because the farm is located next to several villages where these animals wander freely. An earlier paper published in PNG reported the isolation of serovar Tarassovi from a bandicoot, *Echymipera kalubu* (Morahan, 1971) which could be a reservoir host for this serovar. Bandicoots are widely distributed in the Markham-Ramu Valley and are a common source of protein for the villagers.

The seroprevalence of serovar Pomona infection in cattle in PNG was significantly lower than that of serovar Hardjo in all age groups, indicating that the leptospiral vaccine containing the Pomona antigen was effective in controlling Pomona infection on Lae farms where the serovar occurs (Figure 3.16). The leptospiral vaccine given over a substantial period is able to trigger the production of protective antibodies in the cattle
and antibody titres of 50-200 in over 20% of the animals are indicative of vaccination (NerVig et al., 1980). However, results of this study showed a low seroprevalence of Pomona at this antibody titre (50-200); Trukai and Ramu beef farms had 3% (11/369) and 5% (12/224) respectively. A possible explanation as to why there were hardly any detectable antibody titres to serovar Pomona in this study could be that perhaps Pomona simply is not a common serovar in this region. An absence of agglutinating antibodies is not necessarily an indication that there is no protection in the animals, as vaccinated animals have been shown experimentally to be protected from natural challenge with Leptospira for many months after their agglutinating antibody titres could not be detectable (Mackintosh et al., 1980a; Allen et al., 1982).

Cross-reactions can occur between serovars of the same serogroup or closely related serovars as they possess the same agglutinating epitopes (Faine et al., 1999). Serovars Szwajizak and Medanensis are believed to demonstrate cross reactivity with serovar Hardjo (Faine et al., 1999; Black et al., 2001). Serovars Hardjo, Szwajizak and Medanensis were originally group in the serogroup Hebdomadis but are now distributed in two new serogroups. Serovars Hardjo and Medanensis are in the Sejroe serogroup while Szwajizak belongs to the Mini serogroup (Faine et al., 1999). In this study evidence suggests that serovar Hardjo cross-reacts with serovars Szwajizak and Medanensis. The majority of animals that were positive for serovar Hardjo had higher titres for Hardjo than for serovars Szwajizak and Medanensis. The situation was further complicated by the high titres of serovar Szwajizak in the range of 200-1,600 in cattle in PNG. Serovars Szwajizak and Medanensis are thought to be rodent-associated in Australia (Smythe et al., 2000) and may be involved in abortion, as rodent-associated serovars can cause overt disease in cattle (Thiermann, 1982).
Leptospires were isolated from two kidneys and subsequent typing showed them to be *L. borgpetersenii* serovar Hardjo (type hardjobovis). This serotype is probably maintained by the cattle in PNG, however it is not possible to conclude that this organism is associated with bovine abortions and other reproductive problems in PNG. Further studies are needed to isolate the bacteria from aborted foetuses and placentae to confirm this.

Vaccination is the only effective way to control bovine leptospirosis. Manufacturer’s instructions for use of the vaccine should be strictly followed. Current protocols are that healthy cattle should receive 2 doses 4-6 weeks apart. New born calves from 4 weeks of age should receive a primary dose followed by a second dose 4-6 weeks later and annual re-vaccination with a single dose is recommended. All new animals should begin a two-dose course of vaccination upon arrival on the farm. A high level of persistent Hardjo infection present on infected farms is likely to result in a high challenge to the vaccinated animals. Therefore, any animal which has not received a full primary course, or adequate booster vaccination, is likely to suffer from leptospirosis (Gordon, 2002).

Control programs involving herd management and isolation of infected animals can assist in preventing the introduction of *Leptospira* and thus reducing the level of challenge to vaccinated animals on the farms. In Ramu farm, all young heifers are separated from their dams after weaning and are transported to Gusap Downs farm at a different location. Consequently, the chance of these heifers being exposed to significant challenge from older cattle, who are chronic carriers is reduced. Herd management practices such as limiting sharing of bulls between farms, as bulls are believed to be
carriers of serovar Hardjo in their genital tract, can be used to prevent the transmission of serovar Hardjo (Ellis et al., 1986a). The isolation and strategic culling of infected animals is another practice used to prevent transmission of Leptospira. New animals that are purchased should be quarantined, tested for leptospirosis and given a full course of vaccination before being released into the herd.

The public health importance of serovar Hardjo and other serovars is due to their involvement in infections with Leptospira in abattoir workers, meat inspectors and farmers (Smythe et al., 2000). Thus, there is a strong possibility that handling of infected kidneys and splashing of infected urine, may result in the transmission of leptospires to cattle handlers or abattoir workers in PNG. To minimize the risk of infections in abattoirs and farms, workers should be encouraged to wear personal protective equipment such as overalls, boots, gloves and safety glasses, and cattle potentially infected with leptospirosis should be properly handled to reduce the possibility of infection.

3.6 Conclusion

In conclusion, this study has shown that infections with Leptospira are endemic in the PNG cattle population, with a seroprevalence of over 30%. The main infecting serovar is Hardjo and the seroprevalence was found to increase with age. Isolation of the bacteria and typing confirmed the presence of L. borgpetersenii serovar Hardjo (type hardjobovis) in PNG. Serovar Tarassovi also appears to be prevalent in cattle but its significance in PNG is uncertain. Cattle are potential zoonotic reservoirs for serovars Hardjo, Tarassovi and Pomona. Other findings from this study showed that pigs from commercial piggeries in PNG were not infected with any leptospiral serovars and that pigs are thus probably not a significant reservoir of infection for cattle.
CHAPTER 4
SURVEY OF DOMESTIC ANIMALS TO DETECT THE PRESENCE OF INFECTION WITH LEPTOSPIRA IN SMALLHOLDER FARMS AND VILLAGES IN THE MARKHAM VALLEY IN PAPUA NEW GUINEA

4.1 Introduction

This study forms the second part of a broader survey conducted to determine the epidemiology of *Leptospira* in livestock in PNG. The first part of this livestock survey in 2004 showed that there was a high prevalence of infection with serovar Hardjo in cattle from commercial and smallholder farms in Lae and Kimbe.

The result from Trukai farm indicates that infection with Hardjo is common in all cattle that have a Trukai farm brand and those with other brand numbers (not born on Trukai). Trukai farm has been vaccinating for leptospirosis but the results showed that cattle with Trukai brand were actively being infected with serovar Hardjo. There is a strong possibility that Hardjo was being introduced onto Trukai farm from the smallholder farms from where they purchased their replacements. As smallholder farmers do not vaccinate against leptospirosis, their cattle may play a role in the transmission to commercial farms.

Most smallholder farms usually have between 50 to 200 cattle, which are commonly grazed next to the village. The cattle yards are in a semi-bush setting and the soil is often poorly drained. Cattle are exposed to pigs, dogs, bandicoots and rodents and thus they may be infected with leptospiral serovars that are maintained by these animals including
serovars Pomona and Tarassovi (pigs) and serovars Canicola and Tarassovi (dogs) (Faine et al., 1999). There is no data on the presence of leptospiral serovars in dogs and pigs in the villages in the Markham Valley in Lae.

From the public health perspective; there is also a risk that people in villages could be infected with Leptospira as a result of their close relationship with animals. The daily activities of village people such as walking, gardening, feeding pigs, washing clothes or bathing in wells or streams will put them directly or indirectly in contact with these animals. Thus there was a need to investigate the status of smallholder and village animals in the Markham Valley.

4.1.1 Objectives

The aims of this second study were:

1. To determine if cattle from the smallholder farmers in the Markham Valley were infected with species of Leptospira.

2. To determine if the village pigs and dogs in the Markham Valley are infected with leptospiral serovars and what role they might play as reservoirs for bovine leptospirosis.

4.2 Materials and Methods

4.2.1 Survey

The survey was conducted at the end of the wet season in June 2006. Smallholder villages were selected based on the results of the survey in 2004. Two groups of villages were selected based on their proximity to the commercial farms. The first group were close to Trukai farm (Lower Markham) and the second were close to Ramu Sugar farm in the Upper Markham. The villages in these areas sell animals to either Trukai or Ramu.
The smallholder villages in the Markham Valley were identified from information supplied by the Regional Veterinary Officer in Lae. A total of 11 villages were selected with the assistance of the President of the Smallholder Cattleman’s Association in the Markham Valley and the Trukai farm manager. Seven villages were selected in the Upper Markham Valley, Mampim, Maranafun (smallholder cattle farmer), Ragizaria, Mutzing, Guntu, Guaruk (smallholder cattle farmer) and Mangiang. Four villages were selected in the lower Markham Valley, Wanpup (smallholder cattle farmer), Arisisi (smallholder cattle farmer), Gavtitif and Zifasing. In addition, Rumion piggery, Clean Water cattle farm in Zifasing and Trukai farm were included. The locations of the villages and farms are shown in Figure 4.1.

Figure 4.1   Location of villages and farms in the Markham Valley
During the survey, the president of the Smallholder Cattlemen Association in the Markham Valley accompanied the survey team to the Upper Markham Valley villages and a representative from Trukai farm in Erap accompanied the team to the villages in the Lower Markham Valley. In each village, the villagers were informed about the purpose of the study and blood was collected from their animals once their consent was obtained. Only one villager, a nurse in Zifasing, had heard of leptospirosis.

In addition to cattle, blood samples were also collected from pigs and dogs in all the villages except Maranafun (only cattle) and Guaruk (horses were also sampled). Only cattle were bled in Wanpup and Arisisi villages and Clean Water farm. Horses were bled at Trukai farm. Blood samples were collected from pigs from Rumion piggery when they were slaughtered at the Lae abattoir. A summary of the number of blood samples collected from the animals is shown in Table 4.1.

4.2.2 Maranafun village profile

There are 8 houses and about 30 people in village of Maranafun. Most people are subsistence farmers although one villager had approximately 50 cattle. In the evening the cattle are kept in a yard next to the village. Several families own 3-5 pigs each which are kept in backyard pens. Dogs are owned by individual families and are an integral part of the village environment. During the day, cattle, pigs and dogs roam freely around the village and nearby bush (Figures 4.2–4.5). The description of this village is typical to all the other villages.
Table 4.1  Summary of animal species and number of blood samples collected from the villages in the Markham Valley

<table>
<thead>
<tr>
<th>Location</th>
<th>Area</th>
<th>Village</th>
<th>Animal</th>
<th>Number sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper Markham</td>
<td>Leron</td>
<td>Rumion farm</td>
<td>pig</td>
<td>50</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Mutzing</td>
<td>Mampim</td>
<td>pig</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dog</td>
<td>1</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Mutzing</td>
<td>Maranafun</td>
<td>pig</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dog</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cattle</td>
<td>28</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Mutzing</td>
<td>Mutzing</td>
<td>pig</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dog</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Umin</td>
<td>Ragizaria</td>
<td>pig</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dog</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Kiapit</td>
<td>Mangiang</td>
<td>pig</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dog</td>
<td>3</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Kiapit</td>
<td>Guntu</td>
<td>dog</td>
<td>3</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Leron</td>
<td>Guaruk</td>
<td>dog</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cattle</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>horse</td>
<td>4</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Erap</td>
<td>Wanpup</td>
<td>cattle</td>
<td>23</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Erap</td>
<td>Arisisi</td>
<td>cattle</td>
<td>13</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Zifasing</td>
<td>Gavtitif</td>
<td>pig</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dog</td>
<td>5</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Zifasing</td>
<td>Zifasing</td>
<td>pig</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dog</td>
<td>3</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Zifasing</td>
<td>Clean Water farm</td>
<td>cattle</td>
<td>32</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Erap</td>
<td>Trukai farm</td>
<td>horse</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 4.2 A mother and daughter walking barefoot to their house. Pigs are in the background.

Figure 4.3 Pigs and dogs roam the village scavenging for food.
4.2.3 Sample collection

Blood was collected from the jugular vein of pigs and horses, the cephalic vein of dogs and the coccygeal artery or vein of cattle. About 8 to 10 mls of blood were collected from animals and samples left at 4°C overnight to clot. Blood was centrifuged at 3,000 x g for 15 min and serum was removed and stored at -20°C until required.
4.2.4 Microscopic agglutination test (MAT)

All serum specimens were tested at the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis in Brisbane. A panel of 22 live antigens were used in the MAT. This differed from the standard panel used by the addition of *Leptospira borgpetersenii* serovar Ballum, substituted for *L. borgpetersenii* serovar Arborea, and the new serovar *L. interrogans* serovar Topaz. The panel of 22 leptospiral serovars from 19 serogroups represented those identified previously in the Western Pacific Region. In this study an agglutination titre $\geq 50$ was indicative of seropositive for *Leptospira*.

4.3 Results

The results of testing sera with the MAT are shown in Table 4.2. The dominant serovar was Hardjo (18.9%) followed by serovars Tarassovi (5.4%), Arborea (3.6%) and Topaz (0.9%). Cattle from Wanpup, Clean Water and Guaruk were seropositive to serovar Hardjo (Table 4.3). Cattle from Arisisi and Maranafun were seronegative.

Of the 22 dogs tested, only one from Maranafun village was seropositive (Table 4.4). This dog had titres to *L. interrogans* serovar Canicola and *L. interrogans* serovar Djasiman.

Leptospiral antibodies were not detected in any of the village pigs. Only 1 pig from Rumion piggery had titres to two serovars, Canicola and Djasiman (Table 4.5). Horses from Trukai farm and Guaruk village were positive for serovars Arborea (6/15), Topaz (3/15), Hardjo (2/15), Canicola (1/15) and Grippotyphosa (1/15) (Table 4.6). A summary of the individual serovars identified during the survey is presented in Table 4.7.
Table 4.2  Proportion of cattle from smallholder farmers in the Markham Valley in PNG with antibodies to leptospiral serovars determined using the MAT

<table>
<thead>
<tr>
<th>Species</th>
<th>Serovar</th>
<th>Number tested</th>
<th>Number positive</th>
<th>% positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptospira</em></td>
<td>Hardjo</td>
<td>111</td>
<td>21</td>
<td>18.9</td>
<td>11.6, 26.2</td>
</tr>
<tr>
<td><em>L. borgpetersenii</em></td>
<td>Tarassovi</td>
<td>111</td>
<td>6</td>
<td>5.4</td>
<td>1.2, 9.6</td>
</tr>
<tr>
<td><em>L. borgpetersenii</em></td>
<td>Arborea</td>
<td>111</td>
<td>4</td>
<td>3.6</td>
<td>0.1, 7.1</td>
</tr>
<tr>
<td><em>L. interrogans</em></td>
<td>Topaz</td>
<td>111</td>
<td>1</td>
<td>0.9</td>
<td>0, 2.7</td>
</tr>
</tbody>
</table>

Table 4.3  Seroprevalence of serovar Hardjo in 111 cattle from smallholder village farms in the Markham Valley in PNG

<table>
<thead>
<tr>
<th>Location</th>
<th>Village</th>
<th>Number tested</th>
<th>Number positive</th>
<th>% positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower Markham</td>
<td>Wanpup</td>
<td>23</td>
<td>9</td>
<td>39.1</td>
<td>19.2, 59.1</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Arisisi</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Clean</td>
<td>32</td>
<td>7</td>
<td>21.9</td>
<td>7.6, 36.2</td>
</tr>
<tr>
<td></td>
<td>Clean</td>
<td>Water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Guaruk</td>
<td>15</td>
<td>5</td>
<td>33.3</td>
<td>9.5, 57.2</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Maranafun</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0, 0</td>
</tr>
</tbody>
</table>
Table 4.4 Leptospiral serovars detected in 22 dogs from villages in the Markham Valley in PNG

<table>
<thead>
<tr>
<th>Location</th>
<th>Village</th>
<th>Number tested</th>
<th>Number positive</th>
<th>Serovars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Canicola</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Manpim</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Maranafun</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Mangiang</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Guntu</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Guaruk</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Gavitif</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Zifasing</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.5 Leptospiral serovars detected in 69 pigs from villages and Rumion piggery in the Markham Valley

<table>
<thead>
<tr>
<th>Location</th>
<th>Village</th>
<th>Number tested</th>
<th>Number positive</th>
<th>Serovars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Canicola</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Manpim</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Maranafun</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Mangiang</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Mutzing</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Gavitif</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lower Markham</td>
<td>Zifasing</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper Markham</td>
<td>Rumion farm</td>
<td>50</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4.6 Leptospiral serovars detected in 15 horses from Trukai and Guaruk smallholder farms in the Markham Valley

<table>
<thead>
<tr>
<th>Farm</th>
<th>Number tested</th>
<th>Number of serovars positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hardjo</td>
</tr>
<tr>
<td>Trukai</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Guaruk</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.7 Summary of leptospiral serovars detected in all animals sampled

<table>
<thead>
<tr>
<th>Species</th>
<th>Number tested</th>
<th>Leptospiral serovars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hardjo</td>
</tr>
<tr>
<td>Pigs</td>
<td>69</td>
<td>0</td>
</tr>
<tr>
<td>Dogs</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Horses</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Cattle</td>
<td>111</td>
<td>21</td>
</tr>
</tbody>
</table>

4.4 Discussion

This is the first serological evidence of infection with leptospires in smallholder cattle, horses, dogs and pigs in PNG. The results showed that there was a relatively low seroprevalence to serovar Hardjo infection in smallholder cattle.

4.4.1 Serovar Hardjo

The results showing that cattle from three farms were positive for infection with Hardjo should be interpreted with caution, because the majority of cattle tested had a titre of 50. This low titre could be due to either an infection early in life or to non-specific agglutination (Smith et al., 1994; Faine et al., 1999). However, the observation that there
was evidence of seropositive for Hardjo from cattle from Wanpup village and not from Arisisi village is interesting because the village farms are adjacent to each other and to Trukai farm which showed high prevalence of infection with serovar Hardjo in the first survey (see Chapter 3).

The low seroprevalence of Hardjo in the smallholder village farms showed, there is no external transmission of Hardjo from surrounding commercial farms. Due to the geography of the area cattle, from Wanpup and Arisisi villages could potentially be infected with Hardjo originating from Trukai farm during the wet season via contaminated surface water. However, this did not appear to happen as evidenced by the low seroprevalence in village cattle. There were no animals with antibody titres of $\geq 400$ suggesting that there is no active infection within the farms (NerVig et al., 1980). The results show that the level of infection with Hardjo is low and leptospirosis is probably not a problem in smallholder farms in the Markham Valley.

The types of Hardjo present in the cattle can determine the modes of transmission. Two serologically indistinguishable but genetically distinct types of serovar Hardjo are, *Leptospira interrogans* serovar Hardjo (type hardjoprajitno) and *L. borgpetersenii* serovar Hardjo (type hardjobovis) (LeFebvre et al., 1987). Both types are transmitted differently with *L. interrogans* usually acquired from contaminated surface water, whereas epidemiological data supports a host-to-host transmission cycle for *L. borgpetersenii* through contaminated urine (Bulach et al., 2006). In the first survey in March 2004 in the Markham Valley, *L. borgpetersenii* serovar Hardjo was isolated and typed (see Chapter 3). This could explain why there is no infection with Hardjo in the 2 smallholder farms. A recent study showed *L. borgpetersenii* cannot be transmitted
through contaminated surface water or moist soil because it is undergoing genomic reduction and lacks the capacity to acquire nutrients and cannot survive in the environment (Bulach et al., 2006). For transmission to occur the village cattle would need to come into direct contact with infected cattle.

### 4.4.2 Serovar Tarassovi

There is serological evidence of infection of cattle in villages with serovars Tarassovi and Arborea. Cattle in PNG from village farms in Markham Valley appear to be incidental hosts for serovar Tarassovi. Pigs are commonly believed to be the reservoir for Tarassovi (Davos, 1977) but this does not appear to be the case in PNG, because the pigs tested in this study and the previous study showed no serological evidence of infection with Tarassovi. An earlier study in PNG reported that infection with Tarassovi was associated with rodents (Morahan, 1971). The clinical relevance of this serovar in PNG is not clear.

### 4.4.3 Serovar Arborea

Serovar Arborea of the genomo-species *L. borgpetersenii* was detected for the first time in PNG from cattle and horses. There was no serological evidence of infection with serovar Arborea in pigs and dogs. Arborea is one five members of the Ballum serogroup (Kmety and Dikken, 1993). The main carriers of this serovar throughout the world are rodents, mainly *Mus domesticus* and *Rattus rattus* (Everard et al., 1995; Matthias and Levett, 2002).

### 4.4.4 Serovar Canicola

The results from this study showed there was limited evidence for infection with serovar Canicola in dogs, pigs and horses in the villages. Dogs are reported to be the reservoir
host for serovars Canicola and Icterohaemorrhagiae (Faine et al., 1999). Pigs and horses can be infected with this serovar by coming into contact with contaminated urine from dogs. The low prevalence may be a consequence of the acute nature of canine leptospirosis which often results in high mortality (Faine et al., 1999). However none of the villages reported any significant mortality in dogs and the low prevalence of serovar Canicola suggests that it is not clinically significant in these villages.

### 4.4.5 Serovar Topaz

Serovar Topaz is a newly recognised serovar, isolated in 1994 from urine from a dairy cow on the Atherton Tablelands in Queensland, Australia. Serovar Topaz belongs to the genomic-species \textit{Leptospira weilii} (Monash, http://www.med.monash.edu.au/microbiology/staff/adler/tsc-minutes-november-2005/ accessed 2006). Serological evidence of this serovar was demonstrated in horses and cattle in PNG.

### 4.5 Conclusion

This survey of villages in the Markham Valley showed that seroprevalence in domestic animals like dogs, pigs, horse and cattle is low. The absence of titres in pigs in these villages does not mean that there is no infection with \textit{Leptospira}. The animals could be infected with other leptospiral serovars that are not included in the panel of serovars used in the MAT. One such example is serovar Icterohaemorrhagiae, which is believed to be associated with dogs (Faine et al., 1999) but which was not included in the panel.

The results of this study highlights that cattle from the smallholder farms in the valley are unlikely to be reservoirs of infection with Hardjo for cattle on commercial farms as was previously assumed because of the practice of buying cattle from smallholders. It is
probable therefore that most of the active infections with Hardjo are endemic on the commercial farms and that the greatest risk is for infection of introduced cattle. Preventive measures such as vaccination should be undertaken by commercial farms when new cattle are introduced.

In this study village pigs were not commonly infected with *Leptospira* and appear not to play a significant role in the transmission of leptospirosis in the Markham Valley. Whether this finding applies to other village communities in Morobe province and other parts of PNG is not known. The role of village dogs in the transmission of leptospirosis to other animals and humans is also not clear. Of the nine villages sampled, only one village had a dog with evidence of infection with serovar Canicola. This dog could play a role in transmitting leptospirosis to humans and village animals due to their close contact with each other. A serological survey performed in several Fijian villages reported that dogs are commonly infected with serovar Canicola and that this serovar is an important cause of human leptospirosis (S. Angus, pers. comm). To date, there have been no reported cases of leptospirosis in villagers however the risk of infection from dogs should be a concern to the public health authorities.

In addition, there is a lack of knowledge of the part played by the type of reservoir hosts such as rodents and bandicoots, of the leptospiral serovars that they maintain in the different locations in the Markham Valley.

In conclusion, this study has shown smallholder cattle have minimal infection with serovar Hardjo and are unlikely to be the source of infection for commercial farms in the Markham Valley. Furthermore, pigs appear to be an insignificant host for leptospiral
serovars; however dogs may have played a role as reservoirs of *Leptospira* for humans in leptospirosis in the villages in Markham Valley.
CHAPTER 5
DEVELOPMENT AND EVALUATION OF AN ANTIBODY-DETECTION ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF INFECTION WITH LEPTOSPIRA SPECIES IN CATTLE AND PIGS

5.1 Introduction

Leptospires belonging to serovars Hardjo and Pomona are the major cause of bovine leptospirosis worldwide. Infection with these serovars is responsible for considerable financial losses to farmers as a consequence of agalactia, abortion, stillbirth, birth of weak calves and reduced fertility (Ellis, 1994; Dhaliwal et al., 1996a). In addition, infection with both Hardjo and Pomona are zoonotic hazards for cattlemen (Mackintosh et al., 1980b).

The laboratory diagnosis of leptospirosis is predominantly achieved by either culture of the organism or by the detection of specific host antibodies using serological tests. However, the direct detection of leptospires by microscopic examination or culture is impractical because the techniques lack sensitivity and are labour intensive (Surujballi and Mallory, 2001).

The microscopic agglutination test (MAT) is the most commonly used serological test and is considered to be the standard reference test for leptospirosis (WHO and International Leptospiral Society, 2003). The MAT is an effective test with high specificity for individual serovars however it is relatively insensitive when used to identify cattle infected with serovar Hardjo type hardjobovis because some infected
animals have low antibody titres (Thiermann and Garrett, 1983). The MAT is difficult to perform in many laboratories because it requires the use of live cultures of pathogenic *Leptospira*, which are difficult to maintain and pose a risk to laboratory staff. In addition, the MAT is labour intensive because of the need to test each serum sample against multiple serovars and it is difficult to standardise because there are no standard operating procedures or reference strains and the test is subjectively assessed (Cousins et al., 1985; Cho et al., 1989). Thus, there is a limited capacity for diagnostic laboratories in most developing countries to use the MAT.

Attempts have been made to develop an antibody–detection enzyme-linked immunosorbent assay (ELISA) as an alternative method to investigate leptospiral infection. A variety of different antigen preparations have been assessed, including a carbohydrate antigen produced by phenol extraction of whole cell preparations (Thiermann and Garrett, 1983), outer sheath protein (Cho et al., 1989), whole lysed bacteria (Bercovich et al., 1990), formalin-fixed whole culture extracts (Ribotta et al., 2000) and proteinase K-resistance antigens (Ribeiro et al., 1995). Assays using protein and carbohydrate antigens were shown to have similar sensitivities and specificities for the detection of antibodies (Dhaliwal et al., 1996b). However, irrespective of the antigen used, the specificity of the ELISA is limited to the detection of genus level antibodies (Thiermann and Garrett, 1983; Bercovich et al., 1990).

None of the existing ELISAs have been rigorously validated. This is partly because there is no ‘gold standard’ test that can be used to infer unbiased estimates of the accuracy of the tests. The use of the MAT as a reference test may result in biased estimates of the sensitivity and specificity of the ELISA because the MAT does not in itself have perfect
sensitivity and specificity. Bayesian methods have been described that overcome the
problems associated with the use of a ‘gold standard’ for validation of diagnostic tests
(Branscum et al., 2005).

5.1.1 Aim of the study

The aim of this study was to develop and validate a *Leptospira* genus-specific antibody–
enzyme linked immunosorbent assay (ELISA) that had adequate sensitivity and
specificity for the detection of leptospiral infections in livestock and domestic animals in
PNG. This study was performed in two parts; the first was to develop a suitable antigen
of *Leptospira* for use in an Ab-ELISA and the second was to test cattle sera from PNG
with the Ab-ELISA and to evaluate the diagnostic sensitivity and specificity for the test
in the absence of a reliable ‘gold standard’ using a Bayesian approach.

5.2 Materials and Methods

5.2.1 Stains of *Leptospira* and culture media

The following reference strains of *Leptospira* and culture media were kindly provided by
the Animal Health Laboratory of the Department of Agriculture and Food, Western
Australia (DAFWA).

1. Three times subculture of *Leptospira interrogans* serovar Pomona in 5.0 mL
   liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) media and 3X 330 ml
   liquid EMJH media containing 5-fluorouracil for growing the bacteria.

2. Three times subculture of *Leptospira biflexa* serovar Patoc in 5.0 mL liquid
   EMJH media and 1L EMJH media containing 5-fluorouracil for growing the
   bacteria.
The sterility of the liquid EMJH medium was assessed by incubating the medium at 37°C for 48 hours. If the medium remained clear (not cloudy) it was considered to be sterile and was stored in at 4°C until required.

5.2.2 Subculturing of *Leptospira interrogans* serovar Pomona and *Leptospira biflexa* serovar Patoc

The 3X subcultures of *L. interrogans* serovar Pomona and *L. bifexa* serovar Patoc were incubated at 28°C until a maximum cell density of 1x10^8 leptospires / ml was achieved approximately 8-10 days. In a PC2 cabinet, using aseptic techniques 5 ml of the three cultures of serovar Pomona were transferred into each of three flasks containing 330 ml EMJH media and 15 ml serovar Patoc suspension was added to the 1L of EMJH media. The flasks were gently swirled and incubated at 28°C. Cultures were gently swirled and checked daily for contamination as evidenced by a cloudy solution. The cultures were harvested when the maximum cell density of 1x10^8 leptospires per ml was achieved.

5.2.3 Bacterial counts

One ml of cell suspension was removed from each flask and placed into a sterile bottle and 2 to 3 drops of 10% formalin was added to each culture sample to inactivate the bacteria. Each culture was diluted 1:10 with PBS and 4 to 5 µl was pipetted onto a slide counter so that the liquid completely filled the circle. A cover slip was gently applied and with care being taken to avoid air bubbles.

Each preparation was examined under a light microscope with a 0.8 dark field condenser, at x150 magnification. The bacteria in the squares were counted and the average number of bacteria per square was calculated. The average bacterial count was
then multiplied by a dilution factor of $2.0 \times 10^7$ to obtain the number of bacterial cells per ml of original suspension.

### 5.2.4 Microscopic agglutination test

All serum specimens were tested at the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis in Brisbane. The MAT panel used by the laboratory consisted of 21 live *Leptospira* serovars from 19 serogroups representative of those found in Australia and the Western Pacific Region (Table 5.1).

A panel of 21 live leptospiral serovars was used in this test and antibodies were measured by incubating cultures of specific serovars with serial dilutions of each test serum. Doubling dilutions of 1:50 to 1:6,400 were prepared in 96-well trays using phosphate buffer saline, pH 7.4. An equal volume of *Leptospira* culture was added to each dilution. After 90 min at 30°C, the trays were examined by dark field microscopy for agglutination of the cells of *Leptospira*. The end point is the highest dilution of serum at which 50% agglutination occurs (WHO and International Leptospiral Society, 2003).
Table 5.1  Serovars of *Leptospira* used in the MAT panel at the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
<th>Genomo-species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pomona</td>
<td>Pomona</td>
<td>Pomona</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Sejroe</td>
<td>Hardjo</td>
<td>Hardjoprajitno</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>Copenhageni</td>
<td>M20</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Australis</td>
<td>Australis</td>
<td>Ballico</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Zanoni</td>
<td>Zanoni</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Robinsoni</td>
<td>Robinsoni</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Canicola</td>
<td>Canicola</td>
<td>Hond Utrect IV</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>Kremastos</td>
<td>Kremastos</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Mini</td>
<td>Szwajizak</td>
<td>Szwajizak</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Sejroe</td>
<td>Medanensis</td>
<td>Hond HC</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Bataviae</td>
<td>Bataviae</td>
<td>Swart</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Djasiman</td>
<td>Djasiman</td>
<td>Djasiman</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Tarassovi</td>
<td>Tarassovi</td>
<td>Perepelitdin</td>
<td><em>L. borgpetersenii</em></td>
</tr>
<tr>
<td>Ballum</td>
<td>Ballum</td>
<td>Mus 127</td>
<td><em>L. borgpetersenii</em></td>
</tr>
<tr>
<td>Javanica</td>
<td>Javanica</td>
<td>Poi</td>
<td><em>L. borgpetersenii</em></td>
</tr>
<tr>
<td>Autumnalis</td>
<td>Bulgarica</td>
<td>Nikolaevo</td>
<td><em>L. kirschneri</em></td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>Grippotyphosa</td>
<td>Moskva</td>
<td><em>L. kirschneri</em></td>
</tr>
<tr>
<td>Cynopteri</td>
<td>Cynopteri</td>
<td>3522 C</td>
<td><em>L. kirschneri</em></td>
</tr>
<tr>
<td>Celledoni</td>
<td>Celledoni</td>
<td>Celledoni</td>
<td><em>L. weilii</em></td>
</tr>
<tr>
<td>Panama</td>
<td>Panama</td>
<td>CZ214 K</td>
<td><em>L. noguchii</em></td>
</tr>
<tr>
<td>Shermani</td>
<td>Shermani</td>
<td>1342 K</td>
<td><em>L. santarosai</em></td>
</tr>
</tbody>
</table>
5.2.5 Antigen Preparation

5.2.5.1 Washing of *Leptospira* cells

Each 500 ml culture was divided and placed into two 500 mL capped polystyrene centrifuge tubes. One ml of 10% formalin was added to each centrifuge tube and the tubes were centrifuged at 13,400 x g (Beckman) at 4°C for 45 min. The supernatant was discarded and the pellet was resuspended in 20 ml sterile Dubelcco’s PBS (pH 7.4) and carefully transferred to a 40 mL polystyrene centrifuge tube. These tubes were then centrifuged at 15,000 x g (Beckman) at 4°C for 20 min. The supernatant was discarded and the pellet resuspended in 20 ml sterile Dubelcco’s PBS (pH 7.4). The pellet was then washed a further two times as described. After washing, the final supernatant was carefully removed and the pellets were stored at -80°C for antigen extraction.

5.2.5.2 Ultrasonication

A pellet of *L. interrogans* serovar Pomona was resuspended in 1.0 ml of the extraction solution [1% Triton X-114 (Sigma, USA), 150 mM NaCl, 20 mM Tris (pH 8.0) (Sigma, USA) and 2 mM EDTA]. Nine volumes of Dubelcco’s PBS (pH 7.4) were added and the suspension was sonicated (BioLogics, Inc.) twice at a setting of 50 kHz for 10 sec on ice. The solution was then shaken gently in a rotary shaker for 10 min, transferred to a number of 2.0 mL eppendorf tubes and these were centrifuged at 13,000 x g at 4°C for 20 min. The supernatants were collected, pooled and then stored in 10 µl aliquots at -80°C (labelled L1).

A pellet of *L. biflexa* serovar Patoc was resuspended in nine volumes of Dulbecco’s PBS. The pellet was sonicated (BioLogics, Inc) twice at a setting of 50 kHz for 10 sec on ice and then transferred to a number of eppendorf tubes which were centrifuged at
20,800 x g at 4°C for 20 min. The supernatants were removed, pooled in a sterile test tube and stored at 4°C. Five volumes of 0.1% v/v Triton X-100 were added to each pellet and homogenised. All samples were pooled in a 5.0 mL sterile test tube and freeze/thawed three times to disrupt the bacteria. The bacterial suspension was transferred to 2.0 mL eppendorf tubes and centrifuged at 14,000 x g for 20 min at 4°C. The supernatants were collected, pooled with the sonicated antigen and stored in 10 µl aliquots at -80°C (labelled L2).

5.2.5.3 French press

A pellet of *L. biflexa* serovar Patoc was thawed and resuspended in 5 volumes of Dulbecco’s PBS. The cell suspension was disrupted twice using a French press at 1,400 psi. The resulting lysate was dispensed into a number of 2.0 mL eppendorf tubes and centrifuged at 20,800 x g for 20 min at 4°C. The supernatants were pooled and stored in 10 µl aliquots at -80°C (labelled L3).

5.2.6 Test sera

A positive control serum was obtained from five pigs experimentally challenged with *Leptospira interrogans* serovar Pomona at Murdoch University. A sample of pig serum donated by the Animal Health Laboratory of the Department of Agriculture and Food, Western Australia with no MAT titre was used as a negative control. A collection of sera from pigs from a farm in Western Australia was tested with the MAT and a reference collection of 21 positive and 96 negative pig sera were chosen to further examine with the ELISA. A sample was considered to be positive if it had a MAT titre of $\geq 50$.

5.2.6.1 ELISA test procedure

The optimal dilution of each antigen and horseradish peroxidase conjugated Protein G (HRP-PG) (Jackson Immunoresearch Laboratories, USA) was determined by
checkerboard titration using the positive and negative control sera. The optimal dilutions for the three whole cell antigens were 1:4,000 for L1 (Appendix 3, Figure 9.1.1) and 1:250 for L2 and L3. The optimal concentration of HRP-PG for the ELISA using L1 was 1:10,000 (Appendix 3, Figure 9.1.1) and using L2 and L3 was 1:2,000.

The three antigen preparations were diluted in 0.05 M carbonate/bicarbonate buffer (pH 9.6) and 100 µl was added to each well of “U”-bottom microtitre plates (Microtest, Sarstedt) and incubated overnight at 4°C in a humid chamber.

Plates were allowed to warm to room temperature. The antigen solution was discarded and plates were washed three times with TEN-T (Tris 0.05 M, EDTA 0.006 M, sodium chloride 0.003M with 0.05% Tween 20) before 100 µl of test sera diluted 1:100 with TEN-TC (TENT-T with 0.2% casein) (TropBio, Townsville) was added to duplicate wells. Plates were sealed in a humid chamber and incubated for 60 min at 37°C. The plates were then washed three times as above and 100 µl of HRP-PG (Jackson Immunoresearch Laboratories, USA) diluted in TEN-TC was added to each well and incubated in a humid chamber for 60 min at 37°C. Plates were then washed three times and 100 µl of 2,2’-azino-di-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) peroxidase substrate (Kirkegaard and Perry Laboratories, USA) was added to each well and plates were incubated in a humid chamber at room temperature for 90 min.

The optical density (OD) in each well was measured using a UV microplate reader (Bio Rad, USA) at dual wavelengths of 412 and 490 nm. A sample was considered positive if its mean OD was greater than the mean (OD) plus two standard deviations (2SD) of the sera from seronegative animals (i.e. sera with a MAT titre < 50) (OIE, 2001).
Each plate contained duplicate wells with positive and negative control sera and a reagent blank.

5.2.6.2 Evaluation of the antigen preparations

The three antigen preparations were evaluated to determine which antigen preparation was most suitable for use in an Ab–ELISA. Two repeats of the Ab–ELISA using the antigens were tested using the 21 positive and 96 negative pig sera. The parameters calculated were the agreement test (Kappa), the sensitivity and specificity and the area under the receiver-operating characteristic (ROC) curve.

5.2.6.3 Antigen stability

The stability of the L1 antigens stored at -80°C for 10 months was tested twice by running a checkerboard titration using the positive and negative control sera. Two-fold dilutions of the L1 antigens from one in 500 to 4,000 dilution and horseradish peroxidase conjugated Protein G from 500 to 32,000 were used.

5.2.6.4 Bovine and porcine serum used to validate the Ab-ELISA

Sera from three populations of cattle and pigs were used to validate the Ab-ELISA. They were:

1. A total of 200 serum samples were collected from beef cattle on Kimbe island east of mainland Papua New Guinea that had reported episodes of infertility characterised by abortion, stillbirth and weak calves that died shortly after birth. The presence of infection with *Leptospira* Hardjo was confirmed using the MAT, with antibody titres ranging from 50 to 6,400. A further 200 samples were obtained from cattle from two farms on the mainland of PNG that had not experienced signs of leptospirosis and did not have any MAT titres greater than 50.
2. A total of 159 serum samples were collected from cattle from a farm in Pinjarra, Western Australia that had experienced an outbreak of leptospirosis based on the presence of typical clinical signs and a high prevalence of MAT titres ranging from 50 to 3,200.

3. Sera were collected from 96 pigs seropositive with *Leptospira* from a farm in Western Australia with MAT titres of > 50 and 196 seronegative pigs from PNG that had no MAT titre.

5.2.6.5 Cattle sera from eight populations used to evaluate the Ab-ELISA

The Ab-ELISA using the L1 antigen was used to test 1,465 sera from eight cattle populations from PNG and WA and the results were compared with the MAT to evaluate the performance of the two tests. Two of these cattle farms reported clinical signs of leptospirosis while five farms did not. The eighth group was from cattle slaughtered at the Lae abattoir.

Each population was stratified into 3 age groups (<2, 2-5 and >5 years), which were systematically sampled to provide approximately equal numbers of samples from each age stratum. Table 5.2 summarises the number of blood samples from the female cattle in the three age groups collected from each farm.
<table>
<thead>
<tr>
<th>Cattle population</th>
<th>Farm</th>
<th>Number sampled in age group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;2 years</td>
<td>2-5 years</td>
</tr>
<tr>
<td>1</td>
<td>Numundo</td>
<td>47</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>Pinjarra, WA</td>
<td>0</td>
<td>159</td>
</tr>
<tr>
<td>3</td>
<td>Trukai</td>
<td>126</td>
<td>122</td>
</tr>
<tr>
<td>4</td>
<td>Ramu</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>Rumion</td>
<td>47</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td>Prhalda</td>
<td>67</td>
<td>45</td>
</tr>
<tr>
<td>7</td>
<td>Rearaguntu</td>
<td>45</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Abattoir</td>
<td>16</td>
<td>40</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.2.7. Data analysis

5.2.7.1 Evaluation of the antigens

To select the optimal antigen for the ELISA, receiver operating characteristic (ROC) curve analysis was performed and the area under the ROC curve (AUC) was calculated for the ELISA using Win Episcope 2.0 (ftp://ftp.zod.wau.nl/pub/qve/wepi21.exe). The AUC is a summary statistic of the overall diagnostic accuracy (discriminatory power) of each test (Greiner et al., 2000). The agreement (kappa) and its 95% confidence interval (CI) between two repeat tests for each ELISA were also determined using Win Episcope 2.0. The sensitivity and specificity of the Ab-ELISA using each antigen preparation were calculated using a reference collection of 21 positive and 96 negative pig sera. The 95% CI was calculated using the binomial method (Smith, 1995).
5.2.7.2 Selection of a cut-off value for the Ab-ELISA

The sensitivity and specificity (and their 95% CI) including the positive predictive value (PPV) and negative predictive value (NPV) at different cut-off values were calculated using Win Episcope 2.0.

Four methods used to calculate the cut-off values were evaluated. They were the mean plus 2SD of the OD of serum from seronegative cattle and pigs; two values from TG-ROC (http://city.vetmed.fu-berlin.de/~mgreiner/TG-ROC/tgrocm.htm) representing the value where test sensitivity and specificity are equal (d₀); and a value equal to the upper limit of the intermediate range (IR) where specificity equals 90% (Greiner, 1995); and 20% of the high positive control (20 PP) (Davison et al., 1996).

Receiver operating characteristic curves were constructed for Ab-ELISA (L1) using cut-off values of OD = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1, 1.5, and 2. The optimum cut-off value for each curve was determined as the value that gave the highest combined sensitivity and specificity (i.e. highest value of sensitivity plus specificity divided by 2).

Frequency histograms and scatter plots of the OD for both seropositive and seronegative populations were constructed to visually determine the effects of different cut-off values using Excel 2002 (Microsoft).

5.2.7.3 Performance of the Ab-ELISA on sera from cattle on two farms diagnosed with clinically leptospirosis

The results from testing sera from cattle originating from PNG or WA were used to determine the accuracy of the Ab-ELISA. The post-test probabilities that cattle were infected with *Leptospira* given a positive (PPV) or negative (1-NPV) test result were
calculated and plotted for prevalences (pre-test probability) of 0-100% for these data (Smith, 1995). The kappa values were calculated (Gardner et al., 2000).

5.2.7.4 Comparison of the Ab-ELISA and the MAT

Any statistical association between the results of testing sera from 8 populations (7 from PNG and 1 from WA) with the Ab-ELISA and MAT were determined by calculating the Spearman Rank Correlation using SPSS Ver. 14.0 (SPSS Inc). Frequency histograms of the correlation between the MAT titre and ELISA OD values for the 8 populations were plotted using Excel 2002 (Microsoft).

A Bayesian analysis framework was used to make inferences about the prevalence, sensitivity and specificity of each test and the level of conditional dependence between the ELISA and MAT, using the results from testing serum from cattle from PNG. Two models were constructed and run in WinBUGS 2.2 (Thomas et al., 2006). Each model was run with a burn-in phase of 5,000 iterations and another 20,000 iterations were run to obtain estimates.

The first model used code prepared by Branscum (http://www.epi.ucdavis.edu/diagnosticstests/AB2deptests2popns.html, accessed 2006) to determine the sensitivity and specificity of two diagnostic tests using data from two cattle populations in the absence of a gold standard (Appendix 1). Informative priors were used for the sensitivity and specificity of each test. The prior estimates of the sensitivity and specificity of the ELISA and MAT were 0.9 (s.d. = 0.1). This model was used initially on the assumption that there would be a moderate degree of conditional dependence between the MAT and ELISA because they were designed to directly detect the presence of leptospiral antibodies. A pairwise comparison of the ELISA and MAT
was performed and estimates of the sensitivity and specificity covariances were inferred to evaluate the level of dependence. The tests were shown to be conditionally independent because the covariance estimates were low, with 95% confidence intervals that included 0. Based on this result, further analysis was completed on the assumption that the tests were conditionally independent.

A second model was adapted from a model provided by Nils Toft (per comm), which was constructed using the Hui and Walter model for four independent tests using data from two different populations (Hui and Walter, 1980) (Appendix 2). Uninformative priors were used for prevalence (Beta 1,1).

5.3 Results

5.3.1 Antigen stability

There was no significant change in the OD values of the positive control serum at the optimum antigen and HRP-protein G dilutions of 1:4,000 and 1:10,000 respectively after 10 months of storage at -80°C (Appendix 3, Figure 9.1.2).

5.3.2 Evaluation of an Ab-ELISA using 3 crude antigen fractions for the detection of antibodies to *Leptospira* in pigs

The observed agreements between two repeats of an Ab-ELISA using three antigen preparations are shown in Table 5.3. The highest agreement was observed for the Ab-ELISA using the L1 fraction (kappa = 0.96) compared to the L2 and L3 fractions with values of 0.57 and 0.68 respectively.
The sensitivities and specificities of an Ab–ELISA using three antigen preparations are displayed in Table 5.4. The sensitivity of an Ab–ELISA using the L1 antigen was significantly higher than the sensitivity of an Ab-ELISA using the L2 antigen. There was no significant difference between the specificity of the Ab–ELISA for each of the three antigen preparations.

Table 5.3  Agreement (kappa) between 2 repeats of an Ab–ELISA using three antigen preparations to test serum from 21 MAT-positive pig sera and 96-MAT negative pig sera

<table>
<thead>
<tr>
<th>Antigen fraction</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>0.96</td>
</tr>
<tr>
<td>L2</td>
<td>0.57</td>
</tr>
<tr>
<td>L3</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Table 5.4  Sensitivity and specificity of an antibody ELISA using three different antigen preparations for testing 21 MAT-positive pig sera and 96 MAT-negative pig sera

<table>
<thead>
<tr>
<th>Antigen preparation</th>
<th>% Sensitivity*</th>
<th>% Specificity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>95.4 (90.4, 100)</td>
<td>97.9 (95.1, 100)</td>
</tr>
<tr>
<td>L2</td>
<td>61.9 (41.0, 82.7)</td>
<td>97.9 (95.1, 100)</td>
</tr>
<tr>
<td>L3</td>
<td>71.4 (56.1, 90.8)</td>
<td>97.9 (95.1, 100)</td>
</tr>
</tbody>
</table>

*95% confidence intervals are given in parentheses.

Receiver operating characteristic curves were constructed for the Ab–ELISA using the L1, L2 and L3 antigen preparations (Figure 5.1). The AUC of the Ab-ELISA using the
L1 antigen was significantly higher, then the AUC of the Ab–ELISA using the L2 and L3 antigen preparations (P < 0.05) (Table 5.5). The L1 antigen was used to further validate the Ab-ELISA for testing sera from cattle and pigs.

Table 5.5  Area under the ROC curve for the three antigen preparations

<table>
<thead>
<tr>
<th>Antigen preparation</th>
<th>AUC</th>
<th>95% CIs</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>0.97</td>
<td>0.96, 0.99</td>
</tr>
<tr>
<td>L2</td>
<td>0.90</td>
<td>0.87, 0.95</td>
</tr>
<tr>
<td>L3</td>
<td>0.93</td>
<td>0.91, 0.97</td>
</tr>
</tbody>
</table>

Figure 5.1  Receive operating characteristic curve obtained from analysis of the Ab-ELISA using L1, L2 and L3 antigens for the detection of leptospiral antibodies in 21 MAT–positive pig sera and 96 MAT–negative pig sera
5.3.3 Evaluation of an Ab-ELISA using the L1 antigen for the detection of antibodies to Leptospira species in cattle

There was very high to perfect agreement between two repeats of an L1 Ab–ELISA used to test sera from PNG cattle and WA cattle (Table 5.6).

Table 5.6 Agreement (kappa) between two repeats of the L1 Ab-ELISAs used to test sera from 200 seropositive and 200 seronegative cattle from PNG and WA cattle

<table>
<thead>
<tr>
<th>Species</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNG cattle</td>
<td>0.97</td>
</tr>
<tr>
<td>WA cattle</td>
<td>1.00</td>
</tr>
</tbody>
</table>

5.3.4 Evaluation of an Ab-ELISA using the L1 antigen for the detection of antibodies to Leptospira in cattle and pigs

The sensitivity and specificity of an L1 Ab-ELISA are shown in Table 5.7. There was no significant difference between the sensitivity and specificity of an L1 Ab–ELISA for the detection of antibodies to Leptospira in cattle and pigs.
Table 5.7  Sensitivity and specificity of the L1 Ab-ELISA for the detection of antibodies to *Leptospira* in sera from 200 seropositive and 200 seronegative cattle from PNG, 159 seropositive cattle from WA and 96 seropositive pigs from WA and 196 seronegative pigs from PNG

<table>
<thead>
<tr>
<th>Species</th>
<th>Repeats</th>
<th>% Sensitivity*</th>
<th>% Specificity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNG cattle</td>
<td>1</td>
<td>89.0 (84.7, 93.3)</td>
<td>98.5 (96.8, 100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>84.0 (78.9, 89.1)</td>
<td>98.5 (96.8, 100)</td>
</tr>
<tr>
<td>WA cattle</td>
<td>1</td>
<td>76.1 (69.5, 82.7)</td>
<td>98.5 (96.8, 100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>76.1 (72.9, 85.6)</td>
<td>99.0 (97.6, 100)</td>
</tr>
<tr>
<td>WA pigs</td>
<td>1</td>
<td>87.5 (80.9, 94.1)</td>
<td>96.9 (94.5, 99.4)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>87.5 (80.9, 94.5)</td>
<td>96.9 (94.5, 99.4)</td>
</tr>
</tbody>
</table>

*95% confidence intervals are given in parentheses

5.3.5 Selection of the cut-off values for the Ab–ELISA for cattle

The cut-off values calculated by each of the four methods were $2\sigma = 0.40$, TG-ROC $d_{(0)} = 0.32$, TG-ROC >IR = 0.22 and 20 PP = 0.55 of an L1 Ab-ELISA using sera from 200 cattle seropositive with *Leptospira* and 200 seronegative cattle from PNG. There was no significant difference between the sensitivity, specificity, PPV and NPV estimates of the two Ab–ELISAs at each of the four cut-off values (Table 5.8). The specificity values with the repeat at each of the four cut-off values were identical.
Table 5.8  Comparison of sensitivity (%), specificity (%) and positive and negative predictive values (%) of the L1 Ab–ELISA and their repeats at four methods for calculating cut-offs using sera from 200 cattle seropositive with *Leptospira* and 200 seronegative cattle

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>ELISA</th>
<th>% sensitivity*</th>
<th>% specificity*</th>
<th>PPV#</th>
<th>NPV#</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pos</td>
</tr>
<tr>
<td>2SD</td>
<td>1</td>
<td>89.0 (84.7, 93.3)</td>
<td>98.5 (96.8, 100)</td>
<td>98.3</td>
<td>90.0</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>84.0 (78.9, 89.1)</td>
<td>98.5 (96.8, 100)</td>
<td>98.3</td>
<td>86.0</td>
<td>134</td>
</tr>
<tr>
<td>TG-ROC d(_0)</td>
<td>1</td>
<td>97.0 (94.6, 99.4)</td>
<td>96.5 (94.0, 99.1)</td>
<td>96.5</td>
<td>97.0</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>97.0 (94.6, 99.4)</td>
<td>96.5 (94.0, 99.1)</td>
<td>96.5</td>
<td>97.0</td>
<td>64</td>
</tr>
<tr>
<td>TG-ROC &gt;IR</td>
<td>1</td>
<td>100 (100, 100)</td>
<td>85.0 (80.1, 90.0)</td>
<td>87.0</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100 (100, 100)</td>
<td>85.0 (80.1, 90.0)</td>
<td>87.0</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>20 PP</td>
<td>1</td>
<td>78.5 (72.8, 84.2)</td>
<td>100 (100, 100)</td>
<td>100</td>
<td>82.3</td>
<td>∞</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65.5 (58.9, 72.1)</td>
<td>100 (100, 100)</td>
<td>100</td>
<td>74.4</td>
<td>∞</td>
</tr>
</tbody>
</table>

* 95% confidence intervals are given in parenthesis

# assumed 30% prevalence

A ROC curve was constructed for cattle using an OD from 0.1 to 2. The sensitivity and false-positive rate (1-Specificity) of the Ab-ELISA do not change until the cut-off value exceeds 0.55 (Figure 5.2). A frequency bar graph (Figure 5.3) and a scatter plot (Figure 5.4) were constructed to visualise the cut-off values to discriminate seronegative and seropositive cattle. A cut-off value of 0.50 differentiated clearly between negative and positive animal.
Figure 5.2  ROC curve for an L1 Ab-ELISA using OD cut-off values of 0.03, 0.05, 0.1, 0.2, 0.35, 0.4, 0.55, 0.6, 0.7, 1.0, 1.2, 1.4, 1.6, 1.8, 1.9 and 2 for seropositive and seronegative cattle. Optimum cut-off OD values (0.35 to 0.55) are marked by the arrow.
Figure 5.3 Frequency histogram of OD from cattle seropositive with *Leptospira* and seronegative cattle tested using an L1 Ab-ELISA. The arrow marks the cut-off value calculated using the mean OD plus 2SD.

![Frequency histogram](image1)

Figure 5.4 Scatter plot of OD values from cattle seropositive with *Leptospira* and seronegative cattle tested using the L1 Ab-ELISA. The broken line shows the cut-off value of 0.40 calculated as mean OD plus 2SD.

![Scatter plot](image2)
5.3.6 Selection of the cut-off values for the Ab–ELISA for pigs

Four methods were used to calculate the cut-off values for the seropositive and seronegative pigs: $2SD = 0.30$, $d_{(a)} = 0.20$, $IR = 0.23$ and $20 \, PP = 0.50$ (Table 5.9). There was no significant difference between sensitivity, specificity and positive and negative predictive values as well as the repeats using the four cut-off values. The sensitivity, specificity, PPV and NPV values of an Ab–ELISA (L1) using the 2SD were identical.

A ROC curve was constructed for pigs using an OD from 0.1 to 1.6. The sensitivity and false-positive rate (1-Specificity) of the Ab-ELISA did not change until the cut-off value exceeded 0.50 (Figure 5.5). A frequency bar graph was constructed to visualise see the cut-off value from the seropositive and seronegative pigs (Figure 5.6). A cut-off value of 0.50 clearly differentiated between positive and negative animals.
Table 5.9  Comparison of the sensitivity (%), specificity (%) and positive and negative predictive values (%) of the L1 Ab-ELISA using four methods for calculating the cut-off points with sera from 96 seropositive and 196 seronegative pigs

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>ELISA</th>
<th>% sensitivity*</th>
<th>% specificity*</th>
<th>PPV#</th>
<th>NPV#</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pos</td>
</tr>
<tr>
<td>2SD</td>
<td>1</td>
<td>87.5 (80.9, 94.1)</td>
<td>96.9 (94.5, 99.4)</td>
<td>93.3</td>
<td>94.1</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>87.5 (80.9, 94.1)</td>
<td>96.9 (94.5, 99.4)</td>
<td>93.3</td>
<td>94.1</td>
<td>32</td>
</tr>
<tr>
<td>TG-ROC d(0)</td>
<td>1</td>
<td>88.5 (82.2, 94.9)</td>
<td>91.8 (88.9, 95.7)</td>
<td>84.2</td>
<td>94.2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>89.5 (83.5, 95.7)</td>
<td>91.8 (88.9, 95.7)</td>
<td>84.3</td>
<td>94.7</td>
<td>12</td>
</tr>
<tr>
<td>TG-ROC &gt;IR</td>
<td>1</td>
<td>88.5 (82.2, 94.9)</td>
<td>91.8 (88.0, 95.7)</td>
<td>84.2</td>
<td>94.2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>89.6 (83.5, 95.7)</td>
<td>91.8 (88.0, 95.7)</td>
<td>84.3</td>
<td>94.7</td>
<td>12</td>
</tr>
<tr>
<td>20 PP</td>
<td>1</td>
<td>78.1 (69.9, 86.4)</td>
<td>100 (100, 100)</td>
<td>100</td>
<td>90.3</td>
<td>∞</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>72.9 (64.0, 81.8)</td>
<td>100 (100, 100)</td>
<td>100</td>
<td>88.3</td>
<td>∞</td>
</tr>
</tbody>
</table>

* 95% confidence intervals are given in parenthesis
# assumed 30% prevalence
Figure 5.5 Receiver operating characteristic curve for an Ab-ELISA L1 using OD cut-off values of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6 for seropositive and seronegative pigs. Optimum cut-off OD values (0.28 to 0.50) are marked by the arrow.

Figure 5.6 Frequency plot of OD values of sera from 96 pigs positive for leptospirosis and 196 negative pigs tested using an L1 Ab-ELISA. The arrow marks the cut-off value.
5.3.7 Pre-test and post-test probabilities for an Ab-ELISA L1 using sera from cattle from a farm seropositive with *Leptospira*

The post-test probabilities for positive and negative test results for an Ab-ELISA L1 are displayed in Figure 5.7. There is a high probability of detecting animals infected with *Leptospira* using the Ab-ELISA.

Figure 5.7 The pre-test and post-test probabilities of detecting cattle infected with *Leptospira* using an Ab-ELISA with the L1 antigen

![Graph showing pre-test and post-test probabilities](image-url)
5.3.8 *Comparison of an Ab-ELISA L1 with the MAT using cattle sera from a farm in PNG and a farm in WA believed to be infected with Leptospira*

There was moderate agreement (kappa) between the results of testing cattle sera from PNG and WA using an Ab–ELISA and the MAT (Table 5.10).

<table>
<thead>
<tr>
<th>Country</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNG</td>
<td>0.61</td>
</tr>
<tr>
<td>WA</td>
<td>0.73</td>
</tr>
</tbody>
</table>

5.3.9 *Correlation between an Ab-ELISA and MAT from eight cattle populations*

There was a significant correlation (Spearman correlation coefficient = 0.68) between the Ab-ELISA and the MAT titre in all eight cattle populations (P < 0.01). In Figure 5.8 the correlation between the MAT titre and the Ab-ELISA ODs is displayed. There is also a significant correlation between the MAT and Ab-ELISA results from the 8 cattle populations when the animal ages were stratified into three groups (P < 0.01) (Table 5.11). Within the 5 farms and an abattoir in PNG there was a significant correlation between the MAT titre and Ab-ELISA ODs as shown in Table 5.12.
Figure 5.8 Correlation between MAT titres and Ab-ELISA OD of 8 cattle populations

Table 5.11 Correlation between the results of the MAT and the Ab-ELISA to test serum from cattle on 8 properties that were stratified by age

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Number of sera</th>
<th>Spearman Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2</td>
<td>418</td>
<td>0.72*</td>
</tr>
<tr>
<td>2-5</td>
<td>571</td>
<td>0.66*</td>
</tr>
<tr>
<td>&gt;5</td>
<td>475</td>
<td>0.66*</td>
</tr>
</tbody>
</table>

* Correlation is significant (P < 0.01)
Table 5.12 Correlation between the results of testing cattle from 6 farms and an abattoir in PNG using the MAT and ELISA

<table>
<thead>
<tr>
<th>Farms</th>
<th>Number of sera</th>
<th>Spearman Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numundo</td>
<td>200</td>
<td>0.32*</td>
</tr>
<tr>
<td>Trukai</td>
<td>369</td>
<td>0.77*</td>
</tr>
<tr>
<td>Ramu</td>
<td>224</td>
<td>0.89*</td>
</tr>
<tr>
<td>Rumion</td>
<td>172</td>
<td>0.82*</td>
</tr>
<tr>
<td>Prhalda</td>
<td>134</td>
<td>0.49*</td>
</tr>
<tr>
<td>Rearaguntu</td>
<td>128</td>
<td>0.18</td>
</tr>
<tr>
<td>Abattoir</td>
<td>79</td>
<td>0.39*</td>
</tr>
</tbody>
</table>

* Correlation is significant (P < 0.01)

5.3.10 Bayesian analysis of an Ab-ELISA and MAT

Data used for the pair-wise cross classification of an Ab-ELISA and MAT for the 8 cattle populations is displayed in Table 5.13.

The median estimate of the sensitivity and specificity of each test data is shown in Table 5.14. The Ab-ELISA was significantly more sensitive compared to the MAT for the detection of antibodies to *Leptospira* in cattle (P < 0.05). There was no significant difference in the specificity between the two tests.

The prevalence of antibodies to *Leptospira* in cattle from the 8 populations calculated using the Bayesian analysis is displayed in Table 5.15. The seroprevalence on Numundo farm was significantly higher than the 7 other cattle populations.
The sensitivity and specificity covariances for test dependence were -0.016 (-0.073, 0.207) and 0.149 (-0.032, 0.552) respectively. Comparison of test dependence between the Ab-ELISA and MAT indicated low sensitivity and specificity covariance’s with 95% confidence intervals that included zero, which shows that the Ab-ELISA and MAT are conditionally independent.

Table 5.13 Cross-classified results for Ab-ELISA (T1) and MAT (T2) for the detection of Leptospira from 8 cattle populations

<table>
<thead>
<tr>
<th>Population</th>
<th>T1+/T2+</th>
<th>T1+/T2-</th>
<th>T1-/T2+</th>
<th>T1-/T2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>159</td>
<td>18</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
<td>25</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>226</td>
<td>35</td>
<td>3</td>
<td>105</td>
</tr>
<tr>
<td>4</td>
<td>114</td>
<td>3</td>
<td>2</td>
<td>105</td>
</tr>
<tr>
<td>5</td>
<td>98</td>
<td>14</td>
<td>2</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>13</td>
<td>23</td>
<td>76</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>9</td>
<td>47</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td>TOTAL</td>
<td>725</td>
<td>131</td>
<td>112</td>
<td>498</td>
</tr>
</tbody>
</table>

Table 5.14 Median (95% CI) sensitivity and specificity of the Ab-ELISA and MAT for detection of antibodies to Leptospira in cattle estimated using Bayesian analysis

<table>
<thead>
<tr>
<th>Tests</th>
<th>% Sensitivity (95% CI)</th>
<th>% Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab-ELISA</td>
<td>97.3 (95.1, 99.4)</td>
<td>90.1 (86.0, 93.2)</td>
</tr>
<tr>
<td>MAT</td>
<td>90.4 (87.5, 92.9)</td>
<td>84.1 (80.4, 87.2)</td>
</tr>
</tbody>
</table>
Table 5.15 Results of Bayesian analysis of testing sera from cattle on 8 properties using the MAT and an Ab-ELISA

<table>
<thead>
<tr>
<th>Population</th>
<th>Farm</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Numundo</td>
<td>92.5</td>
<td>86.2, 97.0</td>
</tr>
<tr>
<td>2</td>
<td>Pinjarra (WA)</td>
<td>76.2</td>
<td>66.8, 84.6</td>
</tr>
<tr>
<td>3</td>
<td>Trukai</td>
<td>67.5</td>
<td>62.2, 72.5</td>
</tr>
<tr>
<td>4</td>
<td>Ramu</td>
<td>51.1</td>
<td>44.0, 57.9</td>
</tr>
<tr>
<td>5</td>
<td>Rumion</td>
<td>61.8</td>
<td>54.2, 69.8</td>
</tr>
<tr>
<td>6</td>
<td>Prhalda</td>
<td>17.3</td>
<td>10.9, 25.6</td>
</tr>
<tr>
<td>7</td>
<td>Rearaguntu</td>
<td>3.4</td>
<td>0.4, 8.5</td>
</tr>
<tr>
<td>8</td>
<td>Abattoir</td>
<td>8.2</td>
<td>2.3, 16.9</td>
</tr>
</tbody>
</table>

5.4 Discussion

The objective of this study was to determine the usefulness of an antibody ELISA test for screening livestock in PNG and other Pacific Island countries to detect animals that are positive for *Leptospira*. It is important to use a leptospiral antigen for the ELISA that is stable and that cross-reacts with all leptospiral serovars. The other parameters that the ELISA must possess are a high repeatability and a high sensitivity and specificity. Of the two leptospiral strains used, for the antigen preparation the pathogenic *Leptospira interrogans* serovar Pomona was chosen because it possessed the above parameters and had higher discriminatory power, compared to the non-pathogenic strain *L. Biflexa* serovar Patoc.

It was necessary to obtain a collection of sera from cattle and pigs positive with *Leptospira* and negative animals for use in the validation tests. Cattle from PNG are the target population so a reference population from PNG was needed. The cattle from
which sera were obtained to determine the sensitivity and specificity of the test originated from a beef farm on an island in PNG. The farm was chosen based on a history of clinical disease suggestive of active leptospirosis. The results from the MAT from this herd confirmed the presence of high antibody titres (100-6,400) to leptospiral serovars, with serovar Hardjo predominating. Cattle sera from a beef farm in Western Australia with confirmed antibody titres to *Leptospira* were used as a comparison because of the different climate, farming system and breed of cattle.

The seronegative cattle were from herds farmed on the mainland of PNG whose sera were negative for *Leptospira* when tested using the MAT (Titre < 50).

Sera from a population of seronegative pigs were obtained from abattoirs in PNG and sera from seropositive pigs were obtained from a pig farm in Western Australia to evaluate the ability of the tests to detect *Leptospira* infection in pigs.

### 5.4.1 Evaluation of an Ab-ELISA using 3 different antigens for the diagnosis of leptospirosis

#### 5.4.1.1 Receiver operating characteristic (ROC) curve for antigen selection

The ELISA data for antigens L1, L2 and L3 were subjected to ROC curve analysis. The Ab–ELISA using the L1 antigen preparation was chosen for further evaluation because it had the highest AUC compared to the L2 and L3 antigen preparations. This type of analysis has been used to evaluate the ability of a test to discriminate between infected and healthy animals and to compare the diagnostic performance of two or more tests (Greiner *et al.*, 2000). With the ROC curve analysis, the sensitivity and specificity values are estimated for every possible cut-off point that is selected to distinguish between a positive and negative result. However, depending on the intended application
of the test, a higher sensitivity or specificity than that recommended may be desired, which can be achieved by an appropriate adjustment of the cut-off value. The AUC can be used as a measure of the accuracy of the test and it summarises the ROC curve as a whole. If the test cannot distinguish between infected and normal populations the AUC will be equal to 0.5 and the ROC curve will coincide with the diagonal. On the other hand, if the test is 100% sensitive and specific, the AUC will be equal to 1 and the curve will reach the upper left corner (Greiner et al., 2000).

5.4.1.2 Crude antigens

The L1 antigen preparation derived from the pathogenic *L. interrogans* serovar Pomona was selected for use in the Ab-ELISA in this study because the test detected high levels of antibodies in the serum of animals positive for *Leptospira*. Pathogenic *Leptospira* possess both lipopolysaccharide and proteins in their cell walls. Antigenic proteins are conserved and are expressed in high concentrations during infection and become targets of the host immune response (Haake et al., 2000). Serovar Pomona possesses the conserved proteins LipL31, LipL32, LipL41/42, p62 and p76 (Guerreiro et al., 2001). The p62 and p72 proteins are identified as heat shock proteins and are conserved in all *Leptospira* including *L. biflexa* serovar Patoc (Guerreiro et al., 2001). Therefore the antigenic preparation should be suitable for detection of all pathogenic leptospires.

The use of the non-ionic detergent Triton X 114 can selectively release proteins from the outer membrane and the cytoplasm, including lipopolysaccharides, into the crude extract (Zuerner et al., 1991; Haake et al., 2000). The high sensitivity of the L1 antigen preparation was possibly due to the increased number of antigenic binding sites available, comprised of proteins and lipopolysaccharides, whereas the L2 and L3 antigen preparations from the non-pathogenic strain contained the lipopolysaccharides (Cullen et
al., 2003; Priya et al., 2003). High concentrations of proteins and lipopolysaccharides will theoretically enable an Ab-ELISA based on the L1 antigen preparation to detect all *Leptospira* infections regardless of the infecting leptospiral serovars.

### 5.4.2 Repeatability of an Ab-ELISA using the L1 antigen to test sera from cattle and pigs

The L1 Ab-ELISA was used to test the same sera on two occasions 10 months apart, using antigen stored at -80°C, and results showed that the antigen preparation is stable for at least 10 months if frozen. This is a useful attribute because the production of large batches of antigen will reduce inter-batch variation.

### 5.4.3 Selection of cut-off values

The purpose of a diagnostic test is to differentiate positive from negative animals. Tests such as ELISA giving results that fall on a continuum require a value, the cut-off value, above which an animal is considered infected (Smith, 1995). The cut-off value must provide a balance between sensitivity and specificity and its selection must consider the purpose for which the test is to be used (i.e. diagnosis of clinical cases or screening for disease in a general population), the relative cost of a false-negative or false-positive result and the homogeneity of the result variable in the population under study (Smith, 1995).

There is much debate about the most appropriate method for selecting the cut-off value for an ELISA, particularly for test design in tropical countries (Greiner et al., 1994). Construction of a frequency histogram and scatter plot of the OD values of the two populations provides a graphical representation of the ability of an Ab-ELISA to differentiate the two populations and the likely impact that altering the cut-off will have
on test sensitivity and specificity. The method is subjective however and is not widely used for this reason. A commonly used cut-off value is calculated as the mean plus 2 or 3 standard deviations of the OD values of the sera that serve as a negative reference population (OIE, 2001). If the test variable is normally distributed in the population this method will theoretically provide tests with 95.4% and 99.7% specificity respectively (i.e. 95.4% and 99.7% are the proportion of individuals in the test population who have a test value that falls 2 or 3 standard deviations either side of the mean of the test variable). However, as antibody levels in a population of animals are unlikely to be normally distributed, it cannot be assumed that these theoretical values of specificity will be achieved.

Another method for selecting a cut-off value is to accept a value equal to a percentage (e.g. 20%), which is chosen because it provides the highest combined test sensitivity and specificity for a positive control serum with a high test value (Davison et al., 1996). This is known as percentage positive (PP).

The sensitivity of an Ab-ELISA using such approaches however may not reflect an important function of a screening test, which is differentiating between sub-populations of infected individuals (Greiner et al., 1995). To have a greater confidence that the cut-off value chosen will fulfil this requirement, receiver operating characteristic (ROC) curves are used to demonstrate the ability of a test to discriminate directly between infected and uninfected individuals over the complete spectrum of cut-off values (Smith, 1995). Because it is not possible to calculate a cut-off value directly from a ROC curve, a new approach to defining test cut-off has been described, and this approach uses the conventional ROC principle modified in such a way that the sensitivity and specificity...
can be read directly from the plots. This technique is known as two-graph ROC curves (TG-ROC) and the approach can be used to determine numerical values for test cut-offs that give equal weight to test sensitivity and specificity (Greiner et al., 1995).

Of the four cut-off values calculated for results in this study for cattle using Ab-ELISA (L1), TG-ROC were the lowest (d_{(0)} = 0.32 and >IR = 0.22 respectively). This reduced specificity but numerically increased sensitivity (mean 97% and 100% respectively) compared to values for 2SD or 20 PP. The cut-off values calculated using 2SD and 20 PP were high (0.40 and 0.55 respectively). The sensitivity for 20 PP was significantly reduced compared to 2SD (mean 86.5%) (P < 0.05) but the specificity increased numerically (mean 98% and 100% respectively). The specificity values for d_{(0)}, 2SD and 20 PP were not significantly different (P > 0.05).

Test performance in this study was compared by calculation of the likelihood ratio for positive and negative test results and PPV and NPV. Comparison of the LRs for a positive or negative ELISA result over a range of cut-off OD values allows evaluation of the impact of the cut-off value on test performance. The Ab-ELISA using the 2SD or 20 PP cut-off value is more likely to correctly identify cattle infected with *Leptospirae* species than when using either the cut-off values TG-ROC cut-off values calculated using TG-ROC (higher PPV and LR for a positive test result). Furthermore, of the OD values from the seronegative population, all except four are above the 2SD cut-off, and those animals in the population which were seropositive were found positive in the ELISA have OD values above the 20 PP cut-off OD value. The positive cattle that were found to be negative in the ELISA had OD values within the distribution of the seronegative cattle. It is thus possible in this case to improve the test characteristics by
manipulation of the cut-off value. The cut-off value at which this ELISA is most accurate in detecting leptospiral antibodies is 20 PP. A value within this range of OD values would also have been chosen intuitively from the graphical representation of frequency of OD values for known positive and negative sera.

5.4.4 Comparison of the Ab-ELISA with the MAT

There was good agreement (kappa) between the Ab–ELISA and the MAT. Kappa is a widely used measure of test agreement (Maclure and Willet, 1987) and is calculated as the ratio of the difference between the observed agreement and the chance agreement to the maximal possible agreement beyond chance. The magnitude of kappa in a mixed population of infected and uninfected animals is dependent on several factors: sensitivity and specificity of each test, prevalence of infection, sensitivity and specificity covariances between the tests (Gardner et al., 2000). Because prevalence strongly affects kappa, comparison and interpretation of kappa values can be problematic (Maclure and Willet, 1987).

The correlation between the MAT antibody titre and the Ab-ELISA ODs were calculated using the Spearman rank coefficient or a non-parametric equivalent to the Pearson correlation coefficient (Petrie and Watson, 2006). This provides a measure of the association (not necessary linear) between the two tests. The results from analysis of the eight cattle populations showed that there was significant correlation between the MAT antibody titres and Ab-ELISA OD values for the positive results. This clearly indicates that animals which had been, or were currently positive for *Leptospira* with MAT at a high antibody titre of > 200 yielded high Ab-ELISA OD values. This finding agrees with another study that reported a high correlation between the two tests (Bercovich et al.,
1990) but differs from a study that showed that the levels of antibody detected by the MAT and the ELISA did not correlate well (Cousins et al., 1985).

When animals are tested using more than one diagnostic test, the test outcomes can be dependent (correlated) within the infected and/or uninfected sub-population. In this study the two test outcomes were correlated. A conditional dependence approach was used to compare the MAT and the Ab-ELISA to see if the tests were independent or dependent (Gardner et al., 2000). The magnitude of the covariance is directly affected by the magnitude of the sensitivity and specificity values. Low covariance values of sensitivity and specificity are desirable if tests are to be used in combination, but for the test to be conditionally independent the calculated 95% confidence intervals (CIs) for sensitivity covariances should include 0 (Gardner et al., 2000).

The diagnostic sensitivity and specificity of both tests and the prevalence of infection were estimated in the absence of an accurate ‘gold standard’ test for *Leptospira* using Bayesian methods. The MAT is considered to be the standard reference test for leptospirosis (WHO and International Leptospiral Society, 2003). Bayesian analyses have been widely used in the veterinary field and offer a method of incorporating prior information about test performance to assist in diagnostic evaluation (Branscum et al., 2005). The estimate of the sensitivity of the Ab-ELISA was significantly higher than the MAT. The high sensitivity and specificity of the Ab-ELISA is reflected in high positive predictive values, which shows that positive test results are highly likely to occur in animals that are truly infected with species of *Leptospira*. 

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The increased sensitivity of the Ab-ELISA could be due to the high titre of antibodies in the serum directed to the detecting antigen. Such a situation could arise if the antigen used in the test has high immunogenicity. The procedure used to prepare the antigen for the test also might have improved the binding sites, making the antigen more recognizable to antibodies directed against them. It is also possible that the cattle population used for evaluating the test had animals chronically infected with *Leptospira*, which induced a higher level of IgG compared to IgM. The Ab-ELISA can detect IgG, which may have been at levels below the detection threshold of the MAT.

The sensitivity and specificity covariances included 0, which showed that the tests were conditionally independent. This means that the sensitivity (or specificity) of the second test does not depend on whether results of the first test are positive or negative among infected (or uninfected) animals (Gardner *et al.*, 2000).

The Ab-ELISA using the L1 antigen developed and validated in this study was shown to be highly sensitive and specific. The ELISA shows a high level of agreement and correlation with the MAT, which suggests that it could be used to replace the MAT without loss of test accuracy. Based on these test parameters and its performance, the Ab-ELISA using the L1 antigen preparation described in this study is a suitable test to be used for testing infection with *Leptospira* in countries with less well developed laboratory facilities.

The MAT is not suitable for use in PNG because it is laborious, hazardous (requiring the handling of live leptospires), requires the continuous culture of leptospires and is subjective. In addition, the assay may be less sensitive for detecting chronically infected
animals (Faine et al., 1999). The Ab-ELISA is therefore probably the preferred assay since it is accurate, safe and inexpensive to perform and it measures serum antibody objectively. However, the Ab-ELISA does have a major limitation in that it can only detect infection to the genus level and is not serovar specific. It addition, it cannot differentiate vaccinated from the un-vaccinated animals. If these limitations are taken into account then the assay can be easily transferred to PNG and other Pacific Island countries.
CHAPTER 6

USE OF THE L1-ANTIBODY-ELISA TO INVESTIGATE AN OUTBREAK OF BOVINE LEPTOSPIROSIS IN WESTERN AUSTRALIA

6.1 Introduction

In Western Australia the clinically important serovars of Leptospira in cattle are Hardjo and to a lesser extent, Pomona. Serovar Tarassovi is commonly identified but its significance as a cause of disease in animals is unclear although it is responsible for about 5% of human cases reported in Australia (C. Mayberry, Dept. Agri. WA, pers. comm). The seroprevalence of serovar Hardjo infection in Western Australian cattle is approximately 50%. In addition, gross lesions suggestive of leptospirosis (white spots) are commonly observed in cattle at slaughter (C. Mayberry, Dept. Agri. WA, pers. Comm.).

6.1.1 Aim

The aim of this study was to evaluate the L1-antibody detecting ELISA to assist in the investigation of an outbreak of bovine leptospirosis.

6.1.2 Case history

In November 2004 a report was received from a beef farm in Pinjarra, Western Australia. The herd had symptoms of bovine leptospirosis in which a group of pregnant heifers failed to calve. This group was from a mob of 250 heifers aged between 2-3 years that were mated in late June/July 2003 to yearling bulls. Pregnancy testing carried out in November 2003 showed that the heifers in this mob were pregnant. In April/May 2004, 40 of the heifers failed to calve. No dead foetuses and foetal membranes were
observed by the farm workers. In July/August 2004 the 40 were-mated and pregnancy testing in September 2004 showed all but one were pregnant. Sera collected in September and October 2004 from 14 of the 40 heifers were tested with MAT at the Animal Health Laboratory, Department of Agriculture and Food, Western Australia (DAFWA). The result of the MAT confirmed that these cattle were infected with *L. interrogans* serovar Hardjo.

### 6.2 Materials and Methods

#### 6.2.1 Study area

The property is located in Pinjarra, Western Australia (Figure 6.1). This farm has approximately 800 cattle which were not vaccinated against leptospirosis.

![Location of the farm in Pinjarra, Western Australia](image)

Figure 6.1  Location of the farm in Pinjarra, Western Australia

#### 6.2.2 Sample Collection

Sera were collected in September and October 2004 from 14 of the 40 heifers that aborted in June/July 2004.
In December 2004 further blood samples were collected from 159 of 250 heifers from the mob suspected of having leptospirosis. Heifers from the group that aborted were also included. Blood (10 ml) was collected from the coccygeal artery or vein with a vacutainer and samples were stored upright in a refrigerated container with ice bricks and transported back to the laboratory. Blood samples were left at 4°C overnight to clot and were centrifuged the next day at 3,000 x g for 30 min. Serum was collected and stored at -20°C until required.

6.2.3 Antibody ELISA

The antibody-detection ELISA L1 was performed according to the protocol described in Chapter 5 page 100-101. A sample was considered positive if its mean OD was > 0.40.

6.2.4 Microscopic agglutination test

All sera were also tested using the MAT at the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis in Brisbane. The MAT panel used by the laboratory consisted of 21 live *Leptospira* serovars from 19 serogroups representative of those found in Australia and the Western Pacific Region (Table 6.1). The protocol for the MAT is described in Chapter 5 page 97.

6.2.5 Interpretation of MAT titres

This farm had not vaccinated cattle against leptospirosis so a MAT titre of ≥ 50 was used as the cut-off to classify animals having been infected. Sera with positive titres to more than one serovar were regarded as seropositive for all those serovars unless the serovars were known to cross react, in which case the sera were scored as positive to the serovar with the highest titre (Black *et al.*, 2001).
6.2.6 Statistical analysis

Data were analysed using the SPSS (Version 14.0, SPSS Inc) and Excel 2002 (Microsoft) programs. Spearman rank correlation and the agreement (kappa) between the two tests were calculated (Petrie and Watson, 2006). The association between the Ab-ELISA and MAT tests for leptospiral infection was analysed with the bivariate correlation and Spearman rank correlation using SPSS was utilised to determine whether there was any significant difference between the results of the two tests.
Table 6.1  Leptospiral serovars used in the microscopic agglutination test panel at the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
<th>Genomo-species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pomona</td>
<td>Pomona</td>
<td>Pomona</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Sejroe</td>
<td>Hardjo</td>
<td>Hardjoprajitno</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>Copenhageni</td>
<td>M20</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Australis</td>
<td>Australis</td>
<td>Ballico</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Zanoni</td>
<td>Zanoni</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Robinsoni</td>
<td>Robinsoni</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Canicola</td>
<td>Canicola</td>
<td>Hond Utrecht IV</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>Kremastos</td>
<td>Kremastos</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Mini</td>
<td>Swajizak</td>
<td>Swajizak</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Sejroe</td>
<td>Medanensis</td>
<td>Hond HC</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Bataviae</td>
<td>Bataviae</td>
<td>Swart</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Djasiman</td>
<td>Djasiman</td>
<td>Djasiman</td>
<td><em>L. interrogans</em></td>
</tr>
<tr>
<td>Tarassovi</td>
<td>Tarassovi</td>
<td>Perepelitdin</td>
<td><em>L. borgpetersenii</em></td>
</tr>
<tr>
<td>Ballum</td>
<td>Ballum</td>
<td>Mus 127</td>
<td><em>L. borgpetersenii</em></td>
</tr>
<tr>
<td>Ballum</td>
<td>Arborea</td>
<td>Arborea</td>
<td><em>L. borgpetersenii</em></td>
</tr>
<tr>
<td>Javanica</td>
<td>Javanica</td>
<td>Poi</td>
<td><em>L. borgpetersenii</em></td>
</tr>
<tr>
<td>Autumnalis</td>
<td>Bulgarica</td>
<td>Nikolaev</td>
<td><em>L. kirschneri</em></td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>Grippotyphosa</td>
<td>Moskva</td>
<td><em>L. kirschneri</em></td>
</tr>
<tr>
<td>Cynopteri</td>
<td>Cynopteri</td>
<td>3522 C</td>
<td><em>L. kirschneri</em></td>
</tr>
<tr>
<td>Celledoni</td>
<td>Celledoni</td>
<td>Celledoni</td>
<td><em>L. weilii</em></td>
</tr>
<tr>
<td>Panama</td>
<td>Panama</td>
<td>CZ214 K</td>
<td><em>L. noguchii</em></td>
</tr>
<tr>
<td>Shermani</td>
<td>Shermani</td>
<td>1342 K</td>
<td><em>L. santarosai</em></td>
</tr>
</tbody>
</table>
6.3 Results

6.3.1 Seroprevalence of leptospiral infection using the L1 Ab-ELISA and MAT

The overall seroprevalence of leptospiral infection in animals tested with the Ab-ELISA was 79.9%. There was no significant difference between the prevalences estimated using the Ab-ELISA or the MAT (Table 6.2).

Table 6.2 Seroprevalence of leptospiral infection using the Ab-ELISA and the MAT

<table>
<thead>
<tr>
<th>Test</th>
<th>Total sera</th>
<th>Number positive</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab-ELISA</td>
<td>159</td>
<td>127</td>
<td>79.9</td>
<td>73.6, 86.1</td>
</tr>
<tr>
<td>MAT</td>
<td>159</td>
<td>134</td>
<td>85.3</td>
<td>78.6, 89.9</td>
</tr>
</tbody>
</table>

6.3.2 Correlation and agreement between the results of the Ab-ELISA and MAT

Data used for the pair-wise cross classification of the Ab-ELISA and MAT to calculate the test agreement is displayed in Table 6.3.

There was a significant correlation (Spearman correlation coefficient = 0.24) between the Ab-ELISA optical densities (ODs) and the MAT titre from the animals tested (P < 0.01). Figure 6.2 displays the proportion of correlation between the MAT titre and the Ab-ELISA ODs. There was an association between the MAT titre ≥ 50 and the Ab-ELISA ODs. As the MAT titre increased the Ab-ELISA ODs also increased. The agreement between tests (kappa value) was 0.40.
Table 6.3  Cross-classified results for Ab-ELISA (T1) and MAT (T2) for infection with *Leptospira* from 159 cattle tested from WA

<table>
<thead>
<tr>
<th>Farm</th>
<th>T1/T2</th>
<th>T1+/T2-</th>
<th>T1-/T2+</th>
<th>T1-/T2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>WA</td>
<td>100</td>
<td>25</td>
<td>14</td>
<td>20</td>
</tr>
</tbody>
</table>

Figure 6.2  Association between MAT titres and Ab-ELISA ODs from the 159 cattle from a farm in WA

6.3.3  Seroprevalence of leptospiral serovars using the MAT

The prevalence of leptospiral serovars detected using the MAT is displayed in Table 6.4. The most common serovars were Hardjo (seroprevalence 71.3%), followed by Medanensis (47.8%), Szwajizak (29.9%), Tarassovi (20.4%) and Arborea (11.5%). The
apparent seroprevalence of serovar Hardjo was significantly higher than all the other serovars (P < 0.05).

Table 6.4 Proportion of cattle from the farm in WA with antibodies to leptospiral serovars determined with the MAT (n = 159)

<table>
<thead>
<tr>
<th>Leptospira species</th>
<th>Serovar</th>
<th>Positive sera</th>
<th>% positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. interrogans</td>
<td>Hardjo</td>
<td>112</td>
<td>71.3</td>
<td>64.3, 78.4</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Medanensis*</td>
<td>75</td>
<td>47.8</td>
<td>40.0, 55.6</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Szwajizak*</td>
<td>47</td>
<td>29.9</td>
<td>22.8, 37.1</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Kremastos*</td>
<td>1</td>
<td>0.6</td>
<td>0, 1.9</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Tarassovi</td>
<td>32</td>
<td>20.4</td>
<td>14.4, 26.7</td>
</tr>
<tr>
<td>L. borgpetersenii</td>
<td>Arborea</td>
<td>18</td>
<td>11.5</td>
<td>6.5, 16.4</td>
</tr>
<tr>
<td>L. borgpetersenii</td>
<td>Ballum</td>
<td>3</td>
<td>1.9</td>
<td>0, 4.1</td>
</tr>
<tr>
<td>L. santarosai</td>
<td>Shermani</td>
<td>4</td>
<td>2.5</td>
<td>0.1, 5.0</td>
</tr>
<tr>
<td>L. interrogans</td>
<td>Pomona</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cross reacting serovars. When a sera had titres to more than one of these serovars, the highest titre was regarded as the correct one in these animals. When these titres were equal to the Hardjo titre, the sample was recorded as a Hardjo positive. If titres were equal for both Szwajizak and Medanensis the sample was recorded as positive for both (Black et al., 2001).

A total of 11 of the 159 serum samples were positive only to serovar Medanensis and no cross reaction with no other serovars. The antibody titres detected were generally low, with only 1 animal with a titre of 200 and 2 animals with a titre of 100.

6.3.4 Antibody titres of leptospiral serovars determined by MAT

The frequency of antibody titres for serovars Hardjo, Tarassovii and Arborea are displayed in Figure 6.3. There were significantly more animals with antibody titres of 100 to 400 for serovar Hardjo than those with titres of 800 and 1,600 implying evidence
of active leptospirosis. The mean antibody titre for serovar Hardjo were significantly higher \( (P < 0.05) \) than titres for serovars Tarassovi and Arborea.

Figure 6.3 Frequency of antibody titres to serovars Hardjo, Tarassovi and Arborea in 159 cattle from the WA farm

The Ab-ELISA and MAT results for 13 of the 14 heifers that aborted and were re-mated in June / July 2003 is shown in Table 6.5. There was a significant decline in the antibody titres from September 2004 to December 2004.
Table 6.5  Ab-ELISA results and serovar Hardjo titres measured with MAT for 14 cattle which were pregnant in November 2003 and aborted in June 2004. Blood was collected in September, October and December 2004 after re-mating in July 2004.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Stage of pregnancy</th>
<th>MAT titre Sep 04</th>
<th>MAT titre Oct 04</th>
<th>MAT titre Dec 04</th>
<th>ELISA OD Dec 04</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5 months</td>
<td>5,000</td>
<td>5,000</td>
<td>0</td>
<td>neg</td>
</tr>
<tr>
<td>2</td>
<td>2 months</td>
<td>5,000</td>
<td>5,000</td>
<td>N/B</td>
<td>N/B</td>
</tr>
<tr>
<td>3</td>
<td>3 months</td>
<td>5,000</td>
<td>5,000</td>
<td>N/B</td>
<td>N/B</td>
</tr>
<tr>
<td>4</td>
<td>2 months</td>
<td>5,000</td>
<td>5,000</td>
<td>N/B</td>
<td>N/B</td>
</tr>
<tr>
<td>5</td>
<td>2.5 months</td>
<td>5,000</td>
<td>5,000</td>
<td>200</td>
<td>pos</td>
</tr>
<tr>
<td>6</td>
<td>2.5 months</td>
<td>1000</td>
<td>1,000</td>
<td>400</td>
<td>pos</td>
</tr>
<tr>
<td>7</td>
<td>N/P</td>
<td>5,000</td>
<td>5,000</td>
<td>400</td>
<td>pos</td>
</tr>
<tr>
<td>8</td>
<td>3 months</td>
<td>5,000</td>
<td>5,000</td>
<td>200</td>
<td>pos</td>
</tr>
<tr>
<td>9</td>
<td>3 months</td>
<td>5,000</td>
<td>5,000</td>
<td>50</td>
<td>neg</td>
</tr>
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<td>10</td>
<td>2.5 months</td>
<td>5,000</td>
<td>5,000</td>
<td>100</td>
<td>pos</td>
</tr>
<tr>
<td>11</td>
<td>2 months</td>
<td>5,000</td>
<td>5,000</td>
<td>100</td>
<td>pos</td>
</tr>
<tr>
<td>12</td>
<td>2 months</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>pos</td>
</tr>
<tr>
<td>13</td>
<td>3 months</td>
<td>5,000</td>
<td>5,000</td>
<td>0</td>
<td>pos</td>
</tr>
<tr>
<td>14</td>
<td>3 months</td>
<td>5,000</td>
<td>5,000</td>
<td>800</td>
<td>pos</td>
</tr>
</tbody>
</table>

N/P – not pregnant; N/B – not bled

6.4 Discussion

Leptospirosis was first reported in Australia in 1933 (Morrisey, 1934; Johnson, 1951). Since then several leptospiral serovars have been isolated in Australia including; *L. interrogans* serovars Australis, Zanoni, Kremastos, Robinsoni, Broomi, Pomona, Szwajizak; *L. kirschneri* serovar Valbuzzi and *L. weilli* serovar Celledoni (Morrisey, 1934; Johnson, 1951; Wellington et al., 1951). *Leptospira interrogans* serovars Pomona and Hardjo have been regularly isolated from cattle in Australia (Sullivan and Callan,
1970; Hoare and Claxton, 1972). Other serovars that have been isolated include Australis (Campbell and Stallman, 1975), Zanoni (McClintock et al., 1993), Celledoni and Grippotyphosa (Abdollahpour et al., 1996).

The Ab-ELISA that was developed and validated in Chapter 5 was used to test sera collected from cattle from a farm in Western Australia after an outbreak of leptospirosis. The ELISA detected a seroprevalence of 79.9%, and the MAT a seroprevalence of 85.3% however there was no significant difference between these seroprevalences. This indicates that the Ab-ELISA is sensitive in detecting cattle that are infected with *Leptospira* and that there is good agreement between the Ab-ELISA and the MAT. This was demonstrated by the Spearman rank correlation test that showed a significant association between both tests (P < 0.01). Graphically it was shown that as the MAT titres increased the Ab-ELISA ODs also increased. The kappa value for both tests was moderate to good (Maclure and Willet, 1987).

These results suggest that the Ab-ELISA can be used as a diagnostic test for leptospirosis in an outbreak on a cattle farm when Hardjo is suspected. However, as previously stated the ELISA is genus specific and does not give information on specific leptospiral serovars.

### 6.4.1 Serovar Hardjo

Serovar Hardjo was the dominant serovar infecting the cattle on this farm. This agrees with other reports that Hardjo is specifically associated with cattle and world-wide (Amatredjo et al., 1976; Ellis et al., 1985b). Although most species of *Leptospira* have a rodent reservoir, this is apparently not the case with serovar Hardjo, which has never been isolated from rodents (Ellis et al., 1981; Faine et al., 1999). This organism is an
extremely well adapted bacterium of cattle (Ellis et al., 1981; Thiermann, 1982). Transmission of this serovar on a farm is mainly from cattle to cattle following the introduction of infected animal onto the property.

It is likely therefore that serovar Hardjo was the causal agent of the initial problem identified on this farm in April/May 2004. The farm did not have a history of vaccination against leptospirosis. The source of infection may have been a recent introduction of an infected animal however no records to support this were available.

Of the 14 heifers that initially aborted, 13 had Hardjo titres of 1:5,000 in September and October 2004 but by December 2004 the Hardjo titre had dropped significantly to a range of 1:50-1:800 (Table 6.6). The sudden decline in the titre in these heifers, which were 2-3 months pregnant, could be due to the chronicity of the infection and a decline in the level of circulatory IgM antibodies. Another reason for the observed decline in the titres could have been because the MAT was performed by two different laboratories and the recording of agglutination titres is subjective and prone to operator bias (Smith et al., 1994; O'Keefe, 2002).

The antibody titres to serovar Hardjo in the other heifers in the mob that were 2 to 3 months pregnant in December 2004 ranged from 50 to 1,600. The number of animals with Hardjo titres of 100, 200 and 400 was significantly higher than the animals with titres of 800 and 1,600 (Figure 6.2). If these Hardjo titres persisted throughout the gestation period this might have resulted in abortion or other reproductive problems. There is no specific antibody titre for serovar Hardjo that is associated with abortion.
However, there are reports of high Hardjo titres of up to 30,000 after two weeks of infection which drop rapidly to low titres (100 to 1,000) at the time of abortion (Sullivan, 1970; Hoare and Claxton, 1972; Ellis and Michna, 1977; Thiermann, 1982).

With over 50% of cattle were seropositive there is a high chance that these pregnant heifers could infect their foetuses as shown by a study in UK (Ellis et al., 1982). As such, control measures should be taken by the farm management to prevent the spread of infection. Vaccination is the only way to prevent any reproductive problems that may be caused by this serovar (Little et al., 1992b).

### 6.4.2 Serovar Tarassovi

Antibodies to serovar Tarassovi was recognised in this cattle mob on Pinjarra farm. This serovar belongs to a pathogenic group and may cause infection and disease in animals. Of the 159 cattle that were pregnant and bled in December 2004, 13 had antibody titres to only Tarassovi (titre range 50-400) and 19 had antibody titres to Hardjo (50-800). One animal had a higher antibody titre to Tarassovi than Hardjo indicating active infection with this serovar in the animals. Whether this serovar had some significance as a cause of reproductive failure on this farm is not known. It is possible that Tarassovi alone might not cause any severe disease in cattle but it might act together with serovar Hardjo to cause abortions and stillbirths in the cattle.

The significance of serovar Tarassovi is uncertain as the organism has not been isolated from cattle in Australia (Durham and Paine, 1997) despite numerous accounts providing serological evidence of its presence (Amatredjo et al., 1976; Milner et al., 1980). Attempts to isolate Tarassovi from the urine of seropositive cattle in Queensland were unsuccessful (Black et al., 2001).
6.4.3 Serovar Arborea

There is evidence to suggest that cattle had been infected with serovar Arborea of the genomo-species *L. borgpetersenii* on the farm. Of the 18 cattle that had an antibody titre to this serovar 3 cross-reacted with *L. borgpetersenii* serovar Ballum. Given that both *L. borgpetersenii* serovar Ballum and *L. borgpetersenii* serovar Arborea react similarly in the MAT, the identification of these cases as *L. borgpetersenii* serovar Arborea is based upon the lack of evidence that *L. borgpetersenii* serovar Ballum exists in Australia because it has never been isolated (Slack *et al.*, 2006).

*Leptospira borgpetersenii* serovar Arborea is one of five known members of the Ballum sero-group and was first isolated in Europe in 1944 (Kmet and Dikken, 1993). The main carriers of serovar Arborea throughout the world are rodents, in particular *Mus domesticus* and *Rattus rattus* (Everard *et al.*, 1995; Matthias and Levett, 2002). The Reference Laboratory in Brisbane has included members of the Ballum serogroup in their routine MAT panel since 1991 to monitor the presence of this serovar which was thought to be exotic to Australia.

The presence of serovar Arborea in WA may be linked to the introduction of domestic rodents via boats. It remains to be seen whether the emergence of *L. borgpetersenii* serovar Arborea in cattle in Western Australia will become a permanent feature in the future or whether it is merely a short-term aberration.

6.4.4 Serovar Pomona

There is no serological evidence of serovar Pomona on this farm. Earlier studies in Australia showed that Hardjo and Pomona can occur together (Elder and Ward, 1978). It is not clear as to why Pomona was not found but it is possible that cattle may no longer
be a reservoir for Pomona in Western Australia. It is also possible that there are no suitable reservoir host for this serovar like pigs (Chappel et al., 1998) in the locality of this farm. Another reason for the absence of Pomona could be the low rainfall in the area, as Pomona has usually been associated with areas of high rainfall (Carroll and Campbell, 1987).

6.4.5 Serovar Medanensis

The results showed that cattle on the farm were seropositive to L. interrogans serovar Medanensis. The positive responses to Medanensis was believed to result from cross-reaction with serovar Hardjo as both belong to the Sejroe serogroup (Black et al., 2001). However, not all of the serum samples cross-reacted with serovars Hardjo and Szwajizak, with 11 of the 157 animals were seropositive to only Medanensis with titres of 50-200. This suggests that there is a reservoir host for this serovar within the vicinity of the farm that is infecting the cattle. Rodents are believed to be associated with this serovar (Faine et al., 1999) however no work has been carried out in WA to test rodents for leptospirosis.

6.4.6 Serovar Szwajizak

There is evidence of cattle with antibodies to serovar Szwajizak on the farm. Rodents are also the main carrier of this serovar (Thiermann, 1982). No single antibody titre to this serovar was observed in the sera tested but cross-reactions were seen with serovars Hardjo and Medanensis at low antibody titres. One of the reasons for this could be that they share the same antigenic epitopes. There is serological evidence of this serovar in Australian cattle but the organism has not been isolated under field conditions (Black et al., 2001). In heifers experimentally infected with serovar Szwajizak in the USA the
organism was isolated from blood and kidney and antibody cross-reactions were seen with Hardjo at low titres (NerVig et al., 1978).

In conclusion the Ab-ELISA used in this study was shown to be a useful diagnostic test to assist in testing for leptospirosis in an outbreak on a cattle farm but cannot provide specific serovar information. Although the Ab-ELISA has the advantages of not requiring maintenance of handling of live cultures and of being a less suggestive test to read, the MAT provides insight into possible sources of Leptosira infection in the herd through its ability to identify serovars.
CHAPTER 7
USE OF PCR TO DETECT *LEPTOSPIRA* SPECIES IN KIDNEY AND URINE SAMPLES FROM INFECTED ANIMALS

7.1 Introduction

Leptospires can be isolated from the urine, kidneys and reproductive tracts of infected animals and from aborted foetuses (Ellis *et al.*, 1982; Thiermann, 1982; Ellis and Thiermann, 1986b). However, isolation of *Leptospira* by culture is unsuitable as a diagnostic test because culture is labour intensive and takes several weeks to complete. It is therefore, difficult to diagnose sub-fertility caused by species of *Leptospira* with any degree of certainty. The polymerase chain reaction (PCR) offers a sensitive and rapid alternative to culture for unequivocally demonstrating current infection with *Leptospira*. In addition, PCR can differentiate pathogenic from non-pathogenic leptospires and can rapidly confirm the diagnosis of leptospirosis in early stages of the disease (Brown *et al.*, 1995; Romero *et al.*, 1998). However, as with the ELISA for serology PCR assays are, currently not able to differentiate between leptospiral serovars.

This study aimed to evaluate a PCR-based assay to detect leptospires from samples of kidneys collected from livestock in PNG. A second component of the study was designed to assess the use of a real-time PCR assay based on a TaqMan® assay (Smythe *et al.*, 2002) for detecting leptospiral DNA in urine from cattle. This was performed in collaboration with the Animal Research Institute, Queensland Department of Primary Industry and Fisheries, Moorooka Brisbane, Queensland.
7.2  Materials and Methods

7.2.1  Kidney samples from PNG

A total of 72 kidney samples were collected from cattle slaughtered in Lae (n = 61) and Kimbe (n = 11). Seventy four kidney samples were collected from pigs slaughtered in Lae (n = 54) and Port Moresby (n = 20).

7.2.2  Urine samples from a beef farm in WA

One hundred and ninety-three urine samples were collected from beef cattle in Pinjarra, WA. Each urine sample was collected into a sterile 50 mL specimen container and chilled for transportation to Murdoch University.

7.2.3  Culture and isolation

The culture and isolation was performed according to the protocol described in Chapter 3 pages 55-56.

7.2.4  Preparation of genomic DNA from kidney samples

Whole genomic DNA was extracted from a 25 mg sample of each kidney using the QIAamp DNA Mini Kit (Qiagen, USA). The kits were used according to the manufacturer's instructions.

Briefly, a 25 mg piece from each sample of kidney was cut into small pieces and placed in a 1.5 ml micro-centrifuge tube, and 180 µl of buffer ATL was added. Twenty microlitres Proteinase K (20 mg/ml, Qiagen) was added and mixed by vortexing, and the mixture digested for up to 20 h at 56°C until tissue was completely lysed. The tube was vortexed occasionally during digestion to disperse the sample. After completion of the digestion, the tubes containing the samples of kidney were briefly centrifuged to remove
drops from inside the lid and 200 µl of buffer AL was added to the sample, mixed by vortexing for 15 sec, and incubated at 70°C for 10 min. Each tube was briefly centrifuged and 200 µl of ethanol (96-100%) was added to the sample, and mixed by vortexing for 15 sec followed by a brief centrifugate to remove drops from inside the lid. The mixture was carefully transferred to a QIAamp Spin Column fitted in a 2 mL collection tube, without wetting the rim, and micro-centrifuged at 8,000 x g for 1 min until the solution had completely passed through the membrane. The column was transferred to a clean 2 mL collection tube, and 500 µl of buffer AW1 were added to the column and the tube was centrifuged at 8,000 x g for 1 min. The column was then transferred to a clean 2 mL collection tube and 500 µl of Buffer AW2 were carefully added to the column and centrifuged at 14,000 x g for 3 min. The column was removed and placed in a sterile 1.5 mL microfuge tube and 200 µl of buffer AE was added and the mixture was incubated at room temperature for 5 min. The column was centrifuged at 8,000 x g for 1 min and the eluate labeled and stored at -20°C. Approximately 10 ng of purified DNA was extracted from each sample.

7.2.5 Extraction of DNA from urine

Whole genomic DNA was extracted from clear urine samples using heat lysis and from urine contaminated with faeces using the QIAamp Mini Kit (Qiagen, USA). Prior to extraction 5 ml of each urine sample were centrifuged for 30 min at 3,000 x g. The supernatant was discarded and approximately 0.5 ml retained. The pellet was resuspended in the retained supernatant, transferred to a 1.5 mL micro-centrifuge tube and centrifuged at 7,500 x g for 10 min. The supernatant was carefully removed without disturbing the pellet. Fourteen urines were prepared using both heat lysis and QIAamp DNA Mini columns.
7.2.5.1 Heat lysis

Each pellet was resuspended in 50 µl of sterile water (Multigrade PCR water) by vigorous pipetting, and was incubated at room temperature for 2 min and 95°C for 10 min. Each tube was briefly centrifuged to remove drops from inside lid and then labelled and stored at -20°C until required. The method was obtained from the Animal Research Institute, Queensland Department of Primary Industry and Fisheries, Moorooka Brisbane, Queensland.

7.2.5.2 QIAamp DNA mini kit for contaminated urine

Twenty microlitres of Proteinase K (Qiagen, USA) was added to each pellet and mixed by pulse vortexing for 15 sec. Each tube was incubated at 56°C for 10 min and spot centrifuged to remove drops from lid. Two hundred microlitres of Buffer AL was added to each tube and mixed by vortexing for 15 sec and incubated at 70°C for 10 min. Two hundred microlitres of absolute ethanol was then added and vortexed for 15 sec and the tube spot centrifuged. The mixture was then transferred to a QIAamp spin column and micro-centrifuged at 8,000 x g for 1 min. Each column was placed into a clean 2 mL collection tube and the used collection tube was discarded. Five hundred microlitres of buffer AW1 was added to each column and centrifuged at 8,000 x g for 1 min. Each column was then placed in a clean 2 mL collection tube and the used collection tube was discarded. Five hundred microlitres of buffer AW2 was added to each column and centrifuged at 14,000 x g for 3 min. Each column was placed in a sterile 1.5 ml microfuge tube and the used collection tube was discarded. Fifty microlitres of buffer AE was added to each column and incubated for 5 min at room temperature. The columns were centrifuged at 8,000 x g for 1 min and the eluates labeled and stored at store at -20°C until required.
7.2.6 Polymerase chain reaction

The nested PCR-based to detect pathogenic *Leptospira* was developed by Phuektes (unpublished). The PCR was designed to amplify a variable sized fragment of the *rfb* locus from *Leptospira interrogans* serovar Hardjo type hardjopratjino, serovar Pomona, serovar Copenhageni and *Leptospira borgspetersenii* serovar Hardjo type hardjobovis using sequences obtained from GenBank (www.ncbi.nlm.nih.gov) and aligned using Clustal W. A pair of primers that produced different predictive sizes of PCR products from these serovars were designed using Amplify (Bill Engels, Genetics Department, University Winconsin, Madison, WI 53706). The 23 base pair (bp) forward primer was designated from conserved sequences in these serovars at approximately 97 bp before the stop codon of ORF 29 of *rfb*, and designated FLepto (5’-AACGTATCTTCATATTCTTTGCG-3’). The 23 bp reverse primer designated RLepto (5’-AGCCCTCAAGTAAATTATCAG-3’) was designed from approximately 31 bp before the start codon of ORF 30 of *rfb*.

The PCR was carried out in 0.2 mL tubes in a reaction volume of 25 µl. The PCR reaction mixture contained 200 µM of dATP, dTTP, dCTP and dGTP, 67 mM Tris HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/ml gelatin, 2.5 mM MgCl₂, 0.55 U Tth Plus DNA polymerase (Fisherbiotec), 12.5 pmol primers and 5 µl of purified DNA.

The second round of the nested PCR was prepared with the same PCR primers and reagents used in the first reaction except that the volume of the DNA template used from the first PCR was 2 µl. The PCR was performed in an automated thermocycler (Perkin elmer) and the conditions used are tabulated in Table 7.1.
Table 7.1  Conditions employed in the nested PCR assay

<table>
<thead>
<tr>
<th>PCR steps</th>
<th>1st PCR</th>
<th>2nd PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial DNA denaturation</td>
<td>94°C for 5 min</td>
<td>94°C for 5 min</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>45 cycles</td>
<td>45 cycles</td>
</tr>
<tr>
<td>DNA denaturation</td>
<td>94°C for 30 s</td>
<td>94°C for 30 s</td>
</tr>
<tr>
<td>Primers annealing</td>
<td>58°C for 20 s</td>
<td>64°C for 20 s</td>
</tr>
<tr>
<td>DNA extension</td>
<td>72°C for 45 s</td>
<td>72°C for 45 s</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 7 min</td>
<td>72°C for 7 min</td>
</tr>
</tbody>
</table>

7.2.7 Analysis of PCR products

Seven microlitres of each PCR product was electrophoresed in a 1.5% agarose gel containing 0.1 µg/ml ethidium bromide at 6 V/cm for 45 min in 1X TAE buffer. The PCR products were visualized using ultraviolet light (UV) transillumination. A 100 bp DNA Ladder (Invitrogen) was used in each gel as a molecular weight marker.

7.2.8 DNA sequencing

The PCR products were purified using the UltraClean Tm GelSpin DNA purification kit (Mo Bio Laboratories) according to the manufacturer’s protocol. The PCR products were sequenced directly in both directions using an automated DNA sequencer (Applied Biosystems) according to the manufacturer’s protocol.
7.2.9 Real-time PCR (TaqMan)

The method, reagents and primers were obtained from the Animal Research Institute, Queensland Department of Primary Industry and Fisheries, Moorooka Brisbane, Queensland.

The assay was modified from the TaqMan\textsuperscript{\textregistered} assay published by Smythe \textit{et al.} (2002). The modifications included an increase in the annealing and extension temperatures from 60 to 64°C to improve discrimination between the negative and weak positive samples; reducing the probe concentration from 200 nM to 50 nM and extending the number of cycles from 40 to 50 (Corney \textit{et al.}, unpublished). This assay used 2 µl of template per 25 µl reaction, and used Platinum Quantitative PCR Super Mix UDG (Invitrogen). All urine samples were tested in duplicate and results were expressed as the mean threshold cycle (C\textsubscript{T}).

The forward primer used was 5’-CCC-GCG-TCC-GAT-TAG-3’ and the reverse primer was 5’-TCC-ATT-GTG-GCC-G A/G A-CAC-3’. The sequence of the probe was 5’-(FAM)-CTC-ACC-AAG-GCG-ACG-ATC-GGT-AGC-(TAMRA)-3’. The master mix was prepared in a PCR clean area in a reaction tube. Volumes of the PCR reagents for one reaction were as follows: PCR water 9.75 µl, forward and reverse primers 0.25 µl each, probe 0.25 µl and platinum PCR supermix UDG 12.5 µl. Twenty three microlitres of the master mix was added to each reaction tube with 2 µl of DNA template. Amplification reactions were performed using a Rotor Gene 3000 (Corbett Research). The programmed cycle was Stage 1 (x 1 cycle) 50°C for 2 min and 95°C for 2 min and stage 2 (x 50 cycles) the annealing and amplification step 95°C for 15 sec and 64°C for 1 min. The entire program was completed in 67 min.
7.2.10 Criteria for interpreting C\textsubscript{T} values

The following criteria for interpreting the C\textsubscript{T} values were as follows: positive \(\leq 37\), 37 < suspect \(\leq 40\), negative > 40. Samples for which one reaction has a C\textsubscript{T} value of \(\leq 40\), and the other reaction a C\textsubscript{T} value of > 50 were retested or scored as suspect (Corney \textit{et al.}, unpublished).

7.2.11 Multicentre study

Samples of the DNA extracted from cattle urine in this study were provided to the Animal Research Institute, Queensland Department of Primary Industry and Fisheries, Moorooka Brisbane, Queensland to be used for inclusion in a multicentre study to validate the real-time PCR-assay for the detection of leptospires.

The multicentre study involved four laboratories in Australia, three of which used Rotor-Gene 2000 or 3000 platforms, whilst the other used an iCycler (Bio-Rad). The assays were performed without any modifications to the protocol. The iCycler data were analysed with the threshold adjusted manually so that the C\textsubscript{T} value for the positive control was similar to that obtained by the Animal Research Institute. Correlations between the results for the laboratories were determined from pairwise comparisons between the sets of C\textsubscript{T} values (Fleiss, 1981). The C\textsubscript{T} values > 50 were ignored. The results were also classified as positive, suspect, or negative using the criteria established (Corney \textit{et al.}, unpublished), and the degree of agreement between the laboratories was assessed using the kappa statistic (Fleiss, 1981). A kappa value of \(\geq 0.75\) indicated excellent agreement, and a kappa value between 0.45 and 0.75 indicated fair to good agreement.
7.3 Results

7.3.1 Genomic DNA from kidneys

Results of testing genomic DNA extracted from the kidneys of cattle and pigs using the PCR are shown in Table 7.2. 300-350 bp product was amplified from DNA extracted from bovine and pigs kidneys, respectively. The DNA sequences of the PCR products from the bovine kidneys shared 98% homology with *L. borgspetersenii* serovar Hardjo (type hardjobovis). Attempts to sequence the PCR products amplified of DNA from pig kidneys were unsuccessful.

Table 7.2 Results of nested PCR testing of genomic DNA extracted from the kidneys of cattle and pigs from PNG

<table>
<thead>
<tr>
<th>Species</th>
<th>Number tested</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>72</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Pigs</td>
<td>54</td>
<td>12</td>
<td>40</td>
</tr>
</tbody>
</table>

Viable leptospires were only isolated from the kidneys of 2 cattle from PNG that were positive using PCR. The remainder of samples were either negative on culture (n = 23) or contaminated (n = 11).

7.3.2 Real-time PCR

A total of 8.3% (16/193) of urine samples were positive and 8.3% (16/193) were considered suspect following testing using the TaqMan real-time PCR assay.
There is no difference between the $C_T$ values from urines extracted using heat lysis and QIAamp Mini kit (Qiagen, USA) (Table 7.3).

Table 7.3  Comparison of $C_T$ values from 14 of the field urines tested by RT PCR following heat lysis and Qiagen Mini kit extraction

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Heat lysis ($C_T$)</th>
<th>Qiagen ($C_T$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>38.96</td>
<td>38.98</td>
</tr>
<tr>
<td>7</td>
<td>42.71</td>
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<td>15</td>
<td>29.23</td>
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<td>20</td>
<td>32.16</td>
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</tr>
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<td>44.37</td>
<td>42.31</td>
</tr>
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<td>37.91</td>
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<td>38.95</td>
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<td>28.83</td>
<td>27.35</td>
</tr>
<tr>
<td>77</td>
<td>38.69</td>
<td>38.08</td>
</tr>
</tbody>
</table>
Table 7.4  \( C_T \) values and classification of 21 urine samples tested with RT PCR by four Australian laboratories (Results from this study are presented under Lab 2)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lab 1</th>
<th>Lab 2</th>
<th>Lab 3</th>
<th>Lab 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( C_T )</td>
<td>Status</td>
<td>( C_T )</td>
<td>Status</td>
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<tr>
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<td>3</td>
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<td>30.6</td>
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<td>28.4</td>
<td>Pos</td>
</tr>
<tr>
<td>9</td>
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<td>44.4</td>
<td>Neg</td>
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<td>43.4</td>
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</tr>
<tr>
<td>11</td>
<td>&gt; 50</td>
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<td>44.7</td>
<td>Neg</td>
</tr>
<tr>
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<td>&gt; 50</td>
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<td>Neg</td>
</tr>
<tr>
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<td>&gt; 50</td>
<td>Neg</td>
<td>&gt; 50</td>
<td>Neg</td>
</tr>
<tr>
<td>14</td>
<td>&gt; 50</td>
<td>Neg</td>
<td>&gt; 50</td>
<td>Neg</td>
</tr>
<tr>
<td>15</td>
<td>38.6</td>
<td>Sus</td>
<td>38.1</td>
<td>Sus</td>
</tr>
<tr>
<td>16</td>
<td>38.7</td>
<td>Sus</td>
<td>37.1</td>
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</tr>
<tr>
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<td>33.1</td>
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</tr>
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<td>Pos</td>
<td>34.5</td>
<td>Pos</td>
</tr>
<tr>
<td>19</td>
<td>37.6</td>
<td>Sus</td>
<td>37.1</td>
<td>Sus</td>
</tr>
<tr>
<td>20</td>
<td>37.4</td>
<td>Sus</td>
<td>35.1</td>
<td>Pos</td>
</tr>
<tr>
<td>21</td>
<td>37.6</td>
<td>Sus</td>
<td>N/T</td>
<td>N/T</td>
</tr>
</tbody>
</table>

N/T – not tested; Sus - suspected
7.3.3 Multicentre evaluation of TaqMan® assay

The \( \text{C}_\text{T} \) values obtained from the four laboratories and their classifications are shown in Table 7.4. There was good agreement in terms of \( \text{C}_\text{T} \) classification between the three laboratories using the Rotor-Gene platform (kappa = 0.70, \( P < 0.001 \)) and a high level of agreement between the three laboratories in terms of \( \text{C}_\text{T} \) values (Table 7.5). The results from laboratory 4 (iCycler) were not included in the analyses because the result from samples classified as negative by other laboratories had lower \( \text{C}_\text{T} \) values when tested on the iCycler at laboratory 4.

<table>
<thead>
<tr>
<th>Laboratory 1</th>
<th>Laboratory 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory 2</td>
<td>0.978</td>
</tr>
<tr>
<td>Laboratory 3</td>
<td>0.898</td>
</tr>
</tbody>
</table>

7.4 Discussion

The purpose of this study was to evaluate the use of a PCR to detect leptospires from samples collected from livestock in PNG and Australia. Early detection of leptospires in livestock increases the chance of a successful intervention to prevent the spread of infection by culling, isolation and/or vaccination. At present there is no rapid and practical means for early detection of leptosiral infections. This report describes the use of a simple PCR assay for the detection of pathogenic species from kidney samples of healthy cattle and pigs slaughtered in abattoirs in PNG.
The results show that a nested PCR was able to detect DNA from pathogenic species of *Leptospira* in more samples of kidney than following culture in EMJH medium. Culture failed to detect the presence of *Leptospira* in 34 of the 36 PCR-positive samples. This may have been due to the organism’s susceptibility to contamination with extraneous bacteria and the inherent requirement for the survival of live *Leptospira* during transportation to the laboratory. The sample storage conditions and transport time prior to laboratory processing are likely to be crucial factors related to the success of microbiological tests. Culture can be a sensitive technique if it is performed under ideal conditions such as during experimental studies. However, it is totally unsuitable for use as a diagnostic tool on samples collected from livestock in PNG due to the lack of a reference laboratory in the country.

The primers used are from a conserved region in the chromosomal *rfb* loci of *L. interrogans* and *L. borgpetersenii* and this region is restricted to the pathogenic leptospires. This makes the PCR more specific for pathogenic leptospires than previously reported PCRs, which are genus specific and detect both pathogenic and non-pathogenic leptospires in urine and tissue samples (Merien *et al.*, 1992; Gravekamp *et al.*, 1993; Kee *et al.*, 1994; Kawabata *et al.*, 2001). The nested PCR used in this study on kidney samples can complement other diagnostic tests for the rapid detection of *Leptospira* in infected cattle herds.

Animal tissues collected in remote locations need to be preserved in suitable storage media prior to testing with the PCR. In this study, portions of kidney samples were stored in a solution of saturated NaCl and 10% DMSO. Kidney samples were stored at room temperature for up to 6 weeks before DNA extraction and testing. Storage of
samples in the saturated NaCl and 10% DMSO solution was sufficient to preserve the tissues without any bacterial contamination. Freezing of fresh kidneys has been suggested as a method of preservation but this is not suitable in PNG because unexpected power disruption frequently occur that can cause the samples to thaw.

Two cycles of PCR were employed because the first cycle of PCR produced weak bands on the agarose gel. This could be due to the biological nature of the field samples and that approximately 10 ng of genomic DNA extracted from a 25 mg of kidney sample using the QIAamp Mini kit (Qiagen, USA) isn’t sufficient to amplify the target DNA with one PCR cycle. This was overcome by performing the second cycle of PCR at a higher annealing temperature. The annealing temperature of the first round PCR was 58°C and this was increased to 64°C for the second PCR which was sufficient to amplify the target DNA so as to ensure good visibility on the agarose gel.

The TaqMan® assay was performed as described by Smythe et al., (2002), but the probe concentration was re-optimized due to the use of the Rotor-Gene 3000 (Corney et al., 2006 unpublished). The modified TaqMan® assay using the Rotor-Gene 3000 was sensitive in detecting *Leptospira* in the urine collected from pregnant heifers on a beef farm in Western Australia. In the sampled herd, over 30% of the pregnant heifers were shown to be positive for leptospires with this rapid diagnostic test. This indicates the TaqMan® assay is suitable for use as a herd test to diagnose bovine leptospirosis. It can also be used in an outbreak of bovine leptospirosis on farms as the assay takes approximately 60-70 minutes to produce results, which can allow prompt rapid interventions such as vaccination or antibiotic treatment.
The two methods used to extract urine from field samples were compared. The \( C_T \) values in the TaqMan\(^{®}\) assay for both methods were similar. There was no significant difference between the results of testing urine extracted using the heat lysis method and the QIAamp Mini kit (Qiagen, USA). Both methods are suitable for the extraction of DNA from urine prior to detection of leptospires using the TaqMan\(^{®}\) assay. However, the heat lysis technique is quicker and less expensive if urine is not contaminated with faeces.

The TaqMan\(^{®}\) assay was also evaluated in three other laboratories using a standard set of urines from a WA farm. The laboratories used three different platforms; two different versions of the Rotor-Gene and an iCycler. No significant difference was observed between the results from the laboratories using the Rotor-Gene. However, the performance of the iCycler was noticeably different from the Rotor-Gene, especially for negative samples. The assay was specifically optimized for the Rotor-Gene (Corney et al., 2006 unpublished) and the multicentre study between the laboratories showed that the TaqMan\(^{®}\) assay is robust and can be transferred to other laboratories provided a similar machine is used.
CHAPTER 8

GENERAL DISCUSSION

The focus of this study was to determine the seroprevalence of leptospirosis in the livestock population and secondly to develop and validate a suitable diagnostic test for active surveillance of this infection in PNG. The impetus for this work came from anecdotal evidence that infection with species of *Leptospira* is one of the causes of poor fertility in commercial cattle in PNG.

The climate in PNG is suitable for the survival of leptospires in the environment and this can increase the chance of transmission to other animals and humans. A total of 13 serovars were identified from the survey and serovar Hardjo was the most prevalent. This serovar is predominant in the large commercial cattle farms compared to smallholders. There was a strong association between age and the seroprevalence of Hardjo infection. A high seroprevalence in older animals may result in a higher risk of transmission to maiden heifers. Vaccination for serovar Hardjo may have had a role, as evidenced by a decrease in the seroprevalence of infection in maiden heifers born on Trukai and Ramu farms. There was evidence of vaccination failure on Numundo farm and the reasons for this are not fully understood. This study has shown a possible role for *Leptospira* in bovine infertility in PNG.

There was a high seroprevalence of serovar Tarassovi in cattle but no evidence for clinical disease was reported. The source of Tarassovi infection in cattle is unknown as pigs do not seem to be the reservoir host in PNG. Small marsupials and rodents could be the source of infection for cattle. The low seroprevalence of serovar Pomona in pigs is
surprising because pigs have been implicated as the source of infection in other studies (Chappel et al., 1998; Mason et al., 1998; Faine et al., 1999). The low seroprevalence of Pomona in cattle is important as a number of animals from commercial properties were vaccinated for Pomona. This could suggest a failure in the vaccine to induce a high antibody response or that there was no challenge with this serovar from the environment.

Significant cross-reaction was seen between serovars Hardjo, Szwajizak and Medanensis in this study. Serovars Szwajizak and Medanensis are reported to be rodent-associated and may be involved in bovine leptospirosis because many rodent-associated serovars can cause overt disease in cattle (Thiermann, 1982; Faine et al., 1999). This study confirms the presence of a number of leptosporal serovars in livestock that are potentially significant to public health.

The relatively low prevalence of serovar Hardjo in smallholder cattle showed that there is very little infection with this serovar circulating outside the large commercial farms. The presence of *L. borgpetersenii* serovar Hardjo in PNG suggests that the main transmission route is through direct contact with contaminated urine because it was shown this serovar does not survive in the environment like *L. interrogans* serovar Hardjo (Bulach et al., 2006). This could explain why infection is high in commercial properties where management brings cattle into close proximity on many occasions. Cattle from smallholder farms introduced to commercial properties may thus be at risk of becoming infected and developing clinical disease. The newly isolated serovar Topaz was detected in cattle and horses for the first time in PNG (Monash, http://www.med.monash.edu.au/microbiology/staff/adler/tsc-minutes-november-2005/)
accessed 2006). In the villages surveyed in the Markham Valley pigs do not appear to play a role in leptospirosis; however dogs may be a source of infection for humans.

There is a need for a better diagnostic test for active surveillance of leptospirosis in PNG because the MAT is not a suitable test. For reasons stated earlier the Ab-ELISA developed using the crude pathogenic antigen is highly sensitive and specific. In the absence of a true ‘gold standard’ test for Leptospira a Bayesian approach was used to infer the estimates of sensitivity and specificity of the MAT and the Ab-ELISA. Both tests were highly sensitive and specific. There is significant correlation between MAT titres and Ab-ELISA OD values which suggests that the Ab-ELISA is a suitable test for use in PNG. Although the test cannot provide information on leptospiral serovars, the Ab-ELISA will be used mainly in cattle where serovar identification will not be a concern because Hardjo was found to be the dominant serovar infecting the cattle in PNG.

The utility of the Ab-ELISA was demonstrated by testing sera from a cattle farm in WA after an outbreak of bovine leptospirosis during the study. There was a high level of agreement between the results of the ELISA and the MAT which reiterates the practicality of using the Ab-ELISA to detect Leptospira infection in cattle in PNG. It is interesting to note that in this outbreak serovar Tarassovi was present with serovar Hardjo. Serovar Tarassovi has no clinical significance in cattle to date but it might, in the future, be of clinical concern.

The isolation of leptospires from kidney or urine is essential proof that an animal is infected with Leptospira. However, the sensitivity of leptospiral isolation techniques
used to achieve such proof is limited. Culture is the most practical test to use but its sensitivity is very low as a result of contamination with extraneous bacteria and the inherent requirement for leptospiral survival during transportation to the laboratory. The PCR-based assay is highly sensitive and is a more useful test for PNG. Storage of kidney samples in DMSO and NaCl at room temperature has been shown to be suitable for preserving samples from remote locations such as PNG.

Two methods used to extract DNA from urine were compared. The results showed that there was no significant difference in the results obtained after extraction of DNA from urine using heat lysis or the QIAamp Mini kit. However, the heat lysis method is simple and inexpensive, which again makes it suitable for use in PNG. The multicentre study between the four Australian laboratories using either version of the Rotor-Gene that showed the TaqMan® assay is robust and can be easily transferred to other laboratories for Leptospira detection. The real-time PCR is not recommended for diagnostic test in an animal health laboratory in PNG because it is not feasible for routine use.
APPENDICES

Appendix 1  WinBUGs code for Bayesian framework assuming conditional dependence between tests

model;
{
  y1[1:Q, 1:Q] ~ dmulti(p1[1:Q, 1:Q], n1)
  y2[1:Q, 1:Q] ~ dmulti(p2[1:Q, 1:Q], n2)
  p1[1,1] <- pi1*eta11 + (1-pi1)*theta11
  p1[1,2] <- pi1*eta12 + (1-pi1)*theta12
  p1[2,1] <- pi1*eta21 + (1-pi1)*theta21
  p1[2,2] <- pi1*eta22 + (1-pi1)*theta22
  p2[1,1] <- pi2*eta11 + (1-pi2)*theta11
  p2[1,2] <- pi2*eta12 + (1-pi2)*theta12
  p2[2,1] <- pi2*eta21 + (1-pi2)*theta21
  p2[2,2] <- pi2*eta22 + (1-pi2)*theta22
  eta11 <- lambdaD*eta1
  eta12 <- eta1 - eta11
  eta21 <- gammaD*(1-eta1)
  eta22 <- 1 - eta11 - eta12 - eta21
  theta11 <- 1 - theta12 - theta21 - theta22
  theta12 <- gammaDc*(1-theta1)
  theta21 <- theta1 - theta22
  theta22 <- lambdaDc*theta1
  eta2 <- eta11 + eta21
  theta2 <- theta22 + theta12
  rhoD <- (eta11 - eta1*eta2) / sqrt(eta1*(1-eta1)*eta2*(1-eta2))
  rhoDc <- (theta22 - theta1*theta2) / sqrt(theta1*(1-theta1)*theta2*(1-theta2))
  pi1 ~ dbeta(1, 1) ##uninformative
  pi2 ~ dbeta(1, 1)
  eta1 ~ dbeta(7.2, 0.8)
  theta1 ~ dbeta(7.2, 0.8)
  lambdaD ~ dbeta(7.2, 0.8) ## Mean 0.90 SD = 0.1
  gammaD ~ dbeta(7.2, 0.8)
  lambdaDc ~ dbeta(7.2, 0.8) ## Mean 0.90 SD = 0.1
  gammaDc ~ dbeta(7.2, 0.8)
}
list(n1=200, n2=369, Q=2, y1=structure(Data=c(159, 18, 15, 8), Dim=c(2,2)),
y2=structure(Data=c(226, 35, 3, 105), Dim=c(2,2)))
list(pi1=0.5, pi2=0.5, eta1=0.9, theta1=0.90, lambdaD=0.90, lambdaDc=0.90,
     gammaD=0.90, gammaDc=0.90)
model{
    # Priors for Se and Sp
    # T1=ELISA, T2=MAT
    for (i in 1:2){
        se[i] ~ dbeta(1,1);
        sp[i] ~ dbeta(1,1);
    }

    # The model
    for (i in 1:8) {
        pop[i,1:4] ~ dmulti(par[i,1:4],n[i]);
        par[i,1] <- se[1]*se[2]*p[i] + (1-sp[1])*(1-sp[2])*(1-p[i]);
        par[i,2] <- se[1]*(1-se[2])*p[i] + (1-sp[1])*(sp[2])*(1-p[i]);
        par[i,3] <- (1-se[1])*(se[2])*p[i] + (sp[1])*(1-sp[2])*(1-p[i]);
        par[i,4] <- (1-se[1])*(1-se[2])*(1-sp[2])*(1-p[i]);
        n[i] <- sum(pop[i,1:4])
        # prior for the prevalence
        p[i] ~ dbeta(1,1);
    }

    # DATA MUST BE ORDERED ++| +|--|--|--
    #numundo, pinjarra, trukai, ramu, rumion, prhalda, reara abattoir
    159 18 15 8
    96 25 18 20
    226 35 3 105
    114 3 2 105
    98 14 2 58
    21 14 23 76
    5 9 47 67
    6 12 2 59
    END

    #INITS HERE
    list(se=c(0.9,0.9), sp=c(0.9,0.9), p=c(0.5,0.5,0.5,0.5,0.5,0.5,0.5,0.5,0.5))
}
Appendix 3  Checkerboard titration curves of the L1 antigen after extraction

Figure 9.1.1  Checkerboard titration curve of the L1 antigen after extraction. The antigen concentration used was 1:4,000

Figure 9.1.2  Checkerboard titration curve of the L1 antigen 10 months after extraction
CHAPTER 10

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