Advancing leptospirosis diagnostics in human and animal systems

Sarah Jane Wynwood

B. Sc. (Human Life Science)
Grad. Dip. (Health Science)

Submitted to the University of the Sunshine Coast in fulfilment of the requirements for the degree of Doctor of Philosophy

July 2015
Synopsis/Extended Abstract

Leptospirosis, a zoonotic disease with a world-wide distribution, causes significant disease in humans and animals. This thesis presents experimental work dealing with the serological diagnosis of leptospirosis in humans and animal populations. Chapters 2-5 of this thesis have been published, Chapter 6 has been accepted for publication pending acceptance of minor changes, and Chapter 7 is currently in review for publication. This thesis is produced as a collection of papers, with the intent that the work described herein is a coherent body work.

The first chapter of this thesis presents a brief overview of leptospirosis and seeks to put the disease into historical context. It provides an overview of the classification of leptospires, as well as some general aspects of leptospirosis, including its transmission, incidence and clinical signs and symptoms in both humans and animals. This chapter also introduces the current methods of diagnosis, highlighting some major disadvantages of these assays and therefore the need for improved diagnostic assays and methods of detection.

The second and third chapters of this thesis form what would be regarded as a literature review in a traditional thesis, summarising mostly contemporary work. Chapter 2 is a review focusing on the epidemiology and transmission of the disease in humans and animals. This chapter also delineates the symptoms observed in humans and animals and the conclusions section outlines unresolved and evolving issues for microbiologists, epidemiologists and public health officials. A key theme emergent from Chapter 2, and germane to this thesis, is the reliance of serology in the diagnostics and epidemiology of leptospirosis, in particular the use of the current “gold-standard” in the serological diagnosis of leptospirosis, the microscopic agglutination test (MAT). Chapter 3 focuses on leptospirosis primarily as a water-borne disease. Chapters 2 and 3 highlight the burden of disease in humans and animals as well as showing one of the major routes of transmission, water borne, and some of its associated outbreaks worldwide.
Chapters 4, 5, 6 and 7 are experimentally-based. The fourth chapter seeks to show the need for improved diagnostic methods for leptospirosis. This is illustrated using an outbreak of leptospirosis as a case-in-point, where the MAT is heavily relied upon as an epidemiological tool. This chapter reports the emergence of *Leptospira borgpetersenii* serovar Arborea as the dominant infecting serovar following a summer of natural disasters and the ensuing clean up in Queensland, Australia during 2011. It presents 154 new leptospiral infections during 2011, using the MAT. An emergent idea in this chapter is the need to develop improved methods of serological identification of leptospirosis, particularly in differentiating immunoglobulin classes and using more robust methods allowing automated, quantitative, high throughput analysis of samples.

Chapter 5 outlines the development of an alternative methodology to the MAT, using microsphere immunoassays (MIAs). The work described in this chapter sought to validate the MIA by comparison of the MIA with the MAT. An MIA capable of detecting antibodies to leptospires and differentiating IgG and IgM was developed. The MIA was validated using 200 human samples submitted for routine leptospirosis serology testing. The traditional microscopic agglutination (MAT) method (now 100 years old) suffers from a significant range of technical problems including a dependence on antisera which is difficult to source and produce, false positive reactions due to auto-agglutination and an inability to differentiate between IgG and IgM antibodies. A comparative validation method of the MIA against the MAT was performed and used to determine the ability of the MIA to detect leptospiral antibodies when compared with the MAT. The MIA was able to determine samples in the reactive, equivocal and non-reactive ranges when compared to the MAT and was able to differentiate leptospiral IgG antibodies from leptospiral IgM antibodies. The MIA was more sensitive than the MAT and in true infections was able to detect low levels of antibody in the later stages of the acute phase, as well as detect higher levels of IgM antibody earlier in the immune phase of the infection. The relatively low cost, high throughput platform and significantly reduced dependency on large volumes of rabbit antisera make this assay worthy of consideration for any microbiological assay that currently uses agglutination assays.
Chapters 6 and 7 describe work adopting an applied focus using both MATs and MIAs for diagnosis of leptospirosis in bovine (Chapter 6) and Tasmanian devil (Chapter 7) populations. These chapters seek to draw some conclusions about the incidence of leptospirosis in these disparate populations, as well draw some conclusions about the efficacy of the both MIAs and MATs and to compare and contrast their strengths and weaknesses.

Leptospirosis causes significant economic loss within the cattle industry worldwide, and the aim of Chapter 6 is to show the application of an MIA to 200 bovine serum samples to determine this method’s usefulness in the diagnosis of leptospirosis in comparison with the current gold standard, the microscopic agglutination test (MAT). Although the MAT is the most widely used laboratory test for the diagnosis of leptospirosis, its reliance on live cultures; subjective interpretation of results, and an inability to differentiate between antibody classes suggest the MAT is no longer the best method for the diagnosis of leptospirosis. The MIA was able to determine reactive from non-reactive samples when compared to the MAT and was able to differentiate IgG and IgM classes of antibody. The results suggest increased sensitivity in the MIA and the ability to multiplex up to 500 antigens at one time allows for significant improvements in cost effectiveness as well as a reduced dependency on live cultures. The relatively low cost, high throughput platform and differentiation of antibody class make the MIA worthy of consideration for the diagnosis of leptospirosis in small or large scale bovine populations.

Chapter 7 outlines a diagnostic study of Tasmanian devil samples for the presence of leptospirosis. Tasmanian devil populations have been declining due to a facial tumour disease since the 1990s with ongoing investigations examining potential causative agents. Identifying other causative pathogens which may contribute additively to their decline is important to preserve current and future populations. Leptospirosis has been shown to be present in other Tasmanian wildlife, including wombats, potoroos and deer, in the 1970s. However no contemporary studies are available. Eighty three Tasmanian devil samples were tested using three diagnostic methods. This study shows for the first time, that leptospirosis exists in Tasmanian devil populations across a wide geographical range.
of Tasmania. Antibodies to serovars in the serogroup Javanica, which are not considered endemic to Australia, have been identified in ten Tasmanian devils using the MAT. Serovar Celledoni was identified using the IgG MIA and one sample was detected using PCR. These results suggest that further work clarifying the prevalence and distribution of leptospirosis in Tasmanian devils is needed. More broadly, these technologies would be a beneficial addition to the conservation efforts of other Tasmanian wildlife populations as well.

Chapter 8 seeks to summarise the thesis as a whole. This chapter pulls together the key themes from each chapter and makes general conclusions about leptospirosis as an emerging infectious disease, its diagnosis, and its effect on human and animal populations worldwide, in particular, threatened Australian wildlife.
Declaration of Originality

I declare that this thesis is my own work, except where attribution has been indicated and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Sarah Jane Wynwood
28/7/2015
Acknowledgements

“As I look back at the last ten years I have realised that every time I thought I was being rejected from something good, I was actually being re-directed to something better”.

With this in mind, I would firstly like to thank the three people who not only helped me deliver this PhD but who believed in me enough to take a chance on me. To Dr Scott Craig, I can’t thank you enough for firstly, giving me the opportunity to join your team and, then for your encouragement, motivation and patience supervising this work. You opened my eyes to what could be, and so you have given me the confidence to do what I love, and say what I mean. I am extremely grateful for the time you spent helping me better myself professionally and personally and for teaching me new ways to take in the world around me.

To another great professional and personal role model, supervisor and friend – Dr Glenn Graham – my sincere thanks for your guidance, moral support and common sense - particularly in times of severe drought. Your compassion and understanding has allowed me to complete this chapter of my career with an amazing support system and a sense of self-belief.

To Dr David McKay, thank you for firstly for giving me the opportunity to complete this work and also for your immense support, patience and guidance as my principal supervisor throughout this project. I sincerely appreciate every minute you have spent wading through administrivia, teaching me fancy new words and keeping me on track.

I would also like to express my sincere appreciation and gratitude to Mary-Anne Burns and Michael Dohnt, my work colleagues and friends in the Leptospirosis Reference Laboratory. I loved every minute working with you and will be forever grateful for the learning experience, the language lessons (strudel) and the never ending story time. A huge thank you also, to Corinna Lange, the musical maestro of food chemistry. Working with you has been an absolute pleasure.
To my family, near and far, I thank you. Mum, a special thank you for being such an inspiration, pushing me to strive for the things I want out of life and making sure I never let “those” people bring me down. To Mum and Dad, Alli, Vaughan, Isla and Quade, Bob and Cossie, thank you for your unconditional love and support since forever, but particularly during the last few years. You’re an amazing bunch, I couldn’t ask for a better family. To Sherrie, Chloe and Jasmyn, my Queensland family, thank you for keeping things real, providing wine and entertainment when necessary and taking me out to Sizzler twice a year for Freddo Frog cake. You guys kept me sane and very happy. A big thank you to Wally, my best friend forever, for your ridiculous (but hilarious) jokes, your wonderful advice and for being the listening ears I have needed on so many occasions over the last few years. And to my Queensland Mum, Cathy. Words can’t express how thankful I am to have had you in my corner. You made the tough times easy and the easy times fantastic, a truly amazing woman.

And finally, to Ashley, you wonderful human being, thank you from the bottom of my heart for just being you. You’ve kept me on track and made sure I had the time, space and anything else I needed to complete this work. You’ve kept a smile on my face, food in my belly and a wine in my hand. Thank you for your love and support, your understanding and your patience. For this and so many other things, I love you.
# Table of contents

<table>
<thead>
<tr>
<th>Title Page</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synopsis/Extended Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Declaration of Originality</td>
<td>5</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>6</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>8</td>
</tr>
<tr>
<td>List of Tables</td>
<td>12</td>
</tr>
<tr>
<td>List of Figures</td>
<td>13</td>
</tr>
<tr>
<td>Publications arising from the thesis</td>
<td>14</td>
</tr>
<tr>
<td>Conference Presentations from thesis</td>
<td>15</td>
</tr>
<tr>
<td><strong>Chapter 1:</strong> Introduction</td>
<td>16</td>
</tr>
<tr>
<td>References</td>
<td>23</td>
</tr>
<tr>
<td><strong>Chapter 2:</strong> Leptospirosis and leptospires (the silent assassins)</td>
<td>26</td>
</tr>
<tr>
<td><em>(Springer Publications, 2014)</em></td>
<td></td>
</tr>
<tr>
<td>Publication Details</td>
<td>26</td>
</tr>
<tr>
<td>Statement of Contribution</td>
<td>26</td>
</tr>
<tr>
<td>Preface</td>
<td>27</td>
</tr>
<tr>
<td>Abstract</td>
<td>28</td>
</tr>
<tr>
<td>Leptospirosis and leptospires (the silent assassins)</td>
<td>29</td>
</tr>
<tr>
<td>References</td>
<td>41</td>
</tr>
</tbody>
</table>
Chapter 3: Leptospirosis from water sources
(Pathogens and Global Health, 2014)
Publication Details 51
Statement of Contribution 51
Abstract 52
Leptospirosis from water sources 53
References 64

Chapter 4: The emergence of *Leptospira borgpetersenii* serovar Arborea
as the dominant infecting serovar following the summer of natural
disasters in Queensland, Australia 2011
(Tropical Biomedicine, 2014)
Publication Details 71
Statement of Contribution 71
Preface 72
Abstract 73
The emergence of *Leptospira borgpetersenii* serovar Arborea
as the dominant infecting serovar following the summer of natural
disasters in Queensland, Australia 2011 74
References 82
<table>
<thead>
<tr>
<th>Chapter 5: Validation of a microsphere immunoassay for serological leptospirosis diagnosis in human serum by comparison to the current gold standard</th>
<th>85</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PLOS Neglected Tropical Diseases, 2015)</td>
<td></td>
</tr>
<tr>
<td>Publication Details</td>
<td>85</td>
</tr>
<tr>
<td>Statement of Contribution</td>
<td>85</td>
</tr>
<tr>
<td>Preface</td>
<td>86</td>
</tr>
<tr>
<td>Abstract</td>
<td>87</td>
</tr>
<tr>
<td>Validation of a microsphere immunoassay for serological leptospirosis diagnosis in human serum by comparison to the current gold standard</td>
<td>88</td>
</tr>
<tr>
<td>References</td>
<td>109</td>
</tr>
</tbody>
</table>

| Chapter 6: Serological diagnosis of leptospirosis in bovine serum samples using a microsphere immunoassay | 115 |
| (In Review, Veterinary Record Open, 2015) | |
| Publication Details | 115 |
| Statement of Contribution | 115 |
| Preface | 116 |
| Abstract | 117 |
| Serological diagnosis of leptospirosis in bovine serum samples using a microsphere immunoassay | 118 |
| References | 136 |
List of Tables

Chapter 1
1.1 Distribution of occupation among leptospirosis patients in West Bengal, India...pg. 20

Chapter 4
4.1 Leptospiral species used in the MAT panel...pg. 76

Chapter 5
5.1 Leptospiral cultures (antigens) used for validation assay...pg. 91
5.2 Positive sera used for standard curve...pg. 95
5.3 Cut-off points for reactivity equivalents of samples...pg. 96
5.4 Comparison of leptospirosis serology results for validation samples...pg. 98
5.5 MIA reactive, MAT non-reactive samples...pg. 99
5.6 Comparison of MAT and MIA sensitivity in paired samples...pg. 101
5.7 Assay repeatability...pg. 102

Chapter 6
6.1 Leptospira cultures (antigens) used in the MAT and bovine microsphere immunoassay with associated bead-set numbers...pg. 125
6.2 Reactive samples results...pg. 130

Chapter 7
7.1 List of Leptospiral Antigens...pg. 148
7.2 List of Serogroup Javanica Antigens...pg. 148
7.3 Tasmanian devil serum sample results...pg. 150
7.4 Summary of reactive samples...pg. 151
7.5 MAT reactive serovar Javanica samples against serogroup Javanica...pg. 152
List of Figures

Chapter 1
1.1 Scanning electron micrograph of *Leptospira interrogans* strain RGA...pg. 17

Chapter 4
4.1 Leptospirosis notifications for 2011...pg. 77

Chapter 5
5.1 Standard curve for cut-off points...pg. 96

Chapter 6
6.1 A comparison of the MIA and MAT reactive samples...pg. 127
6.2 Analysis of reactive samples by antibody type measured by MIA...pg. 128
6.3 Serovar analysis of a typical Hardjo reactive bovine sample...pg. 129
6.4 Non-specific cross reacting IgM sample...pg. 130

Chapter 7
7.1 Areas of sample collection...pg. 146
7.2 PCR Results...pg. 150
Publications arising from the work presented in this thesis


Publications submitted and in review from the work presented in this thesis


Conference presentations from the work presented in this thesis

1. 61st Annual Scientific Meeting of the **Australian Mammal Society**, 2015
   Presenting Speaker

**Abstract** A diagnostic study of Tasmanian devil samples for the presence of leptospirosis was undertaken. Tasmanian devil populations have been declining due to a facial tumour disease since the 1990’s with ongoing investigations examining potential causative agents. Identifying other causative pathogens which may contribute additively to their decline is important to maintain current and future populations. Leptospirosis has been shown to be present in other Tasmanian wildlife, including wombats, potoroos and deer, in the 1970’s, however no recent research is available. 83 Tasmanian devil samples were tested using three diagnostic methods. This study shows, for the first time, that leptospirosis exists in Tasmanian devil populations across a wide geographical range of Tasmania. Antibodies to serovar Javanica, which is not endemic to Australia, have been identified in ten Tasmanian devils using the MAT. Serovar Celledoni was identified serologically using the IgG MIA and one sample was PCR detected. These results suggest that further work on the prevalence and distribution of leptospirosis in Tasmanian devils, and more broadly, Tasmanian wildlife would be beneficial for the maintenance of Tasmanian devil populations.
CHAPTER 1

INTRODUCTION – Leptospirosis
Leptospirosis is the disease caused by organisms belonging to the genus *Leptospira*, which is in the family Spirochaetales. Organisms belonging to this family are characteristically slender, being only a fraction of a micron in diameter, but 5 to 250 microns long, and include the well-known pathogens, *Treponema pallidum* and *Borrelia Burgdorfi* (the causative agents of syphilis and Lyme disease, respectively). Leptospires were first isolated in Japan by Inada and Ido in 1915 (Inada, *et al.* 2016), almost 30 years after the clinical disease was described by Weil in 1886 (Weil, 1886). Although only relatively recently discovered, leptospirosis perhaps has a much longer history, possibly known earlier as Autumn fever (Dutta and Christopher, 2005), Yellow fever, Harvest fever or infectious jaundice.

Figure 1.1 Scanning Electron Micrograph of *Leptospira interrogans* strain RGA

(Carr, 2005)
Genus *Leptospira* is broadly divided into 20 species. There are nine pathogenic species, those that have been isolated from humans or animals, six saprophytic species, environmental non-pathogenic strains, and five intermediate species which are distinct from pathogens and saprophytes by their rRNA 16S sequence (Picardeau, 2012). There are currently over 300 serological variants of leptospires with more than 250 of these serovars known to be pathogenic. Serovars are currently classified into 24 serogroups by antigenic relatedness (Kmety, 1978) with the lipopolysaccharide (LPS) structure being the main serovar determinant.

Described as a zoonotic disease of global importance (Bharti, *et al.* 2003), leptospirosis occurs in urban environments in industrialised and developing countries as well as rural areas. It is currently considered an emerging zoonosis as the incidence is increasing worldwide (Vijayachari, *et al.* 2008) although misdiagnosis and underreporting suggest the incidence is perhaps much higher than what is actually reported. The incidence is significantly higher in warm climate countries than in temperate regions due mainly to the longer survival of leptospires in the environment in warm and humid conditions (Levett, 2001). Leptospirosis is a seasonal disease with outbreaks most often occurring in summer in temperate regions or in periods of heavy rainfall in warm climate regions. Factors associated with endemic leptospirosis include tropical climates, stagnant waters, poor levels of environmental sanitation and personal hygiene, occupational or recreational exposure and proximity of mammalian hosts to human populations (Levett, 2001). Extreme weather events which cause flooding are also risk factors for leptospirosis and are associated with numerous outbreaks worldwide (Lau, *et al.* 2010).
Rodents and domestic mammals including cattle, pigs, dogs and horses serve as the main animal reservoirs for pathogenic Leptospires (Aslantas, 2005), however leptospires have been isolated from many mammalian species, domestic and wild. Infected animals may excrete leptospires intermittently or regularly for months, years or a lifetime (Faine, et al. 1999) and vaccinated animals can also excrete leptospires in urine. In 1942, Tiffany and Martorana noted that in all parts of the world where research had been undertaken, 10-30% of the wild rats were shown to harbour leptospires in the kidneys. They also noted an occupational link with sporadic cases seen in what was known as the “civilised world” in sewer workers, fish cutters, miners, dock workers, restaurant workers and anyone exposed to rat-infested work places.

Human infection can occur as a result of exposure to infected urine or by direct contact with contaminated soil or water. Leptospires can also enter the host via the conjunctiva or the lungs following the inhalation of aerosols or via the mucosal membranes in the respiratory tract (Levett, 2001). Infections are commonly seen among workers in outdoor labouring jobs and agricultural industries who are continuously exposed to potential animal hosts (Table 1.1) and infected water and soil sources and infections are also commonly linked to recreational water activities (Monahan, et al. 2008).
Table 1.1 *Distribution of occupation among leptospirosis patients in West Bengal, India (Data from Majumdar, et al. 2013)*

<table>
<thead>
<tr>
<th>Work Category</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outdoor Manual Labourers</td>
<td>41 (53.24)</td>
</tr>
<tr>
<td>Agricultural Workers</td>
<td>18 (23.37)</td>
</tr>
<tr>
<td>Indoor Workers</td>
<td>11 (14.28)</td>
</tr>
<tr>
<td>Housewives</td>
<td>3 (3.89)</td>
</tr>
<tr>
<td>Unemployed</td>
<td>2 (2.59)</td>
</tr>
<tr>
<td>Students</td>
<td>2 (2.59)</td>
</tr>
</tbody>
</table>

The clinical signs and symptoms of leptospirosis can range from asymptomatic to fatal in humans and animals. The majority of infections are sub-clinical and result in mild self-limiting systemic illness (Levett, 2001) with non-specific symptoms including fever, vomiting, headache, abdominal pain, jaundice and muscle aches. If not diagnosed and treated early, leptospirosis can progress to a severe disease characterised by hepatic failure, renal and pulmonary dysfunction or haemorrhagic manifestations (Guerra, 2013). It has been suggested that many, if not most, cases of leptospirosis go unrecognised due to the lack of specificity of the signs and symptoms (Katz, 2001) and unreported due to poor surveillance due to inadequate laboratory capacity and clinicians inability to recognise the disease (Guerra, 2013). Confirmation of the diagnosis of leptospirosis has also proven difficult because of the problems associated with diagnostic methods, in particular, isolation of the organism and current serological methods.

Diagnosis of leptospirosis occurs at two stages. In the acute phase, which generally lasts from three to ten days, leptospires can be detected in blood in decreasing numbers until 15 days post onset of symptoms. Two methods are currently routinely used for diagnosis
in this phase. Polymerase chain reaction (PCR) for detecting specific leptospiral DNA is a relatively rapid method for detection but relies on specific storage conditions of samples and specific technology for analysis of results. A second method for acute phase diagnosis is culture isolation, followed by direct visualisation of the organism under dark field microscopy. This method requires specialised laboratory techniques and specially prepared culture media, and again relies on the correct sample collection and storage prior to analysis. Successful culture isolation can take anywhere up to 8 weeks due to the relatively slow growth of leptospires.

Serological methods are utilised for diagnosis in the immune phase which is characterised by the presence of antibodies in the blood. Enzyme-linked immunosorbent assays (ELISAs) and the current gold standard method, the microscopic agglutination test (MAT) are the two most common serological methods used for leptospiral antibody detection. These methods both suffer from many disadvantages. A reactive leptospirosis ELISA result gives no indication of the infecting serovar or serogroup, and it is not sufficient to diagnose a case of leptospirosis; it must be confirmed by MAT, PCR, or culture isolation (Picardeau, 2012). Whilst the MAT has a high degree of sensitivity and specificity (Cole, et al. 1973), it is a complex test that requires a large panel of live leptospiral antigens to provide adequate coverage of the antigenic diversity represented in a given testing area. The MAT is also a time consuming and costly test and interpretation of results must be performed by an analyst with specialised training.
Many challenges exist in the diagnosis of leptospirosis in both the acute and immune phases of the disease. Over time, better methods and new assays have improved diagnostic capabilities; however, current methodologies can still be immensely improved.
References


CHAPTER 2

Leptospirosis and leptospires (the silent assassins)

Publication Details


Statement of Contribution

Substantial contributions to the conception and design of this publication were made by SJW in consultation with all other authors. This paper was initially jointly-drafted by SBC and SJW. All authors contributed equally to reviewing, revising and approving the final version submitted for publishing.
Preface to CHAPTERS 2 and 3

The previous chapter gave a brief overview of leptospirosis. Chapters 2 and 3 are reviews of the (mostly) contemporary literature from different perspectives, and seek to highlight current problems and issues in leptospirosis. Chapter 2 gives a broad perspective of leptospirosis, and Chapter 3 examines leptospirosis from the perspective of leptospirosis as a water-borne disease.

One key theme emergent from Chapters 2 and 3—and germane to this thesis—is the reliance of serology in the diagnosis and epidemiology of leptospirosis, in particular the use of the current “gold-standard”, the microscopic agglutination test (MAT), in the serological diagnosis of leptospirosis in both animal and human populations.
Abstract

Leptospirosis is one of the most common yet under reported zoonoses. Leptospires, the etiological agents of leptospirosis are ubiquitous pathogens, with a world-wide distribution, causing a spectrum of disease ranging from a mild influenza-like illness to Weil’s disease, which manifests itself in multi-organ failure. The following chapter reports on the epidemiology and transmission of the disease in humans and animals. The chapter will also delineate the symptoms observed in humans and animals and in concluding outline unresolved and evolving issues for microbiologists, epidemiologists and public health officials.
Introduction

Leptospires are 6 to 20 μm in length and 0.1 to 0.2 μm in diameter and have optimal growth at 30°C (Levett, 2001). In the genus *Leptospira*, there are some 20 species (nine pathogenic, six saprophytic/environmental and five intermediate species). Serologically, there are more than 300 serovars and leptospirosis has been reported in over 150 mammalian species (Picardeau, 2013, Ko, Goarant and Picardeau, 2009). The main animal reservoirs include rodents, dogs, cattle, horses and pigs. These animals may act as maintenance hosts for adapted serovars such as serovar Canicola in dogs or serovars Ballum, Icterohaemorrhagiae or Copenhageni in rodents. Renal colonization and shedding of leptospires in the urine of infected animals sets the scene for the transmission of the organism to infect humans and other animals who are incidental hosts (Adler and de la Pena Moctezuma, 2009, Faine, et al. 1999).

Epidemiology in Animals

Epidemiological studies in animal populations, particularly in endemic regions, have relied on the use of serosurveys. Roqueplo, et al. (2013) conducted a cross sectional survey to estimate the prevalence leptospiral antibodies in wild and domestic animals in New Caledonia. This study reported that 43% of cattle, 72% of Rusa deer, 80% of horses, 43% of dogs and 100% of cats (n=8) had reactive leptospiral titres. Interestingly, members from the serogroups, Icterohaemorrhagiae, Australis, Canicola, Ballum and Cynopteri appeared to circulate in the majority of hosts investigated.

Similarly, Desvars, et al. (2013) conducted a serosurvey of 574 animals belonging to 12 species on Reunion Island and reported reactive leptospiral titres in approximately, 80%
of rats, 34% of cattle, 48% of pigs, 26% of cats and 47% of dogs. This study also investigated the renal carriage (leptospires in kidney tissues) of 10 animal species by qPCR and reported renal carriage in approximately 18% of cattle, 16% of pigs, 66% of rats, 85% of mice and 30% in cats and dogs. Leptospiral DNA was also detected in 2 samples of bat urine, consistent with previous suggestions of a rodent-bat infection cycle (Matthias, et al. 2005).

In a meta-review of leptospiral serosurveys undertaken over a 20 year period in Rio de Janeiro, Brazil, Martins and Lilenbaum (2013) reported seroprevalence rates of approximately 36% of rats, 73% of dogs, 38% of cattle, 40% of horses, 66% of pigs, 40% of wild animals excluding felines, 15% of wild felines and tamarins. The predominant infecting serogroups were Icterohaemorrhagiae, Sejroe, Australis and Pomona. Another study from Brazil also reported high seropositivity (71%) in 119 racehorses to serovar Copenhageni (Hamond, et al. 2012). Leptospirosis in horses is of interest because of the association with post infection recurrent uveitis which has been postulated to cost the United States equine industry in the vicinity of 100-250 million dollars per year (Verma and Stevenson, 2012). The high prevalence of reactive antibody titres and carriage of leptospires in rodents is well known in leptospirosis community. Recently in an ecological study of Leptospira interrogans in rats in Vancouver it was noted that increase weight, body fat and bite wounds increased the probability of infection in rodents (Himsworth, et al. 2013).
Epidemiology in Humans

The global burden of human leptospirosis is currently unknown, however, estimates of the annual incidence range from 0.1–1 case per 100,000 people in temperate areas to 100 cases per 100,000 during epidemics in tropical regions (Everard and Everard 1993; Levett, 2001). In addition, the incidence of leptospirosis is also higher in those environments prone to flooding (Lau, et al. 2010a). World-wide, an estimated 300,000–500,000 severe cases occur each year, with case fatality reports of up to 30% (WHO, 2003; Hartskeerl, 2006). In an attempt to develop a better understanding of the burden of leptospiral disease, the WHO (2011) estimate the global incidence in endemic areas exceeds 5 severe cases per 100,000. Given the lack of reporting in many developing areas, misdiagnosis, lack of awareness, patients failing to present for treatment, and those with subclinical infections, it is almost impossible to determine the true incidence.

The source of infection in humans is usually through either direct or indirect contact with the urine of an infected animal. Further, the usual portal of entry is via compromised cutaneous or mucosal membranes (Levett, 2001). Occupation is a significant risk factor as dairy and cattle farmers, veterinarians, abattoir workers, meat inspectors, rodent control workers and other occupations where intermit contact with animals is required, all have a greater chance of direct contact with the urine of infected animals. Occupations that bring humans into indirect contact with animal urine are also at risk of infection for example, sewer workers, miners, soldiers, septic tank cleaners, fish farmers, gamekeepers, canal workers, rice field workers, taro farmers, banana farmers and sugar cane workers. (Faine, et al. 1999, Levett, 2001, Tulsiani, et al. 2011). Recreational activities while travelling are also considered a risk factor for the disease (Lau, et al. 2010b).
In Europe as a whole, the overall incidence rate in 2010 was 0.13 per 100,000 inhabitants (Dupouey, et al. 2014). In Germany, there were 2694 reported cases of leptospirosis from 1962-2003. During this time period, the highest mean annual incidence was 0.11 per 100,000 in 1962-1967. The lowest mean annual incidence was 0.04 per 100,000 between 1992 and 1997 (Jansen, et al. 2005). In the Netherlands, there were 2553 (mainly severe) cases of leptospirosis reported from 1925-2008 although the average incidence was 0.25 per 100,000 of population. The incidence in the Netherlands showed a small decrease over the 84 year period and like Germany, male patients accounted for the majority of infections (Goris, et al. 2013b; Jansen, et al. 2005). In France, approximately 600 cases per year are diagnosed however half of these cases are from French overseas principalities. The incidence of 0.5 per 100,000 in mainland France is similar to that seen in Germany and the Netherlands. However, in the overseas territories, an average incidence of 1060 per 100,000 was reported between 2007 and 2009. In French Polynesia, the average incidence is 39 per 100,000 and in New Caledonia, the average incidence is 45 per 100,000 (Picardeau 2013).

The Asia Pacific region has some of the highest incidence rates for leptospirosis since high population densities are potentially at risk of leptospirosis (Victoriano, et al. 2009). This is not surprising given the frequent climatic calamities, overcrowding, poor sanitation, proximity of domestic and wild animals and occupational risks. In China over the past two decades, the average annual incidence was 0.7 per 100,000 inhabitants (Zhang, Wang & Yan, 2012).
Major outbreaks have occurred following flooding and heavy rainfall. Interestingly, 60% of cases in China are due to infection with *L. interrogans* serovar Lai. The principal vector for transmission is rats, such as *Apodemus agrarius* (Zhang, Wang & Yan, 2012). In South Korea, the predominate infecting serovar and vector are similar to that seen in China however, the incidence is lower. Between 1998 and 2011 in South Korea there were 1528 reported cases of leptospirosis giving rise to an incidence rate of 0.22 per 100,000 inhabitants (Kim 2013).

In Australia, the annual incidence is 8.9 cases per million (Pappas, *et al.* 2008). In North Queensland, leptospirosis is endemic as agriculture (such as banana and sugar cane farming), common to these areas, are high-risk industries. Seasonal changes have a direct impact on the incidence of the disease as the frequency of infection is highest during the wet season (January–April) whilst being relatively low during the dry season (June–December). Although the organism was only identified in the late 1990s, *L. borgpetersenii* serovar Arborea has emerged as the most dominant infecting serovar in Queensland (Tulsiani, *et al.* 2011; Wynwood, *et al.* 2014).

Leptospirosis is endemic in the Caribbean Islands and many parts of Central and Southern America. Pappas, *et al.* (2008) reported that the incidence in Trinidad and Tobago is 120.4 per million, Barbados 100.3 per million and Jamaica 78 per million. In El Salvador, Brazil and Argentina the incidence is 358, 12.8 and 9.5 per million respectively.

**Evidence of Human to Human Transmission**
Currently, reported evidence of human to human transmission is scarce. However, diagnosis of such transmission has been confirmed by serological testing. Bolin and Koellner (1988) reported the case of a 29 year old breast feeding mother who worked as a veterinarian and had a confirmed *L. interrogans* serovar Hardjo infection. The mother continued to breast feed during her illness and twenty-one days post-onset of symptoms, the infant displayed clinical signs consistent with leptospirosis. A positive result was confirmed by the microscopic agglutination test (MAT). In another report detailing possible human to human transmission, Harrison and Fitzgerald (1988) discussed the possibility of sexual transmission of *L. interrogans* serovar Icterohaemorrhagie. The diagnosis of this condition was also confirmed serologically by MAT.

**Evidence of Animal to Human Transmission**

Reports of human to human transmission are rare and as such Adler and de la Pena Moctezuma (2010, p.289) contend that ‘human to human transmission for practical purposes is non-existent and that leptospirosis is recognised globally as a zoonosis’. Since cases and outbreaks of leptospirosis are either unreported or misdiagnosed, it is not surprising that reports, which attempt to identify and track the course of leptospirosis outbreaks are rare. Recently, Li et al. (2013a) and Li et al. (2013b) used molecular methods, such as multi locus sequence typing and multiple locus variable-number tandem repeat analysis, to type isolates recovered from rodents in the Guizhou province in China. The authors found that the newly sequenced strains were consistent with serological investigations undertaken in leptospirosis patients from this province.
Desvars et al. (2013) reported that 16S gene sequencing identified four pathogenic genospecies which are responsible for human leptospirosis, have also been isolated in Mayotte rats.

**Disease Symptoms in Humans**

At present, the minimum infecting dose leading to leptospirosis is unknown, however, the incubation period is assumed to be inversely correlated with the size of the inoculum. For example, a high infecting dose may engender a short incubation period when compared to a low infecting dose. Conversely, small doses may result in prolonged incubation times which may extend into the immune phase. It is anticipated that these small infecting doses maybe responsible for mild or even sub-clinical infection (Faine, et al. 1999).

Once in the blood, leptospires are capable of circulating to all tissues. Leptospires that evade phagocytic cells of the reticuloendothelial system grow in an exponential manner doubling every eight hours (Faine, et al. 1999). There is evidence to suggest that phagocytosed leptospires do not survive long within the interior of the phagocyte (Vinh, Adler and Faine, 1982, Wang, et al. 1984). Virulent strains have the ability to attenuate phagocytic responses by activating apoptosis in the macrophage (Merien, Baranton and Perolat, 1998). Moreover, Adler and de la Pena Moctezuma (2009) write that the ability to resist complement and death by neutrophilic destruction may be a feature of virulent leptospires in non-immune hosts. Central to the pathology observed in leptospirosis is the damage caused to the endothelium of small blood vessels. This causes ischemia in target organs resulting in the renal, hepatic and pulmonary damage and thrombocytopenia. A number of leptospiral virulence factors, such as haemolysins, fibronectin binding proteins
and numerous surface proteins such as LipL32, Lig A, Lig B, lipoprotein Loa22 and the 6 Len proteins (LenABCDEF) are postulated to play a role in pathogenesis (Adler and de la Pena Moctezuma, 2009; Bulach et al. 2006; Hoke, et al. 2008; Matsunaga, et al. 2003; Merien, et al. 2000; Picardeau, et al. 2008; Ristow, et al. 2007; Stevenson, et al. 2007). Recently, L. interrogans catalase KatE and HtpG (high-temperature protein G is the bacterial homolog to the highly conserved molecular chaperone Hsp90) have also been shown to be virulence factors in leptospirosis (Eshghi, et al. 2012; King, et al. 2014).

Following the initial incubation period, the infection enters the acute phase of the disease which can last up to 10 days (Tulsiani, et al. 2011). Clinically, during the acute phase, patients typically present with headache, fever, excruciating myalgia and arthralgia and sometimes rigours, vomiting, photophobia and a mucosal rash (Faine, et al. 1999). Haemoptysis, hypotension and bradycardia are also common presentations. These symptoms are considered non-specific thereby making the diagnosis of leptospirosis difficult. Hepatosplenomegaly, jaundice (produced as a result of hepatocellular damage, increased erythrocyte destruction and the resulting increase in circulating haemoglobin and bilirubin), renal failure, liver failure and acute respiratory distress are common features of the more acute form of the disease (Sutliff, Shepard and Dunham, 1953; Solbrig, Sher and Kula, 1987; Faine, et al. 1999, Levett, 2001). Host factors, or more specifically, the activation of the innate immune system in which a myriad of cytokines are released (cytokine storm) in response to the invading pathogen, also play a central role in the clinical outcome (Reis, et al. 2013). Following the acute phase, patients enter the immune phase where immunoglobulins, specific for the destruction of leptospires, are produced to resolve the infection (Levett, 2001).
Disease Symptoms in Animals

Canines

The severity of Leptospirosis in canines may depend upon the size of the infecting dose, infecting serovar, age and health of the dog as well as vaccination status. Clinical signs may vary from the sub-clinical or asymptomatic infection with infections due to *L. interrogans* sv Canicola) to chronic infection characterised by chronic hepatitis and uveitis, sub-acute disease accompanied by pyrexia, anorexia, vomiting, renal failure and petechiae (Prescott, 2008; Sykes, *et al.* 2011). The acute and peri acute disease while uncommon, may result in coagulopathies, vascular injury and death (Prescott 2008). Other clinical signs include arched back, swollen tender kidneys, depression, melena and blood stained urine. Death can occur 36 hours to four days after the onset of symptoms. Serovars causing the more sinister clinical picture include serovars Australis, Grippotyphosa, Icterohaemorrhagiae, Autumnalis and Pomona. Regular vaccinations may assist to prevent severe disease however, the vaccines are serovar specific and do not engender protection against all possible infecting serovars (Faine, *et al.* 1999).

Felines

Given the predatory activities of cats towards rodents, it is reasonable to conclude that cats are at high risk for contracting leptospirosis. Surprisingly, the clinical presentation of diseased cats is rare even though there is greater seroprevalence of leptospiral antibodies in cats than in dogs (Roqueplo, *et al.* 2013). In addition, renal insufficiency and hepatic
inflammation may be evident in those animals that present with leptospirosis (Arbour, et al. 2012; Bryson and Ellis 1976, Lapointe, Plamondon and Dunn, 2013).

Equines

Clinical features of leptospirosis disease in horses include fever, anorexia, jaundice, mucosal petechiae and depression. Severe forms of the disease (respiratory failure) are more likely to occur in foals than adult horses. Reproductive catastrophes are common in infected mares. Antibodies directed toward leptospiral LruA and LruB proteins have been shown to cross-react with structures in the eye, thus, resulting in an auto-immune basis for equine recurrent uveitis or moon blindness (Verma and Stevenson, 2012, Verma, Stevenson and Adler, 2013).
Bovines

Cattle infected with serovars for which they are not maintenance hosts are more likely to display clinical disease especially if the host is young i.e. a calf. Clinical signs in cattle with acute disease include fever, pulmonary congestion, jaundice, haemoglobinuria and anaemia. Renal lesions may be observed at slaughter. In cows, the milk drop syndrome has also been observed. Chronic infections may also engender reproductive catastrophes (Faine, et al. 1999, Pearson, Mackie and Ellis, 1980).

Swine

Younger pigs are more likely to display acute leptospirosis compared with more mature swine. Clinical features are similar to those observed in other animals and include jaundice, weakness, haematuria, anorexia, renal failure and convulsions. Adult pigs are usually asymptomatic, however, may have renal lesions. Again reproductive catastrophes (abortions and stillbirths) occur as a result of maternal infection (Baker, et al. 1989, Faine, et al. 1999).

Unresolved issues

There are numerous unresolved issues that need to be addressed. Firstly, for nearly a century, culture and serology have underpinned the diagnostic practices of laboratories with an interest in this field (Martin and Petit, 1918). With the dawn and rapid advances in molecular diagnostics, there is now a plethora of molecular techniques available for
laboratories to undertake primary diagnostic and reference services. However, as Goarant (2014) argued, reconciling historical serological knowledge with modern molecular epidemiological practices remains a challenge as does identifying the most appropriate DNA targets and techniques for *Leptospira* spp. typing. As whole genome sequencing becomes less costly, less time consuming and less technically demanding, we are hopeful of gaining consensus and resolving these issues.

Secondly, the issue of chronic illness and the occurrence of post infection symptoms in patients needs a more comprehensive investigation. While neuropathies and mental illness may be considered persistent sequelae, 10% of patients may complain of uveitis and headaches for years (Faine, *et al*. 1999, Shpilberg, *et al*. 1990). In an interesting and much needed attempt to add framework to understanding the burden of human leptospirosis, Goris *et al*. (2013b) reported that 21.1% of their patient cohort frequently reported complaints such as myalgia and headache beyond 24 months post infection.

Finally, governments around the world are encouraged to invest more in public health initiatives focussing on surveillance and reporting structures for leptospirosis and educating medical officers and the public of the disease. Until such initiatives are universal, leptospires will remain silent assassins and serious attempts to understand and prevent leptospirosis will be futile.
References


CHAPTER 3

Leptospirosis from water sources

Publication Details


Leptospirosis from water sources, Pathogens and Global Health, Vol 108 (7) p. 334-338

Statement of Contribution

Substantial contributions to the conception and design of this paper were made by SJW in consultation with all other authors. This paper was initially drafted by SJW. All authors contributed equally to reviewing, revising and approving the final version submitted for publishing.
Abstract

Leptospirosis outbreaks have been associated with many common water events including water consumption, water sports, environmental disasters and occupational exposure. The ability of leptospires to survive in moist environments makes them a high risk agent for infection following contact with any contaminated water source. Water treatment processes reduce the likelihood of leptospirosis or other microbial agents causing infection, provided they do not malfunction and the distribution networks are maintained. Notably, there are many differences in water treatment systems around the world, particularly between developing and developed countries. Detection of leptospirosis in water samples is uncommonly performed by molecular methods.
Introduction

Leptospirosis is a worldwide zoonosis caused by spirochaetes from the genus *Leptospira*. The genus currently contains twenty species containing nine pathogenic, six saprophytic and five intermediate species (Picardeau, 2013). Leptospirosis infections in humans vary from asymptomatic to severe. Two phases of infection, acute and immune, are routinely characterised by a range of non-specific symptoms including fever, chills, headaches, conjunctival suffusion, excruciating myalgia and arthralgia and sometimes rigours, vomiting, photophobia, a mucosal rash, haemoptysis, hypotension, bradycardia, hepatosplenomegaly and jaundice are also common. Death can occur from kidney failure, pulmonary haemorrhage or other serious organ dysfunction (Fentahun and Alemayehu, 2012, Kasper, et al. 2005 and Craig, Greene and Sykes, 2006). However, the extent of organ damage is dependent on the virulence of the organism and host susceptibility (Faine, et al. 1999).

Laboratory findings show significant differences in haemoglobin concentrations, haematocrits, counts of erythrocytes, leucocytes, neutrophils, platelets and concentrations of creatinine, urea, protein and albumin when comparing those with mild to those with severe disease (Craig, et al. 2009).

Transmission of leptospirosis was first recognised as an occupational hazard in industries related to agriculture, sewer maintenance and animal husbandry and results from direct or indirect contact with the urine of infected animals (Picardeau, 2013 and Fentahun and Alemayehu, 2012). Other common modes of transmission include exposure to urine-contaminated water through recreational activities, adventure travel and ingestion...

Leptospires enter the body via small cuts or abrasions, through mucous membranes such as the conjunctiva and through wet skin (Fentahun and Alemayehu, 2012). Indirect exposure, and/or contact with contaminated water and soil, has been a major factor in numerous outbreaks and plays a crucial role in endemic settings.

This paper provides a review of leptospirosis cases with transmission linked to potentially contaminated water sources, the public health implications of leptospirosis and the current methods of diagnosis.

Outbreaks of leptospirosis have been associated with common water events such as rural and urban flooding, swimming and other water sports as well as occupational exposure involved predominantly with farming and drinking contaminated water (Cacciaputoi, et al. 1987, Wynwood, et al. 2014, Morgan, et al. 2002 and Faine, et al. 1999).

Both pathogenic and saprophytic strains of leptospires have been isolated from water sources including rivers and lakes as they are able to survive in moist soil and fresh water for long periods of time. Leptospires require fresh water to remain viable in the environment and can survive for several months in running water but only several weeks in stagnant water, while some halophilic strains may be recovered from brackish and salt water (Faine, et al. 1999, Trueba, et al. 2004, Smith and Self, 1955 and Khairani-Bejo, et
al. 2004). Recently, two strains of *Leptospira kmetyi* (MS432 and MS422) were shown to survive for 3 days in artificial seawater and natural seawater. When the seawater was mixed with soil the strains were able to survive for four days (Saito, et al. 2004). This finding warns of the possible risks the leptospiral infections in areas prone to ocean storm surges or tsunami.

Areas with high rainfall and warm climatic conditions provide optimal environments for the survival of leptospires. Most urban communities collect water from natural water-bodies such as rivers, streams or underground aquifers and then store this water for long periods of time in a reservoir. Hospital data and seroprevalence surveys in the United States indicates that more than 70% of leptospirosis infections can be attributed to physical contact with contaminated water supplies (Hawkins, et al. 2013). This shows that environmental detection is important in the development of adequate control measures.

Currently, detecting pathogenic leptospires in water samples is difficult due to filtering problems with the volume of the sample water and leptospiral concentration in the sample and the number of other potential bacteria present in water samples which can contaminate culture media. Further, there is currently no DNA-based methodology universally accepted to test water samples for the presence of leptospires and the effect of inhibitors on these molecular techniques also requires investigation. A number of molecular methods have recently emerged that will allow microbial agents to be detected in water samples.

A DNA microarray has been developed to detect leptospires and 10 other commonly occurring pathogens in drinking water (Zhou, et al. 2011). Another study which has
recently detected *Leptospira interrogans* in drinking water has used 454 pyrosequencing and Illumina sequencing to investigate bacterial virulence in drinking water (Huang, *et al.* 2014). The methodology this study used to collect bacterial cells for DNA extraction required the use of water purifiers to filter approximately 1000 litres of water. Collection and processing of such large samples may be problematic for many laboratories. Other studies have used smaller volumes with centrifugation to concentrate samples prior to extraction and PCR detection of pathogenic leptospires by targeting the *lipL32* gene (Munoz-Zanzi, *et al.* 2014).

**Transmission**

Transmission of leptospirosis is facilitated by the survival of pathogenic leptospires in moist environments outside of their mammalian hosts (Trueba, *et al.* 2004). Seasonal weather patterns involving flooding have long been recognised as a potential source of leptospirosis outbreaks and more recently, the contamination of drinking water and urban water supply has been implicated (Reynolds, *et al.* 2008). It is estimated that in the United States, water borne illness rates are approximately 16 million cases per year (Messner, *et al.* 2006). Worldwide, there have been many outbreaks specifically associated with water contamination, most commonly in areas where sanitation is poor.

Leptospires can survive for up to 152 days in fresh water by means of cellular aggregation and therefore water sanitation and hygiene are important factors in preventing and controlling the transmission of leptospirosis (Trueba, *et al.* 2004). One study found that the levels of leptospires in urban water sources (underground, streams and open gutters) were significantly higher than the levels of leptospires found in rural water sources in the
Peruvian Amazon regions of Iquitos Ganoza, et al. 2006). Further, the authors found that the incidence of leptospirosis infection (and the corresponding serovar) was a direct reflection of these results. Similarly, it was found that transmission of leptospirosis in Iquitos most likely occurs as a combination of environmental factors and human behaviour (Johnson, et al. 2004).

The importance of controlling environmental factors is highlighted in a recent study investigating household and environmental water source contamination by pathogenic leptospires in Chile (Munoz-Zanzi, et al. 2014). This study revealed that nearly 20% of human drinking sources and puddle water samples tested were contaminated with pathogenic leptospires. Not surprisingly the study also reported that lower income, increased temperature and the signs of the presence of dogs and rodents were associated with the contamination of some samples.

A number of studies have suggested that leptospirosis can be acquired from drinking contaminated water. Thirty-three confirmed cases of leptospirosis were attributed to contaminated drinking water in 1984 in a small town in Italy (Cacciapuoti, et al. 1987). Two deaths were attributed to this outbreak which was believed to be caused by drinking water from a fountain contaminated with leptospires of the serogroup Australis. There was evidence that a hedgehog became stuck and drowned in a water reservoir leading to contamination of the fountain water system. Although the fountain was not connected to the municipal water supply, people often drank directly from the fountain which is fed by rain water from the mountain where water reservoirs are located. There was no indication of water treatment post outbreak other than the removal of the dead animal from the
water reservoir. Samples were not taken from the hedgehog as it was believed that it had been dead for quite some time.

A similar event occurred in a nurses hostel in Chennai, South India when 69 residents tested positive serologically by microscopic agglutination testing (MAT) and their drinking water source tested positive by polymerase chain reaction (PCR) for leptospires in 2002 (Ramakrishnan, et al. 2003). The drinking water was sourced from an underground storage tank that filled a water tanker weekly. Collection was performed using a bucket on a rope and the tank was usually left open. Control measures were introduced to remove any further contamination of water sources including chlorination, boiling, education and the removal of large numbers of rats and mice in the area. Following the implementation of water sanitation and control methods, no further cases were detected.

A 48 year old man in Japan, who had a laboratory confirmed diagnosis of leptospirosis, infection (serovar *L. autumnalis*) was also believed to be infected after drinking water from a well following an earthquake (Aoki, et al. 2001). Although water testing was not performed, the well was slightly muddy and many rats had inhabited the area around the well following the earthquake.

Natural disasters including earthquakes, floods, typhoons, landslides and tsunamis have been linked to communicable disease outbreaks generally as a result of a lack of clean drinking water and sanitation facilities. Following typhoon Dina in 1987, an outbreak of leptospirosis occurred in two groups of US military personnel in Okinawa, Japan—one
group was exposed during training exercises, and the other group was exposed whilst engaging in recreational swimming (Corwin, *et al.* 1990).

In 1998 an outbreak of leptospirosis (52 cases) occurred following a triathlon in Illinois, USA (*Morgan, et al.* 2002). Investigations suggested that swallowing a mouthful of contaminated water was the only factor significantly associated with an increased risk of developing leptospirosis as sero-positivity was demonstrated in the full cohort of racers. Although it is not known whether a heavy rainfall event contributed to this outbreak, it shows that leptospires are able to survive in fresh water and be transmitted in this type of environment and circumstance.

Early guinea pig studies from Japan showed that the animals could be infected with leptospires by intraperitoneal, subcutaneous, or oral injection routes and death resulted in 5-10 days depending on the route of infection (*Inada, et al.* 1916). Given that oral infection has been reported it is surprising there is a paucity of research outlining more outbreaks of leptospirosis from drinking water. Whether this is due to a lack of reporting systems in developing countries, sub-clinical infections or protection/attenuation of infection from natural host defences, or a combination of these, is difficult to determine.

Recent research has shown that low passage, pathogenic leptospires rapidly agglutinate in saliva and the mucosal surface of the mouth is an effective barrier as submucosal injection of leptospires caused death but infection by drinking contaminated water did not (*Asoh, et al.* 2014). This research also revealed the utility of gastric acid in preventing infection as intragastrically infected animals displayed no sign of illness.
Public Health Perspectives

Outbreaks of leptospirosis have been attributed to a number of factors with a large proportion of infections resulting from contaminated water sources. *Leptospira* can survive in ponds, rivers, lakes, surface water and moist soil when the environmental temperature is warm and are generally transmitted through direct or indirect contact with the urine of an infected animal. Current prevention and control methods of leptospirosis consist of source/rodent reduction, environmental and water sanitation and hygienic work and personal practices. There is no universal control method applicable to all epidemiological settings as the characteristics of the environments differ from place to place. Understanding the eco-epidemiological and cultural characteristics of communities where leptospirosis is a problem is an essential prerequisite for evolving effective and acceptable control measures. Global climate change is also considered a factor contributing to leptospirosis as an emerging disease as increased temperatures are able to lengthen the survival of leptospires in the environment and can result in the expansion of habitats into higher elevations and latitudes (Chen, et al. 2011).

The water treatment processes in developing and modernised countries differ significantly. Poor water quality and sanitation accounts for 1.7 million deaths in developing countries each year, mostly in children (Ashbolt, 2004). Most infectious agents in water in developing countries are controlled by economically feasible methods such as chlorine treatment; however recontamination of the treated water is a major problem. In some countries, water is not treated by any methods—it is simply collected from non-reticulated and centrally controlled sources, such as wells, and consumed.
Factors including inadequate reservoir and storage design and construction, inadequate maintenance of storage facilities and poor quality control checks have also lead to the contamination and recontamination of drinking water (Pitkanen, et al. 2011). A study of drinking water sources in rural areas of Beijing found that well construction was a major factor in bacterial contamination of drinking water (Ye, et al. 2013). Shallow wells with open tops and no well housing were found to be most likely to have high bacterial contamination.

Collection of water for consumption in many developing countries is performed by hand, using buckets or urns to carry water from the drinking water source to the community or households which provides a means for contamination from environmental sources.

Informal water distribution supplies, such as private systems or community run systems have also been linked to high levels of microbial contamination in drinking water. In the slums of Mumbai, people rely on community run drinking water systems. Levels of microbial contamination in the water sources were assessed, and it was found that approximately 50% of water was contaminated (Subbaraman, et al. 2013). However, they noted that this contamination occurred post-source.

These are important considerations for the prevention of leptospiral contamination of water systems that may be exposed to animals shedding leptospires in their urine at or
near a water source or open storage area. Diligence should also be applied to feral animal, domestic animal and rodent control around these areas.

**Diagnosis/Detection**

The diagnosis of leptospirosis in blood samples from humans and animals is challenging as the majority of infections are subclinical or mild and leptospirosis usually presents as a non-specific acute, febrile illness (Picardeau, 2013, Fentahun and Alemayehu, 2012 and Faine, et al. 1999). Diagnosis of leptospirosis can occur at two stages of infection. The acute phase, bacteriemia, generally occurs between days 3 and 10 post-infection and can most effectively be diagnosed with molecular diagnostic methods such as PCR and blood culture isolation. During this stage, leptospires are present in blood and remain, in decreasing numbers, until approximately day 15 (Picardeau, 2013). The immune phase begins at approximately day 4 and can last up to day 30. During this phase, an increase in antibody response is correlated to the elimination of *leptospires* in the blood. Serological diagnosis methods including the MAT and enzyme-linked immuno-sorbent assay (ELISA) can determine an infection in this phase (Faine, et al. 1999).

Localised environmental detection is an important process in the development of control measures for leptospirosis. The detection of leptospires in water is mostly performed by molecular methods with culture methods still being utilised in some laboratories. The main issue with using leptospiral culture methods when performing environmental testing is the potential bacterial contamination in general and contamination with non-pathogenic leptospires specifically.
Quantitative real-time PCR and sequencing have been used to identify *Leptospira* species in human samples and water samples in Iquitos, Peru, to compare urban and rural environmental surface waters (Ganoza, *et al*. 2006). The authors found that the distribution of leptospires in human samples mirrored that found in environmental water samples. A PCR-based method has been designed which can differentiate between pathogenic and saprophytic (non-pathogenic) leptospires (Murgia, *et al*. 2006). As outlined previously, a DNA microarray has been developed to detect leptospires and 10 other commonly occurring pathogens in drinking water (Zhou, *et al*. 2011). Recently *Leptospira interrogans* in drinking water was detected using 454 pyrosequencing and Illumina sequencing (Huang, *et al*. 2014).

Currently, testing for leptospirosis in water samples is not common practice. Culture isolation is limited due to the presence of non-pathogenic leptospires in the environment. Whilst PCR-based tests have been developed to differentiate pathogenic from non-pathogenic leptospires, validated methods for testing leptospirosis in water samples have not yet been developed to a point where they are universally accepted or routinely performed. Such tests will be required to be sensitive and specific as well as robust, non-labour intensive and cheap to perform.
References


CHAPTER 4

The emergence of *Leptospira borgpetersenii* serovar Arborea as the dominant infecting serovar following the summer of natural disasters in Queensland, Australia 2011

Publication Details


Statement of Contribution

SJW was responsible for the conception and design of this study in consultation with the other authors. All sample analysis was jointly performed by SJW and MAB. SJW gathered and analysed all sample data presented in this paper and gathered all data from previous sample collection and analysis for review and inclusion in this paper. SJW drafted, edited and revised the initial manuscript. All authors then contributed equally to reviewing, revising and approving the final version submitted for publishing.
Preface to Chapter 4

This chapter seeks to show the need for improved diagnostic methods for leptospirosis. This is illustrated using an outbreak of leptospirosis as a case-in-point, where the MAT is used as an epidemiological tool. Indeed, most, if not all, epidemiological analysis of leptospirosis at the time this work was carried out was based on serology, employing the MAT.

As stated earlier in this thesis, the MAT, though heavily relied upon, has a number of disadvantages, and an emergent idea, though only tacitly stated in this chapter, is the need to develop improved methods of serological identification of leptospires, particularly in differentiating immunoglobulin classes and using more robust methods allowing automated, quantitative, high throughput analysis of samples.

As leptospirosis is a seasonal disease, large outbreaks often occur during times of extreme weather events including floods, cyclones and typhoons worldwide. Diagnosis of leptospirosis can prove difficult in these times due to the reliance on MAT.
Abstract

The following research reports the emergence of *Leptospira borgpetersenii* serovar Arborea as the dominant infecting serovar following the summer of disasters and the ensuing clean up in Queensland, Australia during 2011. For the 12 month period (1 January to 31 December) *L. borgpetersenii* serovar Arborea accounted for over 49% of infections. In response to a flooding event, public health officials need to issue community wide announcements warning the population about the dangers of leptospirosis and other water borne diseases. Communication with physicians working in the affected community should also be increased to update physicians with information such as clinical presentation of leptospirosis and other waterborne diseases. These recommendations will furnish public health officials with considerations for disease management when dealing with future disaster management programs.
Introduction

In late 2010 and early 2011, the state of Queensland in Australia experienced protracted, heavy rainfall which led to widespread flooding and destruction of rural and urban built environments. Subsequent to the widespread river flooding, coastal regions experienced the compounding effects of coastal flooding following the landfall of the Category 5 storm, Tropical Cyclone Yasi. The rain associated with the La Niña event combined with this natural disaster resulted in a yearly average rainfall of 826mm, some 203 mm above the previous 30 year average of 623 mm. In January, the average rainfall was 138 mm and in February the average rainfall was 133 mm. During both months, record levels of rainfall were recorded across many locations across the state. (Australian Bureau of Meteorology, 2012a-c). Following the aftermath and clean up, health complications associated with floodwater exposure were inevitable.

Leptospires, the etiological agents of leptospirosis are bacterial pathogens, ubiquitous in Queensland. Leptospires are tightly coiled (helical) spirochaetes that are approximately 6–20 μm in length and 0.1–0.2 μm in diameter. Leptospires infect the human host via cuts and abrasions in the skin or acquire direct access into the blood or lymphatics of the host via the conjunctivae or the lungs following the inhalation of water or aerosols (Levett, 2001). Leptospiral infections that occur in humans result from direct or indirect contact with infected soil, water, vegetation or body fluids from infected animals. Given optimum temperatures and pH values, leptospires may survive for weeks outside of their hosts. The general incidence of leptospirosis and the frequency of outbreaks of the disease tend to be high in areas where flooding is common (Tulsiani, et al. 2010).
In the following, we show the emergence of *Leptospira borgpetersenii* serovar Arborea as the dominant infecting serovar following the summer of disasters in Queensland, Australia 2011, which is significant since the organism has only been recently identified in Australia (Slack, *et al.* 2006).

**Materials and Methods**

The investigation was undertaken at the World Health Organisation Collaborating Centre for Reference and Research on Leptospirosis, Queensland Health Forensic and Scientific Services, Brisbane, Australia. The investigation began on January 1, 2011 with prospective surveillance and retrospective data collection and concluded on December 31, 2011. To be considered a recent leptospiral infection, patients needed to have a positive blood culture (*Leptospira* grown in Ellinghausen–McCullough–Johnson–Harris [EMJH] medium and observed by dark field microscopy). The leptospires cultured were later typed using the serum agglutination test (SAT) or the cross-agglutinin absorption test (CAAT) (Dikken and Kmety, 1978). Both the CAAT and SAT have high specificity for identifying infecting serovars circulating in Australia. A recent infection was also defined by either a positive IgM ELISA with a microscopic agglutination test (MAT) titre ≥ 400, or a 4-fold or greater rise in agglutination titre between the acute and convalescent (immune-phase) sera, obtained at least 10 days apart and tested in parallel. The end-point of the MAT reaction was deemed to be the dilution of serum that caused 50% agglutination (observed under dark-field microscopy), leaving 50% of the leptospires free (Faine, 1982, Anon, 1984, Tulsiani, *et al.* 2010). The MAT also has high specificity for identifying infecting serovars circulating in Australia. The MAT panel consisted of the following leptospires shown in Table 4.1.
Table 4.1 Leptospiral species used in the MAT panel.

<table>
<thead>
<tr>
<th>Species</th>
<th>Serovar</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  L. interrogans</td>
<td>Pomona</td>
<td>Pomona</td>
</tr>
<tr>
<td>2  L. interrogans</td>
<td>Hardjo</td>
<td>Hardjoprajitno</td>
</tr>
<tr>
<td>3  L. borgpetersenii</td>
<td>Tarassovi</td>
<td>Perepelitsin</td>
</tr>
<tr>
<td>4  L. kirschneri</td>
<td>Grippotyphosa</td>
<td>Moskva V</td>
</tr>
<tr>
<td>5  L. weilii</td>
<td>Celledoni</td>
<td>Celledoni</td>
</tr>
<tr>
<td>6  L. interrogans</td>
<td>Copenhageni</td>
<td>M20</td>
</tr>
<tr>
<td>7  L. interrogans</td>
<td>Australis</td>
<td>Ballico</td>
</tr>
<tr>
<td>8  L. interrogans</td>
<td>Zanoni</td>
<td>Zanoni</td>
</tr>
<tr>
<td>9  L. interrogans</td>
<td>Robinsoni</td>
<td>Robinson</td>
</tr>
<tr>
<td>10 L. interrogans</td>
<td>Canicola</td>
<td>Hond Utrecht IV</td>
</tr>
<tr>
<td>11 L. interrogans</td>
<td>Kremastos</td>
<td>Kremastos</td>
</tr>
<tr>
<td>12 L. interrogans</td>
<td>Szwajizak</td>
<td>Szwajizak</td>
</tr>
<tr>
<td>13 L. interrogans</td>
<td>Medanensis</td>
<td>Hond HC</td>
</tr>
<tr>
<td>14 L. kirschneri</td>
<td>Bulgarica</td>
<td>Nicolaevio</td>
</tr>
<tr>
<td>15 L. kirschneri</td>
<td>Cynopteri</td>
<td>3522C</td>
</tr>
<tr>
<td>16 L. borgpetersenii</td>
<td>Arborea</td>
<td>Arborea</td>
</tr>
<tr>
<td>17 L. interrogans</td>
<td>Bataviae</td>
<td>Swart</td>
</tr>
<tr>
<td>18 L. interrogans</td>
<td>Djasiman</td>
<td>Djasiman</td>
</tr>
<tr>
<td>19 L. borgpetersenii</td>
<td>Javanica</td>
<td>Veldrat Batavia 46</td>
</tr>
<tr>
<td>20 L. noguchii</td>
<td>Panama</td>
<td>CZ 214</td>
</tr>
<tr>
<td>21 L. santarosai</td>
<td>Shermanni</td>
<td>1342K</td>
</tr>
<tr>
<td>22 L. weilii</td>
<td>Topaz</td>
<td>94-79970/3</td>
</tr>
</tbody>
</table>
Results

For the 12 month period there were 154 confirmed new leptospiral infections. The most common infecting serovars were: *L. borgpetersenii* serovar Arborea with 76 (49%) recorded infections; *Leptospira interrogans* serovar Australis with 20 (13%) recorded infections; *L. interrogans* serovar Zanoni with 19 (12%) recorded infections and *Leptospira borgpetersenii* serovar Hardjo with 16 (10%) recorded infections. The majority of infections for the year occurred immediately following the floods in January, the cyclone in February and the ensuing clean up. The number of reported infections remained higher in the two months following the disasters than in the preceding year and the 10 year average (Figure 4.1).

Figure 4.1 Leptospirosis notifications for 2011
Discussion

The observed increase in the number of notifications of leptospirosis was consistent with previous investigations reporting an increase in leptospirosis following flooding (Lau, et al. 2010). First isolated in 1955 from a wood mouse (*Apodemus sylvaticus*) in Arborea-Sardegna, Italy, *L. borgpetersenii* serovar Arborea has subsequently, been reported throughout the world and has only recently been observed in Australia (Kmety and Dikken, 1993, Slack, et al. 2006).

Explanations as to why *L. borgpetersenii* serovar Arborea has emerged as the dominant infecting serovar in Australia is still in debate. Possible explanations may lie in changes in the distribution and abundance of animal reservoirs or ecological changes (Lau, et al. 2010). The research literature is replete with studies reporting disruptions to ecosystems which can lead to an increased risk of a number of vector borne and zoonotic disease such as Hantavirus and Lyme disease; (Allen et al. 2003, Mills, 2006).

Further, it has been reported that there is a significant negative correlation between the incidence of leptospirosis and the number of terrestrial mammalian species in a location (Derne, et al. 2011). Extinction, either natural (including natural disasters), or by the urbanisation of many regional centres, may provide the genesis for a decrease in the regulation of rodent populations by predatory species. Alternatively, the loss of species in a given area may result in the loss of the ‘dilution effect’ of having less competent host reservoirs in a location to absorb pathogens from the environment resulting in increasing pathogen proliferation and disease risk from the environment (Allan, et al. 2003, Mills, 2006).
A number of molecular variants of serovar Arborea isolated in Queensland have also been reported however it unclear whether the genomic plasticity of this serovar is affording serovar Arborea with a selective advantage (Slack, et al. 2010). Other members of the Ballum serogroup, for example, L. borgpetersenii serovar Ballum, L. borgpetersenii serovar Ballum 3/Guangdong, L. borgpetersenii serovar Kenya, L. borgpetersenii serovar Castellonis, L. borgpetersenii serovar Soccoestomes and L. santarosai serovar Peru, were not considered as they have not been isolated in Australia.

As with serovars Zanoni and Australis, the other infecting serovars with the highest incidence, mice and other rodents are associated with the transmission of serovar Arborea. Therefore, it is prudent for public health officials to advocate for stringent rodent control measures year round not only prior to any wet season. Rodent control measures should also be adhered to during any clean up phase following flooding. In response to a flooding event, public health officials need to issue community wide announcements warning the population about the dangers of leptospirosis and other water borne diseases. The warnings should also be accompanied by advice outlining the need to avoid entering flood waters unless it is absolutely necessary. Communication with physicians working in the affected community should also be increased to update physicians with information such as clinical presentation of leptospirosis and other waterborne diseases.

Given the logistical and compliance difficulties with community broad antibiotic prophylaxis and the lack of a safe multi-valent vaccine to cover the all the possible infecting serovars in a region, preventative control measures during flood recovery should include wearing gloves and other protective equipment such as enclosed foot wear.
Injuries sustained from the flood water should be washed and dressed with waterproof dressings.
References


CHAPTER 5

Validation of a microsphere immunoassay for serological leptospirosis diagnosis in human serum by comparison to the current gold standard

Publication Details


Statement of Contribution

SJW designed all experiments described in this paper after consultation with all other authors. SJW and MAB performed all serological and culture sample analysis. Molecular sample analysis was performed by SJW and SBC. SJW gathered and analysed all sample data presented in this paper. Literature reviews were carried out by SJW and SBC and relevant data extracted for further analysis and inclusion in this paper. The initial draft was written by SJW and reviewed by SBC and GCG. All authors contributed equally to reviewing, revising and approving the final version submitted for publishing.
Preface to Chapter 5

This chapter outlines the development of an alternative methodology to the MAT (microscopic agglutination test) using microsphere immunoassays (MIAs). The work described in this chapter sought to validate the MIA and to compare the MIA with MAT.

This work follows on from the work described in previous chapters in that it seeks to address some of the shortcomings seen with the MAT, which is still regarded as the current gold standard for leptospirosis serology. A thorough rationale for this work is described in the Introduction.
Abstract

A microsphere immunoassay (MIA) utilising Luminex xMap technology that is capable of determining leptospirosis IgG and IgM independently was developed. The MIA was validated using 200 human samples submitted for routine leptospirosis serology testing.

The traditional microscopic agglutination (MAT) method (now 100 years old) suffers from a significant range of technical problems including a dependence on antisera which is difficult to source and produce, false positive reactions due to auto-agglutination and an inability to differentiate between IgG and IgM antibodies. A comparative validation method of the MIA against the MAT was performed and used to determine the ability of the MIA to detect leptospiral antibodies when compared with the MAT. The assay was able to determine samples in the reactive, equivocal and non-reactive ranges when compared to the MAT and was able to differentiate leptospiral IgG antibodies from leptospiral IgM antibodies.

The MIA is more sensitive than the MAT and in true infections was able to detect low levels of antibody in the later stages of the acute phase as well as detect higher levels of IgM antibody earlier in the immune phase of the infection. The relatively low cost, high throughput platform and significantly reduced dependency on large volumes of rabbit antisera make this assay worthy of consideration for any microbiological assay that currently uses agglutination assays.
Introduction

Leptospirosis is considered to be the most widespread zoonotic disease in the world (Levett, 2001) with clinical diagnosis proving challenging due to the non-specific nature of symptoms associated with the disease. There are some 300 leptospiral serovars belonging to a number of different serogroups. Currently there are 24 sero-groups of pathogenic leptospires based on their antigenic relatedness (Kmety and Dikken, 1978).

Leptospirosis was first reported in Australia in 1933 in the state of Queensland and has since been isolated Australia wide (Slack, et al. 2006) with Queensland reporting the majority of these cases (57.6%) (NNDSS, 2005). In 2011 the reported incidence of leptospirosis in Queensland was 3.4 cases per 100,000 people and overall in Australia the incidence was 0.84 cases per 100,000 people (Burns, 2011). At present, 24 serovars of *Leptospira* spp are recognised in Australia and in recent years a dramatic increase in the incidence of leptospirosis cases in Australia (particularly Queensland) has been noted with environmental factors believed to be the main influence on this increase (Tulsiani, et al. 2010).

Diagnosis of leptospirosis occurs at two stages, and during the first (acute) phase the live organism can be detected by two methods. Polymerase chain reaction (PCR) testing is a useful molecular detection tool for rapid qualitative diagnosis of leptospirosis in its earliest stage (Levett, et al. 2004). Serum or blood samples provided for PCR testing must be collected within a precise timeframe (0-8 days post onset) to enable diagnosis. Blood culture isolation can also be utilised in the early stages of leptospiral infection (0-10 days
post onset), however this method is time consuming, requires specialised media and equipment and can take months for a serovar specific result (Levett, 2004).

The second (immune) phase of a leptospiral infection is characterised by the presence of leptospiral antibodies and diagnosis is based on serological methods with the microscopic agglutination test (MAT) considered the current gold standard (Faine, 1982). If the stage of the disease is unknown, both acute and immune phase tests are performed. Other serological test methods have previously been developed including flow cytometry (Yitzhaki, et al. 2004), complement fixation testing (Wolf, 1954), indirect hemagglutination assay (Levett and Whittington, 1998) an IgM dipstick assay (Smits, et al. 1999) and an IgM enzyme-linked immunosorbent assay (ELISA) in a number of formats (Adler, et al. 1980 and Terpstra, et al. 1984). Each of these assays has its advantages and disadvantages (Bajani, et al. 2003) and the type of assay used for diagnosis is generally dependant on the facilities available.

Serological diagnosis of leptospirosis in humans in Queensland, Australia is currently performed by screening with a commercially available leptospirosis IgM ELISA followed by the MAT as a reference and confirmatory test. The MAT method has many disadvantages as it requires specialist expertise, fresh cultures, is labour intensive, costly and is capable of determining total antibody only.

The current endemic routine panel for MAT testing in Queensland, Australia consists of 16 serovars, with representatives from a number of different serogroups. Each sample submitted for MAT is screened against this panel and any reactive samples are then
seriously diluted and retested to determine an end point. Results are reported as a titre with the end point being the final dilution of serum at which 50% or more of the leptospires are agglutinated. This assay permits the testing of up to 20 samples per day on a routine basis. The MIA has the ability to simultaneously test large numbers of samples against large numbers of serovars as well as determine individual IgG and IgM titres. These factors alone would be enormously beneficial in the laboratory diagnostics and epidemiological studies of leptospirosis.

Bead based suspension array technology (xMap, Luminex) has the capacity to multiplex up to 500 individual analytes in a single well and has been shown to be a successful diagnostic tool for serology in many applications (Anderson, et al. 2011, Van Der Wal, et al. 2011 and Dias, et al. 2005). This assay platform is based on magnetic coated polystyrene beads filled with two coloured fluorescent dyes in differing ratios resulting in 500 distinct bead sets. Each bead set can be coated with a different antigen and mixed to allow the simultaneous measurement of antibody response to up to 500 different antigens. This high-throughput screening system allows processing of high numbers of patient samples per day. Its speed, sensitivity, and accuracy of multiple binding events measured in the same small volume have the potential to replace many clinical diagnostic and research methods and deliver data on hundreds of analytes simultaneously Kellar and Iannone, 2002).

The microsphere immunoassay (MIA) that has been validated in this study was adapted from the method described by Luminex Corp (2000) and can be utilised as a routine serology testing protocol for leptospirosis.
The development and validation of a high quality, reliable serological assay is pertinent to the ability of a laboratory to sero-diagnose diseases in humans. Assay development begins with the identification of a need for improved diagnostic capabilities and the benefits that can be obtained from such an assay. A Luminex microsphere immunoassay (MIA) for leptospirosis antibody detection has the potential to function both as a high sensitivity, high throughput screening assay as well as a high specificity assay for determination of serovar level antibodies.

This paper assesses the leptospirosis MIA in human samples as a screening assay to determine reactive, equivocal and non-reactive samples. Validation is performed by comparison to the leptospirosis IgM ELISA and the current gold standard, the microscopic agglutination test (MAT) as the basis for defining the performance characteristics of the MIA.

Materials and Methods

Ethics

The study protocol was approved by the Public and Environmental Health Research Committee and the Humans Ethics Committee, Queensland Health Forensic and Scientific Services. All human samples utilised in this study were de-identified and allocated a generic number.
Antigens

Sixteen Australian endemic pure leptospiral cultures, Table 5.1, were grown for 5-7 days in 3mL EMJH broth at 30°C. These antigens were then quantitated using a Petroff-Hausser grid and centrifuged at 4°C for 25 mins. The supernatant was removed and the pellet resuspended in 500µL phosphate buffered saline (pH 7.5). All cultures were then diluted to obtain a concentration of 1.8 x 10^9 per mL. These diluted antigens were used to coat 16 individual Bio-Plex Pro Magnetic COOH Bead-sets. Coupled beads were then checked for sensitivity and specificity using rabbit anti-sera of known serovar and titre, obtained from MAT results (See method below).

Table 5.1: Leptospiral Cultures (antigens) used for Assay Validation

| L. Interrogans serovar Pomona   | L. Kirschneri serovar Grippotyphosa |
| L. Borgpetersenii serovar Hardjobovis | L. Weillii serovar Celledoni |
| L. Borgpetersenii serovar Tarassovi | L. Interrogans serovar Szwajizak |
| L. Interrogans serovar Australis | L. Interrogans serovar Medanensis |
| L. Interrogans serovar Zanoni    | L. Kirschneri serovar Bulgarica |
| L. Interrogans serovar Robinsoni | L. Interrogans serovar Copenhageni |
| L. Interrogans serovar Canicola  | L. Borgpetersenii serovar Arborea |
| L. Interrogans serovar Kremastos | L. Weillii serovar Topaz |

Serum Samples

This study utilised 200 serum samples which were selected from human serum samples submitted for routine leptospirosis serology to the WHO/FAO/OIE Collaborating Centre for Leptospirosis Reference and Research during 2012 and 2013. These samples were submitted from Queensland hospitals and private laboratories. One hundred and eighty
of these samples had leptospirosis IgM ELISA reactive serology, 12 had non-reactive leptospirosis IgM ELISA serology and the remaining 8 samples were not tested previously using leptospirosis IgM ELISA. All leptospirosis IgM ELISA testing was performed at a Queensland hospital or private laboratory prior to the samples being received at the WHO/FAO/OIE Collaborating Centre for Leptospirosis Reference and Research. Routine diagnostic MAT was performed on all samples at the WHO/FAO/OIE Collaborating Centre for Leptospirosis Reference and Research and results recorded against 16 routinely used, endemic serovars.

Forty-eight additional samples with reactive serology for Dengue Virus (24), Barmah Forest Virus (8), Ross River Virus (8) or Rabies Virus (8) antibodies were obtained from the Queensland Health Public and Environmental Health Virology Laboratory. These samples had previously been tested by ELISA IgM (Dengue virus), ELISA IgG (Rabies virus) or Alphavirus Haemagglutination Inhibition total antibody (HAI) (Ross River virus and Barmah Forest virus) and were used to assess whether cross reactions exist in the leptospirosis MIA.

In addition to the 200 samples used for the validation, 20 sets of paired samples with a non-reactive leptospirosis acute sample and reactive leptospirosis convalescent sample on the MAT were also obtained and analysed using the MIA to determine a timeline for the detection of leptospiral antibody. The results for these twenty additional samples are shown separately in Table 5.6.
**Bead Coupling**

Leptospiral antigens were covalently coupled to individual Bio-Plex Pro Magnetic COOH bead-sets using the Bio-Rad Amine Coupling kit and methods from Luminex Corp. Coupling is achieved via carbodiimide reactions involving the primary amino groups on the protein and the carboxyl functional groups on the bead surface. The bead yield per coupling reaction is approximately 2,500 beads per well (in a 96-well microtitre plate). For optimum results in the MIA, the coupled beads were diluted 1:4 in Triton-X detergent and 100 beads in 100µL buffer were used for the immunoassay. Each individual coupled bead-set was diluted in phosphate buffered saline (PBS) to give a reading of approximately 100 beads per bead-set per well. The working dilution and specificity of each bead-set was validated prior to use in a diagnostic capacity by utilising serovar-specific rabbit antisera and the IgG method as described below, substituting the secondary antibody with an anti-rabbit IgG (RPE). Bead-sets were considered to be valid for use if the targeted serovar produced an antibody response to that specific bead-set.

**Microsphere Immunoassay**

Two microsphere immunoassays (IgG and IgM) were performed on 200 serum samples taken from the routine MAT submissions which included samples with MAT titres (serial dilutions) ranging from < 1:50 (non-reactive) to 1:6400. Samples with an MAT titre between 1:50 and 1:200 were considered equivocal and samples with a titre 1:400 or above were considered reactive. Pooled convalescent serum from patients with recent leptospirosis infections, confirmed by PCR (on acute samples) and MAT, was used as the positive control serum in each microsphere immunoassay. Negative patient serum,
confirmed by negative PCR and serology) was pooled and used as negative control serum. These controls were monitored each run to ensure the assay was consistent.

A 96-well filter plate was pre-wetted with 150µL PBS per well and vacuum applied. One-hundred µL of the diluted coupled beads were then added to each required well of the pre-wetted 96-well microtitre filter plate and vacuum applied. Serum samples for the IgG immunoassay were diluted 1:400 in PBS in 1mL micronic tubes. One-hundred µL of the diluted samples were added to the plate which was then incubated for 45 minutes on a shaker (750rpm) at room temperature. The plate was then vacuum-washed three times with 150µL PBS per well. 100µL of a diluted secondary antibody (anti-human IgG) with a fluorescent tag (RPE) was added to each well followed by a second 45 minute incubation and vacuum wash as per previous step. Finally, 150µL PBS was added to each well and the plate placed back on a shaker at room temperature for at least 10 minutes prior to analysis.

Serum samples for the IgM immunoassay were treated with Siemens Rheumatoid Factor (RF) absorbent (at a dilution of 1:2) and diluted to a final concentration of 1:800 in PBS. The plate was prepared as per the IgG immunoassay. The secondary antibody - anti-human IgM with a fluorescent RPE tag - was used in this assay for conjugation.

**Analysis**

All plate wells were then analysed using Luminex xMap technology on a BioPlex 200 Platform. The MIA results were reported as mean fluorescent intensity (MFI) and were
deemed congruent or incongruent relative to the standard of comparison (MAT). Cut-off values for reactive samples were determined using five reactive sera for each MAT titre ranging from 1:100 to 1:6400 (Table 5.2), and developing a standard curve (R-Biopharm, 2012) using the titres obtained from MAT testing and comparing them with the mean fluorescent intensities from the MIA titrations.

Table 5.2 Positive sera used for standard curve

<table>
<thead>
<tr>
<th>MAT Titre</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:100 (5)</td>
<td>1511</td>
<td>1422</td>
<td>1586</td>
<td>1387</td>
<td>1675</td>
</tr>
<tr>
<td>1:200 (5)</td>
<td>2086</td>
<td>1879</td>
<td>1999</td>
<td>1986</td>
<td>2108</td>
</tr>
<tr>
<td>1:400 (5)</td>
<td>4158</td>
<td>3897</td>
<td>3956</td>
<td>4322</td>
<td>5186</td>
</tr>
<tr>
<td>1:800 (5)</td>
<td>7998</td>
<td>7258</td>
<td>7985</td>
<td>8065</td>
<td>8723</td>
</tr>
<tr>
<td>1:1600 (5)</td>
<td>10078</td>
<td>9985</td>
<td>10203</td>
<td>10406</td>
<td>11005</td>
</tr>
<tr>
<td>1:3200 (5)</td>
<td>12037</td>
<td>11896</td>
<td>11785</td>
<td>12403</td>
<td>13875</td>
</tr>
<tr>
<td>1:6400 (5)</td>
<td>15106</td>
<td>14759</td>
<td>14265</td>
<td>15089</td>
<td>15843</td>
</tr>
</tbody>
</table>

Figure 5.1 shows the reactive sera MAT titres plotted against the MFI’s and the standard curve that resulted. From this curve, cut-off points were determined (Table 5.3). Positive/negative ratios were used to determine the cut-off point for non-reactive samples. During the validation and determination of cut-off points the results reactive high and reactive low were used to ensure that the MAT and the MIA results were comparable. All patient results were reported as reactive, non-reactive or equivocal.
Table 5.3 Cut-off points for reactivity equivalents of samples

<table>
<thead>
<tr>
<th>MAT Titre</th>
<th>MIA IgG and IgM MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Reactive</td>
<td>&lt; 1:50</td>
</tr>
<tr>
<td>Equivocal</td>
<td>1:50 – 1:200</td>
</tr>
<tr>
<td>Reactive Low</td>
<td>1:400 – 1:1600</td>
</tr>
<tr>
<td>Reactive High</td>
<td>1:3200 +</td>
</tr>
</tbody>
</table>

Diagnostic Sensitivity

Sensitivity, the ability of the MIA to correctly determine the presence of leptospiral antibody, was determined by running known reactive (true positive) samples and calculating the proportion of reactive samples detected by the MIA. True positive samples
are samples known to be reactive by paired sample testing with the Gold standard, the microscopic agglutination test.

**Diagnostic Specificity**

Assay specificity was assessed using two methods. The first involves running known non-reactive (true negative) samples and calculating the proportion of non-reactive samples detected by the MIA. True negatives are defined as non-reactive samples known to be non-reactive by paired sample testing with the Gold standard assay, microscopic agglutination test. False positives are reactive samples determined by the test assay (MIA) that are non-reactive by the gold standard.

The second test of specificity ensured that samples that have been shown to have reactive serology for other pathogens are not cross reacting with the leptospirosis MIA.

**Assay Repeatability**

Within-run repeatability was determined by running four samples 20 consecutive times on one assay run for both IgG and IgM assays. Two of these samples had an equivocal result for at least one serovar on both assays, one sample had a reactive result for at least one serovar on both assays and the remaining sample was non-reactive for all 16 serovars for both IgG and IgM assays.

Repeatability is also monitored continuously as a quality control measure by monitoring positive (reactive) and negative (non-reactive) controls with expected and accepted MFI
ranges for each control serum in every assay. If the control serum results were outside of these ranges, the run was deemed to have failed and was repeated.

Results

MAT vs ELISA

Of the 200 samples tested, 180 samples were reactive for leptospirosis IgM by ELISA (Table 5.4). Twelve samples were IgM ELISA non-reactive and eight samples did not have previous IgM ELISA results; comparisons could only be made with the MAT and MIA for these eight samples. The MAT confirmed 27 of the leptospirosis IgM ELISA reactive samples had evidence of leptospiral total antibody and suggested that the remaining 153 IgM ELISA reactive samples were non-reactive (titre of < 1:50). These results suggest a substantial gap in the diagnostic performance of the ELISA and the MAT.

Table 5.4 Comparison of leptospirosis serology results for validation samples

<table>
<thead>
<tr>
<th></th>
<th>MAT (Total Ab)</th>
<th>MIA IgG and IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REACT</td>
<td>NR</td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reactive N = 180</td>
<td>27 (15%)</td>
<td>153 (85%)</td>
</tr>
<tr>
<td>Non- Reactive N = 12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Not Tested N = 8</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>
MIA vs MAT

The MIA results (in mean fluorescent intensity - MFI) for the 27 MAT reactive samples also indicated reactive serology (MFI > 1200). Of the 173 non-reactive MAT samples, 126 were non-reactive on the MIA and the remaining 47 had low reactivity on the MIA, suggesting better sensitivity in the MIA. The results for five of these 47 samples, which have been confirmed as true leptospiral infections by PCR or blood culture, are shown in

Table 5.5. MIA reactive, MAT non-reactive samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>ELISA</th>
<th>MAT</th>
<th>MIA IgG</th>
<th>MIA IgM</th>
<th>PCR</th>
<th>Blood Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>REACTIVE</td>
<td>&lt; 1:50</td>
<td>Equivocal</td>
<td>Non-Reactive</td>
<td>DETECTED</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>2</td>
<td>REACTIVE</td>
<td>&lt; 1:50</td>
<td>Equivocal</td>
<td>Non-Reactive</td>
<td>DETECTED</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>3</td>
<td>REACTIVE</td>
<td>&lt; 1:50</td>
<td>Equivocal</td>
<td>Non-Reactive</td>
<td>DETECTED</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>4</td>
<td>REACTIVE</td>
<td>&lt; 1:50</td>
<td>REACTIVE</td>
<td>Not Done</td>
<td>POSITIVE</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>REACTIVE</td>
<td>&lt; 1:50</td>
<td>Equivocal</td>
<td>Not Done</td>
<td>Not Done</td>
<td></td>
</tr>
</tbody>
</table>

MIA vs ELISA

The MIA detected leptospiral antibody in 74 (41%) of the 180 ELISA IgM reactive samples. The remaining 106 ELISA IgM reactive samples were non-reactive on the MIA and the non-reactive IgM ELISA samples were also non-reactive on the MIA. The 8 samples that were not previously tested by ELISA were non-reactive on the MIA also (Table 5.4).
Paired Sample Testing

Of the 20 sets of additional paired samples with an MAT non-reactive acute sample and MAT reactive convalescent sample, 12 of these pairs demonstrated equivocal or reactive IgM MFI results for the acute samples with a significant rise in MFI in the convalescent samples on the IgM MIA. The results for the remaining eight pairs of samples were consistent between the MAT and the MIA. Table 5.6 shows the results for the paired samples comparing the MAT titre and the MIA IgM and IgG results. These samples were included in this study to show that IgM can be detected earlier or, at least at the same time, by the MIA when compared with the MAT in true leptospiral infections, as determined by a four-fold rise in serology.

Table 5.6 Comparison of MAT and MIA sensitivity in paired samples
<table>
<thead>
<tr>
<th>Sample Pair</th>
<th>Phase</th>
<th>MAT Titre</th>
<th>MIA IgM</th>
<th>MIA IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acute &lt; 50</td>
<td>REACTIVE</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>2</td>
<td>Acute &lt; 50</td>
<td>REACTIVE</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>800</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>3</td>
<td>Acute &lt; 50</td>
<td>Equivocal</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>800</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>4</td>
<td>Acute &lt; 50</td>
<td>Equivocal</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>5</td>
<td>Acute &lt; 50</td>
<td>REACTIVE</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>Equivocal</td>
</tr>
<tr>
<td>6</td>
<td>Acute &lt; 50</td>
<td>REACTIVE</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>1600</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>7</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>800</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>8</td>
<td>Acute &lt; 50</td>
<td>REACTIVE</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>3200</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>9</td>
<td>Acute &lt; 50</td>
<td>REACTIVE</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>&gt; 6400</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>10</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>800</td>
<td>REACTIVE</td>
<td>Equivocal</td>
</tr>
<tr>
<td>11</td>
<td>Acute &lt; 50</td>
<td>Equivocal</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>800</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>12</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>Equivocal</td>
</tr>
<tr>
<td>13</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>Equivocal</td>
</tr>
<tr>
<td>14</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>Equivocal</td>
</tr>
<tr>
<td>15</td>
<td>Acute &lt; 50</td>
<td>Equivocal</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>1600</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>16</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>17</td>
<td>Acute &lt; 50</td>
<td>Equivocal</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>18</td>
<td>Acute &lt; 50</td>
<td>REACTIVE</td>
<td>Equivocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>800</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>19</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>800</td>
<td>REACTIVE</td>
<td>Equivocal</td>
</tr>
<tr>
<td>20</td>
<td>Acute &lt; 50</td>
<td>Non-Reactive</td>
<td>Non-Reactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>400</td>
<td>REACTIVE</td>
<td>REACTIVE</td>
</tr>
</tbody>
</table>

**Cross-reactivity**
Of the 48 reactive viral serology samples only one showed reactive IgG and IgM serology for leptospirosis (this sample was previously reactive for Dengue virus serology) and the remaining 47 samples were non-reactive for both leptospirosis IgG and IgM.

Repeatability

The four samples used to test within-run repeatability showed comparable results in each well across each of the 16 serovars. Table 5.7 shows the mean fluorescent intensity and standard deviation values for each of the four samples used in the repeatability testing for one of the serovars in the IgM immunoassay. Samples 1 and 2 were non-reactive. Sample 3 was reactive and sample 4 was in the equivocal range. The expected values were derived from comparison of the MIA mean fluorescent intensity with MAT titres. Repeatability was assessed across one run with one operator as, at the time of testing, only one operator was available to perform this testing.

Table 5.7 Assay repeatability

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean FI (IgM)</th>
<th>SD</th>
<th>Expected Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>104</td>
<td>4.99</td>
<td>&lt; 1200</td>
</tr>
<tr>
<td>2</td>
<td>795</td>
<td>78.46</td>
<td>&lt; 1200</td>
</tr>
<tr>
<td>3</td>
<td>4626</td>
<td>427.9</td>
<td>&gt; 2000</td>
</tr>
<tr>
<td>4</td>
<td>1626</td>
<td>79.43</td>
<td>1201 – 2000</td>
</tr>
</tbody>
</table>

Discussion
The aim of diagnostic serology is to determine reactive and non-reactive samples for a particular infectious agent. By definition, a validated assay consistently provides test results that identify samples as being reactive or non-reactive for a selected analyte, and, by inference, accurately predicts the disease status of patients with a predetermined degree of statistical certainty (Jacobson, 1998). The aim of this study was to validate a microsphere immunoassay (MIA) using Luminex xMap technology for diagnostic leptospirosis serology screening. The validation process was performed using a comparative method—that is comparing the new assay with the current gold standard assay. Sixteen leptospiral antigens have been coupled to 16 individual magnetic bead-sets and validated as a panel for routine diagnostic leptospirosis serology. This assay gives a qualitative result—Reactive, Equivocal or Non-Reactive—and has the ability to determine recent from past infection by differentiating between IgM and IgG antibodies—something that is not possible to achieve with microscopic agglutination testing (MAT) as this test can only determine total antibody. The class of antibody detected by the MIA can be used to determine the stage of the infection which is valuable for clinicians as it can determine treatment regimens for patients or in the case of a past infection, can suggest that something other than leptospirosis is causing symptoms. Information regarding new infections is also vital from a public health perspective as it can provide information on what serovars of leptospirosis are currently circulating and indicate the areas where these infections are occurring.

All leptospirosis serologically reactive samples by MAT were detected by MIA suggesting that congruence is 100% when compared to the MAT. Results from the non-reactive samples, as well as the paired samples suggest, however, that the MIA is more sensitive
than the MAT. In true infections (as demonstrated by paired sample serology testing with a minimum four fold rise in titre) the MIA was able to detect low level antibody in the later stages of the acute phase as well as pick up higher levels of IgM antibody earlier in the immune phase of the infection. The MAT results indicated that these samples were non-reactive in the acute/early immune phase. The MAT generally becomes positive between day 8 and day 10 of infection (Picardeau, 2013) however, results from this validation suggest that the MIA could detect antibody in the earlier stages of infection development and increase the likelihood of the clinician submitting a convalescent sample for confirmation of infection status.

The leptospirosis IgM ELISA has previously been shown to have poor specificity, as low as 41%, when used according to the manufacturer’s instructions (Picardeau, 2013). All leptospirosis IgM ELISA reactive samples tested in Queensland pathology laboratories are sent to the WHO/FAO/OIE Collaborating Centre for Leptospirosis Reference and Research for confirmation testing. In this study it was found that of 180 leptospirosis IgM ELISA reactive samples only 15% (27/180) of these showed reactive results on the MAT. This could be due to a lower level of antibody which is not detected by the MAT at a dilution of 1 in 50 or a non-specific antibody reaction. In this study, 41% (74/180) of the leptospirosis IgM ELISA reactive samples had reactive IgG and/or IgM serology on the MIA, again suggesting the level of antibody in these particular samples may be too low for the MAT to detect. Also, this again shows that there may be some non-specific reactions occurring in the IgM ELISA, which are not seen on the MIA. The MIA is therefore advantageous as a screening test as it reduces the large numbers of samples that are unnecessarily sent for confirmation testing by MAT. It has also been suggested that false
positivity can also occur in the leptospirosis IgM ELISA due to the presence of persistent IgM from past infections (Tanganunchitcharnchai, et al. 2012). The MIA screening test eliminates these results by looking at the levels of the individual IgG and IgM antibodies across paired specimens. A low level or non-reactive IgM result and a plateaued reactive IgG would be suggestive of a past infection – something not currently visible on the leptospirosis IgM ELISA or the MAT.

The MIA results suggest that the beads coated with leptospiral antigen are specific for leptospiral antibodies and show no cross-reactivity with other viral agents. The one case in this study where a Dengue Virus reactive serology sample also showed leptospiral antibodies is likely to be a true leptospirosis infection occurring simultaneously with a Dengue Virus infection. Leptospirosis and Dengue Virus infections are both common in northern parts of Queensland (where this sample was from) as they are both associated with tropical and sub-tropical regions where extreme weather events occur (Lau and Weinstein, 2010). In many cases samples are submitted for both arbovirus testing (including Dengue virus) and leptospirosis testing at the same time.

The results from the MIA show that reproducibility is possible and accurate when compared to the MAT. A major disadvantage of the MAT is attenuation of the live leptospiral cultures. It has been shown that over time, leptospiral cultures lose their antigenicity and therefore become less effective (Tulsiani, et al. 2011). Also, day to day, the cultures can be different – more or less dense or contaminated - which makes reproducing results accurately a difficult task on the MAT. This issue is overcome with the MIA as the antigens (leptospiral cultures) are wild type cultures with a known passage
number and are all diluted to a known concentration ($1.8 \times 10^9$) prior to the bead coupling process. This ensures that there are equal amounts of each antigen available in every test.

Another major advantage of the MIA over the MAT is that there is no need to maintain stocks of live leptospiral cultures for daily use. Pure cultures are only used in the MIA as antigens for bead coupling and these antigens can be centrifuged, diluted and frozen at $-20^\circ$ C for up to six months (Biorad, 2005). Currently, performing the MAT on a routine basis requires sub-culturing more than 200 tubes per week, maintaining four stocks of cultures.

When comparing the MAT and the MIA the advantages of the latter are obvious. Firstly, the MIA is less time consuming – a full plate of 88 samples can be run in around three hours. To run the same number of samples on the MAT, it would take twice the time for a full panel of 16 serovars excluding analysis. The MIA is also less labour intensive as it does not require adding 16 individual cultures to each well on a 96 well plate for each individual patient. These savings combined as well as the reagent costs suggest that the MIA is also less costly than the MAT. An analysis of laboratory and assay costs shows that the current diagnostic serology method (MAT) is performed at a cost of $AUD6.95 (excluding labour) to the leptospirosis reference laboratory per sample per 16 serovars (Craig, 2014). In comparison, the MIA costs $AUD4.95 per sample (excluding labour) per 16 serovars. Secondly, the MIA uses a total of 7µL of serum (2µL for the IgG assay dilutions and 5µL for the IgM assay dilutions) compared with 50µL of serum used in the MAT. Thirdly, the MIA has the ability to detect and differentiate both IgG and IgM antibodies whereas the MAT can only detect total antibody and cannot give an accurate indication of the stage of infection in a single sample. The MIA can potentially include up to 500
analytes in the one assay, therefore, there is potential to be able to include all known leptospirosis serovars (~250) in one test at one time. Given the number of bead-sets available for microsphere immunoassays other applications could potentially involve the inclusion of a number of different viral and bacterial agents in one assay. For example, leptospirosis antibody detection and Dengue Virus antibody detection could be combined into one routine diagnostic test.

In conclusion, the results from this validation suggest that the leptospirosis MIA is a beneficial diagnostic screening tool for leptospirosis serology testing. This assay is able to determine reactive, equivocal and non-reactive samples when compared to the MAT. It is able to differentiate leptospiral IgG antibodies from leptospiral IgM antibodies which will provide vital diagnostic information as well as provide a better epidemiological picture. Further investigations will include validation of each individual serovar to enable serovar specific results to be reported and validation of a microsphere immunoassay for detection of leptospiral antibodies in animal samples will also be looked at in the future.

Data Availability

Due to patient confidentiality laws, data will not be made public but is available on request from the QHFSS Ethics Committee: Contact: Dr Charles Naylor, Phone: +61 7 3274 9051. Email: Charles.naylor@health.qld.gov.au
Acknowledgments

Technical advice

Taylor, C.T. Queensland Health Public and Environmental Health Virology Laboratory (Serology), PO Box 594, Archerfield, QLD 4108.

Dohnt, M.F. WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis, Queensland Health Forensic and Scientific Services, Po Box 594, Archerfield, Queensland, 4108


R-Biopharm (2012) www.r-biopharm.com
References


CHAPTER 6

Serological diagnosis of leptospirosis in bovine serum samples using a microsphere immunoassay

Publication Details

Statement of contribution
SJW designed all the experiments described in this paper in consultation with all other authors. SJW, with technical support from M-AB, performed all sample testing and analysis. SJW gathered and analysed all data presented in this paper. SJW performed literature reviews and extracted data relevant to this study for presentation. The initial draft of this paper was written by SJW and reviewed by SBC and GCG. All authors then contributed equally to reviewing, revising and approving the final version submitted for publishing.
Preface to Chapters 6 and 7

Chapters 6 and 7 describe work adopting an applied focus using both MATs and MIAs for leptospirosis detection in bovine (Chapter 6) and Tasmanian Devil (Chapter 7) populations. These chapters seek to draw some conclusions about the incidence of leptospirosis in these disparate populations, as well draw some conclusions about the efficacy of the both MIAs and MATs and to compare and contrast their strengths and weaknesses.
Abstract

Leptospirosis causes significant economic loss within the cattle industry worldwide. Current diagnostic methods are generally inadequate for dealing with large numbers of samples, are outdated and provide limited diagnostic and epidemiological information.

The aim of this study was to apply a microsphere immunoassay (MIA), utilising Luminex xMap technology, to 200 bovine serum samples to determine this method’s usefulness in leptospirosis diagnosis in comparison with the current gold standard, the microscopic agglutination test (MAT).

Although the MAT is the most widely used laboratory test for the diagnosis of leptospirosis, its reliance on live cultures; subjective interpretation of results and an inability to differentiate between antibody classes suggest the MAT is no longer the best method for the diagnosis of leptospirosis. The MIA was able to determine reactive from non-reactive samples when compared to the MAT and was able to differentiate IgG and IgM classes of antibody. The results suggest increased sensitivity in the MIA and the ability to multiplex up to 500 antigens at one time allows for significant improvements in cost effectiveness as well as a reduced dependency on live cultures. The relatively low cost, high throughput platform and differentiation of antibody class make this assay worthy of consideration for the diagnosis of leptospirosis in small or large scale bovine populations.
Introduction

Leptospirosis infections are widely recognised as a common cause of reproductive failure and economic loss in cattle. *Leptospira Borgpetersenii* serovar Hardjo and *L. Interrogans* serovar Pomona have been shown to be the most important pathogenic serovars in cattle, responsible for systemic illness, abortion, neonatal death, weak calves and various production losses throughout the world (Bolin and Alt, 2001).

Serovars causing infection in cattle have been split into two groups, those adapted to and maintained by cattle (serovar Hardjo) and incidental infections caused by serovars maintained by other domestic and wild animals (Aslantas, 2005). In Australia, serovars Hardjovis, Pomona and *L. Borgpetersenii* serovar Tarassovi are the most commonly serologically diagnosed serovars (Cousins, *et al*., 1985) with infection rates varying from state to state. *L. Weilii* serovar Topaz has also become a commonly diagnosed infecting serovar in Australia since its isolation in cattle in 1994 (Corney, *et al*. 2008).

Leptospiral infections in cattle are generally subclinical, produce low antibody titres and can affect animals with rapid transmission rates. Infections in cattle can occur through mucous membranes or through abrasions in the skin with direct transmission occurring among animals through exposure to infected urine, post abortion uterine discharge or through milk (Hairgrove, 2004). Serovar Hardjo is associated with a prolonged renal carrier state and may also be associated with chronic renal disease (Hairgrove, 2004) suggesting that leptospires are present in the urine for long periods of time. Leptospires within the proximal renal tubules, genital tract and mammary glands of cattle have been shown to be protected from circulating antibodies (Ellis, 1994) which allows persistence and
multiplication in these areas. Serum antibody levels often decline to undetectable levels in chronic leptospirosis infections in cattle, making diagnosis extremely difficult in many cases.

Serological diagnosis of leptospirosis in cattle is most often performed by microscopic agglutination testing (MAT) and can also be performed by enzyme-linked immunosorbent assay (ELISA). These methods have been shown to have many disadvantages. In a review of laboratory techniques for diagnosing leptospiral infections in cattle, Smith, *et al.* (1994) highlighted the many issues with the current diagnostic methods, in particular the microscopic agglutination test (MAT). Leptospiral antibodies can persist in cattle with titres of >1:400 for up to 12 months and in some cases for up to two years (Smith, *et al.* 1994). However, agglutinating antibodies commonly wane over time and the sensitivity of the MAT in detecting these antibodies in animals infected for more than two years is low (Allen, *et al.* 1982).

Previous serologic studies (Prescott, *et al.* 1988) have shown that due to the low sensitivity of the MAT, seroprevalence may in fact be double the reported figures for specific serovars in cattle. It has been suggested that increasing the sensitivity by decreasing the initial serum dilution (Blackmore, 1985) may resolve the under-reporting of seroprevalence, however, this may also increase the rate of false positives. Smith *et al.* (1994) also points out that a major concern with MAT testing of cattle samples is that there is no differentiation between IgG and IgM antibodies and therefore vaccination status and efficacy cannot be determined. Paired serum samples are currently required to estimate the stage of infection. With the MAT however, determining the stage of infection
can be difficult in cases of bovine abortion or still-birth as infection most commonly occurs 1-4 weeks prior to the expulsion of the foetus and by this time MAT titres have stabilised (Cousins, et al. 1985). Paired sample collection can also be difficult due to the costs involved in sample collection and testing. Few false positive results occur in the MAT as the surface antigens of leptospires are not shared with any other organisms, however cross-reactions do occur within and between some leptospiral serogroups (Kmety and Dikken, 1978).

The use of live leptospires as antigens in the MAT is also a disadvantage of this test. Problems associated with this include the lack of antigen standardisation and quality control and that culture maintenance is both time consuming and costly. The MAT utilises a specific panel of antigens for each test depending on the region of sample collection, availability of antigens and cost effectiveness. Therefore, another disadvantage of this assay is, in areas where endemic strains are unknown, or poorly characterised, diagnosis of leptospirosis may be excluded due to a lack of utilising the correct panel of antigens.

The use of the leptospirosis enzyme-linked immunosorbent assay (ELISA) for testing cattle samples has some advantages over the MAT, including the use of inactivated antigens and objective analysis and determination of antibody class. However, there are still some issues with this type of test. The ELISA has been shown to be more sensitive than the MAT (Cousins, et al. 1985) but in human samples it is often too sensitive and produces large numbers of false positive results. Although the ELISA has some advantages over the MAT, it is not used routinely as a diagnostic test for leptospirosis as it is unable to provide serovar specific results for large numbers of serovars. Bercovich, et al. (1989) evaluated
and confirmed the ELISA method for the specific detection of Hardjo infections and found it to be an advantageous alternative to the MAT. However, cross reactions are often seen with leptospirosis and the ELISA cannot determine the source of cross-reactions in one test. The need for more sensitive, specific and high throughput diagnostic testing for leptospiral infections in cattle has been highlighted previously (Gardner, 1996).

This study aims to show that a microsphere immunoassay (MIA) is a beneficial diagnostic tool in the detection of leptospiral antibodies in bovine serum by comparison to the MAT. The MIA is a highly sensitive, highly specific and high throughput test capable of simultaneously analysing up to 500 analytes in one test. As there are currently more than 300 known leptospira serovars, a multi-plexing assay such as the MIA potentially allows for the comprehensive screening and serovar differentiation for large numbers of samples in a short amount of time, reducing labour, time and costs when compared to the MAT (Wynwood, et al. 2015). In Australia, serovars Hardjo, Pomona, and L. interrogans serovar Zanoni (McClintock, et al. 1993) and serovar Topaz (Corney, et al. 2008) have been isolated in cattle while common serological reactions include serovars Hardjo, Pomona, Tarassovi, L. interrogans serovar Australis, L. interrogans serovar Szwajizak, L. interrogans serovar Medanensis and L. interrogans serovar Kremastos (Corney, et al. 1993). Utilising Luminex xMap technology, the MIA is able to provide quantitative, objective, serovar specific results in a fraction of the time of the MAT and without many of the factors that inhibit the continuing success of the MAT. The MIA utilises inactivated, quantitated antigens which are stable in the long term (BioRad, 2005) and do not require the constant culture maintenance required for the MAT. The ability to manage and monitor quality control of
the antigens is a major advantage over the MAT and provides a stable basis for rigorous method validation and quality assurance.

In Australia, leptospirosis research in bovine populations has halted over the last decade. Thorough serological investigations will provide a better picture of current circulating serovars and the physiological and epidemiological effects of leptospirosis in Australian cattle. Utilising the leptospirosis microsphere immunoassay for this purpose enables cheaper, faster and higher quality testing to be performed on a much larger scale when compared with the MAT.
Materials and Methods

Ethics

The study was approved by the Public and Environmental Health Research Committee and the Humans Ethics Committee, Queensland Health Forensic and Scientific Services. The ethics approval number for this project is HEC 13-17.

Samples

Bovine serum samples for routine leptospirosis serology were submitted to the WHO/FAO/OIE Collaborating Centre for Leptospirosis Reference and Research during 2013. These samples were submitted from veterinary laboratories around Australia. Samples (n=200) were selected, de-identified and tested by the microscopic agglutination test (MAT), with a panel of 12 endemic serovars. The samples had MAT titres (serial dilutions) ranging from < 1:50 (non-reactive) to 1:800 for various endemic serovars. Samples with an MAT titre of 1:50 and above were considered reactive. There was no associated information available for the samples indicating vaccination status, clinical signs and symptoms or reasons for testing.

A microsphere immunoassay (MIA) for leptospirosis diagnosis in humans (Wynwood, et al. 2015) was adapted for use with bovine serum samples. Although this assay can utilise up to 500 antigens, a panel of twelve Australian endemic leptospiral serovars were used in this assay to detect leptospiral IgG and IgM antibody in bovine serum samples. The current MAT panel utilised for routine bovine sample testing at the WHO/FAO/OIE Collaborating Centre for Leptospirosis Reference and Research, Queensland, Australia, consists of only three serovars – Hardjo, Pomona and Tarassovi. Serovar Topaz is also
included for completeness since its isolation in a bovine urine sample in Australia in 1994 (Corney, et al. 2008). Two microsphere immunoassays (IgG and IgM) were performed on the 200 bovine serum samples.

**Antigen Preparation**

Antigen preparation for use in the MIA utilised 12 pure leptospiral cultures, which are shown in Table 6.1. Cultures (antigens) were centrifuged, washed and diluted in phosphate buffered saline (PBS) to give a cell density of $1.8 \times 10^9$ per mL and further diluted 1:4 in Triton-X 100 detergent. Residual cell viability was checked by culture: 25µL of each antigen preparation was suspended in 1mL EMJH broth and the cultures were checked under dark field microscopy every 3 days for 21 days. No leptospires were detected. The diluted antigens were then coupled to individual bead sets as shown in Table 6.1. Coupled beads were checked for sensitivity and specificity using rabbit anti-sera of known serovar and titre, obtained from previous MAT results.
Table 6.1 Leptospira cultures (antigens) used in the MAT and bovine microsphere immunoassay with associated bead-set numbers.

<table>
<thead>
<tr>
<th>Serovar</th>
<th>COOH Bio-Plex Magnetic Bead-set Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. Interrogans serovar Pomona</td>
<td>45</td>
</tr>
<tr>
<td>L. Borgpetersenii serovar Hardjobovis</td>
<td>27</td>
</tr>
<tr>
<td>L. Borgpetersenii serovar Tarassovi</td>
<td>35</td>
</tr>
<tr>
<td>L. Interrogans serovar Australis</td>
<td>26</td>
</tr>
<tr>
<td>L. Interrogans serovar Zanoni</td>
<td>28</td>
</tr>
<tr>
<td>L. Interrogans serovar Robinsoni</td>
<td>34</td>
</tr>
<tr>
<td>L. Interrogans serovar Canicola</td>
<td>52</td>
</tr>
<tr>
<td>L. Interrogans serovar Szwajizak</td>
<td>44</td>
</tr>
<tr>
<td>L. Interrogans serovar Medanensis</td>
<td>43</td>
</tr>
<tr>
<td>L. Kirschneri serovar Grippotyphosa</td>
<td>54</td>
</tr>
<tr>
<td>L. Borgpetersenii serovar Arborea</td>
<td>20</td>
</tr>
<tr>
<td>L. Weilii serovar Topaz</td>
<td>29</td>
</tr>
</tbody>
</table>

Antigen Coupling

Leptospiral antigen preparations were covalently coupled to individual Bio-Plex Pro Magnetic COOH bead-sets (Table 6.1) using the Bio-Rad Amine Coupling Kit, producing 160μL of each coupled bead-set. The bead yield per coupling reaction was approximately 2,500 beads per well (in a 96-well microtitre plate). Each individual coupled bead-set was further diluted in phosphate buffered saline (PBS) to a working dilution, just prior to performing the assay, to give a reading of approximately 100 beads per bead-set per well as per the manufacturer’s instructions.
Microsphere Immunoassays

Samples for the IgG immunoassay were diluted 1:200 in PBS and conjugated with biotinylated Sigma Protein A. Samples for the IgM immunoassay were diluted to 1:400 in PBS and conjugated with KPL Biotin-Labelled Bovine IgM antibody.

100 μL of the working dilutions of coupled beads were added to each required well of a 96-well microtitre filter plate and vacuum applied. The diluted samples (100 μL) were then added to the plate and incubated for 45 minutes on a shaker at room temperature (18 – 24°C). The plate(s) were then vacuum-washed three times with 150 μLPBS per well. Biotinylated secondary antibodies were then added, followed by a second 45 minute incubation and vacuum washed as previously described. 100 μL of diluted streptavidin R-PE, a biotin-binding protein, was added to each well followed by a final incubation and wash step. 150 μL of PBS per well was added to the plate which was placed on the shaker at room temperature (18 – 24°C) for ten minutes for re-suspension of beads. All plate wells were then analysed using Luminex xMap technology on a BioPlex 200 Platform. The MIA results were reported as mean fluorescent intensity and were deemed congruent or incongruent relative to the standard of comparison (MAT). Cut-off points for determination of reactive versus non-reactive results were based on previous human sample assays (Wynwood, 2015) and established using known reactive and known non-reactive bovine serum samples (reactive for serovar Hardjo) and comparing the mean fluorescent intensity levels with the MAT antibody titre levels.
Results

Testing of the 200 bovine serum samples (see Figures 6.1 and 6.2) revealed a greater number of reactive samples using the MIA compared to the MAT; 64 (32%) were reactive by MAT as defined by the cut-off (titre = < 1:50), whereas using the MIA, 110 samples (55%) were deemed reactive. The majority of the MIA reactive samples (75) were IgM reactive only, 13 were IgG reactive only and 22 were reactive for IgM and IgG. The remaining 136 (68%) MAT samples were non-reactive (titre of < 1:50) and the remaining 90 (45%) MIA samples were non-reactive (MFI < 1200).

Figure 6.1 *A comparison of the MIA and MAT reactive samples.*
Anti-leptospiral antibody was detected in an extra 46 samples in the MIA when compared to the MAT. This suggests that the MIA may be more sensitive than the MAT as more reactive samples were detected using the MIA method. The decreased sensitivity in the MAT, compared with the MIA, may also be due to the effects of auto-agglutination if the age of the MAT cultures is not optimal, heavy culture density or as a result of contamination in the microtitre plate. However, of the 64 MAT reactive samples, only 53 (83%) were also reactive on the MIA. The remaining 11 (17%) had low level MAT titres (1:50 – 1:100) and were non-reactive on the MIA.

Serovar Hardjo was the dominant infecting serovar in the reactive MAT (32%) and reactive MIA IgG (54%) samples. Figure 6.3 shows the MIA IgG and MAT results for one bovine sample.
These results show the typical antibody pattern seen in *L. hardjo* infections in cattle in Australia when tested against all twelve serovars. Typical cross reactions are seen with members of the same serogroup (Sejroe) including serovar Medanensis as well as serovar Szwajizak (Mini serogroup) and serovar Pomona (Pomona serogroup) as expected (Corney, *et al.* 1993).

Figure 6.3 *Seroivar analysis of a typical Hardjo reactive bovine sample*

Table 6.2 shows the breakdown of the serovar results for this assay. The majority of the IgM serum samples, 74 (76%), resulted in non-specific reactions as expected with serology samples at an unknown stage. Non-specific reactions are those where a sample has reactive serology for more than one serovar where there is a difference of less than 1.5 times MFI.
Table 6.2 Reactive samples results

<table>
<thead>
<tr>
<th>Serovar</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arborea</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Australis</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Hardjo</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Zanoni</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topaz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robinsoni</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tarassovi</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Medanensis</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Szwajizak</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pomona</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Canicola</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Non-Specific</td>
<td>2</td>
<td>74</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td>35</td>
<td>97</td>
</tr>
</tbody>
</table>

The most commonly seen serovars in non-specific IgM reactions were *L. Interrogans* serovar Australis, *L. Interrogans* serovar Canicola, serovars Topaz and Tarassovi (Figure 6.4) which are all serovars which are associated with Australian domestic animals and wildlife.

Figure 6.4 Non-specific cross reacting IgM sample
Discussion

Diagnostic testing plays an important role in monitoring and maintaining the health of livestock, detecting exposure to and identifying infectious agents in an individual infection or a herd epidemic (Gardner, et al. 1996). The results of this study suggest that using the microsphere immunoassay (MIA) for diagnostic testing of bovine samples is more cost effective, has better quality control, and is capable of providing more valuable diagnostic information when compared with the current routine diagnostic method, the MAT. The MIA has many advantages over other available methods of diagnosis. The main advantage is the high throughput of this assay with the ability to simultaneously include large numbers of serovars with relatively little cost when compared to the MAT (Wynwood, et al. 2015). It can be utilised to test large numbers of cattle samples in a small space of time making it an ideal test for vaccine efficacy studies, management of large cattle populations and disease monitoring. Previous studies have indicated that the ability to increase numbers of bovine samples tested will improve epidemiological information (Hathaway, Little and Pritchard, 1986) and provide better diagnostic information relating to prevalence of the disease and specific serovars than what is currently available with the MAT. Although the ELISA has been shown to be specific for serovars when coated with the desired serovar antigen (Smith, et al. 1994) the multiplexing ability of the MIA allows potentially up to 500 pathogens in one well at one time. The major disadvantage of the ELISA is that to achieve the same results each sample would need to be tested on individually coated plates for each antigen.

Low level antibody was detected in samples on the MIA that were non-reactive on the MAT suggesting that the sensitivity of the MIA may be greater than that of the MAT. The ability of the MIA to detect low level antibody is beneficial in bovine serum sample testing as diagnostic
titres remain present for a limited time and cannot always be detected by the MAT. It has also been shown that low level IgM can persist for up to 2 years in bovine samples (Worthington, 1982) and these titres are not always at a level high enough for the MAT to detect. Using the MIA, these persistent low level antibodies could be detected and monitored, giving a more thorough indication of the progression of leptospirosis in cattle.

Although a reactive serology result does not always indicate current infection (Milner, Wilks and Calvert, 1980) a diagnostic assay with the ability to determine antibody class does make the determination of the stage or progress of infection possible. Agglutinating antibody titres after vaccination are generally lower than those following a natural infection (Kingscote and Proulx, 1986). Given the higher sensitivity of the MIA, it may be possible to pick up post-vaccination IgG antibody as post-vaccination antibodies decrease rapidly (Marshall, et al. 1979). Allen et al. (1982) showed that 95% of vaccinated cattle did not show antibody on the MAT 20 weeks post-vaccination. The absence of the reactive MAT does not mean that protection has waned – it may be that the antibody is at a level below the detectable limit of the MAT. Infections with serovar Hardjo, in particular, can be difficult to diagnose serologically. In one study of abortion caused by infection with serovar Hardjo, 22.8% of aborting cattle had no detectable antibodies (by MAT) at the time of abortion (Ellis, et al. 1982). A similar study (Ellis et al. 1981) found that 19.6% of Hardjo renal carriers had no detectable agglutinating antibodies by MAT. In these types of cases, a more sensitive test, such as the MIA, would be beneficial.

Maintenance host disease is often difficult to diagnose as it is generally subclinical (Hairgrove, 2004), producing low antibody titres and has rapid transmission rates. In maintenance hosts,
leptospirosis is generally characterized by a high prevalence of infection, relatively mild clinical signs, and persistent infection in the kidney and sometimes the genital tract. The serovar Hardjo IgG reactive results in this study are possibly the result of previous vaccinations, which, in Australia, include serovars Hardjo and Pomona (Virbac, 2012), but could also be the result of past infections. In this study, there was no information available on the clinical or vaccination status of each animal therefore it is difficult to confirm the cause of the reactive IgG antibody. However, in the absence of IgM antibody, it is plausible that there was no current infection at the time of sample collection. The high number of samples showing low level IgM may occur as a result of an acute infection but may also be due to a chronic infection. Chronically infected cattle are difficult to diagnose with the MAT as the titres often fall below detectable levels (Smith, et al. 1994) and no differentiation of antibody class is possible.

It is often assumed that a static antibody level in the MAT is indicative of a past infection only, however this may also be the result of chronic infection with persistent IgM. 75% of IgM reactive samples in this study showed non-specific IgM, reflecting the constant challenge within the herd and incidental transmission from other domestic and wild animals. Leptospirosis has been found in many wild animal species in Australia including wallabies, possums, rats (Milner and Wilks et al. 1981), Eastern grey kangaroos (Roberts et al. 2010) and feral pigs (Mason and Fleming, et al. 1998). The most common serovars noted in these cases are Pomona, Tarassovi and Australis (Roberts, et al. 2010) which is consistent with what is being seen in cattle with low level cross-reacting IgM antibody reactions. These reactions may also suggest early-immune phase infections prior to maturation of antibodies.
The MAT is heavily dependent on the experience (read, in-part, subjectivity) of the user, as the results are determined as the titre with the end point being the final dilution of serum at which 50% or more of the leptospires can be seen to be agglutinated. Depending on the experience and the knowledge of the analyst, these results can offer differ from well to well. An advantage of the MIA is that the level of antibody present determines the intensity of machine-detected fluorescence. Whilst arguably, no methodology is entirely objective, the MIA moves in a direction away from the MAT’s heavy dependence on the interpretation of the user.

Another major disadvantage of the MAT is that this test utilises live leptospires as antigens which causes problems in antigen standardisation, loss of antigenicity after frequent sub-culturing, and handling as the pathogens are live and therefore infective. The MIA has the advantage in that the live leptospires are inactivated during the coupling process and there is no need for continuous sub-culturing as the antigens prepared for bead coupling are stable for at least 6 months at -20°C (Biorad, 2005). Further studies will determine whether this shelf life may be extended. Eleven samples that had a reactive MAT result of 1:100 or less were reported as non-reactive by MIA with an MFI of <1200. This may be due to auto-agglutination, poor culture quality, contamination or incorrect subjective analysis – problems which are frequently noted with the MAT.

The results from this study suggest that the MIA is a beneficial diagnostic tool able to differentiate between IgG and IgM antibody classes, reduce costs and test large numbers of samples against many serovars at one time. Further work will need to be performed on this assay for validation purposes and in other animal species however from this data, it is a
suitable and preferable method of diagnosis over the MAT. Future sero-survey data utilising the MIA will assist in determining the sero-prevalence and variations of disease pattern and impact of leptospirosis serovars in cattle in Australia. Utilising the MIA it is possible to process large numbers of samples at minimum cost to gain a better picture of the leptospirosis infections in cattle and potentially, associated wildlife.

Acknowledgements

Technical advice

Taylor, C.T. Queensland Health Public and Environmental Health Virology Laboratory (Serology), PO Box 594, Archerfield, QLD 4108.

Dohnt, M.F. WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis, Queensland Health Forensic and Scientific Services, Po Box 594, Archerfield, Queensland, 4108

Funding

This study was approved by the Forensic and Scientific Services (FSS) Research and Development Advisory Committee, Queensland Health and funded by FSS Operational Funding, Queensland Health Communicable Diseases Department.
References


Chapter 7

Leptospirosis in Tasmanian Devils

Publication Details

Wynwood, SJ, Burns, M-A, Graham GC, Weier, SL, McKay, DB, Peck, S and Craig, SB.


Statement of Contribution

SJW designed all experiments described in this paper in consultation with all other authors. SJW, with technical support from M-AB performed all serological sample testing and analysis. SJW performed all molecular sample testing and initial analysis. SJW gathered and analysed all sample data presented in this paper. The initial draft was prepared by SJW and reviewed by SBC, GCG and SP. All authors then contributed equally to reviewing, revising and approving the final version submitted for publishing.
Abstract

A diagnostic study of Tasmanian devil samples for the presence of leptospirosis was undertaken. Tasmanian devil populations have been declining due to a facial tumour disease since the 1990s with ongoing investigations examining potential causative agents. Identifying other pathogens which may contribute additively to their decline is important to preserve current and future populations. Leptospirosis has been shown to be present in other Tasmanian wildlife, including wombats, potoroos and deer, in the 1970s. However no contemporary studies are available. Eighty three Tasmanian devil samples were tested using three diagnostic methods. This study shows for the first time, that leptospirosis exists in Tasmanian devil populations across a wide geographical range of Tasmania. Antibodies to serovars in the serogroup Javanica, which are not considered endemic to Australia, have been identified in ten Tasmanian devils using the MAT. Serovar Celledoni was identified serologically using the IgG MIA and one sample was detected using PCR. These results suggest that further work is needed on the prevalence and distribution of leptospirosis in Tasmanian devils. More broadly, these technologies would be a beneficial addition to the conservation efforts of other Tasmanian wildlife populations as well.
Introduction

Leptospirosis is a disease affecting humans and animals worldwide. Patterns of infection and clinical manifestations in humans and domestic animals are dependent on a number of factors including climate, location, presence of maintenance and accidental hosts and serovars present (Zwijnenberg, et al. 2008). The genus *Leptospira* is currently made up of 18 species classified on the basis of DNA relatedness and more than 300 serovars based on agglutinating LPS antigens (Victoriano, et al. 2009). The transmission of leptospirosis can occur as a result of direct contact through abrasions and open wounds exposed to infected animal urine or as a result of indirect contact through contaminated water and soil. The disease can range from asymptomatic to acute and fatal in both animals and humans with severe forms of disease characterised by hepatic involvement, acute renal failure, carditis and haemorrhagic syndrome (Yersin, et al. 2000). Diagnosis of leptospirosis is dependent on the stage of the disease. In the acute phase, polymerase chain reaction (PCR) and culture isolation from blood or tissue are the most reliable diagnostic tests as they determine the presence of leptospiral organisms. In the immune phase of the disease, serology is used to determine the presence of specific leptospiral antibodies. The microscopic agglutination test (MAT) is the current gold standard for leptospirosis serology; however technical difficulties with this test have made way for a new, improved diagnostic method, the microsphere immunoassay (MIA) (Wynwood, et al. 2015). If the stage of the disease is unknown, generally all tests are performed. It is often difficult to diagnose leptospirosis in animal populations due to problems associated with finding and or identifying individual sick animals and sample collection.
In Australia, leptospires have been isolated in all states and territories, first noted in Ingham in Queensland in 1933 from human autopsy samples (Morrissey, 1934) and Australia wide by 1950. It has been suggested that Australian wildlife may be of importance in the transmission of leptospirosis to both humans and domestic animals (Milner and Wilks, 1981). In 1968, Corbould isolated serovar Icterohaemorhaggiae from rats (*Rattus norvegicus*) in Tasmania and found serological evidence of the same serovar in greyhound dogs in the same area. A serological study of Tasmanian wildlife, including Gunn’s bandicoots (*Perameles gunnii*), potoroos (*Potorous tridactylus*), wombats (*Vombatus ursinus*), European hares (*Lepus europaeus*) and fallow deer (*Dama dama*) found evidence of serovars Pomona, Grippotyphosa and Hyos (now known as serovar Tarassovi) as well as Icterohaemorhaggiae (Munday, 1972). Whyte and Burke (1973) found evidence of leptospirosis in cattle and pigs in the North West region of Tasmania and serological reactions have also been recorded in sheep (Corbould, 1970). These findings suggest that Tasmania may host many wild and domestic animal reservoirs for leptospirosis. Previous studies into the presence of Serovar Hardjo in Tasmania suggest that leptospirosis occurs widely throughout the beef and dairy industry (Corbould, 1970) and within other Tasmanian fauna including wombats and rats (Milner and Wilks, 1981). Of the three cases presented by Whyte and Burke (1973), all associated with farm animals, serovars Hardjo and Pomona were the most common.

In 1973, it was suggested that leptospirosis may have been one of the significant causes of morbidity among farm workers in Tasmania (Whyte and Bourke, 1973) following the serological diagnosis of serovar Hardjo in beef and dairy cattle in the north of the state. In 1969, four people involved in milking cows were hospitalised with clinically diagnosed
leptospirosis showing a significant rise in titre to serogroup *L. hebdomadis* (now known to be serovar Hardjo) (Corbould, 1970). The cattle associated with these cases were serologically shown to carry the same organism.

Very little research into the prevalence of leptospirosis in Tasmanian animals has been published. The State Department of Agriculture carried out some studies of the distribution of leptospirosis in Tasmania in animals in 1971 (cited in Whyte and Burke, 1973) noting the presence of serovars Pomona and Hardjo in pigs and cows only. Wildlife studies of leptospirosis in Tasmania have so far been limited to wombats, hares, potoroos, bandicoots and deer with Tasmanian devils *Sarcophilus harrisii*, not mentioned here. Tasmanian devils are the largest extant marsupial carnivores and are found only in Tasmania, Australia. They have most recently been affected by devil facial tumour disease, a disease which has diminished wild populations throughout Tasmania by more than 80% (McCallum and Jones, 2006). In order to assist in the long term survival of these animals further research into other potential diseases affecting their populations needs to be conducted. Leptospirosis has been shown to exist in Tasmania in animal populations and it is therefore reasonable to suggest that Tasmania devils may be affected by this disease. This study will be used to provide serological evidence of leptospiral infections in Tasmanian devils and provide the first evidence of leptospirosis in this species.
Materials and Methods

Samples

Eighty-one serum samples and two kidney tissue samples collected from Tasmanian devils between 2008 and 2014 were provided by Department of Primary Industries, Parks, Water and Environment, Tasmania. Samples were collected from one of seven areas of Tasmania (Figure 7.1) – South (10), South-East (28), North (9), North-East (21), West Coast (1), East Coast (10) or North-West (2). The samples collected were from both wild and captive animals, however individual living condition information for each devil was not provided. The tissue samples were provided from deceased devils (2).

Figure 7.1: Areas of sample collection
Analysis

**PCR**

Nucleic acid as extracted from all serum and tissue samples using a QIAGEN DNeasy Blood and Tissue Kit as per manufacturer’s instructions. All samples were then tested with a quantitative real-time polymerase chain reaction (PCR) using a TaqMan probe for the detection of pathogenic leptosiral organisms (Smythe, *et al.* 2004) to determine the presence of an acute infection. Results were reported as detected or not detected.

**MAT**

The current gold standard test, microscopic agglutination test (MAT) (Faine, 1982), for total antibody was performed on 81 of the 83 samples. Twenty-two leptosiral serovar cultures were utilised as antigens in a routine MAT panel (listed in Table 7.1), 18 of which are endemic in Australia and six that are exotic to Australia. A panel of antigens, all exotic to Australia, in serogroup Javanica were also utilised in further testing (Table 7.2). The MAT is specific for serovars or closely antigenically related serovars. All samples were screened at a titre of 1:50 and any samples showing agglutination were then serially diluted from 1:50 to 1:6400 to determine an end-point. Results were reported as a titre with the end point being the final dilution of serum at which 50% or more of the leptospires agglutinated. Titres of 1:50 or higher were deemed reactive, indicating past or present exposure.
Table 7.1: List of Leptospiral Antigens

<table>
<thead>
<tr>
<th></th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>L. Borgpetersenii</em> serovar Arborea</td>
</tr>
<tr>
<td>2</td>
<td><em>L. Interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>3</td>
<td><em>L. Borgpetersenii</em> serovar Hardjobovis</td>
</tr>
<tr>
<td>4</td>
<td><em>L. Interrogans</em> serovar Zanoni</td>
</tr>
<tr>
<td>5</td>
<td><em>L. Weilii</em> serovar Topaz</td>
</tr>
<tr>
<td>6</td>
<td><em>L. Interrogans</em> serovar Robinsoni</td>
</tr>
<tr>
<td>7</td>
<td><em>L. Borgpetersenii</em> serovar Tarassovi</td>
</tr>
<tr>
<td>8</td>
<td><em>L. Interrogans</em> serovar Kremastos</td>
</tr>
<tr>
<td>9</td>
<td><em>L. Interrogans</em> serovar Medanensis</td>
</tr>
<tr>
<td>10</td>
<td><em>L. Interrogans</em> serovar Szwajizak</td>
</tr>
<tr>
<td>11</td>
<td><em>L. Interrogans</em> serovar Pomona</td>
</tr>
<tr>
<td>12</td>
<td><em>L. Interrogans</em> serovar Copenhageni</td>
</tr>
<tr>
<td>13</td>
<td><em>L. Interrogans</em> serovar Canicola</td>
</tr>
<tr>
<td>14</td>
<td><em>L. Kirschneri</em> serovar Bulgarica</td>
</tr>
<tr>
<td>15</td>
<td><em>L. Kirschneri</em> serovar Grippotyphosa</td>
</tr>
<tr>
<td>16</td>
<td><em>L. Weilii</em> serovar Celledoni</td>
</tr>
<tr>
<td>17</td>
<td><em>L. Interrogans</em> serovar Bataviae</td>
</tr>
<tr>
<td>18</td>
<td><em>L. Interrogans</em> serovar Cynopteri</td>
</tr>
<tr>
<td>19</td>
<td><em>L. Interrogans</em> serovar Panama</td>
</tr>
<tr>
<td>20</td>
<td><em>L. Interrogans</em> serovar Shermani</td>
</tr>
<tr>
<td>21</td>
<td><em>L. Interrogans</em> serovar Djasiman</td>
</tr>
<tr>
<td>22</td>
<td><em>L. Borgpetersenii</em> serovar Javanica</td>
</tr>
</tbody>
</table>

Table 7.2: List of Serogroup Javanica Antigens (All exotic)

<table>
<thead>
<tr>
<th></th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>L. Borgpetersenii</em> serovar Javanica</td>
</tr>
<tr>
<td>2</td>
<td><em>L. Borgpetersenii</em> serovar Poi</td>
</tr>
<tr>
<td>3</td>
<td><em>L. Borgpetersenii</em> serovar Sorexjalna</td>
</tr>
<tr>
<td>4</td>
<td><em>L. Weilii</em> serovar Coxi</td>
</tr>
<tr>
<td>5</td>
<td><em>L. Meyeriserovar</em> Sofia</td>
</tr>
<tr>
<td>6</td>
<td><em>L. Borgpetersenii</em> serovar Ceylonica</td>
</tr>
<tr>
<td>7</td>
<td><em>L. Borgpetersenii</em> serovar Menoni</td>
</tr>
<tr>
<td>8</td>
<td><em>L. Santarosai</em> serovar Fluminensi</td>
</tr>
<tr>
<td>9</td>
<td><em>L. Borgpetersenii</em> serovar A85</td>
</tr>
<tr>
<td>10</td>
<td><em>L. Borgpetersenii</em> serovar Dehong</td>
</tr>
<tr>
<td>11</td>
<td><em>L. Weilii</em> serovar Menrun</td>
</tr>
<tr>
<td>12</td>
<td><em>L. Borgpetersenii</em> serovar Yaan</td>
</tr>
<tr>
<td>13</td>
<td><em>L. Weilii</em> serovar Mengma</td>
</tr>
<tr>
<td>14</td>
<td><em>L. Borgpetersenii</em> serovar Zhenkang</td>
</tr>
<tr>
<td>15</td>
<td><em>L. Santarosai</em> serovar Vargonicas</td>
</tr>
<tr>
<td>16</td>
<td><em>L. Santarosai</em> serovar Arenal</td>
</tr>
<tr>
<td>17</td>
<td><em>L. Borgpetersenii</em> serovar Kalimentani</td>
</tr>
</tbody>
</table>
**MIA IgG**

A microsphere immunoassay (MIA) for IgG antibody only (Wynwood, et al. 2015) was performed on 81 of the 83 samples. MIA was not performed on tissue samples. This assay is capable of multiplexing up to 500 analytes in one test per sample. For this purpose, 22 leptospiral antigens (Table 1) were coupled to 22 individual magnetic xMAP microsphere-sets (bead-sets) and combined with diluted test sera in a 96 well filter plate. Biotinylated protein A was used as in place of a secondary antibody followed by treatment with streptavidin-RPE to enable fluorescence detection. A Tasmanian devil-specific IgG-RPE conjugate was not available at the time of testing. Sample analysis was performed on the BioRad MagPix multiplex reader. The results were reported as reactive or non-reactive based on the mean fluorescent intensity (MFI). A MFI of 1000 or greater was deemed reactive and any result less than 1000 was deemed non-reactive.

An IgM antibody MIA was not performed as a Tasmanian devil-specific IgM-RPE conjugate was not available at the time of testing.

**Results**

Of the 81 serum samples tested by all three methods, 11 samples had reactive serology results, ten by MAT and one by IgG MIA, suggestive of past or present leptospirosis. One sample, a kidney tissue sample, was detected by PCR. The sample results are shown in Table 7.3 and Figure 7.2.
Table 7.3: Tasmanian devil serum sample results

<table>
<thead>
<tr>
<th>Test</th>
<th>Reactive/Detected</th>
<th>Non-Reactive/Not Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>1</td>
<td>82</td>
</tr>
<tr>
<td>MAT</td>
<td>10</td>
<td>71*</td>
</tr>
<tr>
<td>MIA</td>
<td>1</td>
<td>80*</td>
</tr>
</tbody>
</table>

N = 83
*Tissue samples not tested with MAT or MAT.

Figure 7.2 PCR results

The 11 serologically reactive samples were from five of the seven regions of Tasmania.

The MAT reactive samples had titres of 1:50 to 1:400 for serovar Javanica, a serovar not
endemic to Australia and the MIA reactive sample had a low level reaction to serovar Celledoni which has been shown to occur in Australia previously, however has not been shown to be present in Tasmania. The PCR detected kidney tissue sample was from the Northern region of Tasmania. Table 7.4 summarises these results.

Table 7.4: Summary of reactive samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Location</th>
<th>Date Collected</th>
<th>MIA Result</th>
<th>Leptospira serovar MAT Titre</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Celledoni</td>
<td>Javanica</td>
</tr>
<tr>
<td>16</td>
<td>NE</td>
<td>2008</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 50</td>
</tr>
<tr>
<td>27</td>
<td>SE</td>
<td>2008</td>
<td>REACT (Celledoni)</td>
<td>&lt; 1: 50</td>
<td>&lt; 1: 50</td>
</tr>
<tr>
<td>31</td>
<td>S</td>
<td>2008</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 50</td>
</tr>
<tr>
<td>32</td>
<td>NE</td>
<td>2008</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 50</td>
</tr>
<tr>
<td>47</td>
<td>NE</td>
<td>2012</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 100</td>
</tr>
<tr>
<td>49</td>
<td>NE</td>
<td>2012</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 100</td>
</tr>
<tr>
<td>55</td>
<td>SE</td>
<td>2012</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 100</td>
</tr>
<tr>
<td>62</td>
<td>N</td>
<td>2012</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 400</td>
</tr>
<tr>
<td>63</td>
<td>EC</td>
<td>2012</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 50</td>
</tr>
<tr>
<td>65</td>
<td>N</td>
<td>2012</td>
<td>NR</td>
<td>&lt; 1: 50</td>
<td>1: 400</td>
</tr>
<tr>
<td>82</td>
<td>N</td>
<td>2012</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Seven of the ten serovar Javanica MAT reactive samples were tested against the 17 available serovars in serogroup Javanica. The results (Table 7.5) show cross-reactivity between serovars in this group. There was an insufficient volume of serum available for further testing of the remaining 3 MAT reactive samples.
Table 7.5: MAT reactive serovar Javanica samples against serogroup Javanica.

<table>
<thead>
<tr>
<th>Sample Serovar</th>
<th>Strain</th>
<th>47</th>
<th>49</th>
<th>55</th>
<th>62</th>
<th>63</th>
<th>65</th>
<th>82</th>
</tr>
</thead>
<tbody>
<tr>
<td>Javanica</td>
<td>Veldrat Bataviae 46</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>400</td>
<td>50</td>
<td>400</td>
<td>50</td>
</tr>
<tr>
<td>Poi</td>
<td>Poi</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>1600</td>
<td>100</td>
<td>1600</td>
<td>400</td>
</tr>
<tr>
<td>Sorexjalna</td>
<td>Sorex Jalna</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>1600</td>
<td>100</td>
<td>1600</td>
<td>200</td>
</tr>
<tr>
<td>Coxi</td>
<td>Cox</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>400</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Sofia</td>
<td>Sofia 874</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>100</td>
<td>400</td>
<td>&lt; 50</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>Ceylonica</td>
<td>Piyasena</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Menoni</td>
<td>Kerala</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>50</td>
<td>400</td>
<td>&lt; 50</td>
<td>400</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Fluminensi</td>
<td>Aa 3</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>200</td>
<td>&lt; 50</td>
<td>400</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>A85</td>
<td>A 85</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>100</td>
<td>&lt; 50</td>
<td>50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Dehong</td>
<td>De 10</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Menrun</td>
<td>A 102</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Yaan</td>
<td>80-27</td>
<td>&lt; 50</td>
<td>200</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Mengma</td>
<td>SS90</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>100</td>
<td>200</td>
<td>&lt; 50</td>
<td>400</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Zhenkang</td>
<td>L 82</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>800</td>
<td>&lt; 50</td>
<td>800</td>
<td>50</td>
</tr>
<tr>
<td>Vargonicas</td>
<td>24</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Arenal</td>
<td>MAVJ401</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>400</td>
<td>&lt; 50</td>
<td>800</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Kailmentani</td>
<td>Amos</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
</tbody>
</table>
Discussion

Tasmanian devil population numbers have drastically declined since the 1990s due to the incidence of the contagious and fatal devil facial tumour (Hawkins, et al. 2006). Tasmanian devils, like other dasyurids have short life spans and often live with injuries due to a lifestyle of constant fighting. They are host to a number of parasites, some of which are known vectors of infectious agents. As devils bite and injure each other during feeding, social and mating interactions, biting is the most plausible route for disease transmission – most notably the facial tumour disease (Hamede, McCallum and Jones, 2008). Other modes of transmission for the facial tumour disease, such as devils scavenging other devils that have died from disease or transfer of tumour cells from co-feeding on prey carcasses, cannot be discounted (Hamede, McCallum and Jones, 2008).

It is possible that these routes of transmission, in particular biting (Luzzi, Milne and Waitkins, 1987) can also be applied to other infectious diseases, such as leptospirosis. Animal disease management is increasingly being recognised as an essential tool for the conservation of many species (Hess, 1996) even though diseases are often viewed as a natural feature, serving to stabilise populations. However, the presence of additional threats can affect the ability of a population to recover. Identifying diseases other than the devil facial tumour and common parasites, which may contribute to the further decline of the Tasmanian devils, will provide important information for preservation of the species and maintenance of current populations.

This study shows that *Leptospira* spp. and leptospiral antibodies are present in Tasmanian devils suggesting that leptospirosis may be contributing to illness and possibly death.
among Tasmanian devil populations. Limited research on leptospirosis in Tasmanian wildlife indicates that a number of leptospiral serovars are present in Tasmania and are dispersed amongst a number of mammalian species (Munday, 1972).

Diagnosis of leptospirosis has previously been performed by MAT (Munday, 1972) utilising only limited leptospiral serovar antigens. The MAT has limitations including a reliance of high levels of expertise of an operator, high costs and a limit on the number of serovars that can be utilised per test (Wynwood, 2015).

In previous studies involving Tasmanian wildlife (Corbould, 1970; Munday, 1972; Burke and Whyte, 1973), the panel of leptospiral antigens utilised in the MAT consisted of up to six serovars only. This creates a potential for missing leptospiral antibodies or misdiagnosing other antibody reactions.

In this study, two diagnostic serological methods (MAT and IgG MIA) and one molecular method (PCR) were utilised. The MAT panel consisted of a large number of antigens representing 20 of the 24 serogroups currently identified worldwide and indicating the presence of total antibody. The MIA was utilised to detect IgG only by using Protein A. Protein A is an immunoglobulin binding protein and has binding sites for the Fc region of mammalian IgG. In humans the affinity is 100% and in bovines, cats, dogs, horses, goats, swine and sheep it has been shown to have 50% reactivity (Thermo Scientific, 2014). Whilst no studies have been performed to specifically determine the reactivity with Tasmanian devils it is assumed that some reactivity is present, given the reactivity levels with other mammals. Further studies with other proteins including Protein A and species
specific monoclonal antibodies will enable more specific results. Currently, an anti-IgM secondary antibody specific to Tasmanian devils is not commercially available therefore, specific IgM testing could not be performed.

Upon initial screening *L. Interrogans* serovar Javanica (reference strain *Veldrat bataviae 46*) was detected in ten (12%) of the 81 Tasmanian devil samples by MAT. This particular serovar has not previously been identified as being endemic in Australia and therefore, not included in any previous MAT panel for testing Tasmanian wildlife. Originating from Java, Indonesia, serovar Javanica is most commonly associated with disease in rats and transmission to humans and other animals via exposure to rat urine. Although serovar Javanica has been found in dairy cattle (Natarajaseenivasan, *et al.* 2011) and canines (Ambily, *et al.* 2013) in India as well as rats in Malaysia (Benacer, *et al.* 2013), there have been no confirmed cases of serovar Javanica in Australian animals. There has, however, been four serologically diagnosed cases of serovar Javanica in humans in Australia (3 in 2007 and 1 in 2009) all travellers returning from South East Asia (Queensland Government, 2014). Remaining serum from seven out of ten of the serovar Javanica MAT reactive samples were then re-tested by MAT against all available serovars (17) in the Javanica serogroup (Table 3). The cross reacting results suggest that the antibodies in these samples belong to the Javanica serogroup but without isolating the organism, a specific serovar cannot be determined. Cross reactions were noted from each sample that was re-tested with the Javanica serogroup for serovars Poi, isolated in Italy in 1941 (Ahmed, *et al.* 2006), and Sorexjalna, isolated in the Czech Republic in 1953 (Ahmed, *et al.* 2006), neither of which are endemic to Australia. Other cross reactions varied among
samples suggesting a strong link to the Javanica serogroup. These results also suggest the possibility of the presence of a new serovar.

Antibodies to *L. weilii* serovar Celledoni were identified by the MIA in one (1.5%) case. As the MIA, in this study, detects IgG, this result may indicate a past infection, cross reactivity between serogroups or an early immune response to a recent infection. Without paired sample testing and IgM testing it is difficult to determine the cause of the presence of these antibodies. Differences between the MAT and MIA serology results may be explained by a number of factors. A lack of an IgM MIA may mean that any early infections or persistent IgM antibodies may have been missed in the IgG assay. These results may also indicate that using Protein A as an immunoglobulin binding protein in place of a secondary antibody is not adequately sensitive for use with Tasmanian devil samples. Other proteins or the development of specific secondary antibodies should be considered in future IgG detection studies.

The PCR detected sample was from a deceased Tasmanian devil that had clinical signs and symptoms of leptospirosis, including nephritis and kidney failure, prior to death. A serum sample was submitted for this devil also (included in the 81 serum samples) and was non-reactive in both the MAT and the MIA. This suggests that this particular devil may have perished in the acute phase of the disease and therefore did not produce any antibodies. This may also suggest that if infected Tasmanian devils are dying in the early stages of the infection that there may be no chance for IgG antibodies to develop which highlights the importance of the development of a specific IgM assay for this population. It should also
be noted that in this study, the remainder of the samples tested showed no clinical signs or symptoms of leptospirosis.

One weakness of this study was that we were unable to isolate leptospires from the Tasmanian devil samples due to the age and storage conditions of these samples. The samples were up to seven years old and all had been in frozen storage conditions. Although the serology results suggests that a serovar from the serogroup Javanica is most likely the infecting serovar, it is possible that a new serovar, not previously isolated, is circulating within the Tasmanian devil populations that belongs to the Javanica serogroup. Regardless, any serovar in this group is not currently endemic to Tasmania and further investigation, both serologically and molecularly, will assist in identifying this particular serovar. A lack of any information regarding the living conditions of the Tasmanian devils (wild versus captive) in this study also provided some challenges. We were unable to determine the differences in disease spread and severity in wild populations compared to captive devils. Further sample collection and investigation comparing the prevalence of disease in wild animals and captive animals will demonstrate any implications for captivity management and translocation of animals into the wild to avoid the spread of disease. Given the large amounts of work currently being undertaken with Tasmanian devils, this information will also assist in monitoring any zoonosis risk to human handlers (keepers and trappers) involved in working with Tasmanian devils.

This study has uncovered the presence of leptospirosis for the first time in Tasmanian devils. Future research would benefit from determining the prevalence and incidence of leptospirosis in Tasmanian wildlife including more focussed research on Tasmanian devil
populations. Further developing the Leptospirosis MIA for Tasmanian devil IgG and IgM testing through the development of specific IgG and IgM antibodies will allow more sensitive and more specific antibody detection. Utilising a large MIA panel of leptospiral antigens will enable a more comprehensive analysis of the serovars currently circulating in Tasmanian wildlife and ensures a better detection system to specially determine the impact that leptospirosis is having on wild and captive Tasmanian devil populations.

References


CHAPTER 8
Conclusions

Leptospirosis is a disease of significance and as such is worthy of study. Although accurate estimates of the incidence of leptospirosis are lacking worldwide, it is believed to be responsible for more than 50,000 deaths per year in humans. Importantly, the study of the disease and its causative organisms, is made difficult because of both scientific and socioeconomic factors. From a scientific standpoint, leptospires are relatively difficult to culture and there are limited molecular methods, such as heterologous expression systems, broad-host-range expression vectors and suicide vectors that facilitate studies. Additionally, from a socioeconomic stand-point, as with many infectious diseases, leptospirosis has a disproportionately high impact on poorer countries, particularly located in tropical areas, making priority funding for study difficult, if not impossible.

The paucity of molecular tools available for analysis of leptospires, a long history of reliance on immunologic tests, and the inherent serologic diversity of this group of organisms, has created a strong dependence on one test, the MAT, and an epidemiological focus on serotypes rather genotypes. Whilst the MAT has been very useful, it is limited in that the technique requires significant expertise, is not automated, and is normally limited to an endemic and arbitrary small panel of serovars.

The non-specific clinical presentation of leptospirosis includes symptoms that make differential diagnosis only possible using methods such as culture and visualisation using dark-field microscopy, PCR (acute phase) or serological detection (immune phase), usually
employing the MAT. All of these methods require significant levels of expertise and specialised, costly equipment or media. This makes them unsuitable for large numbers of samples, particularly for underdeveloped regions where leptospirosis is problematic.

Chapters 1 to 3 of this thesis have provided an overall picture of the burden of leptospirosis in both human and animal populations. The evidence presented in these chapters points to leptospirosis being an under-diagnosed and under-reported disease worldwide due to a combination of poor surveillance methods, lack of analytical facilities, the nature of non-specific symptoms seen in those with the disease, and cost. There are a number of modes of transmission of leptospirosis, all of which can be encountered routinely by humans and animals. In particular, the disease is promoted by the close proximity of humans and animal reservoirs, unclean conditions and lack of suitable provisions for the production of clean drinking water; that is, those conditions most likely to occur in developing countries.

Chapter 4 of this thesis shows the reliance on serological testing during an outbreak situation. Following a spate of natural disasters in Queensland, Australia in 2011, a significant increase in leptospirosis testing and confirmed infections occurred. Agglutination testing (MAT) was utilised to confirm infection as well as identify infecting serovars through a large, judiciously, though arbitrarily-chosen panel of antigens (or antibodies in the reverse MAT – the SAT as discussed in Chapter 4).

Because there will likely be a heavy reliance on serologic diagnosis of leptospirosis in the future due, not least in part, due to the phasic nature of the disease and the amount of
accumulated historical data, an alternative method to the MAT was investigated in which some of the shortcomings of the MAT could be addressed, in particular, the reliance on using live-cultures, lack of automation and very high levels of expertise and training to use the MAT. Chapter 5 reports on the development of a microsphere immunoassay (MIA) which can potentially offer advantages, compared to the MAT, in that it can be automated, simultaneously analyse a large number of serovars and reduce reliance on operator interpretation. An additional advantage of the MIA, compared to the MAT, is its ability to discriminate between IgG and IgM, allowing for a more temporal picture of the infection. The results presented in Chapter 5 clearly show the potential of the MIA as a next generation diagnostic tool for leptospirosis.

Further work using the MIA and MAT was described in Chapters 6 and 7 using very different animal subjects (native Australian population of Tasmanian devils and a domesticated bovine population). These results suggest that the MIA can certainly be a beneficial diagnostic tool – particularly in bovine populations where large numbers of samples are often submitted for testing at one time. Chapter 7 also highlights a current limitation of the MIA compared to the MAT– the lack of species specific secondary antibodies. In this case, the lack of readily-available specific secondary antibody for Tasmanian devils required the use of Protein A, which is limited to detecting IgG. Without the detection of IgM, a definitive diagnosis cannot be made and the stage of disease remains unknown. Further development of the MIA, specifically secondary antibody development, for Tasmanian devil populations will enable large scale testing with serovars not routinely used in the current Australian MAT panel. As this work has uncovered a
potentially exotic leptospiral serovar in Tasmanian devils, MIA development could play a pivotal role in further investigation within this population.

In conclusion, this thesis has presented work that highlights the importance of serologic diagnosis of leptospirosis, a situation which will, in all likelihood, not change in the near future. On this basis, it seems prudent to pursue alternative serologic tests to the MAT. The work presented in this thesis indicates the newly-developed MIA is promising as an alternative to the MAT. Nevertheless, there is still more work to be completed before the MIA could replace or supersede the MAT, particularly with the analysis of non-human populations. However, the benefits of implementing the MIA as a routine test provide incentive for further development and validation.
APPENDICES – REMOVED DUE TO COPYRIGHT RESTRICTIONS

List of Appendices

Appendix A: PDF Document


Appendix B: PDF Document


Appendix C: PDF Document


Appendix D: PDF Document