Use of High Resolution Melting for genotyping *Leptospira* spp.

Ke Zhang (Claire)

2018

Applied Science, Health and Environmental Science, AUT

A thesis submitted to Auckland University of Technology in partial fulfilment of the requirements for the degree of Master of Science.

Abstract

Background
Leptospirosis is a worldwide zoonosis that is endemic in tropical areas. The species *Leptospira interrogans* is the primary agent in human infections, but other pathogenic species, such as *L. kirschner* and *L. borgpetersenii*, are also associated with human leptospirosis.

Methods and Findings
In this study, an unsupervised high resolution melting (HRM) analysis of the products that were amplified with five pairs of primers lfb1 F/R, G1/G2, VNTR-4Bis, VNTR-Lb4 and VNTR-Lb5 facilitated an accurate species classfication of Leptospira reference strains from New Caledonia Institute Pastéur. Next, the genotypes at the subspecies level was identificated by using method with LightCycler®480 instrument and the High Resolution Melting Master kit (04909631001). LightCycler®480 Gene Scanning Software was used to perform a futher analysis results.

Conclusions
This new HRM method enabled the identification of *Leptospira* strains at the species and subspecies levels and support the direct genotyping of Leptospira in biological samples without requiring cultures.

Table of Content

List of Figures

List of Tables

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly de ned in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Ackonwledgements

Foremost, I would like to express my sincere gratitude to my primary advisor Dr. Fabrice Merien for the continuous support of my Master study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Master study. I would also like to thank my second advisor Miomir Vidicki for the encouragement, insightful comments, and hard questions.

Thanks to EpLabs for supplying the clinical samples and Roche for instruments and reagents for the experiments.

Besides, I thank my friends in AUT: Tonya Tong, Olivia Tingting Wang.

Last but not the least, I would like to thank my family: my parents Yongmei He and Weimin Zhang, for giving birth to me at the first place and supporting me spiritually throughout my life.

Ethics Approval

No ethical approval is necessary for this project as there is no human sample involved.

Introduction

Leptospirosis is a universal zoonosis that can have severe economic impact on livestock industries, with endemism in tropical areas. On the other hand, leptospirosis is a fatal disease to human as well. The species *Leptospira interrogans* is the primary agent in human infections, but other pathogenic species, such as *L. kirschner* and *L. borgpetersenii,* are also associated with human leptospirosis. The outcomes of leptospirosis infection are closely connected with pathogenic *Leptospira*. For the better pathological understanding and epidemiological study of this disease, it is essential to do research on the genetic polymorphism of *Leptospira*.

This thesis aimed to introduce a new technique using a High Resolution Melting analysis to genotyping *Leptospira* spp. in New Zealand and furthermore gave a genotping profile that contributed to the study of leptospiral polymorphism in New Zealand.

The clinical materials were amplified with the primer pairs LFB1 F/R primer and G1/G2 to facilitate a particular species classification of *Leptospira* reference strains from New Caledonia Institute Pastéur. Next, the genotypes at the subspecies level were identified by using an unsupervised high resolution melting (HRM) method with Roche LightCycler®480 and the High Resolution Melting Master kit (04909631001). Additionally, three VNTR primers, VNTR-4bis, VNTR-Lb4 and VNTR-Lb5, were involved in. Roche LightCycler®480 Gene Scanning Software was used to give the further analysis results in graphes.

This new HRM approach will enable the identification of *Leptospira* strains at both of the species and subspecies levels, supporting the direct genotyping of *Leptospira* in

biological samples without requiring cultures. It will sharply shorten the time to do investigations of *Leptospira* identification than taking those tranditional methods like pulsed-field gel electrophoresis (PFGE) and restriction enzyme analysis (REA).

Literature Review

1. Leptospirosis

1.1 History of Weil's disease

Leptospirosis is one of the most ubiquitous zoonoses that had a millennia history; it is also known as Weil's disease (Adler, 2015). Adolph Weil, a German physician, first described a particular condition of jaundice accompanied by splenomegaly, renal dysfunction, conjunctivitis, and skin rashes in 1886. It was consequently named after him. Aetiology was not clearly explained at that time though it was apparently more likely to infect in nature and even common when people came into contact with water. Epidemics spread among sewer workers, rice-field workers and coal miners.

There were few firm conclusions from records before the advent of modern medical and scientific pieces of literature talking about this kind of disease; nevertheless, some of the early illness outbreaks described in the ancient texts seemed to be leptospirosis clearly, which can be transparently referred that leptospirosis had existed for millennia. It was indeed recognised as an occupational hazard of rice harvesting in ancient Chinese texts, carrying the name "rice field jaundice"(Faine, 1994). While the Japanese name Akiyama, also called "seven-day fever" or "autumn fever", persists in modern medicine (Kitamura & Hara, 1918). In Australia, Europe and some other places around the world, connections between febrile illness and particular occupations were also well noticed, the syndromes were recognized as "cane-cutter's disease", "swine-herd's disease", and "Schlammfieber (mud fever)", well before the common aetiology was identified and developed (Alston, Broom, & Doughty, 1958; van Thiel, 1948). With hindsight, clear explanation of leptospiral jaundice can be understood as having appeared earlier in

the 19th century, some years before the report by Weil (Faine, 1994). It has been suggested that *Leptospira interrogans* serovar icterohaemorrhagiae was introduced to Western Europe in the 18th century by the westward extension of the range of Rattus norvegicus from Eurasia (Alston et al., 1958).

Stimson (1907) was the first person who made a demonstration of Leptospires after he utilised the lately called Levaditi silver deposition staining technique and saw spirochetes in kidney tissue from a patient described as dying from "yellow fever". The outstanding thing was no spirochete observed in liver, heart or other tissue sections; it was probable that the patient had convalesced from Weil's disease when he was contracted the deadly yellow fever. The organism Spirocheta interrogans named by Stimson survives to modern sciences as the species name; it was suggested by the similarity of the bacterial cells to a question mark, a characteristic hooked end of leptospires as we know now.

A couple of years later, the aetiology of leptospirosis, *Leptospira*, was first isolated independently and almost simultaneously in 1915 in Japan and Germany (Cox, 1996). In Japan, Inada and Ido (1916) detected both spirochetes and specific antibodies in the blood of Japanese coal miners with infectious jaundice; they did an injection of the blood of Weil's disease patients into guinea pigs intraperitoneally and reproduced typical successfully. Inado's and subsequent papers contributed to a tour de force on *Leptospira* research for this period; their studies introduced the transmissibility, routes of infection, pathological changes, tissue distribution, urinary excretion, leptospiral filterability, morphology, and motility. Syndromes may be seen on *Leptospira* infected guinea pigs included jaundice, conjunctivitis, inappetence, anaemia, haemorrhages, as albuminuria. Also, the disease could transfer in guinea pigs for up to 50 generations. In

this practice, most tissues were observed with spirochetes; the greatest numbers were found in kidneys and liver tissues. Similar findings were revealed on postmortem tissues from human bodies. What's more, rabbits, mice, and rats were shown to have comparatively resistance to acute disease, even when they were injected with very volumes of infected guinea pigs tissues. Inada and colleagues (1916) succeeded in propagating the spirochetes in vitro in a medium made from emulsified guinea pigs kidney and found that the organism preferred growing at 25 °C and they would lose viability at 37 °C. This spirochete was named Spirochaeta icterohaemorrhagiae. At the close meantime, two groups of German physicians studied German soldiers afflicted by "French disease" in the trenches of the northeastern France; they did a project to detect spirochetes in the blood of guinea pigs immunised with the blood of infected soldiers. As a result, they transmitted the infection to guinea pigs and demonstrated Leptospires in guinea-pigs tissues successfully (Hubener, 1915; Uhlenhuth & Fromme, 1915). They named the organism *Spirochaeta nodosa* and *Spirochaeta icterogene*s respectively. There were some controversy followed about the priority; however, Inada's group (Inada et al., 1916) had their publication in English predated eight months compared to the German team, so they rapidly obtained the confirmation of the occurrence of leptospirosis from the Western Front after that; the Subcommittee on the Taxonomy of *Leptospira* specified Ictero No. 1 as the Type Strain of *Leptospira interrogans* (Marshall, 1992).

The Japanese group added the finding that rats were renal carriers of *Leptospira* within two years (Ido, Hoki, Ito, & Wani, 1917). They stated a clear connection between rats and Weil's disease, as coal mines where rats frequently infested and kitchen frequented by rats often became the places that ill with spirochetes icterohaemorrhagica frequented. Also, Weil's disease incidence in Japan showed a clear increase in spring and autumn

when the temperatures stayed in 22-25 °C, while in coal mines which had no temperature fluctuation, the prevalence of leptospirosis did not show clear difference all year around. Besides, the incidence in coal mines with neutral or alkaline soil and water was quite higher than it in mines with acidic soil and water, despite similarly elevated levels of rat infestation.

The understanding of leptospirosis advanced a lot in following decades. Leptospirosis was an infectious disease involved all mammalian species, especially rodent species, and infected human from domestic animals as an important source (Alston et al., 1958; van Thiel, 1948). For example, scientists from Netherland reported their work on isolation of a carine strain, Hond Utrecht IV (Klarenbeek & Schüffner, 1933), which remains the type strain for serovar Canicola. Russian firstly reported leptospirosis happened to cattle in 1940 and then referred to as "infectious yellow fever of cattle". A decade later, the range of serovars and host animals had expanded substantially (Alston et al., 1958). By the 1980s, leptospirosis has been well documented as a veterinary disease of high economic importance in dogs, cattle, pigs, horses, and maybe sheep as well; this had a serious impact on the animal husbandry at that time (Ellis, 1990). Isolation of *Leptospira* serovars was developing at the meanwhile; as a result, firm Weil's disease was not considered as the most typical presentation of leptospiral infection any longer. Infecting serovar is a key factor that determines the outcomes of infection; some serovars never causes fatal human infections, such as Hardjo (Gouveia, 2008). However, the host and other factors also play a role in leptospirosis infection; it is possible that serovars most commonly associated with severe fatal leptospirosis only give rise to mild disease syndromes.

It was not sure when the pathogenic leptospires first became established in New

Zealand. There were only two native land mammals in New Zealand and both of them were bats (King, 1990). About 1, 200 years ago, the first land mammals arrived this country were human beings, dogs, and the kiore, *Rattus exulans*. The first confirmed occurrence of leptospirosis in domesticated animals in New Zealand was in 1950 when *Leptospira pomona* (later known as *L. interrogans* serovar *pomona*) was isolated from a calf with haemoglobinuria at Wallaceville Animal Research Station (Anonymous, 1951), but 1953 was the seminal year for publications on leptospirosis.

1.2 Cause and transmission

Both humans and animals can be infected pathogenic strains of *Leptospira*, commonly through contacts with infected animal urine or tissue directly, contaminated water indirectly, or rat bites (Lecour, Miranda, Magro, Gonçalves, & Rocha, 1989). Pathogenic leptospires widely spread in nature, reflecting maintenance in the kidneys of many wild and domestic reservoir hosts. Leptospires need hosts after shedding in the urine and persistence in the ambient environment, and they can disseminate to the kidneys hematogenous via the glomerulus or peritubular capillaries once when the acquisition of a new host. When gained access to the renal tubular lumen of the organ, leptospires would colonise the brush border of the proximal renal tubular epithelium, from which urinary shedding can persevere for an extended duration of time without notable ill effects on the reservoir host. It makes the relationship between leptospires and reservoir hosts commensal. Almost all mammals, including aquatic mammals, and marsupials can be carriers of leptospires; they can be divided into maintenance hosts and accidental hosts (Babudieri, 1958). A species that can infect endemic disease and transfer between animals by contact directly is defined as maintenance host. Small mammals, especially rodent species, are considered to be the most significant support host resulting from transferring infection to domestic farm animals, dogs and human.

For example, rats were reported by the Ido's group to be as the renal carriers of *Leptospira* thanks to the serendipitous findings of leptospire in kidneys and urine of horses and wild rats; utilising a particular Pfeiffer reactivity with immune serum, they had further results that the spirochetes observed and cultured from the rats were identified as S. *icterohaemorrhagiae* (Ido, Hoki, Ito, & Wani, 1917). Moreover, reservoirs of different serovars distinguish from families. To be specific, rats are maintenance hosts for serovars of the serogroups lcterohaemorrhagiae and Ballum, and mice are the maintenance host for serogroup Ballum. Moreover, animals may be maintenance hosts of some serovars but incidental hosts of others, infection with which may cause severe or fatal disease (Levett, 2001).

Domestic animals are also able to be maintenance hosts; dairy cattle may harbour serovars hardjo, pomona, and grippotyphosa; pigs could harbour pomona, tarassovi, or bratislava; sheep may harbour hardjo and pomona; and dogs may harbour canicola (Brown & Bolin, 2000). Additionally, poikilothermic animals, like frogs and toads, could recover leptospirochetes too, and these animals may play a significant role in the transmission of leptospirosis in the nature environment, not including major reservoirs of human infection, however.

Leptospirosis is primarily a zoonosis, while human are those who accidentally suffer from acute and sometimes even fatal infections; they are regarded as normalisation hosts of leptospires consequently (Ko, Goarant, & Picardeau, 2009). The animal source is an integral component of leptospirosis infection in human because there is not a human-to-human transmission in practical purpose existent, and the disease is hence defined as a zoonosis (Adler & de la Peña Moctezuma, 2010). The transmission cycle of the pathogens from animals to human is showing as figure1. Wild animals (especially

rodents) and domestic animals (normally like swine, cattle, dog) cycle leptospires within the population, then human may be transmitted leptospires directly through contact with their infected urine or diffusely by contaminated soil or water, which highly happens after heavy rainfalls and floods. Human leptospirosis may lead to a dead-end infection; transmission between human and human is virtually unknown (Victoriano et al., 2009).

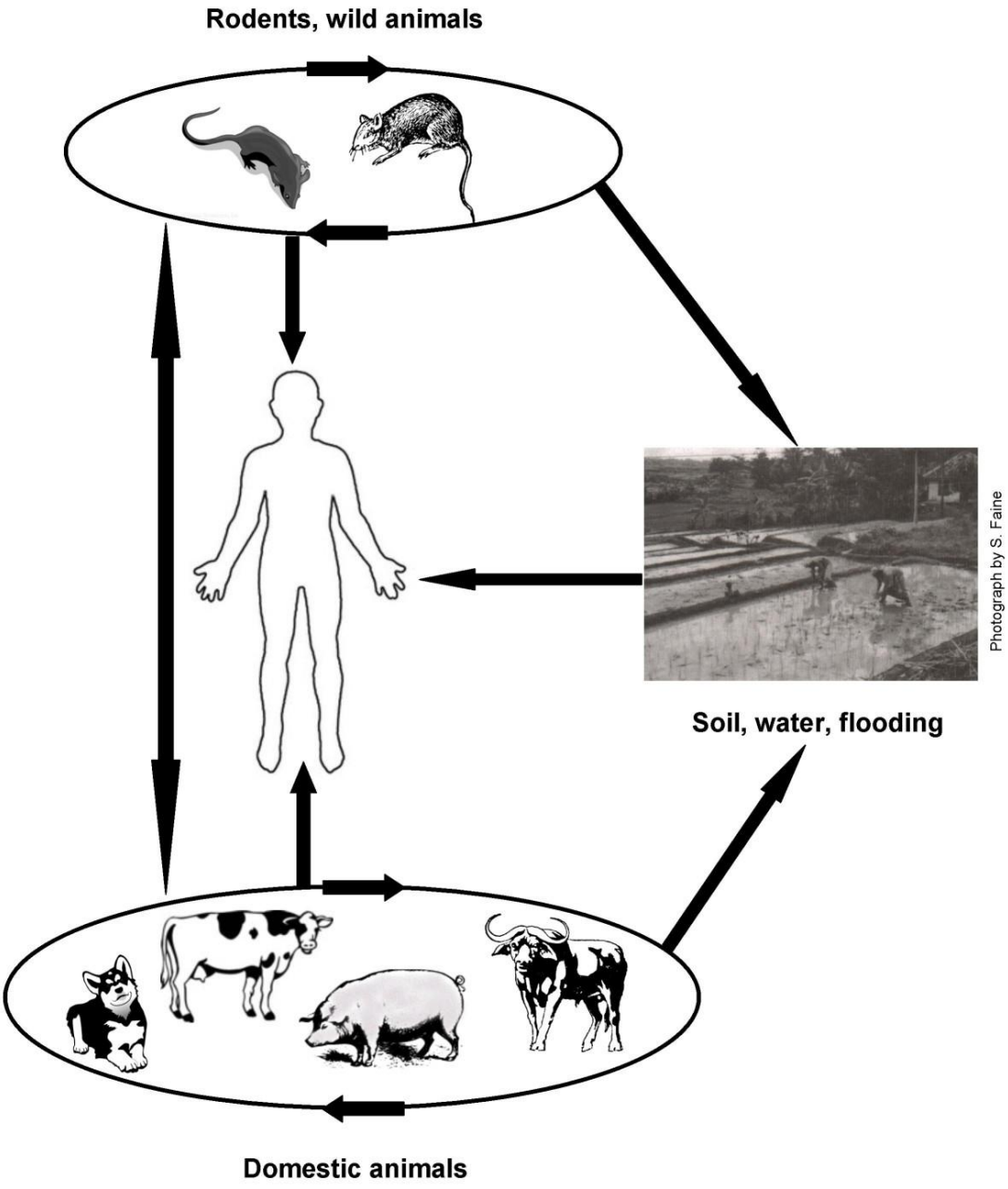

Figure 1 Transmission of Leptospirosis.

1.3 Infection and pathogenesis

The cuts or abrasions on the skin or mucous membranes of the eyes, nose, throat or genital tract are all able to be the usual portals of entry for *Leptospira* to mammal body; infection may even occur through the intact skin after prolonged immersion in water sometimes, such as swallowing while swimming in contaminated water, as a number of studies indicated (Corwin et al., 1990; Levett, 2001; Lingappa et al., 2004; Stern et al., 2010). When leptospires once get access to the renal tubular lumen of the host's kidney, hematogenous dissemination would break out. They would not get the rise of lesions indicating the establishment of infection in the skin like other spirochetes such as *B. burgdorferi* and *T. pallidum*. Pathogenic leptospires take advantages of the bloodstream to disseminate and persist themselves there during leptospiremia phase of the disease. A study detected leptospiremia by quantitative PCR after inoculating blood into the leptospiral medium; its results showed there was more possibility to be positive when the first eight days of the fever symptoms, compared with antibody formation and clearance of organisms from blood (Agampodi, Matthias, Moreno, & Vinetz, 2012). The leptospiremia levels in blood were 10^6/ml as document recorded, which is the same extent of spirochetes burdening in patients' blood when they relapsed fever (Haake & Levett, 2015). Levels of $>10^4$ leptospires/ml in the bloodstream have had the capability to leading to severe complications (Segura, 2005), although recent studies queried that some leptospiral species with lower virulence may not bring about severe outcomes even with higher burdens of leptospires in the bloodstream (Merien, Perolat, Mancel, Persan, & Baranton, 1993). Actually the levels of leptospiremia that occur during disease are not much different from the levels of bacteremia found in infections caused by the relapsing fever Borreliae (Stimson, 1907), but quite distinguished with those found in bacteremia caused by E. coli and other Enterobacteriaceae that typically with

<1 CFU/ml burdens (Jordan & Falk, 1928). Some studies suggested that human innate immune response could account for these differences.

The toll-like receptor 4(TLR4) in human bodies is a protein that encoded by the *TLR4* gene. It can recognize *E. coli* lipopolysaccharide in an extremely low concentration, but is not able to detect leptospiral lipopolysaccharide (Raddi et al., 2012), which is probably because that the lipid A component of *E. coli* and leptospiral lipopolysaccharide are not same in the structure; leptospires has a unique methylated phosphate residue on the lipopolysaccharide, which has not been found in any other form of lipid A (Victoriano et al., 2009). Contrastively, TLR4 in the mouse can identify leptospiral lipopolysaccharide not as that in human, proposing that murine innate immune response can be stimulated by infections of leptospires (Smythe et al., 2013). This notion supported the pathogenesis of leptospirosis from some extents on the differences between people and rodents; people are the accidental hosts that they suffer from potentially fatal complications and rarely transmit the pathogens, while rodents rarely dies from infection, but they serve as natural reservoirs (Faine & Stallman, 1982). TLR4 is the only one element of the innate immune response to leptospirosis. Juvenile mice that are lacking TLR4 are much more susceptible to fatal infection with *L. interrogans*, which emphasised the importance of TLR4 in determining the outcome of infection (Yasuda et al., 1987). TLR4 in both human and mice can recognise the polysaccharide or 2-keto-3-deoxyoctonoic acid component of the leptospiral lipopolysaccharide (Levett, Morey, Galloway, Steigerwalt, & Ellis, 2005).

Innate immune mechanisms eventually attract tissue-based and systemic responses when high levels of bacteremia occur during leptospiral infection, which results in severe conditions like sepsis-like syndrome or organ failure. The liver is usually a target

organ involved in leptospirosis. Many postmortem examinations from fatal leptospirosis cases documented that the Disse's space, located between the sinusoids and hepatocytes, exhibited distention, the sinusoid congested as well (Perolat et al., 1998). Decades saw immunohistochemistry studies found and recorded a mass of leptospires between hepatocytes in animal models. In 2014, an elegant research detected that leptospires infiltrated the space of Disse and invaded the prejunctional area between hepatocytes.

Similar infection process happens when leptospires infect animals via same import ways, plus some oral infection cases reported in predators (Ellis, 2015). An one-week bacteremia may start from 1 to 2 days after infection, following the appearance of circulating antibodies that usually can be identified within 14 days. At this stage, the anti-leptospiral agglutinins in the blood would start to be detectable; at approximately 3-6 weeks, they would achieve the maximum levels. This is the primary bacteremia phase, while there are not many reports about the subsequent bacteremia period (15-26 days) of leptospiral infection so far (Hathaway, Ellis, Little, Stevens, & Ferguson, 1983). When the infection occurs in animal juveniles, the acute clinical disease usually comes with the bacteremic phase, associating with hemolytic disease, hemoglobinuria, jaundice and death. Agalactia may be observed in cattle, sheep and buffalo and renal damage may occur in Canicola infection. These acute diseases can affect single herds/flocks a lot, but fortunately not nationally (Ellis, 2015). Leptospires would localise in the proximal renal tubules after the period of leptospiral bacteremia, which enables them to reproduce and void through urine. The uterus of pregnant females is another place that leptospires prefer to focus on; intrauterine infections in late gestation could result in abortion, stillbirth, and neonatal disease. Transplacental infection from maternal leptospiremia may be the sole cause of these reproductive diseases, which has the evidence that the uterine immunity would wane to lose the ability for preventing

trans placement infection (Ellis, 2015).

What's more, leptospires could trigger hepatocyte apoptosis (Saito et al., 2013). Because of the damaged hepatocellular and disrupt junctions of hepatocyte intercellular, the bile might leak into sinusoidal blood vessels from the bile canaliculi, which can also explain the elevation of indirect bilirubin seen in icteric leptospirosis. Additionally, this may happen to leptospirosis-induced hemolysis occasionally (Avdeeva et al., 2001).

Leptospirosis is also very likely to involve pathological changes in the lung. Quantities of fatal leptospirosis cases documented that patients had pulmonary petechiae on their pleural surfaces and over a half of them had the gross haemorrhage in lung's cut surface accounted by haemorrhage of both the alveolar septa and intra-alveolar spaces (Arean, 1962). Renal infection of leptospirosis varies in severity from mild nonoliguric renal dysfunction, like polyuria, to complete kidney failure, which is a hallmark of Weil's disease. Histologically, changes typically occur in tubular and induce interstitial nephritis. Tubular changes involve thinning and/or necrosis of tubular epithelium and distention of tubular lumen with hyaline casts and cellular debris (Arean, 1962).

The Infection needs an incubation period (2~30days) to produce disease, so the onset varies from 5 days to 2 weeks after environmental exposure (Faine, Adler, Bolin, & Perolat, 1999). The illness may last for more than two weeks, and even up to a half year (Gordon, 1993). Morgan et al. (2002) did a research among 52 athletes who suffered from laboratory-confirmed leptospirosis after taking part in the Springfield Triathalon, and then Morgan's group indicated that there was a noteworthy variability in the length of incubation phase period between exposure and onset of symptoms; it lagged 6-29 days.

1.4 Clinical manifestations

Leptospirosis had been reviewed as a zoonosis of protean manifestations (Peter, 1982). Indeed, the spectrum of symptoms is extremely broad; the classical syndrome of Weil's disease represents only the most severe presentation. It is a systemic illness, characterised by fever, renal and hepatic insufficiency, pulmonary manifestations and reproductive failure. Formerly distinct clinical syndromes were considered to be associated with particular serogroups although this hypothesis has been refuted by more intense studies (Edwards, 1960; Feigin, Anderson, & Heath, 1975). Clinical signs are quite changeable; most cases are probably inapparent and joined with host-adapted serovars such as Canicola in canines, Bratislava in horses and pigs, Hardjo in calves and Australis and Pomona in pigs (Adler & de la Peña Moctezuma, 2010).

Some serovars, lead to more severe disease, like icteric, hemorrhagic, uremic (Stuttgart disease) and reproductive disorders (abortion and premature or weak pups) in dogs (Bolin, 1996). In cattle and pigs, signs of leptospirosis include reproductive failure, abortion, stillbirths, fetal mummification, sick piglets or calves and agalactia. A chronic manifestation of leptospirosis is commonly seen in horses as recurrent uveitis (Rohrbach, Ward, Hendrix, Cawrse-Foss, & Moyers, 2005), but is not unique to this species and may also be seen occasionally in humans. Bilateral or unilateral uveitis, cotton wool spots, and necrotic retinitis, with or without systemic symptoms, have been related to leptospiral infection as the finding suggested (Merien et al., 1993).

Leptospirosis in humans can fluctuate sharply according to the tainting serovar of *Leptospira*, and the age, health and immunological provision of the patient. It ranges

from a mild, influenza-like malady to a severe infection with renal and hepatic failure, pulmonary distress, and death (the classical Weil's disease). At the beginning of infection, leptospirosis patients commonly present some nonspecific febrile syndrome like a sudden onset of fever, which is easy to be confused with those symptoms caused by influenza, dengue fever, or malaria. Rash could be a clear sign that suggests appropriate diagnosis for those febrile diseases mentioned above; it is rare to see the rash in leptospirosis however quite often in dengue fever and chikungunya fever (Burt, Rolph, Rulli, Mahalingam, & Heise, 2012; Zaki & Shanbag, 2010). Headache is also a severe sign that often accompanied by retro-orbital pain and photophobia, presenting a bitemporal, frontal throbbing ache. When meningismus occur additionally, the headache would further lead to lumbar puncture (Berman, Tsai, Holmes, Fresh, & Watten, 1973). It is also commonly observed that muscle pain and tenderness quintessentially occurs in calves and lower back. What is worthy to note, conjunctival suffusion, dilatation of conjunctival vessels without purulent exudate, is a frequent feature in leptospirosis but not in other infectious diseases; this could help identify leptospirosis at this stage. Subconjunctival haemorrhages and icterus are also found in the eyes of leptospirosis patients. Twenty to fifty-seven percent of leptospirosis patients have a nonproductive cough, which potentially confuses clinicians to conclude as influenza or other some respiratory diseases mistakenly. Abdominal symptoms are mainly because of gastrointestinal discomfort, like vomiting, diarrhoea, and abdominal pain performed by acalculous cholecystitis and pancreatitis. Dehydration can also occur in patients with these gastrointestinal illnesses due to some renal functional failure caused by *Leptospira*. Leptospirosis can additionally account for some severe fatal pancreatitis cases, though most of the leptospirosis-associated pancreatitis are self-limited (Spichler, Spichler, Moock, Vinetz, & Leake, 2007). When leptospirosis is admitted to a more advanced phase, multiple organs, such as liver, lungs, and brains, may dysfunction together. For

example, Weil's disease presents jaundice and dysfunction of the kidney, which was described as the most appropriate clinical form of leptospirosis. The main target organ presented in leptospirosis is kidney as a result of the renal-tropic homing competence that leptospires intrinsically have to reserve them in hosts. Serum blood urea nitrogen and creatinine levels commonly manifest elevations in tests, and it also can be found syndromes like pyuria, hematuria, and high levels of urine protein (Katz, Ansdell, Effler, Middleton, & Sasaki, 2001). Severe leptospirosis may trigger acute respiratory distress syndromes (ARDS), like diffuse lung impairment and typically hurt gas exchange function. Pulmonary haemorrhage is a premonitory complication of leptospirosis with high fatal rates (>50%), often presented through massive hemoptysis caused by extensive alveolar haemorrhage (Gouveia et al., 2008). This severe pulmonary haemorrhage syndrome (SHPS) is easily mixed up clinically with viral pneumonitis that also can occur in outbreaks. For instance, an SPHS out broke after 1995's Nicaragua flooding, and it was incorrectly accounted for a hantavirus-associated pulmonary syndrome until postmortem tests revealed *Leptospira* in lung tissues (Trevejo et al., 1998).

Animals, as another target object in leptospiral infection, can have not only environmental impact but also economic problems due to this global zoonosis. Livestock such as ruminants, swine, and horses and donkeys, and pet animals like dogs and cats are like to be the most impacted group suffered from leptospirosis because they are intimately associated with human, which can affect the development of agricultural economics. Agricultural economics is a branch of economics that specially dealt with land usage. Specific clinical signs of leptospirosis presented in ruminants include fever, hemolytic anaemia, hemoglobinuria, jaundice, sometimes meningitis, and death. Infected cows occasionally produce a little blood-tinged milk when they are in lactation period (Higgins, Harbourne, Little, & Stevens, 1980).

1.5 Epidemiology of Leptospirosis

Leptospirosis is a common regional disease but with high intensity widespread in livestock animals and humans. World Health Organisation (WHO) (1999) was the first organisation that attempted to do an investigation on global incidence of leptospirosis. International Leptospirosis Society gathered comprehensive datas and deduced that the impact of severe leptospirosis was 350,000 – 500,000 per year (Ahmed, Grobusch, Klatser, & Hartskeerl, 2012). However, this was considered to be largely underestimated due to some data missing; the notification system in the vast majority of areas was not mature, and some were even not established (Ahmed et al., 2012). The incidence is significantly higher in warm-climate areas than in temperate countries (Pappas, Papadimitriou, Siozopoulou, Christou, & Akritidis, 2008); this is mainly because the leptospires could not longer survive under warmer and more humid conditions. Also, most countries in tropical areas are developing countries, which also contributes to the higher opportunities to expose the human population to infected animals. Seasons have an impact on the incidence of leptospirosis as well; the peak if leptospirosis incedence occurs in summer of fall in temperate areas where the temperature takes the limiting factor in leptospires survival. In warm-climate regions, rapid desiccation helps to prevent its survival (Levett, 2001). The rate of endemic human leptospirosis shows a significant difference from that in Europe (0.5/100,000 population) and Africa (95/100,000 population) (WHO 2011).

Occupation is also a remarkable factor of risk to human leptospirosis (Waitkins, 1986). Direct contacts with infected animal results in the most infections of farmers,

veterinarians, abattoir workers (Campagnolo et al., 2000; Chan, Paul, & Sng, 1987; Terry, Trent, & Bartlett, 2000), meat inspectors (Blackmore, Bell, & Schollum, 1979), rodent control workers (Demers, Frank, Demers, & Clay, 1985), and other work that requires touch with animals (Looke, 1986). High incidence of leptospirosis can also be observed where indirect contact occurs: sewer workers, miners, soldiers (Johnston, Lloyd, McDonald, & Waitkins, 1983), septic tank cleaners, fish farmers (Gill, Coghlan, & Calder, 1985; Robertson, Clarke, Coghlan, & Gill), gamekeepers, canal workers (Andre-Fontaine, Peslerbe, & Ganiere, 1992), rice field workers (Padre, Watt, Tuazon, Gray, & Laughlin, 1988), taro farmers (Anderson & Minette, 1986), banana growers (L. Smythe et al., 2000), and sugar cane cutters (Cotter, 1936).

A study published by Buchanan showed that miners were the group that had the highest risk suffering from Leptospirosis (Buchanan, 1927). In the early 20th century, the occurrence of this disease in sewer workers was the first reported (Fairley, 1934; Stuart, 1939). The data showed nearly one-fifth seroprevalence of sewer workers in Glasgow, Scotland (Stuart, 1939). Another occupational group whose risk of contracting leptospirosis was recognised early was fish farmers. 86% of all leptospirosis cases taken place in the northeast of Scotland between 1934 and 1948 were in fish workers (Smith, 1949). Livestock farming is a major occupational risk factor throughout the world. The highest risk was associated with dairy farming and is related to serovar hardjo, in particular with the milking of dairy cattle (Blackmore & Schollum, 1982; Philip, 1976). What's more, recreational exposures happening in water sports were also connected to a significant risk of leptospirosis (Mumford, 1989). Swimming, canoeing (Shaw, 1992), white water rafting and freshwater fishing (Jevon, Knudson, Smith, Whitecar, & Blake Jr, 1986; van Crevel, Speelman, Gravekamp, & Terpstra, 1994), even potholing and caving had a potential risk (Mortimer, 2005).

It also shows importance when studying on the epidemiology of leptospirosis in animals. Parasitic *Leptospira* can infect any animal species from the theory, but not many of *Leptospira* serovars are endemic, and they usually attempt to maintain in particular maintenance hosts (Hathaway & Blackmore, 1981). The most worth noticed maintenance hosts involved in animal leptospirosis are Icterohaemorrhagiae infected brown rat, Rattus norvegicus, cattle and sheep with Hardjo, and pigs and possibly dogs carried with Canicola and Bratislava. Other host maintained leptospiral infections are not widespread as them due to some limitations in geophysical conditions and host distribution, such as swine leptospirosis infected by serovars Kenniwicki and Tarassovi. Additionally, Hathaway and Blackmore (1981) did a study on brown rats in New Zealand, found that serovar Ballum was only maintained when the brown rat population density was high, like in rubbish dumps, but not under other most conditions. However, an animal species could be infected from other individuals in that species that maintained with the serovars, or even from other animal species in that area. The chances of prevailing social activity, management methods, and environmental elements determine the relative importance of those incidental infections and transmissions occurred between species. Similar with that in human, incidental infections are more likely to happen in warm and moist environments where lack of sanitation and rodent control; these contamination environmental could provide most range of *Leptospira* with more beneficial survival conditions.

2. *Leptospira*

2.1 Leptospiral structure

2.1.1 General morphological features

Leptospira is from family *Leptospiraceae* that belongs to spirochetes. Most members of *Leptospiraceae* are long, thin, highly motile, spiral-shaped bacteria; leptospires are

approximately 0.1 μm in diameter, by 6–20 μm in length, and have distinctive hooked ends (see figure 2 (a)) and spiral shapes by the helical amplitude of 0.1-0.15 μm. No matter what lifestyles of *Leptospira* spp. are, saprophytic or pathogenic, their sizes and appearances maintain overall consistency. Nutrition conditions in the laboratory culture of leptospires are usually set, which could decrease the motility and weaken the cell health and lead to be spherical *Leptospira* that has no motility. See figure 2 for the diverse morphology of *Leptospira*:(a) *Leptospira* with high motility, (b) elongated, limited motile *Leptospira* and (c) spherical *Leptospira* without motility.

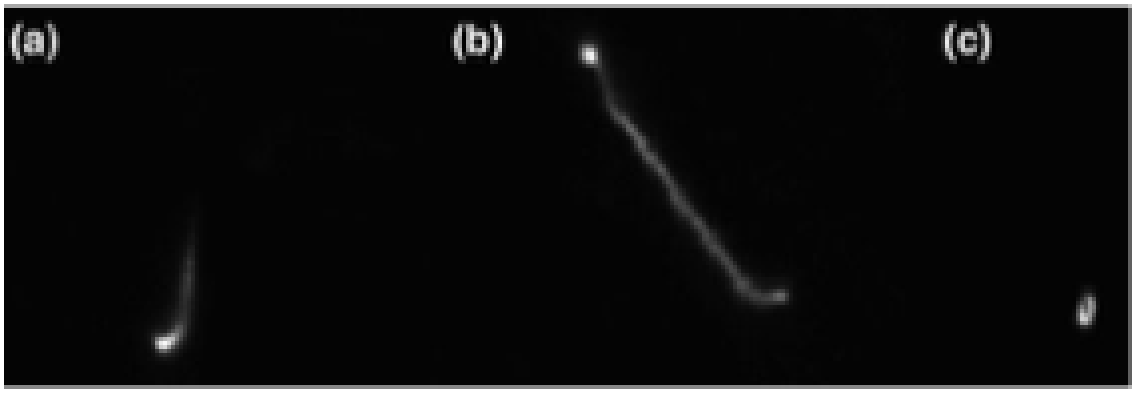

Figure 2 Darkfield image of the three distinct states of laboratory-grown *L. interrogans* cultures, taken using a 100x oil immersion lens: (a) hooked, motile; (b) elongated, semi-motile; (c) spherical (Adapted with permission from Cameron (2015))

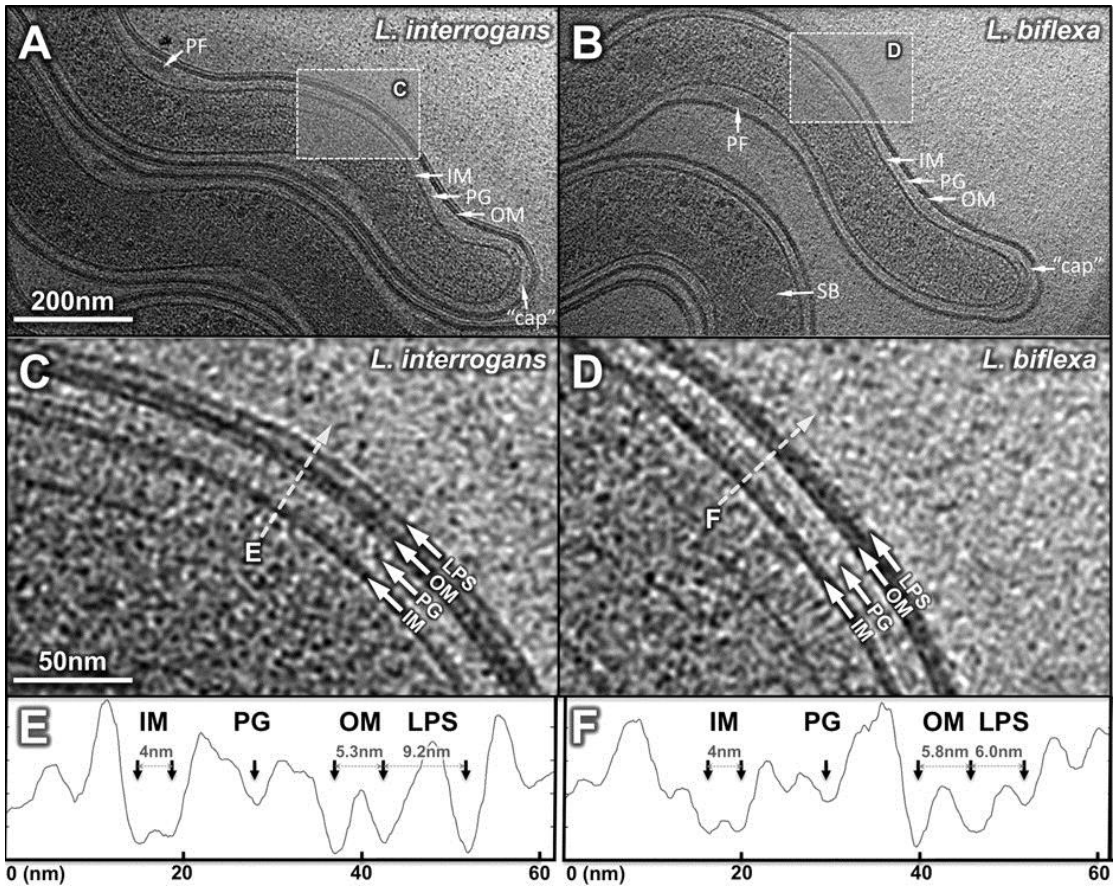

Figure 3 L. interrogans ultrastructure (Adapted with permission from Raddi et al. (2012))

Leptospires have a conventional double membrane structure in which the cytoplasmic

membrane and peptidoglycan cell wall are closely united and are superimposed by an outer layer (Cullen, Haake, & Adler, 2004) presented as figure 3: (a) outer membrane (OM), inner membrane (IM), peptidoglycan layer (PG), periplasmic flagellum (PF), and the "cap" at the cell end (b) magnified image showing the structural details of the cell envelope Within the outer membrane, the LPS institute the first antigen for *Leptospira*, which is structurally and immunologically similar to LPS in Gram-negative organisms (Que-Gewirth, 2004). LPS of *Leptospira* is on the surface, not like *Treponema* or *Borrelia* from spirochete. Actually, LPS structures diverge *Leptospira* and help the taxonomy; *Leptospira* presents 24 serogroups and more than 250 serovars thanks to the diverse leptospiral LPS (Evangelista & Coburn, 2010). The virulence of Leptospires is also well determined by LPS (Murray et al., 2010; Nahori et al., 2005; Werts et al., 2001). The leptospiral outer membrane has three layers, shown in cryo-electron tomography, separating by a space of approximately 5 - 9 nm (Raddi et al., 2012). Leptospiral lipoproteins and transmembrane proteins also locate in the outer envelope. Leptospiral Lipid A contains some unusual features including a mutated glucosamine disaccharide unit, which is phosphorylated and methylated. In addition to LPS, architectural and practical proteins form part of the leptospiral outer membrane. A large proportion of such proteins are lipoproteins with relative abundance on the cell surface: LipL32 > LipL21 > LipL41 (Cullen et al., 2005). Integral membrane proteins such as the porin OmpL1 (Shang et al., 1995) and the type two secretion system (T2SS) secretin GspD (Reyes et al., 2005), are also positioned in the outer membrane of *Leptospira* and have been attested to be antigenic.

2.1.2 Motility of leptospires

Leptospira is the model organism of spirochete for clarifying the motility of these bacteria because it is relatively easier to cultivate than other spirochetes. Leptospires

commonly have fast translational motility by the speed of around 10 μm per second in ordinary media (Faine, Adler, Bolin, & Perolat, 1999b); they can even accelerate in high viscosity (Charon & Goldstein, 2002). The mechanisms of leptospiral motility illustrated by the most convinced theory was that two periplasmic flagella with extreme insertions located in the periplasmic space are responsible for the motility of *leptospiraceae*; the flagella could form a gyrating helical wave by rotating at the cellular leading end, which produces an opposing force for the cell to move along the flagellum (Goldstein & Charon, 1988). Charon, Lawrence, and O'Brien (1981) also found that leptospiral outer envelope presented as a fluid mosaic when doing researches taking on antibody-coated latex beads attaching to the *Leptospira*. The results from this experiment kept the consistency with the theory above; the antibody-coated latex particles would flow from the front to the back end of the motile cell, and always keep the reversed directions with the swimming cell. FlaA and FlaB proteins aggregate the flagellar sheath and core respectively; the FlaB core is surrounded by a sheath and supposed to be a part of FlaA (Li, Wolgemuth, Marko, Morgan, & Charon, 2008). *Leptospira interrogans* genome contains two *flaA* genes and four *flaB* genes that are fully expressed (Malmström et al., 2009). Electron microscopy showed a *flaB* gene could mutate to be deficient in endoflagella and non-motile (Picardeau, Brenot, & Saint Girons, 2001). Later mutation investigations testing in *L. interrogans* showed that if a *flaA1* gene mutant subsequently continued to express FlaA2, the motility would be reduced; while if a *flaA2* mutant expressed neither FlaA protein and formed changed morphology, the *L. interrogans* would lack motility and lose its virulence in an animal infection model. Furthermore, the *flaA2* mutant was found displaying flagella of the similar size with the wild-type flagella, even though it would not express FlaA1 or FlaA2, which stated some other independent components were independent of FlaA (Lambert et al., 2012).

Leptospiral flagellum rotates counterclockwise forms a spiral-shaped end and results in a hook-shape end when it rotates clockwise (Wolgemuth, Charon, Goldstein, & Goldstein, 2006), showed as Figure 4. As the figure showed, a and b are non-translating forms, with either (a) hook-hook ends or (b) spiral-spiral ends; c is a translating form, with hook-spiral ends, moving in the direction of the spiral end. Leptospires might translate their end shapes to either a hook-spiral shape or a spiral-hook shape by rotating the flagella in the counter directions to have them moved directionally or swimming, in the direction of the spiral end.

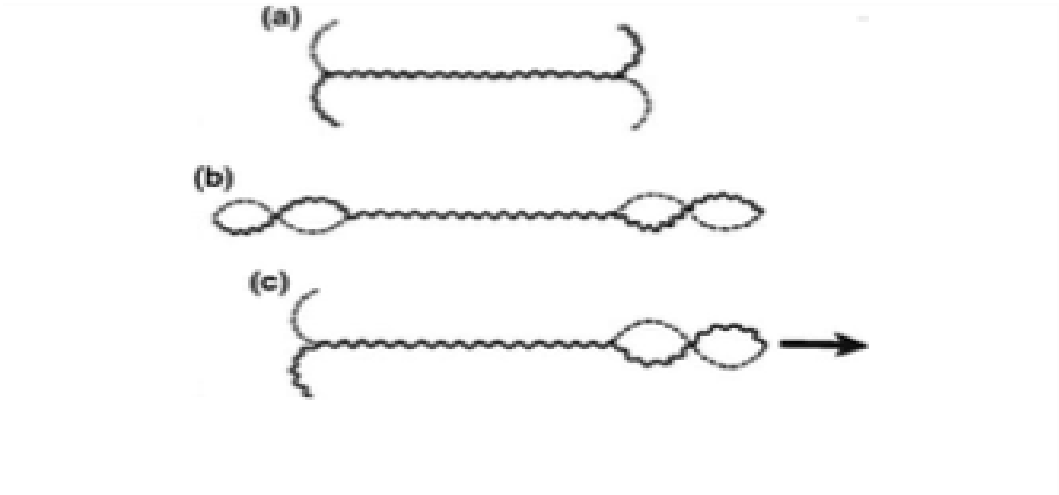

Figure 4 Leptospiral motility. (Adapted with permission from (Goldstein & Charon, 1990)).

Unlike other bacteria wth external flagella, leptospires perform enhanced moving speeds under high viscosity, consistently with other spirochetes (Charon & Goldstein, 2002; Kaiser & Doetsch, 1975). This character facilitates the leptospiral growth in natural environments and invasion of tissues by the leptospiral pathogens (Takabe, Nakamura, Ashihara, & Kudo, 2013). One of the highlighted differences of pathogenic *Leptospira* spp. from saprophyte *L. biflexa* is that leptospiral pathogens survive in both water and mammal bodies, two substantially distinct conditions, and meanwhile maintain their motility upon exist in physiological osmolarity; however, saprophytic

Leptospira do not have this capability (Takabe et al., 2013). This difference also suggests a direct association between the motility of bacteria and leptospiral virulence (Cameron, 2015).

2.1.3 Metabolism

In the contrast of spirochetes *B. burgdorferi* and *T. pallidum*, the genome of *Leptospira* species is responsible for encoding the full process of biosynthesis of amino acid and nucleic acid(Faine, Adler, Bolin, & Perolat, 1999a). While the *L.biflexa* can synthesis of purines and pyrimidines, which facilitates the growth in the condition with the purine analogue 8-azaguanine. The cultivation medium with 8-azaguanine is thus able to differentiate the pathogenic and saprophytic *Leptospira* species (Johnson & Rogers, 1964). The primary sources of energy and carbon that leptospires obtain are from a complete beta-oxidation pathway contained long chain fatty acids (Henneberry & Cox, 1970; Nascimento et al., 2004). Leptospira does not utilise glucose as the major energy source for many of other bacteria because leptospiral genome determines two significant different replacements:(1) pyrophosphate-fructose-6-phosphage 1-phosphotransferase displaces normal phosphofructokinase;(2) hexokinase is substituted by a glucose kinase (Nascimento et al., 2004; Zhang et al., 2011). Another reason explained the lack of glucose utilisation might be the stem from limited glucose transport system (Nascimento, 2004).

Leptospires are aerobic organisms; hence, the leptospiral genome can encode a corresponding tricarboxylic acid cycle and a set of components for respiratory electron transportation. Consequently, oxidative phosphorylation generates ATP for *Leptospira* in the form of F_0F_1-type ATPase (Nascimento et al., 2004; Ren et al., 2003). The

nitrogen sources that leptospires need for growth are frequently from the form of ammonia (Faine et al., 1999a). Sequencing studies on leptospiral genome have stated that pathogenic *Leptospira* species differed from some spirochetes, the *B.burgdorferi* or *T.pallidum*; leptospiral pathogens possess all of the essential genes for encoding the complete pathways for protoheme and vitamin B_{12} biosynthesis (Ricaldi et al., 2012). Diverse from pathogenic leptospiral species, the intermedia pathogenic *Leptospira* that usually causes relatively mild diseases, such as *L.licerasiae*, seems to be easier to cultivate in vitro because its genes can directly express those proteins required in the metabolism of nitrogen, amino acid and carbohydrate (Ricaldi et al., 2012).

2.1.4 Nutritional requirements, growth, and cultivation

The basic nutritional requirements for leptospires to grow include sources of carbon and nitrogen, some certain vitamins, and selected nutritional supplements. The key source of carbon for leptospires is long chain fatty acids. Interestingly, these essential fatty acids are toxic, so the leptospiral culture in vitro needs to add particular detoxicants, e.g., serum with albumin or sorbitol-complexed fatty acids. The albumin aims to absorb superabundant fatty acids and meanwhile control their release at a nontoxic concentration level (Faine et al., 1999a; Stalheim & Wilson, 1964). Glycerol can be added into the medium for accelerating leptospires to grow (Staneck, Henneberry, & Cox, 1973). The only identified source of nitrogen are the ammonium ions, gaining through deamination of amino acids (Faine et al., 1999a). The growth of leptospires also needs some nutritional supplements, including thiamin, biotin, phosphate, calcium, magnesium and iron. Other required compounds, like copper and sulphate, are calculated to isolate and maintain the pathogenic *Leptospira* species (Faine, 1959; Shenberg, 1967; Stalheim & Wilson, 1964). Nascimento et al. (2004) opposed the historical opinion said that the addition of vitamin B_{12} was nutritionally essential for

leptospiral growth in vitro culture at 37°C, demonstrating the fact that pathogenic leptospires composing an operon that can complete the biosynthesis of vitamin B_{12} within the genome, so this nutrition supplement were considered to be redundant. Both of saprophytic and pathogenic *Leptospira* species grow under oxybiotic environment, but they show differences in the speed of growth when they are at low temperatures. They both present the maximum increase in vitro at 28 – 30°C, while, saprophytic leptospires can keep growing under low temperatures (11 – 13°C) but contrast, pathogens exhibit stopping in growth under that conditions. Additionally, pathogenic leptospires can grow at 37°C. The optimal pH range for leptospiral growth is from 7.2 to 7.6. The maximal growth of leptospires using stationary phase seed cultivation might be observed in 4 – 7 days for the pathogens and 2 – 3 days for the saprophytes (Faine et al., 1999a), typically presenting a final density of 10^7 – 10^8 bacteria/ml (Zuerner, 2005). Darkfield microscopic techniques are commonly used to monitor the leptospiral growth in vitro cultures, associating with a Petroff-Hausser chamber counting method. Hemocytometer counting method is not recommended here because the chamber may be too deep for this experiment.

When preparing for the cultivation medium, it is necessary to sterilise completely due to the potential existence of ubiquitous saprophytic leptospires in water sources. It might be considered that filter-sterilisation protocol is not suggested to replace the autoclaving method since the microbiological filters can not block saprophytic leptospires (Cameron, 2015). A successful propagation of pathogenic Leptospira also requires adequate serum albumin to eliminate the toxicity of the fatty acid. Bovine serum albumin (BSA) is constantly added to the medium for this purpose; especially when isolating the leptospires from clinical samples, it becomes mandatory (Cameron, 2015). General leptospiral cultivation medium, according to the states of matter, includes liquid,

semisolid and solid medium. Successful leptospiral growth in the liquid medium depends on the process of seed inoculation to a large extent. An adequate seed inoculum frequently uses 1 – 10% of the volumes of the fresh liquid medium, seeding with the well-adapted laboratory leptospiral strains directly. The refined EMJH medium is the most continually used liquid medium for leptospiral culture, modified by Johnson and Harris (1967) based on the primary Ellinghuasen McCullough medium (Ellinghausen Jr & McCullough, 1965) (EMJH). The rabbit serum with non-toxic could be added into the medium to foster the leptospiral growth. The semisolid medium could also provide a proper condition for some pathogenic leptospires to grow in vitro, which let leptospires load to a dense zone referred to as a Dinger's disk (Lawrence, 1951). The inoculation needs to transfer the Dinger's disk to the semisolid medium aseptically when the disk has been with an apparent density. Solid-media culture is not suitable for all pathogenic *Leptospira* due to the difficulty for those fastidious pathogenic leptospires to grow on the solid medium.

Urine, tissues and blood are the main sources to isolate pathogenic Leptospira from clinical samples. Collecting leptospires from urine usually uses a clean catch approach, placing the urine sample into a sterile container. After a set of vortexes, samples can be cultivated with the semisolid EMJH medium. Postmortem infected animal tissues, such as from kidneys and liver, can be extracted pathogenic leptospires (Zuerner, 2005). The standard process of leptospiral isolation from blood samples is to inoculate 100–200 μl of whole blood into 5–10 ml into semisolid or liquid EMJH medium. It needs to be noticed that the extortionate blood concentration may restrain the leptospiral growth (Wuthiekanun et al., 2007).

2.2 Taxonomy

Leptospiraceae is comprised by three genera: *Leptospira*, *Leptonema*, and *Turneriella*. Saprophytic, pathogenic and intermediate clades consist the genus *Leptospira*; proved capacity to infect a large range of hosts and survive within marine environments and mammalian host conditions were presented in both of them (Adler, 2015). Leptospires are spirochetes, including both free-living saprophytes in the water and pathogenic species that might account for acute or chronic zoonosis, comprising the genus *Leptospira*, which belongs to the family Leptospiraceae, order Spirochaetales (Faine et al., 1999). The family *Leptospiraceae*, first defined in 1979 with two genera, *Leptospira* and *Leptonema*, was included in the Approval Lists of Bacterial Names (Skerman, McGowan, & Sneath, 1980). *Turneriella* was added into it, and now they together composed the family *Leptospiraceae*. *Leptospira* Noguchi is the type genus of *Leptospira* (Noguchi, 1917). Three genera distinguish from each other by the G + C content, DNA-DNA relatedness, and 16S rRNA sequences; the G + C content of the genera *Leptospira*, *Leptonema*, and *Turneriella* are 33-43, 54, and 53.6 mol%, respectively (Stackebrandt et al., 2013). At the meeting of the Subcommittee on the Taxonomy of *Leptospiraceae* held in Quito, Ecuador in 2007, it was decided to give the status of species instead of describing them as genomospecies 1, 3, 4 and 5 previously, resulting in a family spanning 13 pathogenic *Leptospira* species: *L. alexanderi, L. alstonii* (genomospecies 1), *L. borgpetersenii, L. inadai, L. interrogans, L. fainei, L. kirschneri, L. licerasiae, L. noguchi, L. santarosai, L. terpstrae* (genomospecies 3), *L. weilii, L. wolffii*, with wider than 260 serovars (Adler & de la Peña Moctezuma, 2010). It is anticipated that additional new species exist. Saprophytic species of *Leptospira* include *L. biflexa, L. meyeri, L. yanagawae* (genomospecies 5), *L. kmetyi, L. vanthielii* (genomospecies 4), and *L. wolbachii*, and carry over 60 serovars, as showed in Figure 5.

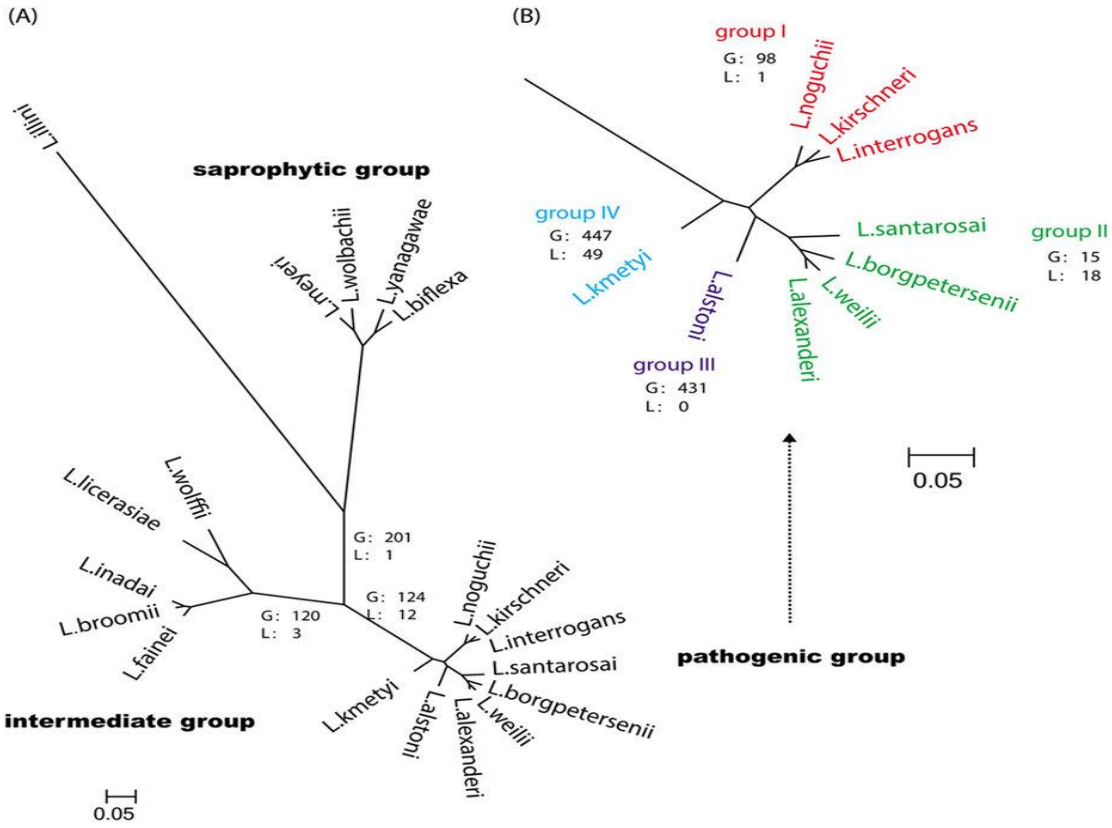

Figure 5 *Leptospira* family tree

Nevertheless, it is relatively less hurtful to cells or animals, being as much as 12 times less poisonous for mice when correlated with *E*. coli LPS (Faine et al., 1999).

2.2.1 Serological classification

According to the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Leptospira*, "Two strains are said to belong to different serovars if after cross-absorption with adequate amounts of heterologous antigen more than 10 % of the homologous titer regularly remains in at least one of the two antisera in repeated tests"(Stallman, 1984). This standardised approach was modified from the first proposal by Wolff and Broom (1954), aimed to the maintenance of cultures and serological characterization, based on the previous work they had established in their laboratory in Amsterdam. As Amsterdam system applied, "two strains are considered to belong to

different serotypes if, after cross-absorption with adequate amounts of heterologous antigen, 10 % or more of the homologous titre regularly remains in each of the two antisera" (Wolff & Broom, 1954); it helped 32 different serotypes to be identified. Additionally. Wolff and Broom further proposed that it is reasonable to cluster closely related serotypes into serogroups for convenience.

It has been recorded more than 60 serovars of *L. biflexa* within the species *L. interrogans* over than 200 serovars were recognised (Levett, 2001). The genus *Leptospira* was separated into two species before 1989; *L. icterohaemorrhagiaes*, comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains isolated from the aquatic environment were recorded in the 7th edition of Bergey's Manual in 1957. Furthermore, *L. icterohaemorrhagiae* was subdivided into serotypes, but *L. biflexa* was not. The meeting of Taxonomic Subcommittee on *Leptospira* in 1962 redefined the classification; it named pathogenic strains as *L. interrogans* and the saprophytic strains as *L. biflexa* (Wolff & Turner, 1963). Characteristically, *L. biflexa* can fluoresce from *L. interrogans* by the germination of the former at 13°C and increase in the presence of 8-azaguanine (225 μg/ml) and by the failure of *L. biflexa* to form spherical cells in 1 M NaCl (Levett, 2001).

Serovars that are antigenically related have traditionally been arranged into serogroups for convenience (Kmety & Dikken, 1993). Although serogroups have no taxonomic standing, they have been proved useful for initial serological diagnosis and epidemiological understanding at regional and population level.

2.2.2 genotypic classification

Genotypic classification of leptospires has replaced the phenotypic one, in which many genomospecies include all serovars of both *L. interrogans* and *L. biflexa* (Brendle, Rogul, & Alexander, 1974). It is because genetic characterisation is possible in only a few labs and reference serological reagents (polyclonal and monoclonal antibodies) capable of defining serovars are not immediately available. Based on the pair ratio, at least two homology groups consist one pathogenic strain; further work expanded the number of groups to six (Brendle et al., 1974). Meanwhile, Brendle *et* al. (1987) also found that serovar Illini was distinct genetically from other leptospires, which inspired Hovind-hougen in the definition of the monospecific genus *Leptonema* (Hovind-Hougen, 1979). A further strain was found to be serologically and genetically distinct both from other leptospires and from *Leptonama illini*, and was named *Leptospira Parva*. This species was later transferred to a new genus, *Turneriella* (Levett et al., 2005) Another new genus named *Turneriella* (Levett et al., 2005) was transferred from the former, *Leptospira parva*, which was first distinguished from other leptospires and even from *Leptonema* illini in 1981. Genetic heterogeneity has been demonstrated for some time; consequently, ten genomospecies of *Leptospira* were defined by using DNA-DNA hybridization studies (Brenner et al., 1999; Smythe et al., 2013; Yasuda et al., 1987). Thanks to the Centres for Disease Control (CDC) that operated an extensive study of several hundred strains (Ramadass, Jarvis, Corner, Penny, & Marshall, 1992), 16 genomospecies of *Leptospira* were described more recently, including those defined previously (Brenner et al., 1999) and five new species (as Table 1). Besides, *L. fainei*, which has been described as a new species, was found to contain a new serovar called hurstbridge (Perolat et al., 1998). DNA hybridization studies also help to discover the taxonomic status of monospecific genus *Leptonema* (Ramadass, Jarvis, Corner, Cinco, & Marshall, 1990). A number of other species have been described: *Leptospira fainei* (Pérolat et al. 1998), *Leptospira broomii* (Levett et al. 2006), *Leptospira wolffii* (Slack

et al. 2008), *Leptospira licerasiae* (Matthias et al. 2008), *Leptospira kmetyi* (Slack et al. 2009b), and *Leptospira idonii* (Saito et al. 2013). There are currently 21 species of *Leptospira*, shown as Table 1.

Species	Valid publication
L. alexanderi	Brenner et al. (1999)
L. alstonii	Smythe et al. (2013)
L. biflexa	Faine and Stallman (1982)
L. borgpetersenii	Yasuda et al. (1987)
L. broomii	(Levett, Morey, Galloway, Steigerwalt, & Ellis, 2005)
L. fainei	(Perolat et al., 1998)
L. idonii	(Saito et al., 2013)
L. inadai	(Yasuda et al., 1987)
L. interrogans	(Faine & Stallman, 1982)
L. kirschneri	(Ramadass, Jarvis, Corner, Penny, & Marshall, 1992)
L. kmetyi	(Slack et al., 2009)
L. licerasiae	(Matthias et al., 2008)
L. meyeri	(Yasuda et al., 1987)
L. noguchii	(Yasuda et al., 1987)
L. santarosai	(Yasuda et al., 1987)
L. terpstrae	(Smythe et al., 2013)
L. vanthielii	(Smythe et al., 2013)
L. weilii	(Yasuda et al., 1987)
L. wolbachii	(Yasuda et al., 1987)
L. wolffii	(Slack et al., 2008)
L. yanagawae	(Smythe et al., 2013)
Turneriella parva	(Levett et al., 2005)
Leptonema illini	(Hovind-Hougen, 1979)

Table 1 Species within the family *Leptospiraceae*

Serovar	Species
Bataviae	*L. interrogans, L. santarosai*
Bulgarica	*L. interrogans, L. kirschneri*
Grippotyphosa	*L. interrogans, L. kirschneri*
Hardjo	*L. borgpetersenii, L. interrogans, L. meyeri*
Icterohaemorrhagiae	*L. interrogans, L. inadai*
Kremastos	*L. interrogans, L. santarosai*
Mwogolo	*L. interrogans, L. kirschneri*
Paidjan	*L. interrogans, L. kirschneri*
Pomona	*L. interrogans, L. noguchii*
Pyrogenes	*L. interrogans, L. santarosai*
Szwajizak	*L. interrogans, L. santarosai*
Valbuzzi	*L. interrogans, L. kirschneri*

Table 2 Leptospiral serovars found in multiple species

The species of *Leptospira* cluster into three big groups, comprising pathogens, non-pathogens and an intermediate group, showed as the family tree of *Leptpspiracae* in Figure 6. Molecular phylogenetic analysis of Leptospiraceae 16S rRNA gene sequences was utilisied by maximum likelihood method, based on the Tamura-Nei model, using MEGA5 (Tamura et al. 2011). The tree with the highest log likelihood is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There was a total of 1,230 positions in the final dataset.

Silmilar phylogenies can be produced using several housekeeping genes, including *rrs* (Morey et al. 2006), *rpoB* (La Scola et al. 2006), and *gyrB* (Slack et al. 2006).

The species of Leptospira currently recognized do not correspond to the previous two species (*L. interrogans* sensu lato and *L. biflexa* sensu lato). Interestingly, both pathogenic and nonpathogenic serovars occur within several species (Table 2). However, it is also clear that some reference strains have been mislabeled, leading to erroneous classification (Slack et al. 2009a). It is likely that some of the serovars listed in Table 2 will in the future be reclassified into a single species. Genetic heterogeneity within serovars has been demonstrated (Brenner et al. 1999; Bulach et al. 2000; Feresu et al. 1999). The presence of the same LPS biosynthetic genes in strains of different species implies genetic transfer; evidence of interspecies transfer has been detected (Haake et al. 2004). Thus, neither serogroup nor serovar of an isolate currently predicts the species of *Leptospira*. In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans* sensu lato from *L. biflexa* sensu lato do not differentiate the genomospecies (Brenner et al. 1999; Yasuda et al. 1987).

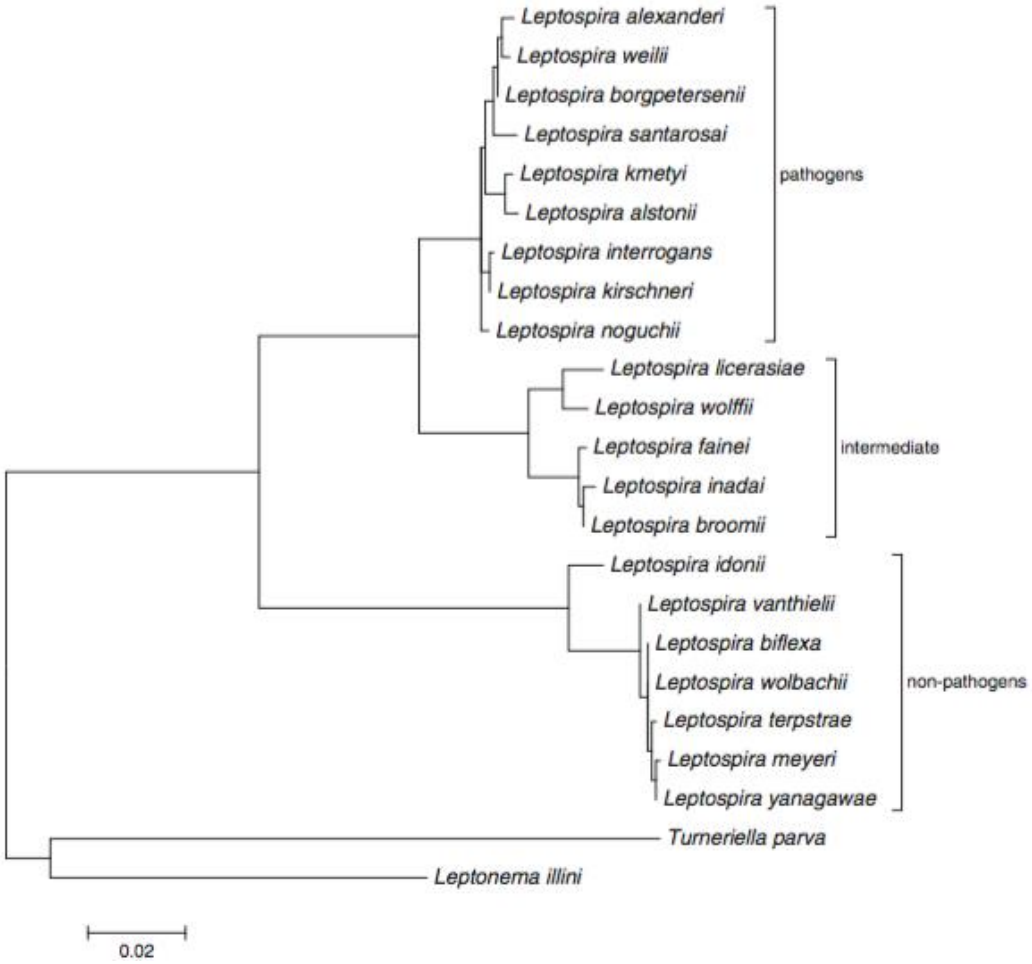

Figure 6 Family tree of *Leotpspiraceae.* Adapted with permission from Levett, 2015.

2.2.3 Correlation and limitation

The genomospecies of *Leptospira* do not correspond to the former two species (*L. interrogans* and *L. biflexa*), and indeed, pathogenic and nonpathogenic serovars occur within the same species. Hence, as Table 2 showed, neither serogroup nor serovar can be responsible for promising the species of *Leptospira* (Levett, 2001). What's more, recent studies have included multiple strains of some serovars and demonstrated genetic heterogeneity within serovars (Brenner et al., 1999; Feresu, Boling, van de Kemp, & Korver, 1999). Also, the phenotypic characteristics formerly used to differentiate *L. interrogans* sensu lato from *L. biflexa* sensu lato do not differentiate the genomospecies.

3. Commonly used assays for identification of *Leptospira* isolates

Characterization of *Leptospira* strains now base on two schemes; one serologically describes serovars as the basic taxon, and the other distinguishes species and subspecies using DNA similarity (Levett, 2015). Serological methods for typing *Leptospira*, such as cross-agglutinin absorption (CAAT) and cross-agglutinin absorption (MAT), are usually used to classify serovars. There exist some molecular approaches that can characterise leptospiral strains into serovars. However, molecular methods are considered lacking repeatability and reproducibility because they are based on highly plastic general genomic characteristics, not on the lipopolysaccharide (LPS) conformational features that actually determine serovars (Rudy A. Hartskeerl & Smythe, 2015). Thus what those general molecular methods present are genome plasticity, but not at a serovar level.

Leptospiremia would occur during the first stage of the disease, beginning before the

onset of symptoms, and has usually finished by the end of the first week of the acute illness (McCrumb Jr et al., 1957). Hence, blood cultures should be carried out as soon as possible after the patient's presentation. Isolated leptospires are identified either by serological methods or, more recently, by molecular techniques. Traditional methods often relied on cross-agglutinin absorption (CAAT) (Dikken & Kmety, 1978) and CAAT was considered as a standard test in serological typing of *Leptospira*. However, there are only a few laboratories where can perform this identification (Levett, 2001). For this reason, other laboratories choose microscopic agglutination test (MAT) as an alternative method that uses panels of monoclonal antibodies (MCAs)(Kim, 1987; Kobayashi, Tamai, & Sada, 1985; Korver, Kolk, Vingerhoed, Van Leeuwen, & Terpstra, 1988). Molecular methods have become widely used (Herrmann, 1993; Perolat et al., 1990) and are discussed below.

3.1. Serological typing

Generally speaking, serotyping is based on antigen-antibody reactions. The antigenic structure is usually complicated in *Leptospira*. Despite the fact that classification by serogroups rarely has actual or official status, it has significant practical meaning that it groups antigenically similar leptospiral strains together among more than 200 reference strains of *Leptospira* that have no access to use or assess individually.

3.1.1 Cross-agglutinin absorption test

CAAT is still regarded as the gold standard for serological typing of leptospiral isolates and identification of serovars despite its complexity and inefficient in operation. The CAAT uses laboratory rabbits to produce hyperimmune sera to process the assay, which commonly costs 6-10 weeks before achieving a suitable hyperimmune titre following the protocols described in the minutes of the Subcommittee on the Taxonomy of

Leptospira (TSC) (Stallman, 1982). Not only aimed at identification of existing serovars, but the CAAT can also denote new serovars. TSC has advised the following theory for defining a serovar: if there is more than 10% of homologous titre remaining in at least one of the two antisera repeatedly and regularly after across absorbing adequate amounts of heterologous antigen, the two sample strains could be considered to belong to different serovar groups. Additionally, it needs a recognition by having CAAT typing identified in a reference laboratory (Stallman, 1984). As the serotyping techniques developing, CAAT shows its limitations in discriminative power. Hartskeerl et at. (2004) demonstrated that CAAT was not able to distinguish some of the serovars in *Leptospira* Grippotyphosa anymore.

3.1.2 Factor serum analysis

Factor serum analysis might be considered as a refinement of CAAT. The anti-*Leptospira* sera, extracted from laboratory rabbits, having a high level of specificity. They absorbed with diverse antigen reference strains until only reacting with a particular serovar, a subgroup, or a serogroup, to achieve that high specificity. MAT can quickly type isolates with the high specific factor sera. Additionally, factor analysis is a quite adequate approach to doing researches about antigenic similarities between leptospiral strains (Kmety, 1967). Kemty's factor serum analysis (1967) further studied the details of each serovar structure; he found the distinguishable combination or mosaic styles that the main and minor antigenic factors form. However, it is no longer being used because the process of preparing factor sera is laborious and lengthy time-consuming. Besides, due to the variation from batch to batch, factor sera lacks reproducibility in practical applications (Korver, 1992).

3.1.3 Typing with monoclonal antibodies

Monoclonal antibodies (MCAs) analysis is primarily a kind of MAT. Using panels

consisted by recognised characteristic antigen patterns, through serial dilutions, the highest serum dilution represented by titres shows the 50% of leptospiral cells agglutinated in the suspension (World Health, 2003). Typing with MCAs has increased the workload per time units than the traditional serological typing; it can recognise plenty of serovars in a relatively short time. Besides, the accuracy of figuring out mislabeled strains enhances instead of using natural rabbit sera (Terpstra, Korver, Van Leeuwen, Klatser, & Kolk, 1985). However, only 70% of all leptospiral isolates can be applied by this method, and it is required to use with caution because MCAs do recognise some of the epitopes but these epitopes are sometimes not unique to belong to one particular serogroup (Hartskeerl et al., 2001). Valverde et al. (2008) and Bourhy et al. (2012) have both reported cases that different serovars had similar patterns in CAAT but showed clear panels when doing typing with MCAs.

3.2. Molecular typing

The difficulties involved in the serological identification of Leptospiral isolates result in that molecular methods for identification and subtyping have aroused great interest (Terpstra, 1991; Terpstra, Schoone, Ligthart, & Ter Schegget, 1987). Serological typing techniques are considered to be arduous, lack of discriminative power, and regularly expensive, so it becomes more frequently to typing using faster molecular technologies based on the nucleic acid. Methods employed have included digestion of chromosomal DNA with restriction endonucleases (REA), restriction fragment length polymorphism (RFLP), ribotyping, pulsed-field gel electrophoresis (PFGE), and many PCR-based approaches, like arbitrarily primed PCR (AP-PCR).

3.2.1 Restriction endonuclease analysis (REA)

REA has been studied extensively (Hathaway, Marshall, Little, Headlam, & Winter, 1985; Hookey & Palmer, 1991; Marshall, Wilton, & Robinson, 1981; Robinson,

Ramadass, Lee, & Marshall, 1982; Tamai, Sada, & Kobayashi, 1988; Thiermann, Handsaker, Moseley, & Kingscote, 1985). The method consists of extraction of DNA from a homogeneous population of organisms, digestion of the DNA with a restriction endonuclease, and electrophoresis of the digested DNA in an agarose gel. Because restriction endonucleases can recognise and cleave double-stranded DNA at specific 4 or 6 base-pair sequences, a set of fragments is generated subsequently. The movement of these fragments in agarose gel is related to their molecular weight; a pattern of bands is presented that can be seen in the gel by ultraviolet light if stained with ethidium bromide, or by autoradiography, when it is labelled with 32P. These patterns constitute a characteristic "fingerprint" for any single DNA (Marshall et al., 1981). Robinson in 1982 demonstrated distinct genotypes within serovar hardjo by using REA (Robinson et al., 1982). Bovine isolates from North America have all been found to be of genotype hardjobovis, of which subtypes A, B, and C could be recognised (Thiermann, Handsaker, Foley, White, & Kingscote, 1986). In Northern Ireland, both genotypes hardjobovis and hardjoprajitno were discovered among bovine isolates (Marshall, Winter, Thiermann, & Ellis, 1985). Antigenic differences were also reported among hardjobovis isolates (LeFebvre, Thiermann, & Foley, 1987). Moreover, serovar balcanica isolates in North America were indistinguishable from genotype hardjobovis isolates by REA (Thiermann et al., 1986). REA also helped Taxonomy of *Leptospira* isolates from pig (Ellis, Montgomery, & Thiermann, 1991); it showed a relatively rapid method when Ellis's group (1991) operated molecular typing for the type strains of Australis serogroup of *Leptospira interrogans* from pigs and gave the reliable conclusion that all the strains were either *Leptospira* brastislava or muenchen. On the other hand, the problems of REA aroused attention. This technology is a labor-intensive work, and large-volume culture is necessary to it, which increased the cost to carry out this experiment. Additionally, the data performed by REA are a significant amount of

bands, which might enhance the difficulty of later process of comparison and interpretation (Cerqueira & Picardeau, 2009).

3.2.2 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) of large DNA fragments produced by rare-cutting restriction enzymes (such as NotI), proposes the advantage of an uncomplicated interpretation combined with a fast and powerful result (Herrmann et al., 1991). Coupled with computer techniques, it is possible to realise dendrograms construction and interlaboratory comparisons, which can further and detailedly contribute to the understanding of the relationship between leptospiral strains (Galloway & Levett, 2008). The genome size of *L. interrogans* was estimated at 5,000 kb by using PFGE after restriction with three separate endonucleases, and the pattern of each of the three serovars analysed was different (Baril & Saint Girons, 1990). PFGE proved concordance with traditional serological typing methods. Herrmann et al.(1994) have reported a new serovar of *Leptospira grippotyphosa*, Dadas I, presented a unique pattern to that serovar in PFGE. Overall, approximate 90% of leptospiral strains can be recognised through their characteristic pulsed-field gel patterns (Galloway & Levett, 2008). While in some cases, the discrepancies were also demonstrated; two serovars belonged to *Leptospira interrogans*, Copenhageni and Icterohaemorrhagiae cannot be distinguished by using PFGE (Tamai et al., 1988). This technique is labor-intensive which is like the handicap of REA. Moreover, in the tropical and sub-tropical areas where leptospirosis is with the highest incidence, PFGE is only available in limited laboratories (Cerqueira & Picardeau, 2009). Nonetheless, PFGE is still regarded as the gold standard of molecular typing for leptospiral serovars.

3.2.3 Ribotyping

Ribotyping has demonstrated reasonably good correlation with the phylogenetic classification of *Leptospira* into 11 genomospecies. The grouping of *Leptospira* using ribotyping, such as determination of the restriction fragment length profiles of digested chromosomal DNA probed with rRNA, is frequently used to help not only on taxonomy study on *Leptospira* but also for the description of different species of leptospiral strains at subgroup level (Grimont & Grimont). It was found that some of the genes are not coupling tightly (i.e. two sets of 16S and 23S rRNA and one or two 5S rRNA) in *Leptospira* spp., but dispersing over the whole chromosome (Baril, Herrmann, Richaud, Margarita, & Girons, 1992; Richard L. Zuerner, Herrmann, & Saint Girons, 1993). Using EcoRI for digestion and 16S and 23S rRNA from Escherichia coli as the probe, which constructed a large database (Perolat et al., 1990; Perolat, Lecuyer, Postic, & Baranton, 1993). However, between some of the serovars, those were proved to relating closely, ribotyping could not be able to tell the difference, such as icterohaemorrhagiae and copenhageni (Hookey & Palmer, 1991). Sometimes ribotyping has less discriminative power when lacking rRNA genes (Kositanont, Chotinantakul, Phulsuksombati, & Tribuddharat, 2007; Perolat, Lecuyer, Postic, & Baranton, 1993).

3.2.4 Insertion sequences

Insertion sequence (IS) elements are considered as of valuable distribution to leptospiral epidemiology researches. From pathogen *Leptospira interrogans*, Boursaux-Eude et al. (1995) and Zuerner's group (2002) first identified IS *1500* and IS *1502*, respectively. IS *1500* was able to be used to differentiate isolates between *Leptospira interrogans* (Zuerner & Bolin, 1997). Another such element, IS *1533* was recognised in *Leptospira borgpetersenii* (Zuerner, 1994); it can discriminate *Leptospira* subspecies at serogroup levels and even tell the difference within a given species (Zuerner, Ellis, Bolin, &

Montgomery, 1993). IS 1502, a new introduction into *Leptospira*, could not be detected in all strains (Zuerner & Huang, 2002).

3.2.5 Randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR)

Randomly amplified polymorphic DNA (RAPD) can provide an easy and rapid identification for Leptospira species (Ramadass, Meerarani, Venkatesha, Senthilkumar, & Nachimuthu, 1997) and also the differentiation between serovars (Corney, Colley, Djordjevic, Whittington, & Graham, 1993). Therefore, RAPD fingerprint technology helped the regions where leptospirosis with high endemicity, such as India, to a large extent on epidemiological studies (Natarajaseenivasan et al., 2005; Roy, Biswas, Vijayachari, Sugunan, & Sehgal, 2005). On the other hand, AP-PCR was proved to have the ability to classify *Leptospira* reference strains into sub-species, and get consistent results with that from 16rRNA gene sequencing and DNA-DNA sequence similarity analysis by Ralph, McClelland, Welsh, Baranton, and Perolat (1993). Later studies additionally identified the discriminative power of this technique (Brown & Levett, 1997; Ciceroni et al., 2002). Both RAPD and AP-PCR utilise low-stringency PCR amplification with primers that have the arbitrary sequence to produce strain-specific fingerprints. They did great work in molecular typing for *Leptospira* species. However, RAPD and AP-PCR sometimes cannot achieve the requirement when doing a large-scale study, due to their relatively poor reproducibility and difficulty in interlaboratory comparison (Cerqueira & Picardeau, 2009).

3.3 Molecular typing in the genome era

In the 21st century, the genomic study has great developments on *Leptospira* species; 6

Leptospira strains have their genome sequences reported: two pathogens *L. interrogans* (Ren et al., 2003) and *L. borgpetersenii* (Bulach et al., 2006), and besides, the *saprophyte L. biflexa* (Picardeau et al., 2008).

The genomic *Leptospira* spp. was reported as 3.9 – 4.6 Mb in size, locating on the two circular chromosomes (Cerqueira & Picardeau, 2009). The percentages of DNA homology differentiate *Leptospira* species using DNA hybridization experiments that is an arduous technique and can only be performed in CDC (Atlanta, USA), which means the shipments of pathogens around the world is meanwhile a nontrivial limitation. Therefore, techniques that allow online comparisons to produce digital data directly will be great choices in evading the need for transporting pathogens (Adler, 2015). Common used approaches include typing arrays (Ahmed, Anthony, & Hartskeerl, 2010), Multiple-locus Variable Number of Tandem Repeat Analysis (MLVA) (Ahmed et al., 2011; Ahmed et al., 2006; Caimi et al., 2012), Fluorescence Amplified Fragment Length Polymorphism (FAFLP) (Vijayachari et al., 2004) and sequence-based characterization.

Leptospiral genomes have been found carrying quantities of repeated sequences, like IS as mentioned above, and numerous short repetitive DNA sequences that have a typical tandem-repeated structure. These different kinds of tandem repeats were named Variable Number of Tandem Repeats (VNTR); they have been already widely used in higher eukaryotes fingerprinting-based tests. In doing *Leptospira* polymorphism study, plenty of tandem repeats located in *L. interrogans* genomes have the small-size sequence motifs that are under 100bp in length, which highly suits the researches based on electrophoresis of PCR products (Grissa, Bouchon, Pourcel, & Vergnaud, 2008). Whereas, VNTR loci do not exist that much in the *L. borgpetersenii*, so the next MLVA

hence needs appropriate primers for each species. In general, typing with VNTR is an accessible approach that can be easily operated even in developing countries with high incidence of leptospirosis, which has excellent distribution not only to the disease researches but also to the leptospirosis public health control (Cerqueira & Picardeau, 2009). Cerqueira and Picardeau (2009) also suggested the further refinements of this method might be necessary to avoid the process of pathogen culture, to make MLVA be able to apply to biological and environmental samples directly.

For subspecies diagnosis or typing of *Leptospira* isolates, there is an uncomplex method that can generate specific profile for discovering polymorphism: single strand conformational polymorphism (SSCP); it relies on the principle of altered conformity of the single-stranded DNA due to single base changes, which can be observed the difference in DNA mobility under specific electrophoresis settings (De Roy, Thavachelvam, Batra, & Tuteja, 2012). This approach has been applied successfully for several years for a great range of microorganisms typing (Manzano et al., 1997). However, in 2004, the correctness of results within former phylogenetic classification based on a single locus sequence has been questioned due to high plasticity and lateral DNA transfer on *Leptospira* genome (Haake et al., 2004; Nascimento et al., 2004). To solve this problem, multiple locus sequences genotyping developed since it can identify a putatively horizontally transferred locus. Multilocus sequence typing (MLST) is a typing technique based on some parts of housekeeping gene sequences, having been carried out to Leptospira spp.(Ahmed et al., 2006). It is not essential to have large quantities of purified DNA so that the difficulty of the experimental preparation would be lowered. MLST is currently the most robust, phylogeny-based typing method for *Leptospira* and additionally, it can be used to analyse new sequences online with *Leptospira* MLST Database (Jolley & Maiden, 2010).

3.3. High Resolution Melting

High resolution melting (HRM) analysis (Gundry et al., 2003), which regulates the melting temperatures of amplicons in real time utilizing a fluorescent DNA-binding dye, is a closed-tube, non-sequencing-based system for genotyping and mutation scanning (Gundry et al., 2003; Wittwer, Reed, Gundry, Vandersteen, & Pryor, 2003). It is refined from earlier, well-established DNA dissociation (or"melting") techniques (e.g., to determine the temperature of a DNA hybrid). Like all melting analyses, the technology subjects DNA samples to increasing temperatures and records the details of their dissociation from double-stranded (dsDNA) to single-stranded form (ssDNA) (Figure 7). Before an HRM analysis can be performed, the target sequence must be available in a high copy number. Performing a DNA amplification reaction (PCR) before HRM is an easy and efficient way to ensure adequate quantity. Both procedures are carried out in the presence of a fluorescent dye that binds dsDNA only. The dye does not interact with ssDNA but fluoresces strongly in the presence of dsDNA. This change in fluorescence can be used both to measure the increase in DNA concentration during PCR and then to measure thermally induced DNA dissociation during HRM directly. For detection of sequence variations, differences in the melting curves of the amplicons are analysed. Heterozygote DNA forms heteroduplexes that begin to separate into single strands at a lower temperature with a different curve shape than homozygote DNA. Depending on the individual sequence, most of the different homozygotes give distinguishable melting curves as well.

This method has emerged as a valuable tool for the rapid testing of several biological specimens and tissues for the presence of micro-organisms and the differentiation of Brucella spp. genetic variants (Wittwer et al., 2003), for example, Chlamydiaceae

(Robertson et al., 2009), Mycoplasma pneumoniae (Schwartz, Mitchell, Thurman, Wolff, & Winchell, 2009), Leishmania (Talmi-Frank et al., 2010), Bordetella pertussis, Staphylococcus aureus (Chan et al., 2009), Bacillus anthracis (Fortini et al., 2007), Mycoplasma synoviae, Pseudomonas aeruginosa adenovirus serotypes, and Aspergillus species. HRM has even been used for the identification of members of the Anopheles funestus group (Vezenegho et al., 2009). Tulsiani et al. developed a random amplification of polymorphic DNA (RAPD) method associated with HRM analysis (RAPD-HRM) using 13 previously published RAPD primers to genotype 10 *Leptospira* strains. Traditional HRM curves are difficult to interpret; thus, the interpretation of HRM results can be arbitrary. In this study, we evaluated the potential of an alternative method based on an unsupervised high-resolution melting curve (HRM) analysis using High Resolution Melting Master (Roche, Auckland, New Zealand) and examined the ability of this new method to type *Leptospira* from New Zealand specimens rapidly and easily.

In a melting experiment, fluorescence is originally high because the sample starts as dsDNA, but fluorescence decreases as the temperatures are raised, and DNA dissociates into single strands. The detected melting behaviour is symptomatic of a distinct DNA sample. Mutations in PCR products are detectable because they modify the shape of the melting curve. These changes could be visible when the mutant sample is compared to a reference "wild type" sample.

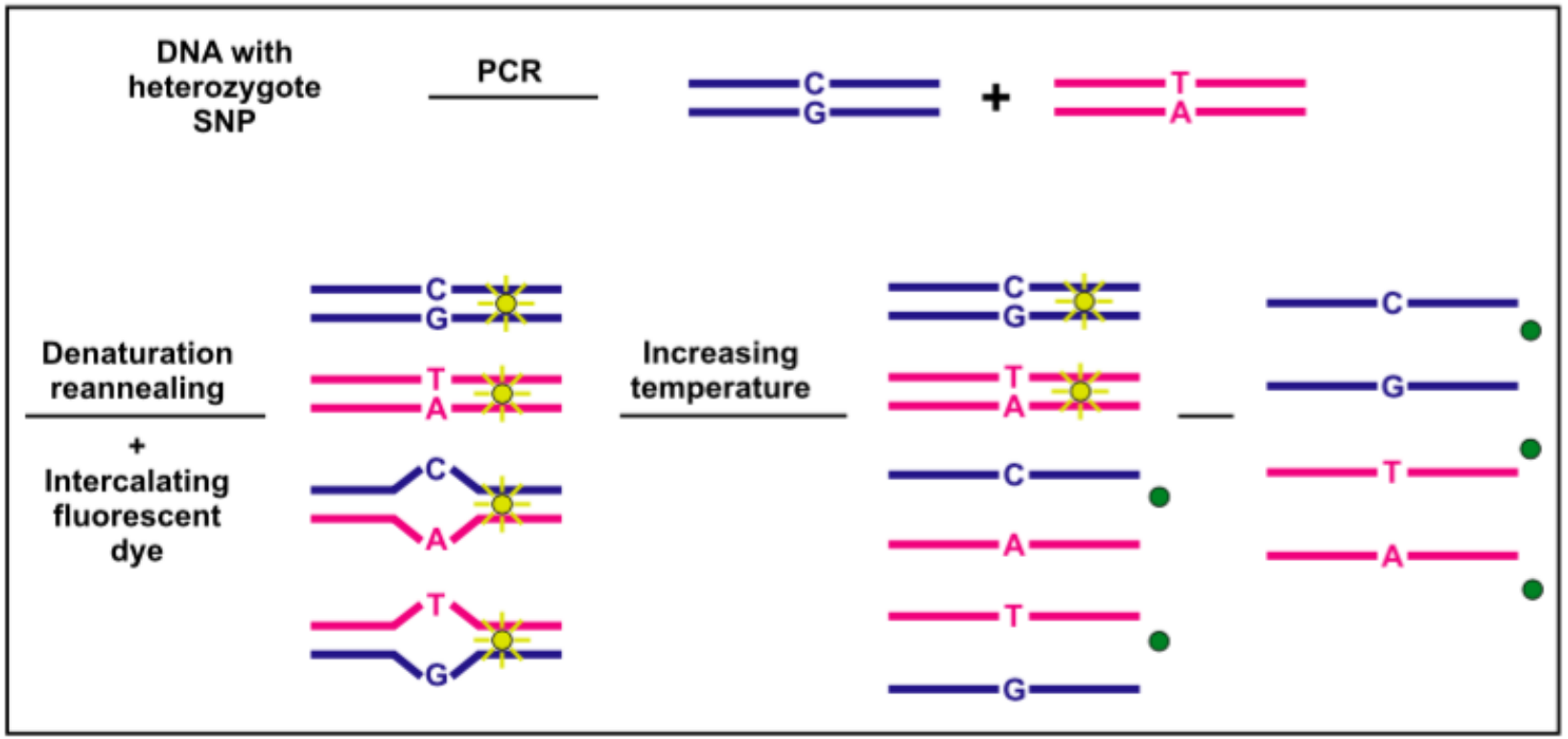

Figure 7 Procedure of High Resolution Melting. DNA with heterozygote SNP was amplified by PCR, then denaturation reannealed and intercalating fluorescent dye. When temperature increased, DNA dissociated into single strands, so the "melting" could be observed.

As HRM based applications became more popular and widespread, new HRM analytic instruments were developed and modified (Herrmann, Durtschi, Bromley, Wittwer, & Voelkerding, 2006; Herrmann, Durtschi, Wittwer, & Voelkerding, 2007). Contemporaneously, new DNA binding fluorescent "saturating" dyes have been developed and commercially offered for HRM experiments. These include LCGreen and LCGreen Plus from Idaho Technology, EvaGreen from Biotium and ResoLight from Roche Applied Sciences (Radvanszky, Surovy, Nagyova, Minarik, & Kadasi, 2015). ResoLight, the new dye used in this experiment, can recognise the presence of heteroduplexes produced during PCR (e.g., if the sample is heterozygous for a particular mutation). The trait is not shared with other dyes traditionally used in real-time PCR (e.g., SYBR Green I or ethidium bromide). LightCycler® 480 High Resolution Melting Dye is not toxic to amplification enzymes. Thus, elevated levels of the dye do not affect the PCR procedures. The dye with high concentrations could fully saturate the dsDNA in the specimen. dsDNA rests dye-saturated throughout the period of the subsequent melting experiment process. Under these circumstances, even small changes in the melting behaviour result in subtle, but reproducible changes in High Resolution Melting Dye fluorescence. Differences occur because the dye cannot redistribute itself from denatured to non-denatured regions of the DNA during melting (Wittwer, Reed, Gundry, Vandersteen, and Pryor, 2003), shown as Figure 8.

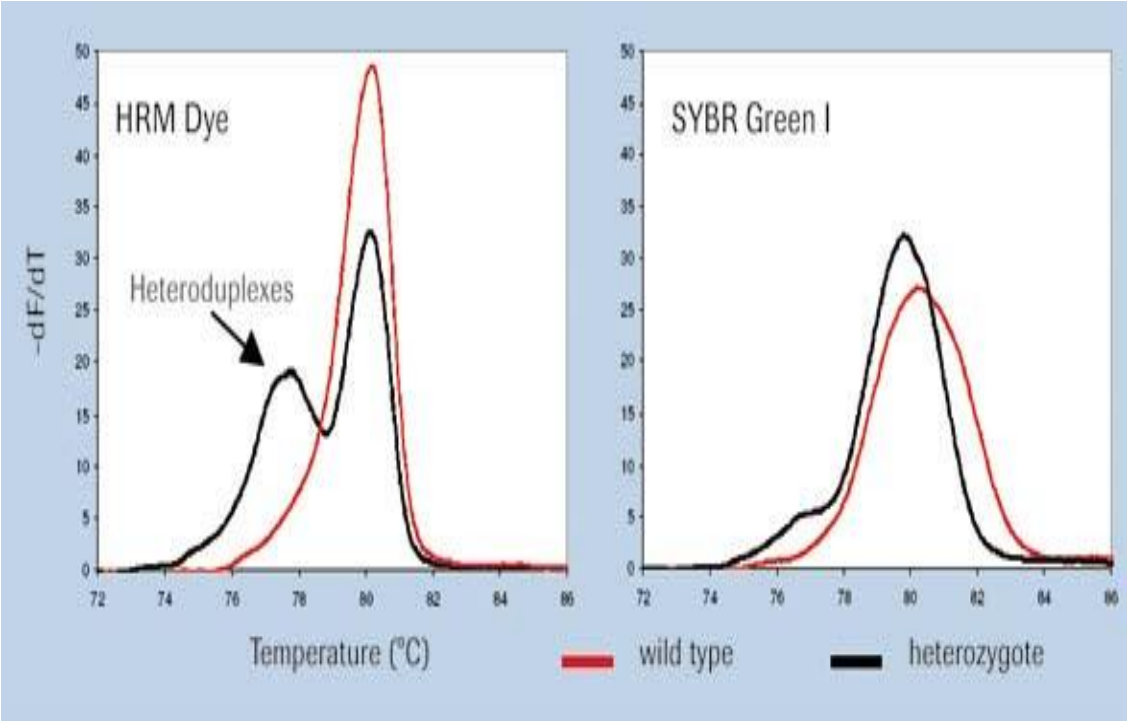

Figure 7 Tm calling profiles obtained by using different dye. Adapted with permission from Wittwer, Reed, Gundry, Vandersteen, and Pryor (2003).

As with any dye employed in melting experiments, the HRM dye has strong fluorescent only when it attaches to dsDNA. This difference of fluoresces during an experiment can be utilised both to measure the increase in DNA concentration during PCR amplification and, afterwards, to measure temperature included DNA dissociation during High Resolution Melting. Saturation of the amplicon leaves no space for redistribution events during melting, which enhances the consistency.

4. Diagnosis

Different kinds of manifestations showed on leptospirosis trouble its clinical diagnosis at the early stage. Leptospirosis may present with non-specific symptoms of fever, myalgia and migraine, which cannot be diagnosis signs suggestive of leptospirosis. Interestingly, it is worth to notice that the requirements are different between human and veterinary diagnosis in some aspects, as follows. When the diagnosis is applying on human, the first requirement is to use appropriate genus specific tests for suiting the individual patient, whereas the veterinary diagnostic researchers value the population from where the infected individual comes more than the individual (Hartskeerl et al., 2011). A diagnostic test of leptospirosis on animals might require not only for the demonstration of leptospirosis as a cause of a clinical disease but also for the reason:(1) to assess the infection and immune status of a herd for control or eradication of this disease on the herd or even the national environment;(2) to survey epidemiology of Leptospirosis;(3) to evaluate the suitability for international trade or for introduction into an uninfected herd (Ellis, 2015). As a result, it is necessary to diagnose infecting serovar at the early stage for control measures that are serovar dependant such as vaccination. Developed diagnostic approaches will help improving case detection and disease control of leptospirosis and, consequently, benefit both veterinary and human public health care and national economies as well to some extent. Evidence of a

clinically suspected leptospirosis case may be succeeded by observation of leptospires from organism or their components in body fluid or tissues using dark field microscopy, by isolation of leptospires in cultures, or by test of specific antibodies (Hartskeerl, Collares-Pereira, & Ellis, 2011; Schreier, Doungchawee, Chadsuthi, Triampo, & Triampo, 2013). A collection of appropriate specimens and selection of tests for diagnosis differ from the time of collection and the period of symptoms (Figure 9). Specimens 1 and 2 are serologically acute-phase samples, 3 is a convalescent-phase collection that might facilitate detection of delayed immune response, and 4 and 5 are follow-up specimens with information on epidemiology, such as the presumptive infecting serogroup.

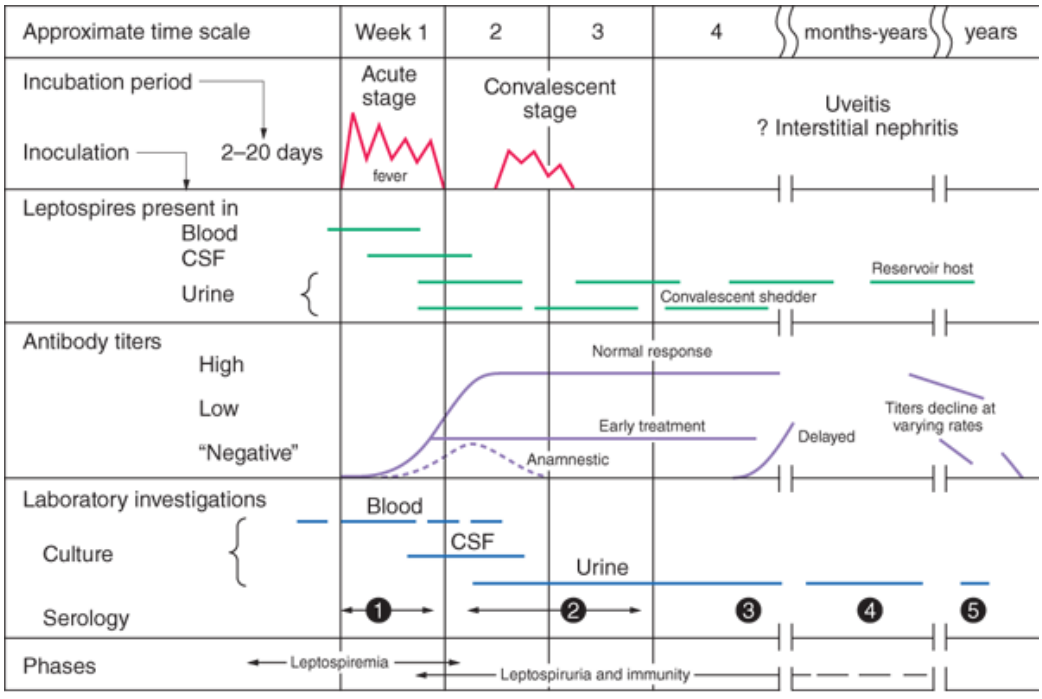

Figure 8 Biphasic nature of leptospirosis and proper investigations of different stages of the disease. Adapted with permission from (Haake and Levett, 2015).

4.1 Historical diagnosis of leptospirosis

Traditional procedures, like the isolation of leptospires, usually need culture. Basic culture media for leptospires is commonly made up of a buffer solution and 5 – 10% of

rabbit serum that has been slightly hemolyzed, some of the culture media were added peptone. Besides, a small amount of yeast extract and vitamin B_{12} may be included in the media, stimulating certain of *Leptospira* to grow. Cultures and isolations are better to be done at the early stage of the disease during the leptospiremia state. Direct cultures from infected materials include blood, CSF and urine. During this phase, patients are generally with febrile. Blood sample for cultures and inoculations should be collected at this stage. The incubation temperature would need to be controlled at 28 – 30 °C. With 5 or 6 week's culture, it could be examined by dark-field microscopical technique. Urine samples are preferred to collect by the second or third week of the disease. It is essential to do 5 – 6 serial dilution of the urine sample with sterile buffered saline or appropriate leptospiral medium, which could effectively avoid the occurring of overgrowth from contaminating organisms. Direct cultures from infected materials, including blood, CSF and urine, are relatively straightforward and more efficient. In some cases, inoculation to laboratory test animals would be recommended to proceed simultaneously. Juvenile hamsters and pigs are majorly used laboratory animals for this injection. Besides observing of the clinical manifestation presented on the test animals, the laboratories technicians need to extract the blood sample from their hearts periodically and do blood culture with Fletcher's semisolid medium.

Microscopic demonstration procedures of leptospiral diagnosis include Dark-field examination, silver staining and fluorescein staining. In facts, all members from *Leptospira* have similar appearances in morphology. They are all slender threadlike, with curved ends and presenting heliciform rotations on the longitudinal axis. Due to the lack of reliability, dark-field examination, therefore, is not recommended to be the only diagnostic test for *Leptospira* but it can be a test for auxiliary reference (Galton, 1962). Silver staining technique has been successfully detected leptospires in aborted

bovine fetuses, which in some extent solved the problem of frequently unsuccessful isolation from fetal animals (Fennestad & Borg-Petersen, 1958). Only a few of investigations using fluorescein-staining techniques had satisfactory results to detect leptospires from contaminated urine and tissue. It needs to notice that all these three approaches are not considered to be ideal to distinguish the serotypes of infected individuals (Galton, 1962).

4.2 Molecular diagnosis

Leptospiral DNAs can be extracted and amplified from serum, urine, aqueous humor, CSF, and many other organs post mortem. PCR can detect *Leptospira* in human when the infection results in a leptospiremia reaching 10^7 bacteria/ml of blood within the first 14 days of exposure (Truccolo, Serais, Merien, & Perolat, 2001). Diagnosis assay approaches at the molecular level fall into two broad categories according to two different kinds of target genes, housekeeping genes like *rrs*, *gyrB*, or *secY*, or pathogenic-specific genes, such as *lipL32*, *lig*, or *lfb1* (Ahmed et al., 2012; Thaipadunpanit et al., 2011). A large case-control study in a high-prevalence population in Thailand evaluated these two types of quantitative assay; it supported the earlier papers that said the samples collected at admission to hospital were more sensitive than those from culture in PCR detection, but MAT is basically used to detect more serological cases (Brown et al., 1995; Thaipadunpanit et al., 2011). PCR becomes an increasingly popular used technology for leptospiral diagnosis thanks to its sensitivity, especially compared to others at the early diagnosis. Real-time PCR methods, such as SYBR Green and Taqman technology, are more rapid than the tranditonal PCR, plus its less sensitivity to the contaminant (Picardeau, 2013). Besides, using real-time PCR assays to quantify the bacterial load in leptospirosis has been carried out (Agampodi et al., 2012; Segura, 2005; Tubiana et al., 2013). 16S rRNA has been used with

amplification and sequencing to identify Leptospira at the species level (Morey et al., 2006; Postic, Riquelme-Sertour, Merien, Perolat, & Baranton, 2000).

PCR-based approaches can only demonstrate the existence of the pathogenic leptospires, not provide a straight recognisation for the leptospiral serovar; it is not a significant limitation on the management of the individual patient, but an inconvenient one for the epidemiology research and public health control (Haake & Levett, 2015). Combining with the melting curve analysis for the amplification products may lead to sucessful species and genotype identification (Cerqueira et al., 2010; Picardeau et al., 2014). In some cases performed with absorted fetal materials, PCR has presented particular problems because that the tissue would autolyse and produce inhibitors (Ellis, 2015). Later refinements of this technology to some extent solved this through testing the contents from stomach, using a fluid to diagonose the abortifacient bacterial infections (Doosti & Tamimian, 2011).

4.3 Serological diagnosis

Culture and isolation are not frequently used now because that leptospire are fastidious and they need a long time to grow. Also, it may not contribute to the early diagnosis process (Ahmed, Grobusch, Klatser, & Hartskeerl, 2012). Dark field microscopy is easier to operate and more timesaving but considered being notoriously unreliable doing on blood samples, so it is not recommended to be the only diagnosis (World Health, 2003). Because of the limitation of capacity for culture and PCR, serological diagnosis plays a critical role in leptospirosis diagnosis. Frequently used serological diagnosis methods are either genus-specific or serogroup-specific. Then, WHO (2003) suggested microscopic agglutination test (MAT) and the IgM ELISA are the two most commonly used methods detecting anti-*leptospira* antibodies as the evidence of infection. IgM antibodies can be detected in the blood within a week after the onset of symptoms.

Agglutination tests have been applied to this organism since the first *Leptospira* isolation was done; microscopic agglutination test (MAT) still stays in the excellent position of serological investigation in both human and animal leptospirosis.

4.3.1. Microscpic Agglutination Test (MAT)

MAT plays the role as the gold standard in leptospirosis diagnosis on account of its unsurpassed serovar specificity (Ahmed et al., 2012). In a MAT practice, sera extracted from patients react with active antigen suspensions of leptospiral serovars, and the mixture of the sera and antigen are incubated and examined microscopically for agglutination and the titers determination. Using darkfield microscopy can read the results from MAT. MAT might usually be a complex test approach in controling, performing, and interpreting (Turner, 1968). It requires the live cultures must cover all the serovars need to be used as antigens and the antigens have to be serologically representative of all serogroups (Faine, 1982; Turner, 1968) and major local serovars (Torten, 1979). Katz et al. (1991) used a large range of antigens in order to discover the infections with the serovars that are not common or have not been detected yet. Since MAT is a test assay that is specific to serogoups, it might not be able to ensure the detection of infecting serovar (Levett, 2003; Murray et al., 2011; Smythe et al., 2009). Detecting the endpoint that the highest dilution of serum in which 50% agglutination occurs is not easy in the practices, so the presence of approximately 50% free unagglutinated leptospires is determined to be the endpoint with the comparison of the control suspension (Faine & Stallman, 1982). When cross-reaction with high degrees occurs between serogroups, especially in acute-phase samples, it is very complicated to tell exact MAT results; patients sometimes though have similar titers to all serovars of a particular serogroup, they react paradoxically (Alston et al., 1958).

It would need paired sera to confirm the diagnosis with certainty, which requires the rise in titre greater than fourfold, regardless of the interval between samples. The interval between the first two samples would be caused by the delay between the symptoms onset and presentation of the patient in a large extent. To be specific, if the symptoms present as typical leptospirosis, then an interval of 3 – 5 days could be enough for detecting the rise of titres. But if the patient performs earlier in the course of the disease, or if the onset time has not been recorded precisely, then a 10-14 day's interval between samples would be more recommended. In some cases, the seroconversion does not happen in that rapid, so it would be necessary to have a longer interval between samplings. MAT cannnot be relied on in the sensitivity in the early acute-phase specimens (Appassakij et al., 1995; Cumberland et al., 1999). Additionally, fulminant leptospirosis may kill patients before the seroconversion occurs (Brown et al., 1995; Cumberland et al., 1999; Ribeiro et al., 1994).

Acute infection can be suggested by a single rise of titre detected within an acute febrile illness. The magnitude of such a titre dose not depend much on the background level of exposure in the population and hence the seroprevalence. The titres results presented in acute infections can be dramaly high, greater than 25600, and may last for months, even years (Alston and Broom, 1958; Blackmore et al., 1984; Cumberland et al., 2001; Lupidi et al., 1991; Romero et al., 1998).

MAT is tedious and laborious (about 3-hour work) and requires a panel of antigens and well-equipped laboratories with expert technicians. The existence of some seronegative carrier animals is also a problem (Hartskeerl et al., 2011). On the other hand, almost all of these serological approaches faced a similar drawback that anti-*leptospira* antibodies are not detectable in the early acute phase of the disease; hence the effective antibiotic

treatment may lose efficacy in the late acute phase when it serologically confirmed (Ahmed et al., 2012). The MAT data cannot draw a conclusion regarding to infecting serovars because it can only give a general impression about the serogroups but without isolates (Everard & Everard, 1993). However, MAT is still the most proper test method to take in epidemiological sero-surveys, since it an be employed to sera samples extracted from any animal species, and because the range of the antigens utilized can be expanded or decreased as required.

4.3.2 IgM detection

Rapid screening tests for antibodies of acute leptospiral infection developed since the complexity of the MAT. IgM antibodies can be detected and confirmed in diagnosis at the very beginning of leptospirosis, the first week of the disease when it is the most valuable to initiate the appropriate treatments (Haake & Levett, 2015). ELISA has been widely used to detect IgM, mostly with the antigens prepared from cultures of *Leptospira biflexa*. Recent developments in IgM detection intend to change the test formats to two dipstick formats (Levett & Branch, 2002), latex agglutination (Smits et al., 2001), laternal flow (Smits et al., 2001) and dual path platform (Nabity et al., 2012); this help get access to the laboratories without extensive instrumentation.

It is not very easy to evaluate serological diagnostic tests for leptospirosis due to the few laboratories have the equipment for MAT; even fewer laboratories can perform isolation and identification of leptospires directly from patients. Contrastly, molecular detection assays can usually be applied in the early acute phase, which helps a lot with the treatment and also understanding of the epidemiology.

Experiment materials and methods

1. *Leptospira* reference strains and animal samples

This study included a minimum of 200 PCR positive animal samples with a viral load of 2,000 copies/mL or greater. The DNA isolation was performed at EpLab of Massey University (Auckland, New Zealand). Institute Pastéur (New Caledonia, France) supplied the reference strains to be matched with for the determination of subspecies.

Forty-six *Leptospira* DNA samples were extracted from the faecal of captive wild animals at the Auckland Zoo (Auckland, New Zealand) between November 2013 and August 2014 and identified by media culture. Presumptive identification and confirmatory identification (by MALDI-TOF-MS) of bacteria of genus *Leptospira* spp. were conducted at AUT Roche Diagnostic Laboratories before the DNA extraction and this project was performed. Seven *Leptospira* reference strains (*L. interrogans* sg. Pyrogenes, *L. borgpetersenii* sg. Mini, *L. kirchneri* sg. Grippotyphosa, *L. noguchii*, *L. santarosai*, *L. biflexa*, *L. weilii*) were provided by the National Reference Center for Leptospirosis, Institut Pasteur (Paris, France), see Table 5 in the results section.

2. PCR amplification and analysis

Three primer pairs specific to *Leptospira* have been tested on seven *Leptospira* reference strains: lfb1 F/R (Merien et al., 2005), secY IV F/R (Ahmed, Engelberts, Boer, Ahmed, & Hartskeerl, 2009), G1/G2 (Gravekamp et al., 1993)(Table3), with the aim of selecting specific primers for *Leptospira* species identification. In all the PCRs, *L. biflexa* sv. Patoc was used as a negative control.

Table 3 Sequences of PCR primers tested on references strains.

Primers	Sequences (5'-3')	Target gene	Size (bp)
Lfb1 F	CATTCATGTTTCGAATCATTTCAAA	*Lfb1*	331
Lfb1 R	GGCCCAAGTTCCTTCTAAAAG		
G1	CTGAATCGCTGTATAAAAGT	*secY*	245
G2	GGAAAACAAATGGTC		
SecY IV F	GCGATTCAGTTTAATCCTGC	*S10-spc-α*	202
SecY IV R	GAGTTAGAGCTCAAATCTA- AG		

The sensitivity of each PCR using the selected primer(s) was evaluated by performing PCRs on 10-fold serial dilutions of the DNA extracted from three in vitro cultured strains from Institute Pastéur (New Caledonia, France): *L. interrogans* sg. Pyrogenes, *L. borgpetersenii* sg. Mini and *L. kirchneri sg*. Grippotyphosa. The quantification of the leptospires was based on genomic DNA mass, taking into consideration that the size of the genome of the *L. interrogans* strain Fiocruz L1-130 is 4.6 Mb (1 genome is approximately five fg). The end-point detection limit was determined by ten repetitions of the measurement of the last positive point with 100% amplification.

A subspecies determination was performed on the matching reference strains, and three primer pairs amplified polymorphic tandem repeat sequences were additionally tested:

VNTR-4bis, VNTR-Lb4, and VNTR-Lb5 (Salaün, Mérien, Gurianova, Baranton, & Picardeau, 2006). These VNTR primers were used in a previously published MLVA study and exhibited a remarkable discriminatory power for the identification of the serovars of *L. interrogans*, *L. borgpetersenii*, and *L. kirschneri* (Naze, Desvars, Picardeau, Bourhy, & Michault, 2015).

Table 4 VNTR primers used in subspecies identification of *Leptospira* samples.

Primers	Sequences (5'-3')	Size (bp)
VNTR-4bis F	AAGTAAAAGCGCTCCCAAGA	425
VNTR-4bis R	ATAAAGGAAGCTCGGCGTTT	
VNTR-Lb4a	AAGAAGATGATGGTAGAGACG	573
VNTR-Lb4b	ATTGCGAAACCAGATTTCCAC	
VNTR-Lb5a	AGCGAGTTCGCCTACTTGC	668
VNTR-Lb5b	ATAAGACGATCAAGGAAACG	

The PCRs were performed using Lightcycler®480 High-Resolution Melting Master Kit (Roche, Auckland, NewZealand) on a LightCycler® 480 System (Roche, Auckland, New Zealand). The 20µl reactions contained 10µl of mix 2X master mix HRM, with a 0.7µM final concentration of each primer (Integrated DNA Technologies, Singapore), 2.5 mM of $MgCl_2$, and 5µl of the extracted nucleic acid solution. The following amplification protocol was used: denaturation at 95°C for 5 min, 45 cycles at 95°C for 10 s, 55°C for 30 s, and 72°C for 10s. These conditions were used for all the primer pairs. For the species determination, a melting curve analysis determined the melting temperature (Tm). To test the reproducibility of the Tm determination, three strains of different species were tested in 10 separate runs with the selected primers.

For the subspecies determination, the samples were heated from 65°C to 95°C with a

continuous acquisition after PCR cycling with the VNTR primers. For each VNTR locus, a normalisation region of the melting curve was selected to improve the analysis per the recommendations of the LightCycler® 480 HRM Software User Guide (Roche, Auckland, New Zealand). Two normalisation regions have been chosen: one before and one after the melting curve transition. The highest fluorescence value was 100, and the lowest fluorescence value was zero. The data were analysed using LightCycler® 480 HRM Software (Roche, Auckland, New Zealand) with Gene Scanning and Tm calling.

We used LightCycler® 480 Gene Scanning Software to identify changes in the shape of the curve based on analysis of the High-Resolution Melting curve data, which indicated the presence of sequence variations in the PCR product. All sample DNA was amplified via real-time PCR with LightCycler®480 High Resolution Melting Dye and immediately analysed with LightCycler® 480 Gene Scanning Software to identify sequence variants on the same LightCycler® 480 Instrument, which could demonstrate the homogeneousness of the entire mutation screening process. That is, the entire experiment can be done on the LightCycler® 480 Instrument; post-PCR analysis does not require a separate device.

The investigation was started with detecting the negatives; LightCycler® 480 Gene Scanning Software automatically used a negative filter to identify negative samples that are with low fluorescence signals and consequently lack of a prominent melting curve. The software also allowed manually identification. Normalisation of the raw melting curve data was by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform values. Pre-melt signals were uniformly set to a relative value of 100%, while post-melt signals were set to a relative value of 0%. The temperature axis of normalised melting curves was shifted at the point where

the entire dsDNA was completely denatured. For this, the software automatically applied a default Temp Shift Threshold of 5% to all data, while we lower some of them to a different value. Then samples with heterozygous SNPs can easily be distinguished from the wild type by the various shapes of their melting curves. With the steps above, a Difference Plot was generated by further analysing the differences in melting curve shape with subtracting the curves from a reference curve (also called "base curve"); this helped cluster samples automatically into groups that have similar melting curves (e.g., those with the same genotype).

The genotype of each *Leptospira* DNA was determined by combining the results of the two clusters that were obtained with the two sets of primers selected by the species in the subspecies characterization.

Simpson index (D) was used as described by Hunter and Gaston (1988) to assess the discriminatory power of the primers, which reads:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

Where D is the index of discriminatory power, N the number of unrelated strains tested, S the number of different types, and x_j the number of strains belonging to the jth type, assuming that strains will be classified into mutually exclusive categories. Hence, an index of 1.0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. Contrastively, a D value of 0.0 would indicate that all members of a strain population belonged to an identical type.

3. Optimisation

The optimisation work aimed to optimise the PCR amplification process, by utilising different concentrations of $MgCl_2$, which is essential to ensure both specificity and robustness of PCR. Recommendation from the LightCycler® 480 User Guide told that the optimal concentration of $MgCl_2$ for this HRM assay could vary from 1.5 to 3.5 mM. Therefore, pre-experiments by titrating the $MgCl_2$ concentration from 1.5 to 2.5 mM, in 0.5 mM steps, were established before the formal test started. Using quantification analysis, the amplification curves showed clearly the lowest C_t value when setting the $MgCl_2$ concentration on 2.5mM.

Additionally, optimisation also proceeded from an adjustment of primer concentration used in the PCR amplification. From the literature review, Naze et. al used a 0.7μM final concentration for all the primers in their experiment. Based on this, three different concentrations (0.5μM, 0.7μM and 1.0μM) were tested on five primers involved in this study: Lfb1 F/R, G1/G2, VNTR-4Bis, VNTR-Lb4, VNTR-Lb5 using with the optimal concentration for MgC_{l2} (2.5mM) as tested before. Using a combination of quantification analysis and HRM melting profiles comparison, 0.7μM was confirmed to be the optimal concentration of primers for this study; it showed better amplification process with lower C_t values and clearer cluster discrimination.

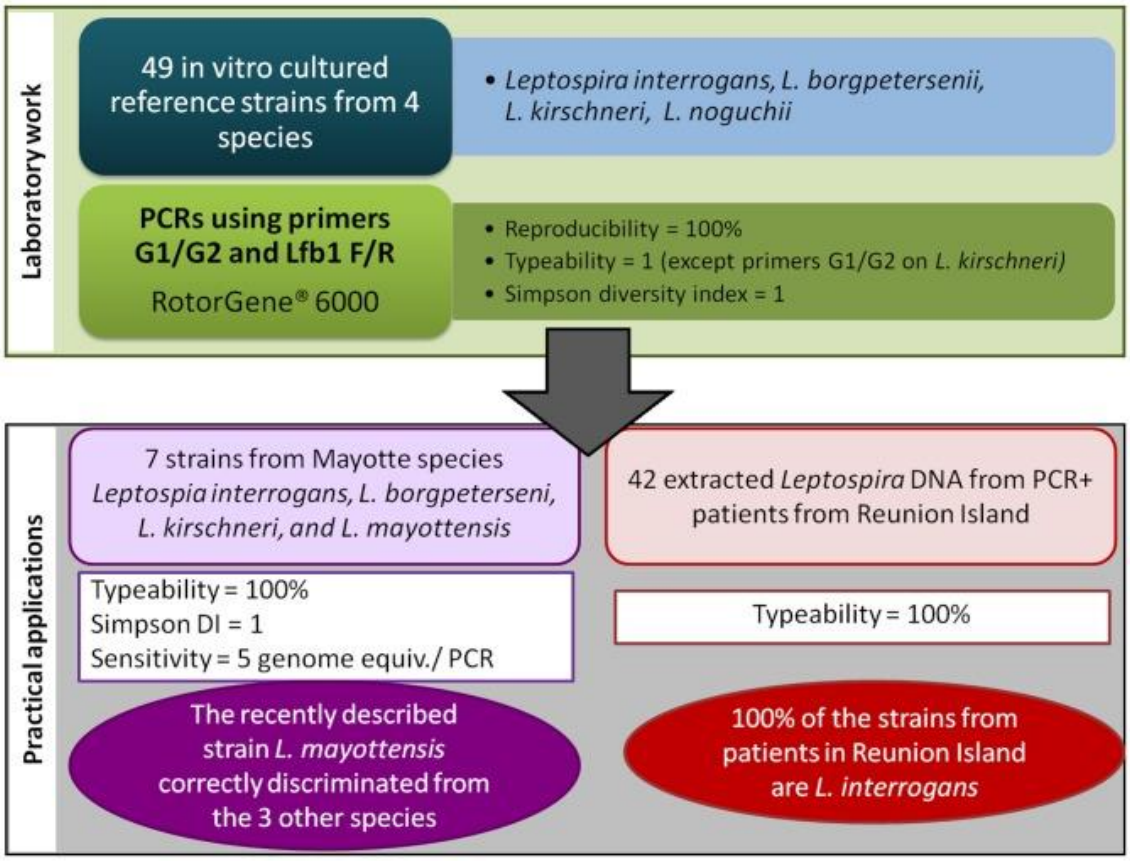

Figure 9 Workflow of the experiments

Results

Table 6 Reference strains and samples and their clusters amplified by real-time PCR with different sets of primers. *na means this strain had not been clustered in this practice.

codes/names	Lfb1 cluster	G1/G2 cluster	VNTR-4Bis cluster	VNTR-Lb4 cluster	VNTR-Lb5 cluster
L. borgpetersenii sg. Mini	3	na	na	na	6
L. noguchii	na	na	na	na	2
L. santarosai	na	na	na	na	na
L. biflexa	na	na	na	na	4
L. kirchneri sg. Grippotyphosa	4	na	3	na	na
L. interrogans sg. Pyrogenes	na	na	1	na	na
L. weilii	1	5	2	na	na
A44K	na	1	na	na	na
A49K	5	na	na	na	5
A54K	na	2	3	1	5
B9K	na	na	1	na	3
C12K	4	na	na	na	na
C18K	na	1	na	3	na
C21K	2	1	na	na	na
C24K	3	na	3	na	na
C26K	4	na	na	6	na
D12K	na	1	na	na	na
D2K	5	na	na	na	6
D21K	na	na	4	5	na
D23K	na	na	2	na	na
D25K	2	na	1	1	na
D26K	4	4	na	na	na
D28K	2	1	na	4	na
D31K	3	na	3	na	na
D32K	2	na	5	na	na
D33K	3	na	4	1	na
D9K	na	1	na	na	na
E2(12)	na	2	5	na	3
E2(1242)	2	3	na	na	na
E23K	na	na	1	na	na
E2(703)	1	na	5	na	2
E3(27)	1	na	na	1	na
E31K	na	na	1	2	na
E33K	1	na	na	na	na
E4(526)	1	2	na	na	1
E44K	na	na	na	na	na
E85(1012)	1	na	na	1	1
E85(1016)	na	na	3	na	na
E85(1017)	1	4	na	na	4
E85(1027)	1	na	na	2	1
E85(1045)	1	na	2	na	2
E85(610)	1	na	na	3	3
E85(616)	na	2	2	na	1

E85(645)	1	na	na	na	na
E85(649)	1	3	na	na	4
E85(666)	na	na	na	1	na
F54K	2	na	na	na	na
K2	na	na	5	2	na
K6	na	na	na	4	5
K11	5	na	na	na	2
128	1	na	4	na	na
28L1&13	1	na	na	na	na

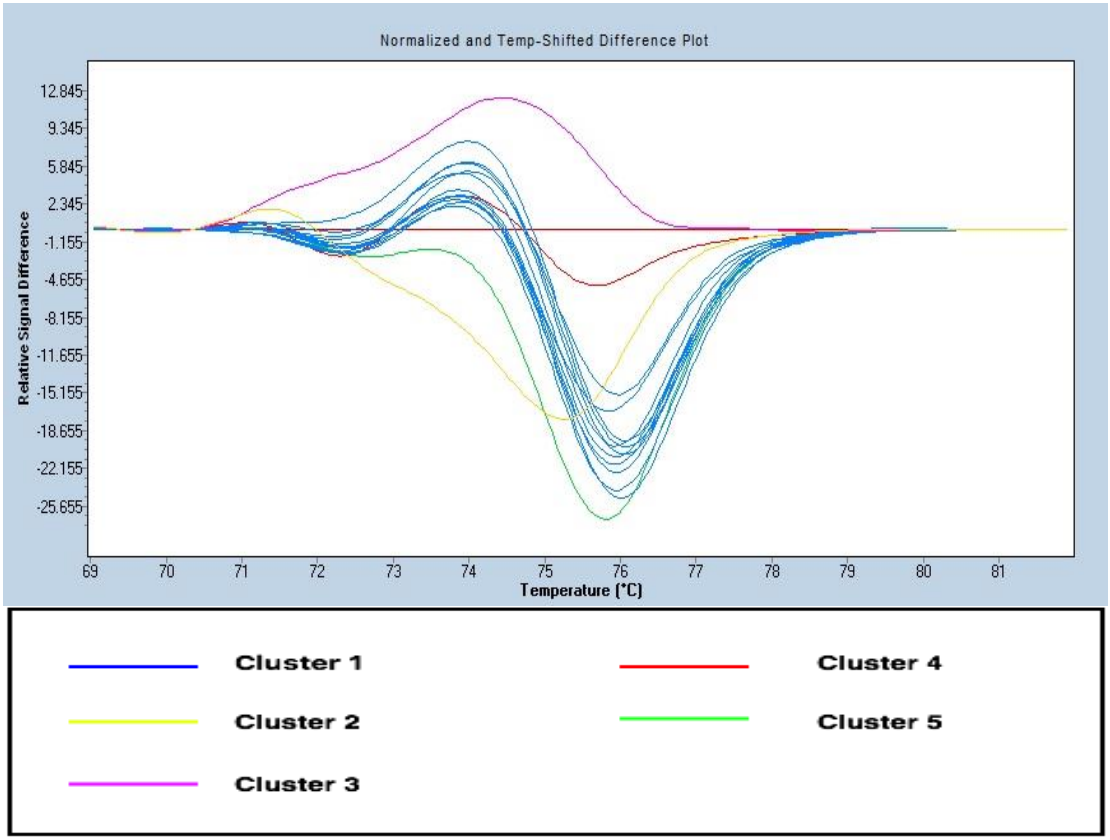

Figure 10 Cluster plot obtained after Gene Scanning using the lfb1 F/R primers.

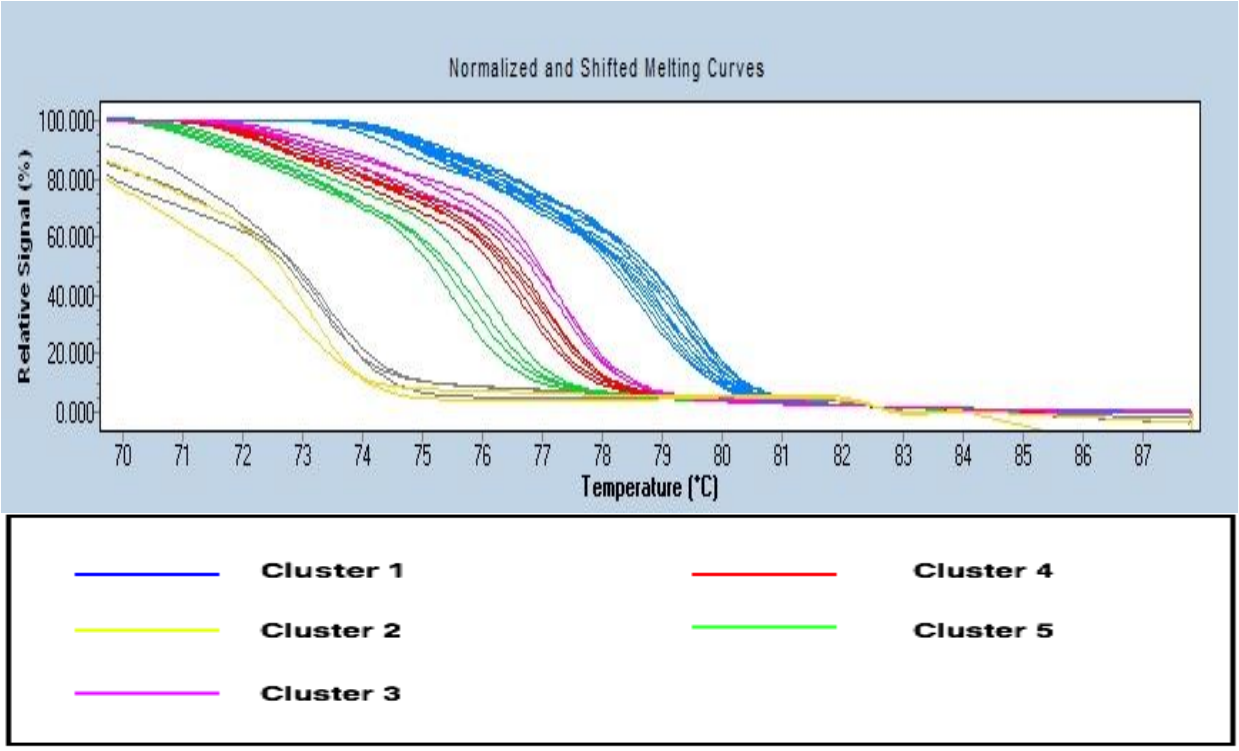

Figure 11 Nomalised melting curves using lfb1 F/R

1. Lfb1 F/R

From Figure 10 and Figure 11, using lfb1 F/R, 34 out of 52 (45 clinical samples and seven reference strains), 65% DNA samples were well genotipically discriminated, showing two main variant groups; one was above the baseline (cluster 3), and other ones were at the bottom (cluster 1, 2 and 5). The different plot was after nomalisation by setting the pre-melt (69.04-79.67), and the post-melt (80.04-81.95) and the threshold shifted with 0.00 (see figure 2). Among the 34 *Leptospira* DNAs, 41% (14 out of 34) exhibited the same melting profile (see cluster 1), including a reference strain *L.weilii*, which even better suggested these 14 leptospiral DNA samples in cluster 1 belong to *L.weilii* genomic species. Cluster 1, 2 and 5 shared similar melting profile; they had similar Gene Scanning shape on the different plot. This may support that cluster 1, 2 and 5 are only slightly different that probably due to mutants in nucleotide sequence; sequencing could help to have further confirmed.

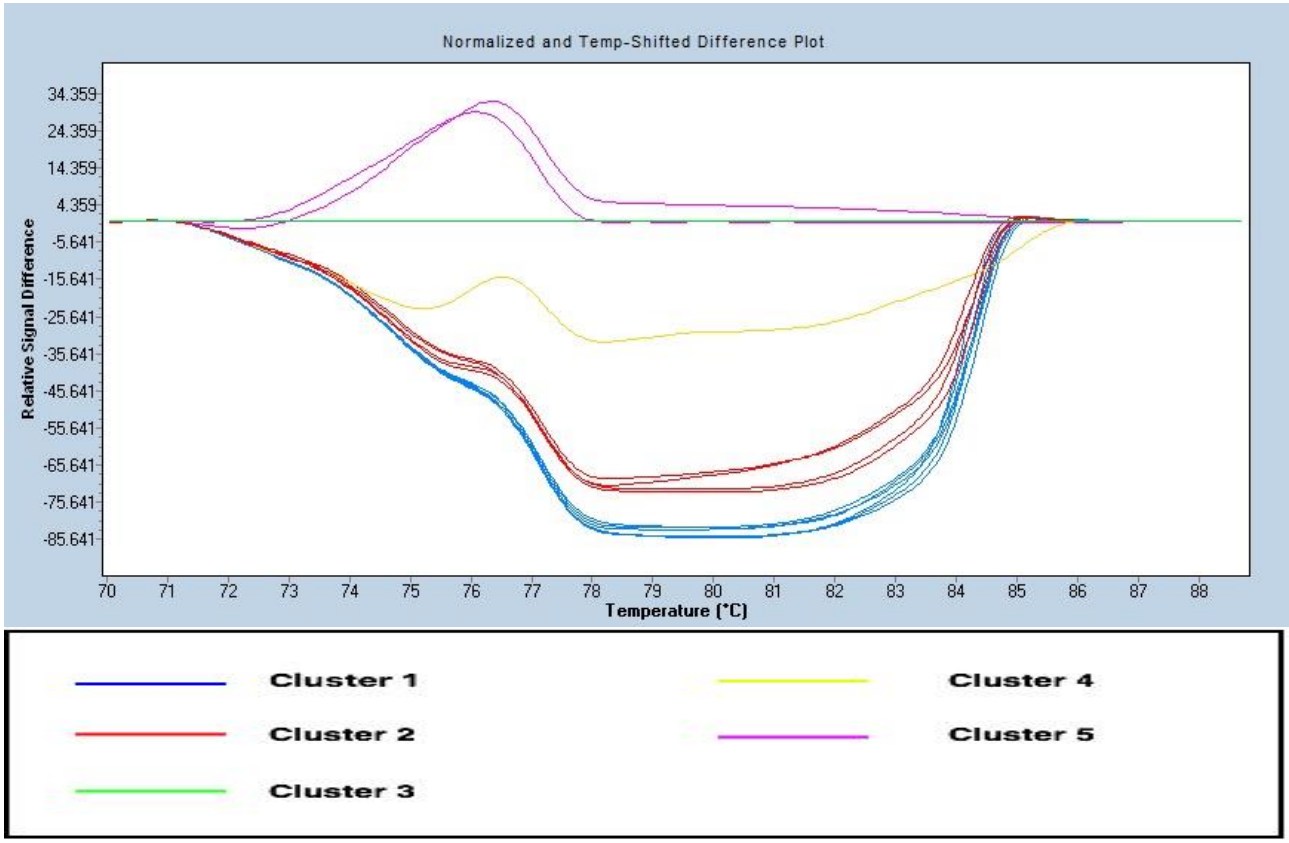

Figure 12 Cluster plot obtained after Gene Scanning using G1/G2 primers.

2. G1/G2

G1/G2 primer set helped to define 31%(15 out of 48) of all animal samples and reference strains, as shown in Figure 12. They were clearly separated into four main groups and 5 clusters with the normalisation (pre-melt: 70.00-71.34, post-melt: 87.07-88.74, threshold: 0.000). Cluster 1 and Cluster 2 were considered to be the same group since they have the pretty similar melting profile; they may be very resemblant with their sequences. After changed a baseline (see Figure 13), cluster 1 showed extreme unity, which indicated all the strains in cluster 1 belonged to the same genome species. However, there was not any reference strain matching with the samples we had in this practice thus it cannot be deduced which particular genome species the clusters belong to.

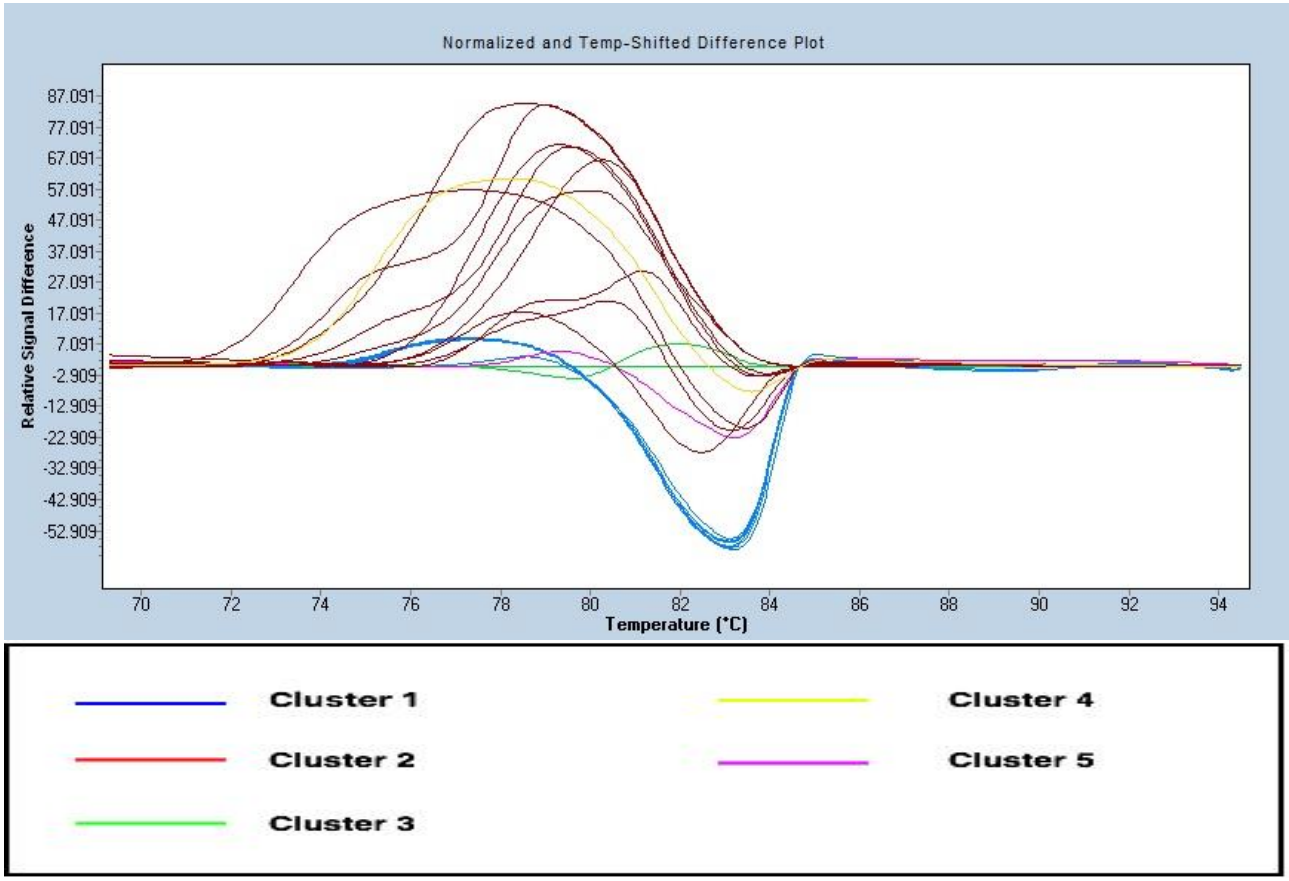

Figure 13 Cluster plot obtained after changing a base curve from Figure 12.

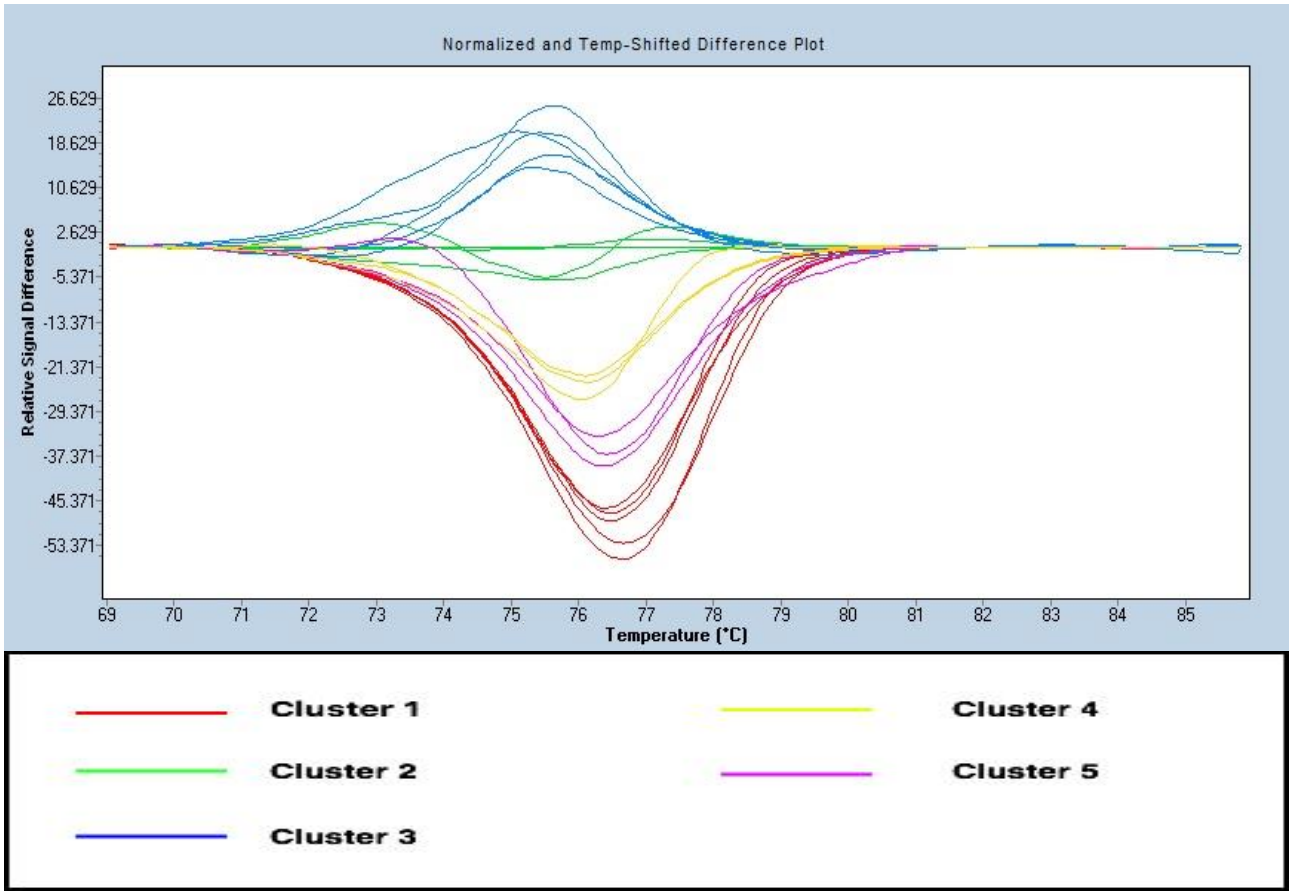

Figure 14 Cluster plot obtained after Gene Scanning using VNTR-4Bis primers.

3. VNTR-4Bis

Shown in figure 14, 44%(21/48) samples were divided into five clusters by using HRM analysis with VNTR-4Bis primer set. The pre-melt and post-melt were set to 69.04-70.92 and 84.63-85.85 for normalisation; meanwhile, the threshold was 1.000. The fact that noteworthy to see was three reference strains (*L. kirchneri*, *L. interrogans* and *L. weilii*) matched with three corresponding clusters. That demonstrated that cluster 1 (four samples), cluster 2 (three samples) and cluster 3 (four samples) were belonging to *L. interrogans*, *L. weilii* and *L. kirchneri*, respectively.

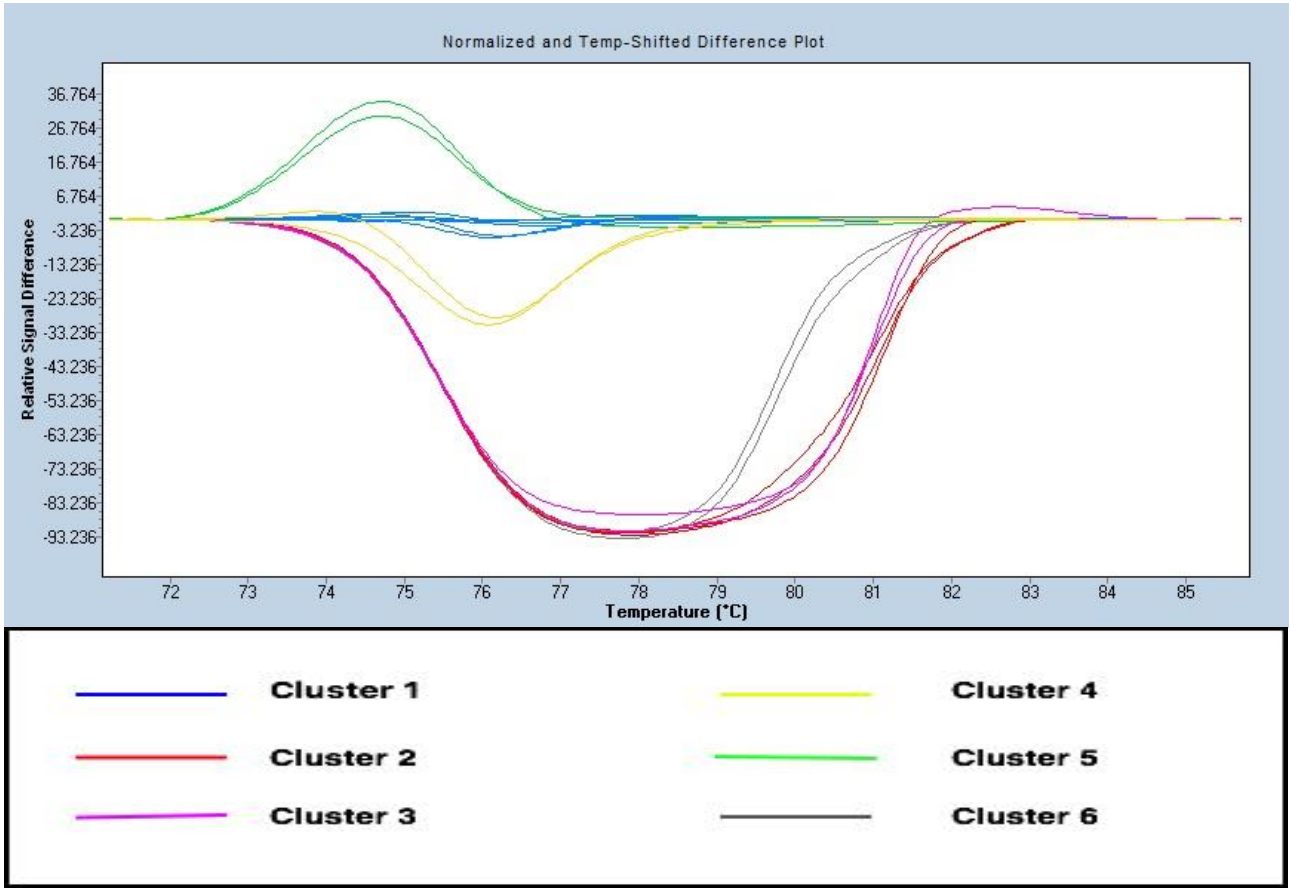

Figure 15 Cluster plot obtained after Gene Scanning using VNTR-Lb4

4. VNTR-LB4

Using VNTR-Lb4, 15 out of 48 (31%) *Leptospira* strains revealed typeability; Gene Scanning Software automatically grouped them into six clusters, from Figure 15. Cluster 1, 4 and 5 were explicitly split from other three clusters and also separated respectively. While, the other three clusters shared similar melting profiles, which means the *Leptospira* DNAs in these three clusters may only have some tiny differences in the gene orders. Unfortunately, no reference strain mated in this experiment so it cannot be sure which species the clusters exactly are.

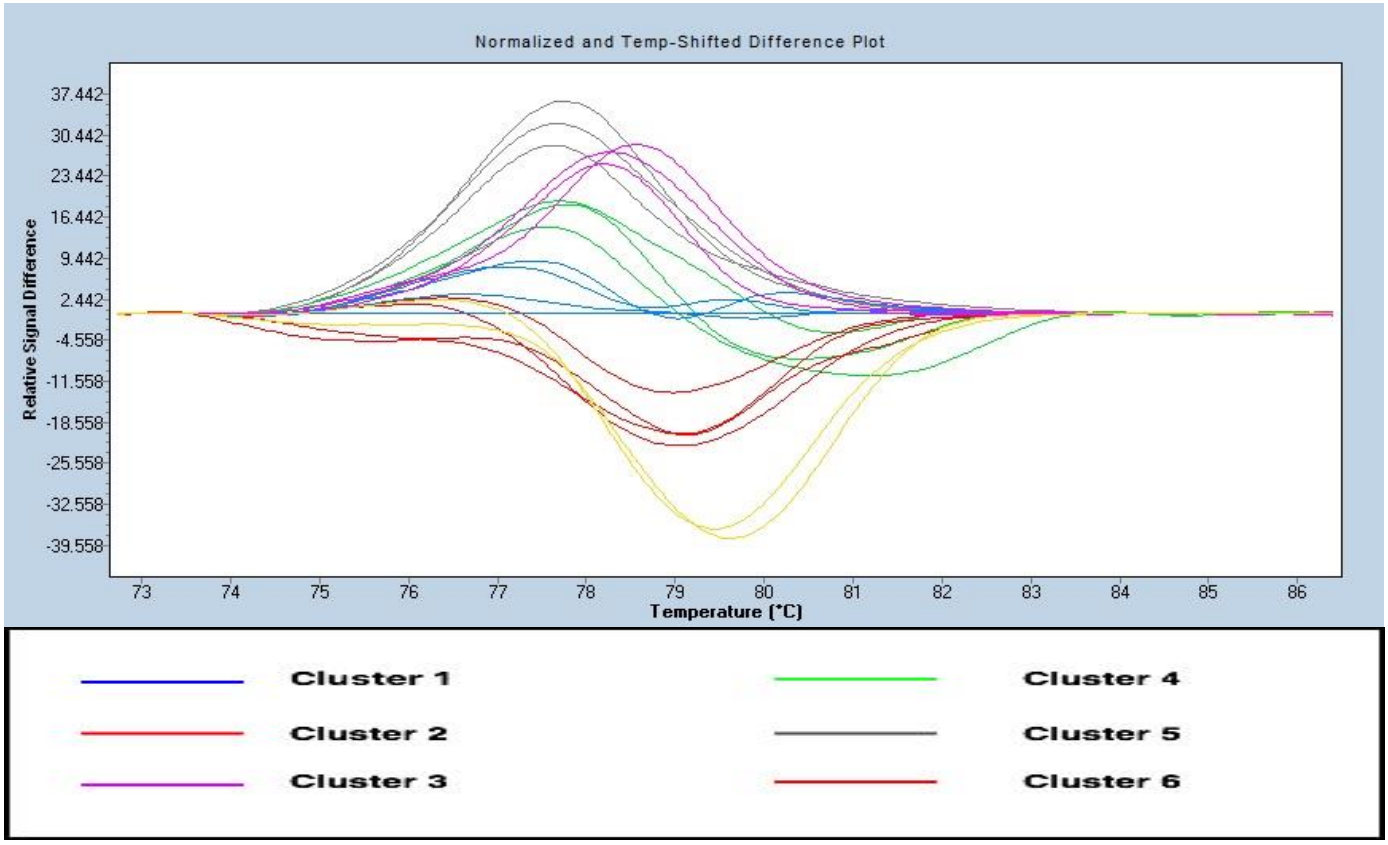

Figure 16 Cluster plot obtained after Gene Scanning using VNTR-Lb5

5. VNTR-Lb5

Using VNTR-Lb5, three *Leptospira* reference strains and sixteen clinical samples were detected to consist six clusters (see Figure 16). In the nomalising, pre-melt temperature was set to 72.73-73.73 and the post-melt was 83.63-86.44. *Leptospira* reference strain *L.noguchii* well matched cluster 2 that had three animal samples: K11, E85-1045 and E2-703. Therefore, these three samples were considered to be of *L.noguchii.*

	Lfb1 F/R	G1/G2	VNTR-4Bis	VNTR-Lb4	VNTR-Lb5
Lfb1 F/R		100%	97.8%	96.4%	94.0%
G1/G2			100%	100%	93.3%
VNTR-4Bis				100%	100%
VNTR-Lb4					100%
VNTR-Lb5					

Table 5 Discriminatory power (D) calculated by combining two clusters that obtained from two primers.

Combinations of each two sets of primers generated the genotypes of the *Leptospira* strains in the subspecies characterization. The discriminatory power of each primer described as shown in Table 6. Over a half of them is 1.00, and most of the others are higher than 95%, which indicated the technique worked with this primer can distinguish each member of a strain population from other members of that strain population very well. When combining the results from Lfb1 F/R and VNTR-Lb5, and G1/G2 and VNTR-Lb5, the discriminatory power were 94.0% and 94.3%, respectively. These two were relatively low but still higher than 90% that many other typing methods could not achieve (Hunter & Gaston, 1988).

Discussion

The primary aim of this study was not to diagnosis precisely pathogenic leptospiral infection, but to introduce and suggest a new developed genotyping way to identify *Leptospira* species and subspecies; this might help for a better knowing of prevalent circulating serovars or genotypes, which could lead to a further understanding of the epidemiology of leptospirosis in New Zealand.

The main character of the present study on HRM was to provide a prompt approach to discriminate between species of pathogenic *Leptospira*, which is an initial step for further levels of genotyping, such as serovar typing. The four most frequent *Leptospira* have been taken into consideration: *L. interrogans, L. kirschneri, L. borgpetersenii, and L.noguchii* (Bourhy, Bremont, Zinini, Giry, & Picardeau, 2011). With using several sets of primers, the melting profiles of the strains were clearly discriminated into distinct groups with reproducibility and specificity. The approach could even recognise the subspecies of the samples via comparing with the reference strains.

Previous methods for the identification of *Leptospira* species, like PCR amplification followed by electrophoresis on a nondenaturing polyacrylamide gel (Olivera et al.), or the sequencing of partial 16rDNAs (Postic, Riquelme-Sertour, Merien, Perolat, & Baranton, 2000), are time-consuming. Contrastly, the HRM method outstands with its ability that allows discrimination of species in a short period. Some methods recently using to diagonose acute infection may have a lower PCR end-point detection than that we are using. However, the PCR-positive samples, detected by those highly sensetive approaches, may not be suitable to use for performing melting temperature analysis. Merien et al. (2005) has a former study used a similar technique for Leptospira

differentiation; they distinguished pathogenic species *L. interrogans*, *L. borgpetersenii*, and *L. kirschneri*, by analysing the melting curves but only with one pair of primer lfb1 F/R. The additional primers used in this study here facilitated the differentiation of *L. noguchii* and *L. kirschneri*, improved the specificity of this method.

In a previous study, Merien et al. (2005) used real-time PCR assay with SYBR Green I for detection of pathogenic leptospires in serum specimens. The use of SYBR Green I dye provided an easy and inexpensive real-time PCR detection technique, but it was sometimes considered to have less specificity and reproducibility (Smythe et al., 2002). In our study, use of LightCycler 480 Resolight Dye that is a saturating fluorescent dye provided improved robustness.

In a recent survey, an HRM assay was applied together with random amplified polymorphic DNA to reference collection strains (Tulsiani et al., 2010). The knowledge of RAPD-HRM specific clustering can be used to identify an unknown serovar rapidly, especially when the isolates of pathogenic leptospires seem to be genetically similar (Vijayachari et al., 2004). In this study, VNTR primers were used to amplification before HRM analysis and then the results were performed using Lightcycler®480 Gene Scanning Software. To the best of the knowledge, this is one of the rare research that genotyping uses VNTR primers. Naze et al. (2015) did the first report of using this technique for genotyping pathogenic *Leptospira* collected from Reunion Island. They used ScreenClust HRM (Qiagen, Courtabeuf, France) to analyse the data obtained after HRM, followed by application of the principal component analysis statistical method (Reja et al., 2010). Thanks to the experience of Naze et al. (2015), this study consulted their method based on similar preparation of experiments and amplification protocol but using a newly applied instrument and the corresponding software for detecting

subspecies of pathogenic *Leptospira* in New Zealand. The new analysis system, Lightcycler®480, permits an easier and prompter process of cluster analysis; results can be performed as normalisation melting difference plots and grouped into genotypic clusters automatically.

Although most of performance measurements of this study were good, presenting discriminatory index greater than 0.95 and reproducibility 100%, not all the strains could be differentiated at serovar level. It might be due to the poor correlation between the leptospiral serological typing and molecular typing methods that are not based on surface-exposed lipopolysaccharides (LPS). The HRM method used here, like MLST, PFGE, and MLVA, is relied on analysis of DNA sequences, which cannot always ensure their correlations to characterization at serovar level. At present, there is no single molecular typing approach that can ideally to satisfy an unambiguous determination if the isolates extracted from numerous individuals are identical; methods like MLST, PFGE, and MLVA are usually required to apply together with others to obtain stronger specificity. While, the HRM analysis realises a simpler and more effective method that can be applied in a single test tube in just a few hours without culture isolation.

However, not all the samples could be matched to reference strains in this practice so we cannot make sure which subspeices they might belong to. In the previous work of Naze, Desvars, Picardeau, Bourhy, and Michault (2015), over 40 reference strains were involved in. Naze et al. (2015) using ScreenClust HRM software with the VNTR-4bis and VNTR-Lb5 primers, found that all the infected patients in Reunion Island were caused by *L. interrogans* strain serogroups Icterohaemorragiae serovar Copenhageni or Icterhaemorrhagiae. So they concluded that the only one genotype that has been

involved in human symptomatic leptospirosis on Reunion Island since 2008 was *L. interrogans* serogroup Icterohaemorragiae. On the other hand, only seven reference strains collaborated in this study. The lack of reference strains could explain for the impossibility of exact identification of *Leptospira* species.

Conclusions

This new genotyping method, high resolution melting (HRM), was able to identify the mutation in genotypes, which was reponsible for veterinary cases in New Zealand. The HRM assay that used LightCycler®480 System enabled a quick, robust technique with high discriminatory power on genotyping of leptospires; *Leptospira* species could be performed without culture isolation and be identified in less than two hours.

For the further study on pathology and epidemiology of leptospirosis in New Zealand, the application of this developed HRM assay might contribute to the research of leptospiral polymorphism in New Zealand in a large extent.

Reference

Adler, B. (2015). History of Leptospirosis and Leptospira. In B. Adler (Ed.), Leptospira and Leptospirosis (pp.1-9). Berlin, Heidelberg: Springer Berlin Heidelberg. doi:10.1007/978-3-662-45059-8_1

Adler, B., & de la Peña Moctezuma, A. (2010). Leptospira and leptospirosis. *Veterinary Microbiology,* 140(3–4), 287-296. doi:http://dx.doi.org/10.1016/j.vetmic.2009.03.012

Agampodi, S. B., Matthias, M. A., Moreno, A. C., & Vinetz, J. M. (2012). Utility of quantitative polymerase chain reaction in leptospirosis diagnosis: association of level of leptospiremia and clinical manifestations in Sri Lanka. *Clinical Infectious Diseases, 54*(9), 1249-1255.

Ahmed, A., Anthony, R. M., & Hartskeerl, R. A. (2010). A simple and rapid molecular method for Leptospira species identification. *Infection, Genetics and Evolution,* 10(7), 955-962.

Ahmed, A., Engelberts, M. F. M., Boer, K. R., Ahmed, N., & Hartskeerl, R. A. (2009). Development and validation of a real-time PCR for detection of pathogenic Leptospira species in clinical materials. *PLoS ONE,* 4(9), e7093.

Ahmed, A., Grobusch, M. P., Klatser, P. R., & Hartskeerl, R. A. (2012). Molecular approaches in the detection and characterization of Leptospira. *Journal of Bacteriology & Parasitology,* 2012.

Ahmed, A., Thaipadungpanit, J., Boonsilp, S., Wuthiekanun, V., Nalam, K., Spratt, B. G., Feil, E. J. (2011). Comparison of two multilocus sequence based genotyping schemes for Leptospira species. *PLoS Negl Trop Dis, 5*(11), e1374.

Ahmed, N., Devi, S. M., De los A Valverde, M., Vijayachari, P., Machangu, R. S., Ellis, W. A., & Hartskeerl, R. A. (2006). Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species. *Annals of clinical microbiology and antimicrobials,* 5(1), 1.

Alston, J. M., Broom, J. C., & Doughty, C. J. A. (1958). *Leptospirosis in man and animals* (Vol. 5): Livingstone Edinburgh.

Anderson, B. S., & Minette, H. P. (1986). Leptospirosis in Hawaii: shifting trends in exposure, 1907-1984. *International Journal of Zoonoses,* 13(2), 76-88.

Andre-Fontaine, G., Peslerbe, X., & Ganiere, J. P. (1992). Occupational hazard of unnoticed leptospirosis in water ways maintenance staff. *European Journal of Epidemiology,* 8(2), 228-232.

Arean, V. M. (1962). The pathologic anatomy and pathogenesis of fatal human leptospirosis (Weil's disease). *The American Journal of Pathology,* 40(4), 393.

Avdeeva, M. G., Moĭsova, D. L., Gorodin, V. N., Kostomarov, A. M., Zotov, S. V., & Cherniavskaia, O. V. (2001). The role glucose-6-phosphate dehydrogenase in pathogenesis of anemia in leptospirosis. *Klinicheskaia meditsina,* 80(6), 42-44.

Babudieri, B. (1958). Animal Reservoirs of Leptospires. *Annals of the New York Academy of Sciences,* 70(3), 393-413. doi:10.1111/j.1749-6632.1958.tb35398.x

Baril, C., & Saint Girons, I. (1990). Sizing of the Leptospira genome by pulsed-field agarose gel electrophoresis. *FEMS Microbiology Letters,* 71(1-2), 95-99.

Blackmore, D. K., Bell, L., & Schollum, L. (1979). Leptospirosis in meat inspectors: preliminary results of a serological survey. *The New Zealand Medical Journal,* 90(648), 415-418.

Blackmore, D. K., & Schollum, L. M. (1982). Risks of contracting leptospirosis on the dairy farm. *The New Zealand Medical Journal,* 95(*9*716), 649-652.

Bolin, C. A. (1996). Diagnosis of leptospirosis: a reemerging disease of companion animals. *Seminars in Veterinary Medicine and Surgery (small animal),* 11(3), 166-171.

Brendle, J. J., Rogul, M., & Alexander, A. D. (1974). Deoxyribonucleic acid hybridization among selected leptospiral serotypes. *International Journal of Systematic and Evolutionary Microbiology*, 24(2), 205-214.

Brenner, D. J., Kaufmann, A. F., Sulzer, K. R., Steigerwalt, A. G., Rogers, F. C., & Weyant, R. S. (1999). Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for Leptospira alexanderi sp. nov. and four new Leptospira genomospecies. *International Journal of Systematic and Evolutionary Microbiology,* 49(2), 839-858.

Brown, C., & Bolin, C. (2000). *Emerging Diseases of Animals*: American Society for Microbiology (ASM).

Brown, P. D., Gravekamp, C., Carrington, D. G., Van de Kemp, H., Hartskeerl, R. A., Edwards, C. N., Levett, P. (1995). Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. *Journal of medical microbiology,* 43(2), 110-114.

Buchanan, G. (1927). Spirochaetal Jaundice. *Special Report Series. Medical Research Council*(113).

Caimi, K., Varni, V., Melendez, Y., Koval, A., Brihuega, B., & Ruybal, P. (2012). A combined approach of VNTR and MLST analysis: improving molecular typing of Argentinean isolates of Leptospira interrogans. *Memorias do Instituto Oswaldo Cruz,* 107(5), 644-651.

Cameron, C. E. (2015). Leptospiral structure, physiology, and metabolism. In *Leptospira and Leptospirosis* (pp. 21-41): Springer.

Campagnolo, E. R., Warwick, M. C., Marx Jr, H. L., Cowart, R. P., Donnell Jr, H. D., Bajani, M. D., Tappero, J. W. (2000). Analysis of the 1998 outbreak of leptospirosis in Missouri in humans exposed to infected swine. *Journal of the American Veterinary Medical Association,* 216(5), 676-682.

Chan, O. Y., Paul, D. R., & Sng, E. H. (1987). Leptospirosis among abattoir workers: a

serological survey. *Singapore Medical Journal,* 28(4), 293-296.

Chan, W. F., Maharjan, R. P., Reeves, P. R., Sintchenko, V., Gilbert, G. L., & Lan, R. (2009). Rapid and accurate typing of Bordetella pertussis targeting genes encoding acellular vaccine antigens using real time PCR and High Resolution Melt analysis. *Journal of Microbiological Methods,* 77(3), 326-329. doi:http://dx.doi.org/10.1016/j.mimet.2009.03.007

Corwin, A., Ryan, A., Bloys, W., Thomas, R., Deniega, B., & Watts, D. (1990). A waterborne outbreak of leptospirosis among United States military personnel in Okinawa, Japan. *International Journal of Epidemiology,* 19(3), 743-748.

Cotter, T. J. (1936). Weil's disease in North Queensland. *The British Medical Journal*, 51-56.

Cox, F. E. G. (1996). *The Wellcome Trust illustrated history of tropical diseases*: Wellcome Trust.

Cullen, P. A., Haake, D. A., & Adler, B. (2004). Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiol. Rev.,* 28(1), 291-318.

Cullen, P. A., Xu, X., Matsunaga, J., Sanchez, Y., Ko, A. I., Haake, D. A., & Adler, B. (2005). Surfaceome of Leptospira spp. *Infection and Immunity,* 73(8), 4853-4863. doi:10.1128/IAI.73.8.4853-4863.2005

De Roy, M., Thavachelvam, K., Batra, H. V., & Tuteja, U. (2012). PCR–SSCP: a tool for molecular diagnosis of leptospirosis. *Int J Pharm Bio Sci,* 3, 179-186.

Demers, R. Y., Frank, R., Demers, P., & Clay, M. (1985). Leptospiral exposure in Detroit rodent control workers. *American Journal of Public Health,* 75(9), 1090-1091.

Dikken, H., & Kmety, E. (Eds.). (1978). Chapter VIII Serological Typing Methods of Leptospires [Article]. 11, 259-307. doi:10.1016/S0580-9517(08)70493-8

Edwards, G. A. (1960). Human leptospirosis. *Medicine,* 39(1), 117-156.

Ellis, W. A. (1990). Leptospirosis-A review of veterinary aspects. *Irish Vet News,* 12, 6-12.

Ellis, W. A. (2015). Animal leptospirosis. In *Leptospira and Leptospirosis* (pp. 99-137). Berlin, German: Springer.

Faine, S. (1994). *Leptospira and leptospirosis*: CRC Press Inc.

Faine, S., Adler, B., Bolin, C., & Perolat, P. (1999). Leptospira and Leptospirosis: Nature Publishing Group.

Faine, S., & Stallman, N. D. (1982). Amended descriptions of the genus Leptospira Noguchi 1917 and the species L. interrogans (Stimson 1907) Wenyon 1926 and L. biflexa (Wolbach and Binger 1914) Noguchi 1918. *International Journal of Systematic and Evolutionary Microbiology,* 32(4), 461-463.

Fairley, N. H. (1934). Weil's disease among sewer workrs in London. *British Medical Journal,* 2(3835), 10-14.

Feigin, R. D., Anderson, D. C., & Heath, C. W. (1975). Human leptospirosis. *Critical Reviews in Clinical Laboratory Sciences,* 5(4), 413-467.

Feresu, S. B., Boling, C. A., van de Kemp, H., & Korver, H. (1999). Identification of a serogroup Bataviae Leptospira strain isolated from an ox in Zimbabwe. *Zentralblatt für Bakteriologie,* 289(1), 19-29.

Fortini, D., Ciammaruconi, A., De Santis, R., Fasanella, A., Battisti, A., D'Amelio, R., & Carattoli, A. (2007). Optimization of High-Resolution Melting Analysis for Low-Cost and Rapid Screening of Allelic Variants of Bacillus anthracis by Multiple-Locus Variable-Number Tandem Repeat Analysis. *Clinical Chemistry, 53*(7), 1377-1380.

Gill, O. N., Coghlan, J. D., & Calder, I. M. (1985). The risk of leptospirosis in United Kingdom fish farm workers. Results from a 1981 serological survey. *The Journal of Hygiene,* 94(1), 81-86.

Gordon, M. E. (1993). Clinical problem-solving: Leptospirosis. *New Engl. J. Med,* 329, 2040-2041.

Gouveia, E. L. (2008). Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. *Emerg. Infect. Dis.*, 14, 505-508.

Gouveia, E. L., Metcalfe, J., Carvalho, A. L. F. d., Aires, T. S. F., Villasbôas Bisneto, J. C., Queirroz, A., & Ko, A. I. (2008). Leptospirosis-associated severe pulmonary hemorrhagic syndrome. Salvador, Brazil:*CDC.*

Gravekamp, C., Van de Kemp, H., Franzen, M., Carrington, D., Schoone, G. J., Van Eys, G., & Terpstra, W. J. (1993). Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *Microbiology,* 139(8), 1691-1700.

Gundry, C. N., Vandersteen, J. G., Reed, G. H., Pryor, R. J., Chen, J., & Wittwer, C. T. (2003). Amplicon Melting Analysis with Labeled Primers: A Closed-Tube Method for Differentiating Homozygotes and Heterozygotes. *Clinical Chemistry,* 49(3), 396-406.

Haake, D. A., & Levett, P. N. (2015). Leptospirosis in humans. In *Leptospira and Leptospirosis* (pp. 65-97): Springer.

Haake, D. A., Suchard, M. A., Kelley, M. M., Dundoo, M., Alt, D. P., & Zuerner, R. L. (2004). Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. *Journal of bacteriology,* 186(9), 2818-2828.

Hartskeerl, R. A., Collares- Pereira, M., & Ellis, W. A. (2011). Emergence, control and re- emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology and Infection,* 17(4), 494-501.

Hathaway, S. C., Marshall, R. B., Little, T. W., Headlam, S. A., & Winter, P. J. (1985). Identification by cross-agglutination absorption and restriction endonuclease analysis of leptospires of the Pomona serogroup isolated in the United Kingdom. *Research in Veterinary Science,* 39(2), 151-156.

Herrmann, J. L. (1993). Genomic techniques for identification of Leptospira strains. *Pathologie-biologie,* 41(10), 943-950.

Herrmann, J. L., Baril, C., Bellenger, E., Perolat, P., Baranton, G., & Saint Girons, I. (1991). Genome conservation in isolates of Leptospira interrogans. *Journal of Bacteriology,* 173(23), 7582-7588.

Herrmann, M. G., Durtschi, J. D., Bromley, L. K., Wittwer, C. T., & Voelkerding, K. V. (2006). Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clinical Chemistry,* 52(3), 494-503.

Herrmann, M. G., Durtschi, J. D., Wittwer, C. T., & Voelkerding, K. V. (2007). Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. *Clinical Chemistry,* 53(8), 1544-1548.

Hookey, J. V., & Palmer, M. F. (1991). A comparative investigation and identification of Leptospira interrogans serogroup icterohaemorrhagiae strains by monoclonal antibody and DNA fingerprint analyses. *Zentralblatt für Bakteriologie,* 275(2), 185-199.

Hovind-Hougen, K. (1979). Leptospiraceae, a new family to include Leptospira Noguchi 1917 and Leptonema gen. nov. *International Journal of Systematic and Evolutionary Microbiology,* 29(3), 245-251.

Hubener, R. (1915). Beitrage zur Aetiologie der Weischen Krankheit. Mitt I. *Deut Med Wochenschr,* 41, 1275.

Hunter, P. R., & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology,* 26(11), 2465-2466.

Ido, Y., Hoki, R., Ito, H., & Wani, H. (1917). The rat as a carrier of Spirochaeta icterohaemorrhagiae, the causative agent of Weil's disease (spirochaetosis icterohaemorrhagica). *The Journal of Experimental Medicine,* 26(3), 341.

Inada, R., Ido, Y., Hoki, R., Kaneko, R., & Ito, H. (1916). The etilology, mode of infection,and specific therapy of Weil's disease. *The Journal of Experimental Medicine,* 23(3), 377.

Jevon, T. R., Knudson, M. P., Smith, P. A., Whitecar, P. S., & Blake Jr, R. L. (1986). A point-source epidemic of leptospirosis: description of cases, cause, and prevention. *Postgraduate medicine,* 80(8), 121-129.

Johnston, J. H., Lloyd, J., McDonald, J., & Waitkins, S. (1983). Leptospirosis--an occupational disease of soldiers. *Journal of the Royal Army Medical Corps,* 129(2), 111-114.

Jolley, K. A., & Maiden, M. C. (2010). *Leptospira* spp. MLST Databases. Retrived

from University of Oxford: http://pubmlst.org/leptospira/

Jordan, E. O., & Falk, I. S. (1928). The Newer Knowledge of Bacteriology and Immunology. *American Journal of Public Health.* 635.

Kim, M. J. (1987). Serovar identification of Korean leptospiral strains with monoclonal antibodies. *Korean J. Intern. Med, 32*, 571-578.

Kitamura, H., & Hara, H. (1918). Ueber den Erreger von "Akiyami". *Tokyo Med J,* 2056, 57.

Klarenbeek, A., & Schüffner, W. A. P. (1933). Het voorkomen van een afwijkend leptospira-ras in Nederland. *Ned Tijdschr Geneeskd, 77*, 4271-4276.

Kmety, E., & Dikken, H. (1993). *Classification of the species Leptospira interrogans and history of its serovars*: University Press Groningen.

Ko, A. I., Goarant, C., & Picardeau, M. (2009). Leptospira: The Dawn of the Molecular Genetics Era for an Emerging Zoonotic Pathogen. *Nature Reviews. Microbiology*, 7(10), 736-747. doi:10.1038/nrmicro2208

Kobayashi, Y., Tamai, T., & Sada, E. (1985). Serological analysis of serogroup icterohaemorrhagiae using monoclonal antibodies. *Microbiology and immunology*, 29(12), 1229-1235.

Korver, H., Kolk, A. H. J., Vingerhoed, J., Van Leeuwen, J., & Terpstra, W. J. (1988). Classification of serovars of the icterohaemorrhagiae serogroup by monoclonal antibodies. *Israel Journal of Veterinary Medicine*, 44(1), 15-18.

Lecour, H., Miranda, M., Magro, C., Gonçalves, V., & Rocha, A. (1989). Human leptospirosis — a review of 50 cases. *Infection*, 17(1), 8-12. doi:10.1007/BF01643489

LeFebvre, R. B., Thiermann, A. B., & Foley, J. (1987). Genetic and antigenic differences of serologically indistinguishable leptospires of serovar hardjo. *Journal of Clinical Microbiology*, 25(11), 2094-2097.

Levett, P. N. (2001). Leptospirosis. *Clinical Microbiology Reviews*, 14(2), 296-326. doi:10.1128/CMR.14.2.296-326.2001

Levett, P. N., Morey, R. E., Galloway, R., Steigerwalt, A. G., & Ellis, W. A. (2005). Reclassification of Leptospira parva Hovind-Hougen et al. (1982). *International Journal of Systematic and Evolutionary Microbiology*, 55(4), 1497-1499.

Lingappa, J., Kuffner, T., Tappero, J., Whitworth, W., Mize, A., Kaiser, R., & McNicholl, J. (2004). HLA-DQ6 and ingestion of contaminated water: possible gene–environment interaction in an outbreak of Leptospirosis. *Genes and Immunity*, 5(3), 197-202.

Looke, D. F. (1986). Weil's syndrome in a zoologist. *The Medical Journal of Australia*, 144(11), 597-600.

Manzano, M., Cocolin, L., Pipan, C., Falasca, E., Botta, G. A., Cantoni, C., & Comi, G.

(1997). Single-strand conformation polymorphism (SSCP) analysis ofListeria monocytogenes iapgene as tool to detect different serogroups. *Molecular and Cellular Probes,* 11(6), 459-462.

Marshall, R. (1992). International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Leptospira. Minutes of the meetings, 13 and 15 September 1990, Osaka, Japan. *Int J Syst Bacteriol,* 42, 330-334.

Marshall, R. B., Wilton, B. E., & Robinson, A. J. (1981). Identification of Leptospira serovars by restriction-endonuclease analysis. *Journal of Medical Microbiology,* 14(1), 163-166.

Marshall, R. B., Winter, P. J., Thiermann, A. B., & Ellis, W. A. (1985). Genotypes of Leptospira interrogans serovar hardjo in cattle in the UK. *Veterinary Record,* 117(25-26), 669-670.

Matthias, M. A., Ricaldi, J. N., Cespedes, M., Diaz, M. M., Galloway, R. L., Saito, M., & Gotuzzo, E. (2008). Human leptospirosis caused by a new, antigenically unique Leptospira associated with a Rattus species reservoir in the Peruvian Amazon. *PLoS Negl Trop Dis,* 2(4), e213.

McCrumb Jr, F. R., Stockard, J. L., Robinson, C. R., Turner, L. H., Levis, D. G., Maisey, C. W., & Smadel, J. E. (1957). Leptospirosis in Malaya. 1. Sporadic Gases among Military and Civilian Personnel. *American Journal of Tropical Medicine and Hygiene,* 6(2), 238-256.

Merien, F., Perolat, P., Mancel, E., Persan, D., & Baranton, G. (1993). Detection of Leptospira DNA by Polymerase Chain Reaction in Aqueous Humor of a Patient with Unilateral Uveitis. *The Journal of Infectious Diseases,* 168(5), 1335-1336.

Merien, F., Portnoi, D., Bourhy, P., Charavay, F., Berlioz-Arthaud, A., & Baranton, G. (2005). A rapid and quantitative method for the detection of Leptospira species in human leptospirosis. *FEMS Microbiology Letters,* 249(1), 139-147.

Morgan, J., Bornstein, S. L., Karpati, A. M., Bruce, M., Bolin, C. A., Austin, C. C., & Davis, B. (2002). Outbreak of leptospirosis among triathlon participants and community residents in Springfield, Illinois, 1998. *Clinical Infectious Diseases,* 34(12), 1593-1599.

Mortimer, R. B. (2005). Leptospirosis in a caver returned from Sarawak, Malaysia. *Wilderness & Environmental Medicine,* 16(3), 129-131.

Mumford, C. J. (1989). Leptospirosis and water sports. *British Journal of Hospital Medicine,* 41(6), 519.

Nascimento, A., Ko, A. I., Martins, E. A. L., Monteiro-Vitorello, C. B., Ho, P. L., Haake, D. A., & Oliveira, M. C. (2004). Comparative genomics of two Leptospira interrogans serovars reveals novel insights into physiology and pathogenesis. *Journal of Bacteriology,* 186(7), 2164-2172.

Naze, F., Desvars, A., Picardeau, M., Bourhy, P., & Michault, A. (2015). Use of a New High Resolution Melting Method for Genotyping Pathogenic Leptospira spp. *PLoS ONE,* 10(7), e0127430.

Noguchi, H. (1917). *Spirochaete icterohaemorrhagae* in American wild rats and its relation to the Japanese and European strains: first paper. *The Journal of Experimental Medicine*, 25(5), 755.

Olivera, M. A. A., Caballero, O. L., Neto, E. D., Koury, M. C., Romanha, A. J., Carvalho, J., & Simpson, A. J. G. Use of nondenaturing silver-stained polyacrylamide gel analysis of polymerase chain reaction amplification products for the differential diagnosis of *Leptospira interrogans* infection. *Diagnostic Microbiology and Infectious Disease*, 22(4), 343-348. doi:10.1016/0732-8893(95)00145-3

Padre, L. P., Watt, G., Tuazon, M. L., Gray, M. R., & Laughlin, L. W. (1988). A serologic survey of rice-field leptospirosis in Central Luzon, Philippines. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 19(2), 197-199.

Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., & Akritidis, N. (2008). The globalization of leptospirosis: worldwide incidence trends. *International Journal of Infectious Diseases*, 12(4), 351-357.

Perolat, P., Chappel, R. J., Adler, B., Baranton, G., Bulach, D. M., Billinghurst, M. L., & Serrano, M. S. (1998). Leptospira fainei sp. nov., isolated from pigs in Australia. *International Journal of Systematic and Evolutionary Microbiology*, 48(3), 851-858.

Perolat, P., Grimont, F., Regnault, B., Grimont, P. A. D., Fournie, E., Thevenet, H., & Baranton, G. (1990). rRNA gene restriction patterns of Leptospira: a molecular typing system. *Research in Microbiology*, 141(2), 159-171.

Perolat, P., Lecuyer, I., Postic, D., & Baranton, G. (1993). Diversity of ribosomal DNA fingerprints of Leptospira serovars provides a database for subtyping and species assignation. *Research in Microbiology*, 144(1), 5-15.

Peter, G. (1982). Leptospirosis: a zoonosis of protean manifestations. *The Pediatric Infectious Disease Journal*, 1(4), 282-288.

Philip, N. A. (1976). Leptospirosis: New Zealand's no. 1 dairy occupational disease. *New Zealand Veterinary Journal*, 24(1-2), 6-8.

Picardeau, M., Brenot, A., & Saint Girons, I. (2001). First evidence for gene replacement in Leptospira spp. Inactivation of L. biflexa flaB results in non-motile mutants deficient in endoflagella. *Molecular Microbiology*, 40(1), 189-199.

Postic, D., Riquelme-Sertour, N., Merien, F., Perolat, P., & Baranton, G. (2000). Interest of partial 16S rDNA gene sequences to resolve heterogeneities between Leptospira collections: application to L. meyeri. *Research in microbiology*, 151(5), 333-341.

Que-Gewirth, N. L. (2004). A methylated phosphate group and four amide-linked acyl chains in Leptospira interrogans lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR. *J. Biol. Chem.*, 279, 25420-25429.

Raddi, G., Morado, D. R., Yan, J., Haake, D. A., Yang, X. F., & Liu, J. (2012). Three-Dimensional Structures of Pathogenic and Saprophytic Leptospira Species Revealed by Cryo-Electron Tomography. *Journal of Bacteriology*, 194(6), 1299-1306.

Radvanszky, J., Surovy, M., Nagyova, E., Minarik, G., & Kadasi, L. (2015). Comparison of different DNA binding fluorescent dyes for applications of high-resolution melting analysis. *Clinical Biochemistry,* 48, 609-616. doi:10.1016/j.clinbiochem.2015.01.010

Ramadass, P., Jarvis, B. D. W., Corner, R. J., Cinco, M., & Marshall, R. B. (1990). DNA relatedness among strains of Leptospira biflexa. *International Journal of Systematic and Evolutionary Microbiology*, 40(3), 231-235.

Ramadass, P., Jarvis, B. D. W., Corner, R. J., Penny, D., & Marshall, R. B. (1992). Genetic characterization of pathogenic Leptospira species by DNA hybridization. *International Journal of Systematic and Evolutionary Microbiology,* 42(2), 215-219.

Reja, V., Kwok, A., Stone, G., Yang, L., Missel, A., Menzel, C., & Bassam, B. (2010). ScreenClust: Advanced statistical software for supervised and unsupervised high resolution melting (HRM) analysis. *Methods,* 50(4), S10-S14.

Robertson, M. H., Clarke, I., Coghlan, J., & Gill, O. N. Leptospirosis in trout farmers. *The Lancet,* 318(8247), 626-627. doi:10.1016/S0140-6736(81)92757-4

Robertson, T., Bibby, S., O'Rourke, D., Belfiore, T., Lambie, H., & Noormohammadi, A. H. (2009). Characterization of Chlamydiaceae species using PCR and high resolution melt curve analysis of the 16S rRNA gene. *Journal of Applied Microbiology*, 107(6), 2017-2028. doi:10.1111/j.1365-2672.2009.04388.x

Robinson, A. J., Ramadass, P., Lee, A., & Marshall, R. B. (1982). Differentiation of subtypes within Leptospira interrogans serovars Hardjo, Balcanica and Tarassovi, by bacterial restriction-endonuclease DNA analysis (BRENDA). *Journal of Medical Microbiology,* 15(3), 331-338.

Rodríguez Reyes, E. A., Cullen, P., Bulach, D., Adler, B., Haake, D., & de la Peña-Moctezuma, A. (2005). Expresión en Escherichia coli del gen gspd l del sistema de secreción tipo II de Leptospira borgpetersenii serovariedad hardjo. *Revista Cubana de Medicina Tropical,* 57(1), 45-46.

Rohrbach, B. W., Ward, D. A., Hendrix, D. V. H., Cawrse- Foss, M., & Moyers, T. D. (2005). Effect of vaccination against leptospirosis on the frequency, days to recurrence and progression of disease in horses with equine recurrent uveitis. *Veterinary Ophthalmology,* 8(3), 171-179.

Saito, M., Villanueva, S. Y. A. M., Kawamura, Y., Iida, K.-i., Tomida, J., Kanemaru, T., & Amako, K. (2013). Leptospiraidonii sp. nov., isolated from environmental water. *International Journal of Systematic and Evolutionary Microbiology,* 63(7), 2457-2462.

Salaün, L., Mérien, F., Gurianova, S., Baranton, G., & Picardeau, M. (2006). Application of multilocus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. *Journal of Clinical Microbiology*, 44(11), 3954-3962.

Schreier, S., Doungchawee, G., Chadsuthi, S., Triampo, D., & Triampo, W. (2013). Leptospirosis: current situation and trends of specific laboratory tests. *Expert Review of Clinical Immunology,* 9(3), 263-280.

Schwartz, S. B., Mitchell, S. L., Thurman, K. A., Wolff, B. J., & Winchell, J. M. (2009). Identification of P1 Variants of Mycoplasma pneumoniae by Use of High-Resolution Melt Analysis. *Journal of Clinical Microbiology*, 47(12), 4117-4120. doi:10.1128/JCM.01696-09

Segura, E. R. (2005). Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden [10.1086/427110]. *Clin. Infect. Dis.,* 40, 343-351.

Shang, E. S., Exner, M. M., Summers, T. A., Martinich, C., Champion, C. I., Hancock, R. E., & Haake, D. A. (1995). The rare outer membrane protein, OmpL1, of pathogenic Leptospira species is a heat-modifiable porin. *Infection and Immunity,* 63(8), 3174-3181.

Shaw, R. D. (1992). Kayaking as a risk factor for leptospirosis. *Missouri Medicine,* 89(6), 354.

Skerman, V. B. D., McGowan, V., & Sneath, P. H. A. (1980). Approved lists of bacterial names. *International Journal of Systematic and Evolutionary Microbiology*, 30(1), 225-420.

Slack, A. T., Kalambaheti, T., Symonds, M. L., Dohnt, M. F., Galloway, R. L., Steigerwalt, A. G., & Harrower, B. J. (2008). Leptospira wolffii sp. nov., isolated from a human with suspected leptospirosis in Thailand. *International Journal of Systematic and Evolutionary Microbiology*, 58(10), 2305-2308.

Slack, A. T., Khairani-Bejo, S., Symonds, M. L., Dohnt, M. F., Galloway, R. L., Steigerwalt, A. G., & Smythe, L. D. (2009). Leptospira kmetyi sp. nov., isolated from an environmental source in Malaysia. *International Journal of Systematic and Evolutionary Microbiology*, 59(4), 705-708.

Smith, J. (1949). Weil's Disease in the North-East of Scotland. *British Journal of Industrial Medicine,* 6(4), 213-220.

Smythe, L., Adler, B., Hartskeerl, R. A., Galloway, R. L., Turenne, C. Y., & Levett, P. N. (2013). Classification of Leptospira genomospecies 1, 3, 4 and 5 as Leptospira alstonii sp. nov., Leptospira vanthielii sp. nov., Leptospira terpstrae sp. nov. and Leptospira yanagawae sp. nov., respectively. *International Journal of Systematic and Evolutionary Microbiology*, 63(5), 1859-1862.

Smythe, L., Dohnt, M., Symonds, M., Barnett, L., Moore, M., Brookes, D., & Vallanjon, M. (2000). Review of leptospirosis notifications in Queensland and Australia: January 1998-June 1999. *Communicable Diseases Intelligence*, 24(6), 153-157.

Smythe, L. D., Smith, I. L., Smith, G. A., Dohnt, M. F., Symonds, M. L., Barnett, L. J., & McKay, D. B. (2002). A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. *BMC Infectious Diseases*, 2(1), 13.

Stackebrandt, E., Chertkov, O., Lapidus, A., Nolan, M., Lucas, S., Hammon, N., . . . Goodwin, L. A. (2013). Genome sequence of the free-living aerobic spirochete Turneriella parva type strain (HT), and emendation of the species Turneriella parva. *Standards in genomic sciences*, *8*(2), 228.

Stallman, N. D. (1984). International committee on systematic bacteriology subcommittee on the taxonomy of Leptospira. *International Journal of Systematic and Evolutionary Microbiology*, 34(2), 258-259.

Stern, E. J., Galloway, R., Shadomy, S. V., Wannemuehler, K., Atrubin, D., Blackmore, C., & Harris, L. (2010). Outbreak of leptospirosis among Adventure Race participants in Florida, 2005. *Clinical Infectious Diseases*, 50(6), 843-849.

Stimson, A. M. (1907). Note on an organism found in yellow-fever tissue. *Public Health Reports (1896-1970)*, 541-541.

Stuart, R. D. (1939). Weil's Disease in Glasgow Sewer Workers. *British Medical Journal*, 1(4076), 324-326.

Talmi-Frank, D., Nasereddin, A., Schnur, L. F., Schönian, G., Töz, S. Ö., Jaffe, C. L., & Baneth, G. (2010). Detection and Identification of Old World Leishmania by High Resolution Melt Analysis. *PLoS Negl Trop Dis*, 4(1), 581. doi:10.1371/journal.pntd.0000581

Tamai, T., Sada, E., & Kobayashi, Y. (1988). Restriction endonuclease DNA analysis of Leptospira interrogans serovars Icterohaemorrhagiae and Copenhageni. *Microbiology and Immunology*, 32(9), 887-894.

Terpstra, W. J. (1991). Typing leptospira from the perspective of a reference laboratory. *Acta Leidensia*, 60(2), 79-87.

Terpstra, W. J., Schoone, G. J., Ligthart, G. S., & Ter Schegget, J. (1987). Detection of Leptospira interrogans in clinical specimens by in situ hybridization using biotin-labelled DNA probes. *Microbiology*, 133(4), 911-914.

Terry, J., Trent, M., & Bartlett, M. (2000). A cluster of leptospirosis among abattoir workers. *Communicable Diseases Intelligence*, 24(6), 158-158.

Thaipadunpanit, J., Chierakul, W., Wuthiekanun, V., Limmathurotsakul, D., Amornchai, P., Boonslip, S., & Day, N. P. J. (2011). Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipL32 genes for human leptospirosis in Thailand: a case-control study. *PLoS ONE*, 6(1), e16236.

Thiermann, A. B., Handsaker, A. L., Foley, J. W., White, F. H., & Kingscote, B. F. (1986). Reclassification of North American leptospiral isolates belonging to serogroups Mini and Sejroe by restriction endonuclease analysis. *American Journal of Veterinary Research*, 47(1), 61-66.

Thiermann, A. B., Handsaker, A. L., Moseley, S. L., & Kingscote, B. (1985). New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar kennewicki. *Journal of Clinical Microbiology,* 21(4), 585-587.

Truccolo, J., Serais, O., Merien, F., & Perolat, P. (2001). Following the course of human leptospirosis: evidence of a critical threshold for the vital prognosis using a quantitative PCR assay. *FEMS Microbiol. Lett.,* 204, 17-321.

Tubiana, S., Mikulski, M., Becam, J., Lacassin, F., Lefèvre, P., Gourinat, A.-C., & D'Ortenzio, E. (2013). Risk factors and predictors of severe leptospirosis in New Caledonia. *PLoS Negl Trop Dis,* 7(1), e1991.

Tulsiani, S. M., Craig, S. B., Graham, G. C., Cobbold, R. C., Dohnt, M. F., Burns, M. A., & Smythe, L. D. (2010). High-resolution melt-curve analysis of random-amplified-polymorphic-DNA markers, for the characterisation of pathogenic Leptospira. *Annals of Tropical Medicine & Parasitology,* 104(2), 151-161.

Uhlenhuth, P., & Fromme, W. (1915). Experimentelle Untersuchungen über die sogenannte Weilsche Krankheit (ansteckende Gelbsucht). *Med Klin,* 44, 1202-1203.

van Crevel, R., Speelman, P., Gravekamp, C., & Terpstra, W. J. (1994). Leptospirosis in Travelers. *Clinical Infectious Diseases,* 19(1), 132-134.

van Thiel, P. H. (1948). *The leptospiroses*.The Leptospirosis.

Vezenegho, S., Bass, C., Puinean, M., Williamson, M., Field, L., Coetzee, M., & Koekemoer, L. (2009). Development of multiplex real-time PCR assays for identification of members of the Anopheles funestus species group [10.1186/1475-2875-8-282]. *Malaria Journal,* 8(1), 282.

Victoriano, A. F. B., Smythe, L. D., Gloriani-Barzaga, N., Cavinta, L. L., Kasai, T., Limpakarnjanarat, K., & Coulombe, C. A. (2009). Leptospirosis in the Asia Pacific region. *BMC Infectious Diseases,* 9(1), 1.

Vijayachari, P., Ahmed, N., Sugunan, A. P., Ghousunnissa, S., Rao, K. R., Hasnain, S. E., & Sehgal, S. C. (2004). Use of fluorescent amplified fragment length polymorphism for molecular epidemiology of leptospirosis in India. *Journal of Clinical Microbiology,* 42(8), 3575-3580.

Waitkins, S. A. (1986). Leptospirosis as an occupational disease. *British Journal of Industrial Medicine,* 43(11), 721.

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G., & Pryor, R. J. (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry,* 49(6), 853-860.

Wolff, J. W., & Broom, J. C. (1954). The Genus Leptospira Noguchi, 1917. Problems of Classification and a Suggested System based on Antigenic Analysis. *Documenta de medicina Geographica et Tropica,* 6(1), 78-95.

Wolff, J. W., & Turner, L. H. (1963). Report of a meeting of the taxonomic

subcommittee on Leptospira Montreal, 16–17 August 1962. *International Journal of Systematic and Evolutionary Microbiology*, 13(3), 161-166.

World Health, O. (2003). Human leptospirosis: guidance for diagnosis, surveillance and control. World Health Organization.

Yasuda, P. H., Steigerwalt, A. G., Sulzer, K. R., Kaufmann, A. F., Rogers, F., & Brenner, D. J. (1987). Deoxyribonucleic acid relatedness between serogroups and serovars in the family Leptospiraceae with proposals for seven new Leptospira species. *International Journal of Systematic and Evolutionary Microbiology*, 37(4), 407-415.