UNIVERSITI PUTRA MALAYSIA

THE GLOBAL PREVALENCE OF INTERMEDIATE LEPTOSPIRA SPP. IN HUMANS: A META-ANALYSIS

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ABSTRACT

The Global Prevalence of Intermediate *Leptospira* spp. in Humans: A Meta-Analysis

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Introduction: Leptospirosis is one of the most widespread zoonotic bacterial diseases caused by pathogenic spirochetes of the genus *Leptospira*. Humans infected with this disease are presented with a wide range of clinical manifestations due to varied pathogenicity of *Leptospira* spp. which can be classified into three clusters such as pathogenic, intermediate, and saprophytic. Intermediate *Leptospira* spp. can either be pathogenic or non-pathogenic and they have been reported to cause mild to severe human leptospirosis in several studies, contributing to the disease burden. As the findings of the pathogenic nature of intermediate *Leptospira* spp. were unclear, it is best to use meta-analysis approach because the prevalence estimates could aid in predicting the pathogenic role of intermediate *Leptospira* spp. **Objective:** This study aimed to estimate the global prevalence of intermediate *Leptospira* spp. in humans using meta-analysis with region-wise stratifications based on United Nations (UN) geo-scheme regions. **Methodology:** The articles were extensively searched according to PRISMA guideline, from three databases which include PubMed, Scopus, and ScienceDirect. Studies obtained were subjected to screening by title and abstract as well as full-text screening. The quality of the relevant studies that fit the pre-determined eligibility criteria were evaluated using the modified Critical Appraisal Checklist recommended by Joanna Briggs Institute. The prevalence data generated using RevMan were recorded after adjusting the effects model which was either random- or fixed-effect model according to the heterogeneity of the included studies. **Results:** There were seven studies included for the analysis involving two out of five United Nations regions, among 469 studies identified initially. Meta-analysis showed that the overall prevalence of intermediate *Leptospira* spp. in humans was 32% (95% CI: -0.09 – 0.73; I^2= 99%; p = 0.12) and pooled prevalence for the American and Asian regions were 62% (95% CI: -0.07 – 1.32; I^2= 96%; p = 0.08) and 17% (95% CI: 0.12 – 0.23; I^2= 47%; p < 0.00001), respectively. The findings also illustrated that *Leptospira wolffii* (n=223/225) was the most predominant species as compared to *Leptospira inadai* (n=1/225) and *Leptospira broomii* (n=1/225). **Discussion:** This is the first meta-analysis summarizing the prevalence of intermediate *Leptospira* spp.

on a global scale. The overall prevalence estimate may not be very high, but it is still indicating that this species contributes to the disease burden. Pooled data based on UN regions also showed the prevalence difference between the two regions which are attributed to several factors including the health surveillance system, access to health care facilities, and diagnostic capabilities for leptospirosis. Meanwhile, the data from the selected studies suggested that *L. wolffii* had the highest pathogenicity than the other intermediates since it could be found in various countries and regions and that most of the samples were collected from the patients presented with common to the severe symptoms. **Conclusion**: The prevalence estimates from this study revealed the insight into the contribution of intermediate *Leptospira* spp. to human leptospirosis and suggested that these species are as important as pathogenic species. Therefore, the data obtained could provide the reference for the health professionals to develop better control and intervention strategies in reducing the burden of the disease.

Keywords: Intermediate *Leptospira*, human leptospirosis, global prevalence, meta-analysis

ABSTRAK

Prevalens Spesies *Leptospira* Perantaraan dalam Manusia Secara Global: Meta-Analisis

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Pengenalan: Leptospirosis merupakan salah satu penyakit bakteria yang disebabkan oleh spiroket yang berpatogen daripada genus *Leptospira*. Manusia yang dijangkiti penyakit ini menunjukkan pelbagai gejala klinikal disebabkan kepelbagaian patogenesis spesies *Leptospira* yang boleh diklasifikasikan kepada tiga kluster, iaitu patogenik, perantaraan, dan saprofitik. Spesies *Leptospira* perantaraan boleh bersifat berpatogenik ataupun tidak, dan telah dilaporkan dalam beberapa kajian bahawa spesies ini boleh menyebabkan penyakit yang ringan dan juga yang berbahaya. Oleh kerana kepatogenannya yang tidak jelas, maka pendekatan yang terbaik untuk meramal peranan spesies perantaraan dalam menjangkiti manusia adalah dengan menggunakan cara meta-analisis. **Objektif:** Kajian ini bertujuan untuk menganggar prevalens spesies *Leptospira* perantaraan secara global dalam manusia menggunakan meta-analisis dengan stratifikasi mengikut rantau Pertubuhan Bangsa-Bangsa Bersatu (PBB atau *United Nations*). **Metodologi:** Artikel-artikel yang berkenaan dicari secara ekstensif mengikut garis panduan PRISMA dan melalui tiga pangkalan data termasuk PubMed, Scopus, dan ScienceDirect. Artikel relevan ditapis mengikut tajuk dan abstrak, dan kemudiannya mengikut teks penuh artikel. Kualiti kajian yang menepati kriteria kelayakan dinilai menggunakan senarai semak Penilaian Kritikal yang telah disyorkan oleh Institut Joanna Briggs. Data prevalens yang dijana oleh program perisian RevMan dicatat selepas mengubah model kesan sama ada model kesan rawak atau tetap, mengikut keheterogenan artikel-artikel yang terkandung untuk analisis ini. **Keputusan:** Tujuh buah artikel yang melibatkan dua wilayah PBB telah dipilih daripada 469 artikel, untuk analisis ini. Meta-analisis menunjukkan bahawa prevalens keseluruhan untuk spesies *Leptospira* perantaraan yang dijumpai dalam manusia ialah 32% (95% CI: -0.09 – 0.73; I^2= 99%; p = 0.12), manakala data terkumpul untuk wilayah Amerika dan Asia ialah 62% (95% CI: -0.07 – 1.32; I^2= 96%; p = 0.08) dan 17% (95% CI: 0.12 – 0.23; I^2= 47%; p < 0.00001), secara respektifnya. Hasil kajian juga mendapati bahawa *Leptospira wolffii* (n=223/225) merupakan spesies yang paling banyak dijumpai dalam tujuh kajian yang terpilih untuk analisis ini, berbanding *Leptospira inadai* (n=1/225) dan *Leptospira broomii* (n=1/225). **Perbincangan**: Ini merupakan meta-analisis yang pertama dalam merumuskan prevalens spesies *Leptospira*

perantaraan pada skala global. Anggaran untuk prevalens keseluruhan mungkin tidak terlalu tinggi, tapi prevalens tersebut masih menunjukkan bahawa spesies ini menyumbang kepada beban penyakit ini. Data terkumpul mengikut wilayah PBB juga menunjukkan perbezaan prevalens antara dua wilayah yang ditentukan atas beberapa faktor termasuklah sistem pengawasan kesihatan, akses kepada kemudahan khidmat kesihatan, dan keupayaan diagnostik untuk leptospirosis. Manakala, data daripada tujuh kajian menunjukkan bahawa *L. wolffii* mempunyai kepatogenan yang tertinggi berbanding *Leptospira* perantaraan yang lain kerana ia boleh dijumpai di pelbagai negara dan wilayah dan kebanyakan sampel dikumpul daripada pesakit-pesakit yang mempunyai simptom yang biasa hingga simptom yang teruk. **Kesimpulan**: Hasil anggaran prevalens daripada kajian ini mendedahkan sumbangan spesies *Leptospira* perantaraan kepada leptospirosis manusia dan mencadangkan bahawa spesies ini adalah sama penting dengan spesies patogenik. Oleh yang demikian, data yang diperoleh boleh dijadikan sebagai rujukan buat pihak profesional kesihatan untuk menghasilkan strategi kawalan dan intervensi yang lebih baik dalam mengurangkan beban penyakit ini.

Kata kunci: Leptospira perantaraan, leptospirosis, global, prevalens, meta-analisis

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µm	Micrometre or micron
16S rRNA (rrs)	16S ribosomal Ribonucleic Acid
CAAT	Cross Agglutination Absorption test
CDC	Centers for Disease Control and Prevention
CEBM	Centre for Evidence-Based Medicine
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
DOI	Digital Object Identifier
ELISA	Enzyme-linked Immunosorbent Assay
Etc	Et cetera
gyrB	DNA gyrase subunit Beta
HIV	Human immunodeficiency virus
I^2	Heterogeneity
IgM	Immunoglobulin M
LAMP	Loop-mediated Isothermal Amplification
LPS	Lipopolisaccharide
M	Molar
MAT	Microscopic Agglutination test
MLST	Multilocus Sequence Typing

mol%	Mole percent
NaCl	Sodium Chloride
No.	Number
PFGE	Pulsed-Field Gel Electrophoresis
PMID	PubMed Identifier
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RevMan	Review Manager
RNA	Ribonucleic Acid
rpoB	Beta subunit of bacterial RNA polymerase
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard Error of Mean
spp	A shorthand for multiple species
SQRT	Square root
UN	United Nations
WHO	World Health Organization
β	Beta
*	To denote multiplication
/	To denote division

CHAPTER 1

INTRODUCTION

1.1 Background

Leptospirosis is one of the most well-known, widespread zoonotic diseases which accounts for high morbidity and mortality particularly in the regions with humid tropical or subtropical climates and in the areas with impoverished populations. Costa *et al*. (2015) reported that leptospirosis affects an estimated of around 1.03 million people and causes 58,900 deaths every year. Even though the reported data is significant, there is no precise estimation of the global burden of human leptospirosis as it is often overlooked and under-reported due to wide range of clinical manifestations such as fever, diarrhea, headache, vomiting, muscle aches, malaise, jaundice, renal failure, pulmonary hemorrhage. Leptospirosis also mimics several other diseases like dengue fever, malaria infection, influenza infection, viral hepatitis, viral hepatitis, many more. Therefore, leptospirosis is recognized as one of the bacterial neglected tropical diseases due to its high disease burden and huge impacts on the public health following the re-emerging of this disease in several parts of the world (Hotez *et al*., 2020).

Humans can get infected through skin cuts or mucous membranes by either direct exposure such as contact with the urine of infected animals or indirect exposure such as contact with the water or soil contaminated with urine of the infected animals. The risk of leptospirosis infection is higher in people that work in water and farms. For example, farmers, veterinarians, sewer workers, mine workers, fishermen, dairy farmers, etc. (Centers for Disease Control and Prevention, 2015). Furthermore, there are various kinds of wild and domestic animals that can serve as the carrier, reservoir, or maintenance host of leptospires which are more likely to be the factor that could increase the burden of leptospirosis. For instance, rodents, cattle, swine, buffaloes, dogs, horses, etc. In addition, recreational activities associated with water and soil such as kayaking, swimming, rafting, hiking, and camping also increase the possibility of getting the infection. The incidence of leptospirosis is also influenced by the climate change which usually occurs in tropical and subtropical climates where there are high frequency changes of the rainfalls and flooding (Lau *et al*., 2010). This can be supported by World Health Organization (WHO) estimation in 2015, by which they expected that leptospirosis morbidity can be up to 100 or more per 100,000 people during the outbreak following flood events. In addition, the climate itself play a role in increasing the risk of the incidence, where it is estimated that the cases will approximately reach 0.1 to 1 per 100,000 population in temperate climates and 10 or more cases per 100,000 people in humid tropics (WHO, 2003).

This disease is caused by the pathogenic spirochetes of the genus *Leptospira*. *Leptospira* can be mainly classified according to methods used which are either based on

serological classification or molecular classification systems. Traditionally, serology-based methods identify leptospires according to their antigenic properties found on the outer membrane of the bacteria, which was based on the difference in the structures of lipopolysaccharides (LPS) (Bharti et al., 2003). This method divides these bacteria into two; *Leptospira interrogans* which contain pathogenic strains and *Leptospira biflexa* which are non-pathogenic. There are more than 300 serovars currently identified using this classification system usually detected using agglutination techniques such as Microscopic Agglutination Test (MAT) and cross agglutination absorption test (CAAT). On the other hand, phylogenetic or genomic classification system based on DNA relatedness using DNA-DNA hybridization and 16S-rRNA based methods has further categorized 22 *Leptospira* species into three different clusters which consist of pathogenic, intermediate, and saprophytic. There are currently, 10 pathogenic species *Leptospira noguchii, Leptospira kirschneri, Leptospira interrogans, Leptospira santarosai, Leptospira mayottensis, Leptospira borgpetersenii, Leptospira alexanderi, Leptospira weilii, Leptospira alstonii, and Leptospira kmetyi*) which could cause disease, 5 intermediate species (*Leptospira broomii, Leptospira wolffii, Leptospira fainei, Leptospira inadai, and Leptospira licerasiae*) which mostly caused moderate symptoms, and 7 saprophytic species (*Leptospira meyeri, Leptospira wolbachii, Leptospira terpstrae, Leptospira vanthielii, Leptospira biflexa, Leptospira yanagawae*, and *Leptospira idonii*) which are commonly found in water and soil and does not have the ability to infect people (Nagraik *et al*., 2020). Although serological classification using techniques which remain as the gold standard method, the overall prediction is not as reliable as genomic classification. This is because molecular methods enable the identification of the species exhibiting both serovars and non-serovars, that is intermediate *Leptospira* spp. Also, a

genomic species may consist the representatives from different serogroups and a serogroup may consist of the serovars from different *Leptospira* species (Levett, 2001).

1.2 Problem Statement and Justification

Intermediate *Leptospira* spp. have been reported to cause mild to severe forms of leptospirosis in humans, however the findings about its pathogenicity status were unclear (Chiriboga et al., 2015). This indicates the need for more studies on the intermediate species since they have also been isolated from the clinical samples and have been proven for its virulence features which have the potential to affect the burden of leptospirosis (Tsuboi et al., 2017). In addition, there have been many individual studies and research that successfully identified the presence of intermediate *Leptospira* spp. in various areas, countries, or regions. But, to date, there is no reported meta-analysis of published data that summarises its prevalence in humans on a global scale. Therefore, it is of essential to conduct a suitable method, that is meta-analysis, to systematically summarise the relevant individual studies in a similar field and to obtain a more precise estimation on the overall effect measure (Haidich, 2010). As such, meta-analysis is the best approach to explore the presence of the intermediate *Leptospira* spp. from various parts of the world. Hence, data obtained from meta-analysis could aid in predicting the pathogenic nature of intermediate *Leptospira* spp.

1.3 Objectives

1.3.1 General Objective

This research generally aimed to study the global prevalence of the intermediate *Leptospira* spp. in humans.

1.3.2 Specific Objectives

i. To quantitatively synthesize the evidence on the frequency of the presence of intermediate *Leptospira* spp. in humans using region-wise stratification.

ii. To determine the type of species of the intermediate *Leptospira* spp. that are significant among human samples from the data of the selected studies.

1.4 Hypothesis

It is hypothesized that the overall prevalence of intermediate *Leptospira* spp. as well as its prevalence by region in humans can be estimated using a meta-analysis approach.

CHAPTER 2

LITERATURE REVIEW

2.1 Leptospirosis

Leptospirosis is one of the widespread zoonotic infections that occur worldwide and is endemic in countries with humid subtropical or tropical climates. It has been identified as an emerging infectious disease with epidemic potential in certain countries depending on the climate changes, occupation, recreational activities, and poor urban slum communities (World Health Organization, 2003).

2.1.1 History

Leptospirosis was first known as Weil's disease after Adolph Weil recognized it in 1886, as a specific type of jaundice together with the dysfunctional of urinary organ, splenomegaly, skin rashes, and conjunctivitis (Adler, 2015). However, this disease has been there since before 1886, only that it was not properly investigated or known with different names. For example, "rice field jaundice" in ancient Chinese texts, and "seven-day fever" or "autumn fever" in Japan (Kitamura & Hara, 1918). Meanwhile, it was recognized as "Schlammfieber (mud fever)", "swine-herd's disease", and "cane-cutter's disease" in the Western countries (Adler, 2015; Alston, Broom, & Doughty, 1958; van Thiel, 1948).

Stimson (1907) was the first to demonstrate leptospires using Levaditi silver deposition staining technique, by which he investigated spirochetes in the kidney tissue sections from a patient that had died of yellow fever. Further, the organism was known as *Spirocheta interrogans* due to its hooked ends which resembled a question mark. Then a few years later, the causative agent of what was known as Weil's disease was then isolated by workers in Europe and Japan (Adler, 2015). Inada and Ido (1916) have identified spirochetes and specific antibodies in the blood samples of Japanese miners with infectious jaundice. Inada et al. named the organism as *Spirochaeta icterohaemorrhagiae* after the Japanese group had successfully grown the spirochetes in vitro using a medium of emulsified guinea-pig kidney. They were also able to show that these organisms can grow at 25-37°C. In German, there were two German groups that have successfully isolated the organism from the guinea pig inoculated with blood of the infected soldiers almost simultaneously as the Japanese group (Hubener, 1915; Unlenhuth & Fromme, 1915). These groups named the spirochetes as *Spirochaeta nodosa* and *Spirochaeta icterogenes*, respectively. Later on, the role of rodents as renal carriers of *Leptospira* was revealed by the Japanese group (Ido, Ito, & Wani, 1917).

Since then, there have been many studies that reported the presence of leptospires from almost all mammalian species in different areas and regions. These bacteria were also found to be significantly contributing to the infection in the domestic animal species. Thus, leptospirosis has been recognized as the most widespread zoonosis occurring around the world.

2.1.2 Transmission Cycle and Mode of Transmission

Leptospirosis is a bacterial disease that can infect both humans and animals. The life cycle of *Leptospira* begins with the shedding in the urine of various animal reservoir hosts, which could be from wild animals like rats and hedgehogs to the livestock and domestic species such as pigs, cattle, dogs, and horses (Centers for Disease Control and Prevention, 2015; Ellis, 2015). Meanwhile, humans serve as accidental hosts which can rarely transmit the infection. Although the chance of human-to-human transmission is low, several reports have shown that leptospiral shedding can also occur through sexual intercourse (Doeleman 1932; Harrison & Fitzgerald, 1988), and during lactation (Bolin & Koellner, 1988). Transplacental transmission could also occur if infection happens during pregnancy, leading to the still birth (Coghlan & Bain, 1969; Faine et al., 1984) or abortion (Chung et al., 1963).

According to Centers for Disease Control and Prevention (CDC) (2015), *Leptospira* can be transmitted to humans directly through contact with urine shed from the infected animals, or indirectly through contact with water, food, or soil that have been contaminated with the urine of infected animals. Skin and mucous membranes such as conjunctival, genital surfaces, or oral are the route of entry of *Leptospira*. The broken skin caused by a cut or scratch can further increase the chance of infection. Moreover, even though it is rare, leptospirosis can also be

occurred following animal bites (Vinholo et al., 2020; Gollop et al., 1993; Luzzi, Milne, & Waitkins, 1987; Barkin, Guckian, & Glosser, 1974; Silverstein, 1953).

2.1.3 Clinical Manifestations

The symptoms could appear as early as two days to four weeks following the exposure to the sources that have been contaminated with the bacteria. Leptospirosis may last from a few days to more than three weeks; however, recovery may take several months if the patients did not receive any treatment.

Leptospirosis can be classified into two forms which are anicteric leptospirosis, a milder form of the disease, and icteric leptospirosis, a more severe disease. Anicteric leptospirosis is the most common disease and occurs in 90% of the cases. Patients with anicteric disease were presented with sudden onset of febrile illness which may occur in two phases. The initial or acute phase may occur for about a week characterized by the presence of *Leptospira* in blood and bodily fluids. The common symptoms appeared during this phase usually include fever, headache, chills, muscle pain, diarrhea, or vomiting. Secondary phase or immune phase is characterized by its presence and production of antibodies in the urine (Levett, 2001) which was accompanied with more severe clinical presentations such as renal failure, liver failure, or even meningitis (Centers for Disease Control and Prevention (CDC, 2017).

On the other hand, patients with icteric leptospirosis usually had rapid disease progressions involving many vital organs such as kidney, lungs, and heart. The symptoms include jaundice, acute renal failure, respiratory distress, cardiovascular collapse, pulmonary hemorrhage, and acute respiratory failure, which can possibly lead to death (Ellis, 2015).

2.1.4 Epidemiology

The incidence of leptospirosis is higher in warm-climate countries than in temperate regions (Ratnam, 1994; Everard, 1993) because warm and humid conditions favor the growth and survival of leptospires. Levett (2001) mentioned that this disease is seasonal, and the number of cases may peak during fall in temperate regions, and during rainy seasons in tropical regions.

The risk factors that could cause human infection include occupational, avocational, or recreational exposure. This disease can be occupational hazard to those that have direct contact with the infected animals such as farmers, veterinarians, rodent control worker, meat inspectors, and abattoir workers, or indirect contact such as miners, soldiers, rice field workers, sugar cane cutters, canal workers, banana farmers, gamekeepers, etc. (Levett, 2001). Recreational activities especially those related to the contact with water have also significant risk for disease transmission such as swimming, rafting, hiking, and fishing. This can be concurred by several outbreaks that have occurred especially during competitive events (CDC, 2001; Baranton & Evans, 2000; CDC, 1998).

There have been several studies reporting the outbreaks in different countries such as in Nicaragua, Brazil, southeast Asia, India, the United States, and several other countries due to the EcoChallenge 2000 competition in Malaysia (Levett, 2001; CDC, 2001; Baranton & Evans, 2000). According to Pan American Health Organization (n.d.), leptospirosis cases can reach to 100 cases within only two weeks following flood in Nicaragua in 2007, more than 40 cases and six deaths within three days of flooding in Guyana in 2005, as well as 3,493 cases in Santa Catarina, Brazil in 2008. This showed that leptospirosis has emerged as one of the public health threats in the world. Munoz-Zanzi et al., (2020) also stated that leptospirosis outbreaks at the regional level occurred mostly in the Latin America and the Caribbean region with 35.8%, which then followed by Southern Asia and North America with 12.9% and 10.7%, respectively. Meanwhile, Cuba had the highest outbreaks at the country level, followed by India, and the United States of America.

Besides, large outbreaks with epidemic potential have also been shown to have significantly associated with severe flooding since flood water caused the movement of rodents or the reservoir hosts of *Leptospira* into the city (WHO, n.d.). The most recent outbreaks have been reported in Fiji with five deaths (Anadolu Agency, 2021), and 1318 confirmed cases and death rate of 53 in Kerala, India (James et al., 2018). Meanwhile, in Malaysia, there were 86 cases with 30 deaths (Abdul Wahab, 2015). Apart from that, outbreaks after heavy rainfall and flooding

were also observed in the non-endemic areas such as Taiwan, the United States, and Australia (Smith et al., 2013; Chiu et al., 2009; Gaynor et al., 2007).

2.2 Leptospira

Dr. Hideyo Noguchi, a Japanese bacteriologist was the first who proposed the genus name of the spirochete, *Leptospira* (Lep.to.spo'ra. Gr. Adj. *leptos* thin, narrow, fine; Gr. N. *spira* a coil; M.L. fem.n. *Leptospira* a fine coil) (Faine & Stallman, 1982; Noguchi, 1918). It was to differentiate Weil's disease spirochete from the other diseases recognized at the time like *Treponema pallidum*, *Spirochaeta* and *Spironema* (later Borrelia) *recurrenti*, which was based on the morphological characteristics (Adler, 2015).

2.2.1 Morphology and Characteristics

Leptospira are spherical as shown in Figure 2.1. They are tightly coiled, rapidly motile spirochetes with length ranging from 0.1 μm by 6 to 0.1 by 20 μm, diameter of about 0.1 μm, wavelength of 0.5 μm, and helical amplitude of 0.1-0.15 μm (Adler & de la Peña Moctezuma, 2010; Fainei et al., 1999). *Leptospira* have either one or both pointed ends with a distinctive hook. It has double membrane structure, such that the peptidoglycan cell wall is closely associated with the cytoplasmic membrane covered by an outer membrane (Cullen, Haake, & Adler, 2004).

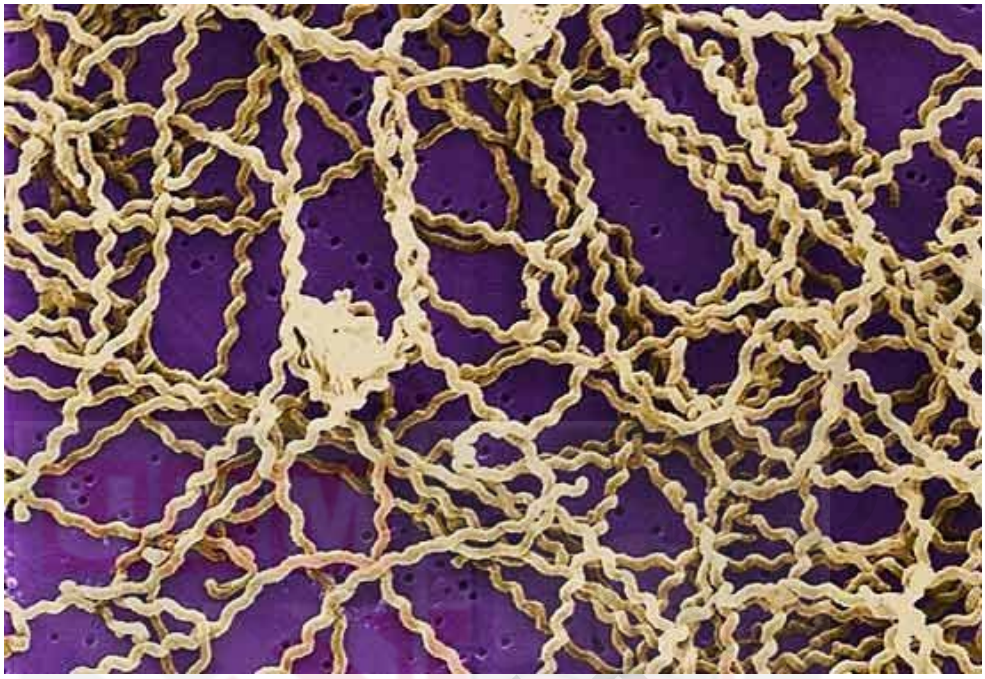

Figure 2.1: Spiral-shaped bacteria of the genus *Leptospira* observed using scanning electron micrograph. Adapted from Leptospirosis in *Encyclopedia Britannica*, 2021, Retrieved July 28, 2021, from https://www.britannica.com/science/leptospirosis.

Lipopolysaccharide (LPS) make-ups of *Leptospira* are like Gram-negative bacteria, nevertheless it has lower endotoxic activity (Faine et al., 1999). *Leptospira* are obligate aerobes that grow in the temperature of 28-30°C with a medium containing vitamins B1 and B12, ammonium salts, and long-chain fatty acids which are produced by β-oxidation, serving as the only carbon source.

2.2.2 Classifications of *Leptospira*

The advancements in the technologies have increased the investigations on leptospirosis and *Leptospira*. Prior to 1989, the subcommittee on the Taxonomy of *Leptospira* had divided *Leptospira* into two species which were named *L.*

interrogans and *L. biflexa*, comprising pathogenic strains and saprophytic strains, respectively.

Serologically, these two species could be distinguished by growth characteristics, such that *L. interrogans* were able to reproduce in the environment with the temperature of 13°C and in the presence of 8-azaguanine. *L. biflexa*, on the other hand, were unable to form spherical cells in 1 M NaCl (Johnson, Faine, & Holt, 1984). *L. interrogans* and *L. biflexa* can be further divided into different serovars by using agglutination techniques such as microscopic absorption test (MAT) and cross agglutinin absorption test (CAAT) after cross-absorption with homologous antigen (Levett, 2001). Serovars that have similar antigens were then classified into serogroups (Kmetyi & Dikken, 1993). According to Nagraik et al. (2020), *L. biflexa* has more than 60 serovars arranged into 38 serogroups, whereas *L. interrogans* has more than 250 serovars arranged into 38 serogorups have been identified.

In subsequent years, the use of a phylogenetic classification system has been used which divided the species of *Leptospira* into three different groups, comprising pathogenic, intermediate, and saprophytic species (Figure 2.2). This classification was done based on DNA relatedness using several housekeeping genes such as rpoB (La Scola et al., 2006), rrs (Morey et al., 2006), and gyrB (Slack et al., 2006), employing DNA-DNA hybridization and 16S rRNA sequence analysis (Picardeau, 2017). Interestingly, this classification system was able to recognize that there were both pathogenic and non-pathogenic serovars within the

same species, which is called the intermediate species. Currently, there are 10 pathogenic species, five intermediate species, and seven non-pathogenic species that have been identified.

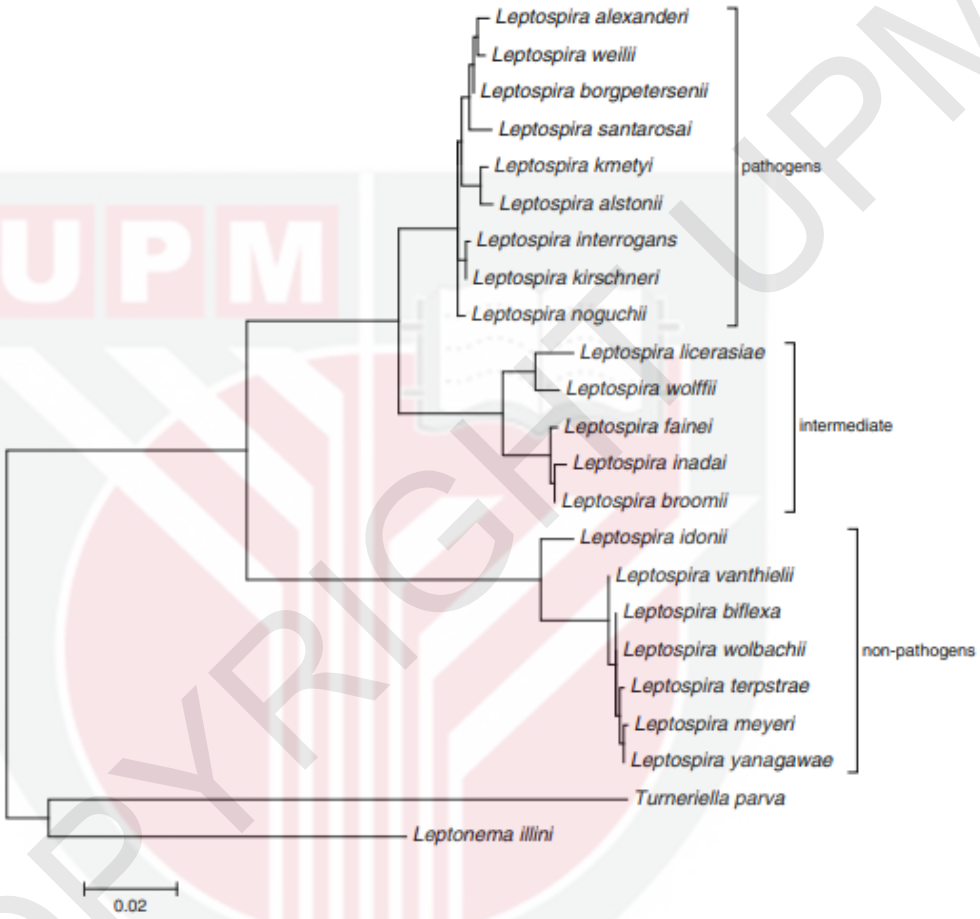

Figure 2.2: Molecular phylogenetic analysis of 16S rRNA gene sequences using MEGA5 based on Tamura-Nei model. Adapted from "History of leptospirosis and leptospira," by B. Adler, 2015, *Current Topics in Microbiology and Immunology, 387*.

2.2.3 Intermediate *Leptospira* spp.

The advancements in the technologies have increased the investigations on leptospirosis and *Leptospira*. Prior to 1989, the subcommittee on the Taxonomy of *Leptospira* had divided *Leptospira* into two species which were named *L.*

interrogans and *L. biflexa*, comprising pathogenic strains and saprophytic strains, respectively.

2.2.3.1 *Leptospira wolffii*

L. wolffiii was first isolated by Slack et al. (2008) from the urine sample of a human patient suspected with leptospirosis in Nakornrachasima, Thailand. The novel species was proposed with a name of *Leptospira wolffii* serovar Khorat. It was named after Prof. Dr. Jan Willem Wolff, who is a bacteriologist from the Netherlands and has made significant contributions to the study related to *Leptospira.* This species has been ratified by the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Leptospiraceae*.

It has been observed under dark-field microscopy that *L. wolffii* of strain Khorat-$H2^T$ possessed similar characteristics to that of typical *Leptospira*, including its morphology and motility. The diameter is 0.2 µm and the length of *L. wolffii* is 10-13 µm, while the wavelength and amplitude were approximately 0.5 µm and 0.3 µm, respectively. (Slack et al., 2008). *L. wolffii* grow in the presence of 8-azaguanine and at the temperature of 30 and 37°C, but it does not have the ability to grow at 13°C. The DNA G+C content was 41.8 mol%. The same study also revealed that this type strain did not cross-react with any recognized *Leptospira* serogroups, as confirmed by serological identification using

MAT. Apart from that, the dendogram shown in Figure 2.3 demonstrated that this type strain falls within the radiation of the genus, using 16S rRNA gene sequencing, which was then confirmed by DNA-DNA hybridization. It also formed a unique lineage within the clade of the intermediates or potentially pathogenic *Leptospira* species.

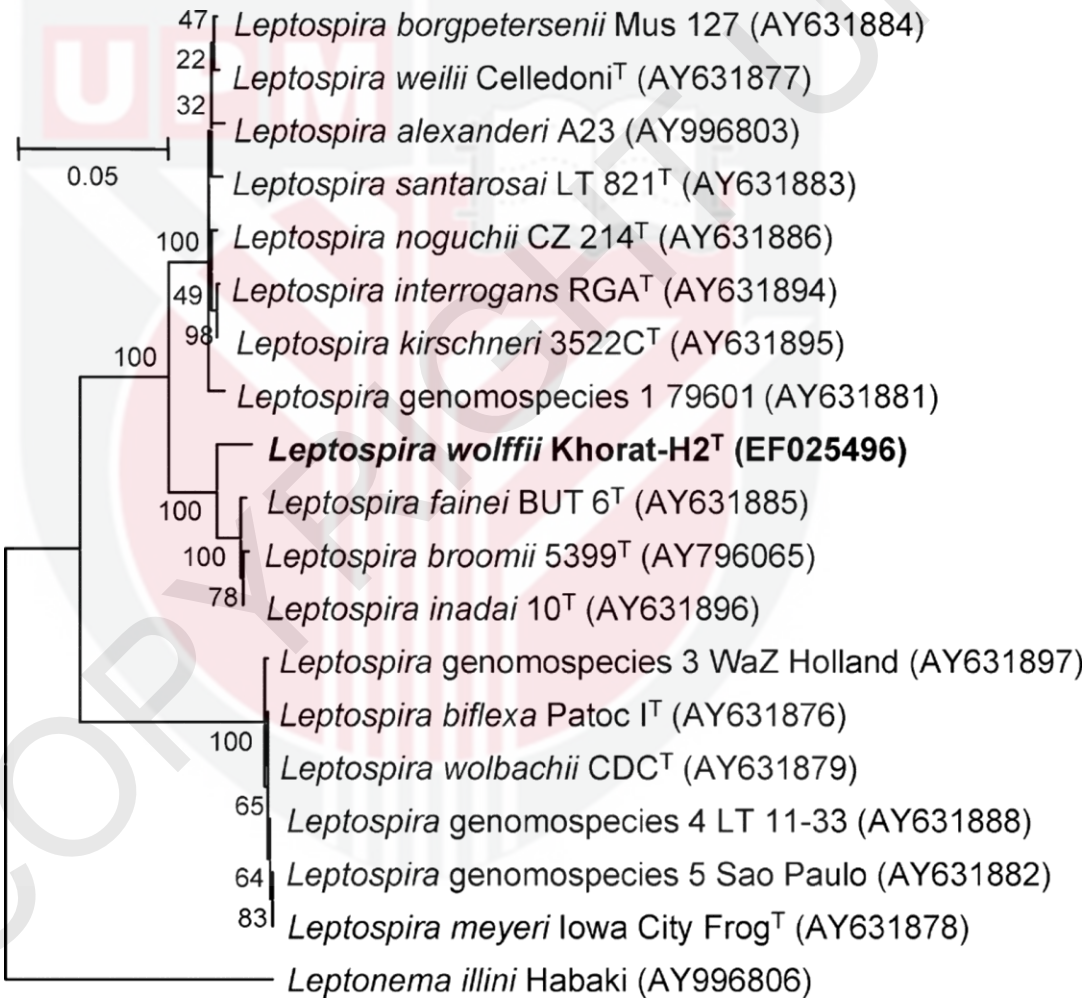

Figure 2.3: Phylogenetic analysis of *L. wolffii* strain Khorat-H2 and representative *Leptospira* species. Adapted from "Leptospira wolffii sp. nov., isolated from a human with suspected leptospirosis in Thailand," by A. T. Slack et al., 2008, *International Journal of Systematic and Evolutionary Microbiology, 58*(10).

There have been several reports that identified *L. wolffii* from human, animal, and environmental samples from different areas in the world. Zakeri et al. (2010) was the first study that successfully discovered the presence of *L. wolffii* from human and animal samples (sheep and dogs) in Iran, and it was found to be the most dominant in the dog samples. This showed the circulation of *L. wolffii* and its crucial part in the disease transmission within both human and wildlife hosts, particularly in dog hosts. This species with serovar Hardjo was later identified using MAT technique, in the patients with febrile disease from Kamalapur, Bangladesh (Kendall et al., 2010).

Subsequently, Benacer et al. (2013) isolated *L. wolffii* from the water and soil samples in University Malaya Lake. Another study conducted in Sarawak, Malaysia has ascertained the circulation of this strain, as they identified that it is the dominant intermediate species found from soil and water samples from the urban areas (Pui et al., 2017). In a recent study carried out in Thailand, *L. wolffii* was isolated from one of the rodent urine samples in Thailand (Krairojananan et al., 2020). All these findings were consistent, suggesting the link between human disease caused by *L. wolffi*, and that this species transmitted among all rodents, environment, and humans.

2.2.3.2 *Leptospira broomii*

A study by Levett et al. in 2006 was the first study that discovered another novel species of intermediate *Leptospira* spp. namely *L. broomii*, from the urine, blood, and cerebrospinal fluid samples of the patients in Denmark and France. It was named after Dr. J.C. Broom, who is a Scottish bacteriologist that has made significant contributions to the study of leptospirosis. It was observed that the length of the cells was 10-15 µm with a slightly smaller diameter than *L. wolffi*i, which was 0.1 µm. Meanwhile, the wavelength and amplitude were approximately 0.5 µm and 0.3 µm, respectively, with the DNA G+C content of 42 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing showed that *L. broomii* placed within the clade of intermediate leptospires (Figure 2.4).

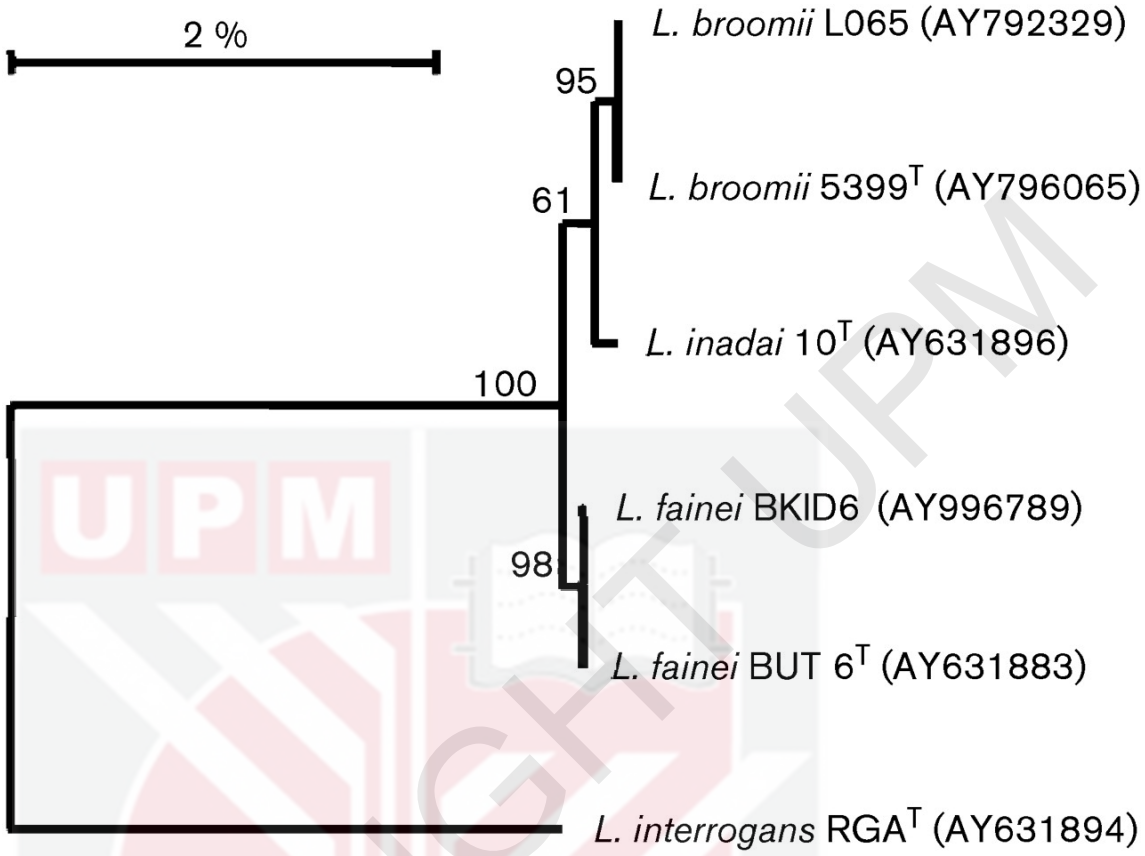

Figure 2.4: A dendogram showing phylogenetic position of *L. broomii* using 16S rRNA gene sequencing. Adapted from "Leptospira broomii sp. nov., isolated from humans with leptospirosis," by P. N. Levett et al., 2006, *International Journal of Systematic and Evolutionary Microbiology, 56*(3), p. 671–673.

Chiani et al. (2016) have isolated *L. broomii* from a suspected patient, but with no sign of severe leptospirosis in Argentina (Chiani et al., 2016). However, there were no other studies that reported the presence of *L. broomii* in human samples from the other areas, as well as in the animal and environmental samples.

2.2.3.3 *Leptospira inadai*

Schmid et al. (1986) was the first to isolate *L. inadai* from a skin biopsy of a patient with Lyme disease and was named after the renowned Japanese physician who contributed to the study regarding the etiologic agent of Weil's disease, Ryokichi Inada.

There have been only a few studies that have isolated *L. inadai* from human samples over the last two decades. It was found from the clinical samples collected from severe human cases in India (Gangadhar et al., 2008) and from a patient with mild symptoms such as jaundice, kidney failure, and hemorrhages in Ecuador (Chiriboga et al., 2015).

Recently, Moreno et al. (2018) discovered the presence of *L. inadai* in the urban rodent sample in Brazil. The identification of this species in animal was supported by several findings, such that it has been isolated from the rodents in India (Gangadhar et al., 2000), and among domestic and peri-domestic animals (cattle, rats, dogs, and pigs) in Ecuador (Chiriboga et al., 2015). Considering that *L. inadai* was widely disseminated, the presence of this species in the patient with febrile disease or asymptomatic leptospirosis might have went unnoticed since it is not part of the routine antigen in many of the diagnostic laboratories (Moreno et al., 2018).

2.2.3.4 *Leptospira fainei*

The novel species of *L. fainei* was first identified from the urine and kidney samples of pigs in Australia and the named in honor for Dr. Solomon Faine, a microbiologist known for research on the epidemiology and physiopathology of leptospirosis (Perolat et al., 1998). This species was able to grow at the temperature of 13°C and 30°C but was partially inhibited in the presence of 8-azaguanine. The cells were 12 µm long with a diameter of 0.2 µm. Serology test showed that *L. fainei* of hurstbridge strain did not agglutinate with any of the reference antisera used in the study.

L. fainei has been reported to cause infection in humans in Australia (Chappel et al., 1998), in the Seychelles (Yersin et al., 1998), in Denmark (Petersen et al., 2001), and in France (Arzouni et al., 2002). Furthermore, it was suggested that *L. fainei* serovar Hurstbridge was involved in the severe leptospirosis with the symptoms of pulmonary hemorrhage, acute renal failure, and possibly death (Yersin et al., 1998). This was supported by the identification of this species from the patients presented with atypical chronic disease due to the symptoms like jaundice, abdominal and back ache, severe headaches, and dizziness experienced by the patients with leptospirosis for months (Petersen et al., 2001). Further, Arzouni et al. (2002) reported the case that confirmed the pathogenic role of *L. fainei* in humans and its geographic distribution to the southern Europe.

2.2.3.5 *Leptospira licerasiae*

L. licerasiae was first isolated from the blood sample of the patient with febrile illness, and also kidney samples collected from peri-domestic rats in Iquitos, Peru. It was named in honor of Professor Julia Liceras de Hidalgo, who has identified the first leptospiral isolates in Peru. *L. licerasiae* serovar Varillal strain VAR 010^T was reported to have 43.9 mol% DNA G+C content, which was within the range of the other *Leptospira* species. This species shared similar characteristics with the pathogenic species, whereby it was sensitive to 8-azaguaniane as well as possessing LipL32-related protein, as confirmed by Southern and Western blots.

L. licerasiae was suggested for being an important cause of acute leptospirosis with undifferentiated fever in that area, and has a *Rattus* species reservoir (Matthias et al., 2008). Based on the results from whole genome analysis, Ricaldi et al. (2012) have suggested that *L. licerasiae* is more likely to be associated with the pathogenic *Leptospira* than saprophytic *Leptospira* species due to the similar protein contents of *L. licerasiae* with that of *L. interrogans* serovar. Lai, which was approximately 67%. Therefore, they described *L. licerasiae* as 'intermediately pathogenic' species. Even so, there were not many studies regarding *L. licerasiae* that can support this finding which may be due to the apparent insignificance to human and animal health. In fact, there has

been only one case that reported the presence of *L. licerasiae* following its first identification in 2008, by which it was isolated from a Japanese traveler returning from Corumba, Brazil (Tsuboi et al., 2017).

2.3 Diagnosis and Identification

The identification of *Leptospira* can be performed directly by either isolating the leptospires in cultures or by detecting the specific antibodies (Schreier et al., 2013; Hartskeerl, Collares-Pereira, & Ellis, 2011).

2.3.1 Differential Diagnosis of Leptospirosis

There are various diseases that share similar clinical characteristics as leptospirosis which need to be taken into consideration during the diagnosis. This includes Hantavirus infection, influenza, malaria, rickettsiosis, aseptic meningitis, malaria, brucellosis, pyelonephritis, viral hepatitis, toxoplasmosis, infectious mononucleosis, typhoid fever, chemical poisoning, primary HIV seroconversion, pharyngitis, etc. (Pan American Health Organization (PAHO), n.d.). This can be confirmed by either serological tests or by using molecular techniques.

2.3.2 Serological Diagnosis

The most frequently and appropriately employed for the diagnosis was serological test, particularly microscopic agglutination test (MAT) and culture tests which remain as gold standard and definitive methods for both animals and

humans (Nick Day, 2019; Adler, 2015). However, MAT could only provide a general interpretation regarding the serogroups present within the population due to the high degree of cross-reaction occurring between different serogroups. There was another alternative test to MAT that is more sensitive and more commercially available such as IgM ELISA (enzyme-linked immunosorbent assay). This rapid assay can be used in especially field settings.

Nonetheless, serological tests are not suitable for early diagnosis. Therefore, it is recommended for the testing of a second sample and for a confirmation test by a reference test (Signorini et al., 2013; Goris et al., 2013; Goris et al., 2011).

2.3.3 Molecular Diagnosis

The advancements in the diagnostic and confirmation methods such as 16S rRNA gene sequencing, DNA-DNA hybridization, G+C content, and rrs gene sequencing have allowed better identification of the *Leptospira* species infecting the patients (Slack et al., 2008). Waggoner (2016) mentioned that molecular methods such as RT-PCR (real-time polymerase chain reaction) and LAMP (loop-mediated isothermal amplification) can increase the ability to detect *Leptospira* which is therefore useful in the acute setting. Another method called pulsed-field gel electrophoresis (PFGE) was also applied in the diagnosis of leptospirosis, in which it was used to identify the serovars (Galloway & Levett, 20210), and Ahmed et al. (2012) stated that it will be likely standardized in the future.

However, the application using these molecular methods, especially in the developing or under-developed countries, is still lacking and not widely available.

2.4 Meta-Analysis

Meta-analysis is a systematic review that synthesizes the findings from previous studies with similar fields to gain a more precise estimation of the overall effects (Haidich, 2010). Meta-analysis is an integrative method that has the highest quality of evidence and it sits on the top of the hierarchy of evidence because it combines the findings from previous related studies (Figure 2.5). Other than that, it also has the lowest level of bias due to the critical protocols in the study setting. An extensive literature search is performed depending on the protocol chosen, such as PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Liberati et al., 2009).

Figure 2.5: Hierarchy of evidence. Adapted from "The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration," by A. Liberati et al., 2009, *Journal of Clinical Epidemiology*, *62*(10).

Most of the researchers utilized meta-analysis in experimental and clinical settings to prove the effect of different interventions by identifying the estimation on the weighted average results from the previous independent studies (Mikolajewicz and Komarova, 2019). For example, risk difference, odds ratio (OR), relative risk, or standardized mean difference. Nevertheless, meta-analysis also allows the researchers to assess the disease frequencies such as prevalence, proportions, and incidence rates (Barendregt et al., 2013). Prevalence follows the same concept as binomial distribution because they have the same definition in which it refers to the proportions of the successes in a sample. Therefore, the binomial equation, which is expressed as proportion, can be used to get the individual study weights based on the inverse variance method. The formula is $Var(p) = (p(1-p))/N$, where p is the prevalence proportion, and N is the size of the population. Additionally, in

accordance with the inverse variance method, the pooled prevalence estimate followed the equation of P= [(P(i)/Var (pi))/ (1/Var(pi)].

The studies are screened based on only the eligibility criteria that have been determined by the researchers before the study selection. The data from the selected studies are then analyzed using statistical software meant for meta-analysis such as RevMan (Review Manager) by Cochrane, Meta-Essentials (Excel workbook), Meta-DiSc, Open Meta-Analyst by CEBM Brown University, etc. Heterogeneity represented as I^2 statistic (expressed in percentage) is also measured to identify the variations between the included studies.

Heterogeneity is very significant in meta-analysis and must be identified because the different studies have different complexities and study designs. Heterogeneity is depending on the amount of overlapped confidence intervals of each of the studies. Therefore, the lower the heterogeneity, the more the amount of CI will be overlapping. The greater the I^2 statistic, the higher the heterogeneity between the studies (Higgins and Green, 2011). It can be an issue if the heterogeneity is significant because the researchers would not be able to assume that the same condition has been investigated in the similar way and that the variabilities in the findings obtained are due to the sampling error only. Even so, the result with considerable heterogeneity can be optimised by adjusting the statistical model for a better data estimation (Ryan 2016; Sedgewick 2015; Barendregt et al., 2013).

CHAPTER 3

METHODOLOGY

Meta-Analysis

3.1 Protocol and Registration

Meta-analysis of this study was conducted according to the PRISMA statement and the study was registered on PROSPERO website (refer Appendix I). PROSPERO or The International Prospective Register of Systematic Reviews is an online database that allows the permanent collection of systematic review protocols that have been registered. It is also recommended to register in this website to prevent duplication with other past or ongoing studies and to enable making comparison with the other studies with similar fields and interests.

3.2 Eligibility Criteria

Eligibility criteria were determined using PICOS format which stands for population, intervention, comparator, outcome, and study design. Table 3.1 shows the inclusion and exclusion criteria for the study.

Table 3.1: Inclusion and exclusion criteria for selection of study.

PICOS ELEMENT	Inclusion Criteria	Exclusion Criteria
Population	Any individual suspected with leptospirosis infection (or co-infected with the other diseases)	Not leptospirosis infection
	Any age and gender	Irrelevant study
		Studies that do not report the origin of the samples or patients
Intervention	Studies specified the species of the intermediate *Leptospira* spp.	Studies that detect the presence only but not identify the type of species
	Studies specified any diagnostic or confirmation methods used	Studies that do not report the method used
		Studies that report the presence of leptospires in animals and environmental samples only
Comparator	Studies reported the number of samples positive for pathogenic *Leptospira* spp.	Insufficient or unclear data
	Studies reported the number of total samples	
Outcome	Studies reported the number of samples positive for intermediate *Leptospira* spp.	
	Studies reported the number of total samples	
Study Design	Research articles of any countries and any publication year	Inaccessible full-text article
		Letter to editor
		Duplicate publications
		Foreign language (other than English)
		Secondary research (review papers, systematic reviews, and meta-analyses)
		Letter to editor

3.2.1 Type of Participants

The subjects of the studies would be any individuals suspected with leptospirosis infection including those that were co-infected with the other diseases. There was no restriction imposed on age, gender, or race. The studies would be excluded if there was no leptospirosis infection and if the studies did not report the origin of the samples or patients. Any irrelevant study will also be removed.

3.2.2 Type of Intervention

The studies must identify the presence of intermediate *Leptospira* spp. which were detected using any recognized diagnostic or confirmation methods. The species of the intermediate *Leptospira* spp. and the methods used must be specified.

3.2.3 Type of Comparator

The absence of intermediate *Leptospira* spp. or the presence of pathogenic *Leptospira* spp.

3.2.4 Type of Outcome

The outcome measures were the frequency of the samples positive for intermediate *Leptospira* spp. and the total number of samples investigated. These data were used to determine the raw prevalence outcome which were measured in

percentage (%) by dividing the number of positive samples with the total number of samples confirmed with leptospirosis.

3.2.5 Type of Study

All research articles of any country and publication year were included. However, inaccessible full-text articles, letter to editor, review papers, duplicated publications, studies using other than English language, and secondary research such as review papers, systematic reviews, and meta-analyses were all removed.

3.3 Search Strategy and Data Source

Comprehensive search related to the human leptospirosis caused by the intermediate *Leptospira* spp. was performed using three electronic databases which include PubMed, ScienceDirect, and Scopus. The combination of the keywords "prevalence", "presence", "epidemiology", "leptospirosis", "intermediate Leptospira", "human", "patient" and species of the intermediate *Leptospira* were included to search for the relevant studies (Table 3.2). Boolean connectors such as 'OR' and 'AND' were applied to connect the terms within the categories and to connect the terms between categories, respectively. In addition, truncations and wildcard operators such as '*' and '~' were also utilized to maximize the search for the related terms of the pertinent studies. The search strategy was slightly adjusted based on the requirements of different databases. There was no restriction for publication dates and language. Then, the reference lists of the included studies were searched manually to seek for additional papers that were not

selected during the initial search. The last database search was carried out on January 10, 2021.

Table 3.2: Search terms and keywords for the literature search.

	PubMed	Scopus	ScienceDirect
Leptospira broomii	(prevalence OR epidemiology) AND leptospir* AND (*leptospira broomii" OR "l.broomii" OR "intermediate leptospir*) AND (human* OR patient*)	TITLE-ABS-KEY (prevalence OR epidemiology) AND leptospir* AND ("leptospira broomii" OR "l.broomii" OR "intermediate leptospir*") AND (human* OR patient*)	(prevalence OR epidemiology) AND leptospirosis~) AND ("leptospira broomii" OR "l.broomii" OR "intermediate leptospira") AND (human~ OR patient~)
Leptospira fainei	(prevalence OR epidemiology) AND leptospir* AND (*leptospira fainei" OR "l.fainei" OR "intermediate leptospir*) AND (human* OR patient*)	TITLE-ABS-KEY (prevalence OR epidemiology) AND leptospir* AND ("leptospira fainei" OR "l.fainei" OR "intermediate leptospir*") AND (human* OR patient*)	(prevalence OR epidemiology) AND leptospirosis~) AND ("leptospira fainei" OR "l.fainei" OR "intermediate leptospira") AND (human~ OR patient~)
Leptospira wolffii	(prevalence OR epidemiology) AND leptospir* AND (*leptospira wolffii" OR "l.wolffii" OR "intermediate leptospir*) AND (human* OR patient*)	TITLE-ABS-KEY (prevalence OR epidemiology) AND leptospir* AND ("leptospira wolffii" OR "l.wolffii" OR "intermediate leptospir*")	(prevalence OR epidemiology) AND leptospirosis~) AND ("leptospira wolffii" OR "l.wolffii" OR "intermediate leptospira") AND (human~ OR patient~)

		AND (human* OR patient*)	
Leptospira licerasiae	(prevalence OR epidemiology) AND leptospir* AND (*leptospira licerasiae" OR "l. licerasiae" OR "intermediate leptospir*) AND (human* OR patient*)	TITLE-ABS-KEY (prevalence OR epidemiology) AND leptospir* AND ("leptospira licerasiae" OR "l. licerasiae" OR "intermediate leptospir*") AND (human* OR patient*)	(prevalence OR epidemiology) AND leptospirosis~) AND ("leptospira licerasiae" OR "l. licerasiae" OR "intermediate leptospira") AND (human~ OR patient~)
Leptospira inadai	(prevalence OR epidemiology) AND leptospir* AND (*leptospira inadai" OR "l. inadai" OR "intermediate leptospir*) AND (human* OR patient*)	TITLE-ABS-KEY (prevalence OR epidemiology) AND leptospir* AND ("leptospira inadai" OR "l. inadai" OR "intermediate leptospir*") AND (human* OR patient*)	(prevalence OR epidemiology) AND leptospirosis~) AND ("leptospira inadai" OR "l.inadai" OR "intermediate leptospira") AND (human~ OR patient~)

3.4 Study Selection

All the search results from the three databases were compiled and sorted out in one Microsoft Excel spreadsheet document. The articles with redundant titles and authors were removed (refer Appendix II for the compilation of studies and removal of duplicated articles using Excel). The title and abstract of the remaining studies were screened by three reviewers independently according to the inclusion and exclusion criteria. The results of the screening by the three reviewers were compared and disagreements were resolved

through consensus. After that, the qualified studies were subjected to full-text screening to further ascertain their relevance (refer Appendix III for the details of the articles for full-text screening). Studies that did not meet the criteria were excluded. The flow of study selection was done by referring to the PRISMA flow diagram (Liberati et al., 2009) (Figure 4.1).

3.5 Data Extraction

Two investigators independently extracted the information from each of the included studies and inserted them into a preliminary table prepared in Microsoft Excel spreadsheet document. The data collected were first author and year of publication, country, region, frequency of samples positive with intermediate *Leptospira* spp., total sample size confirmed with leptospirosis, methods used in the study, and the type of intermediate *Leptospira* species detected. The results of the two reviewers were then compared and re-checked for better accuracy. Disagreements and inconsistencies in data extraction were resolved through consensus and discussion with the third reviewer.

3.6 Methodological Quality Assessment

The quality of each of the included studies was conducted by two reviewers independently using a modified Critical Appraisal Checklist recommended by Joanna Briggs Institute (JBI) for prevalence studies since the use of this tool had been formally evaluated and increased in assessing the prevalence studies (Borges Migliavaca et al., 2020; Munn et al., 2015) (refer Appendix IV for the JBI's checklist). Any disagreements were discussed and resolved through consensus with the third reviewer. The tool was

consisted of nine questions, by which the reviewers referred to, when assessing the studies (refer Appendix IV). The score would be either 0 or 1 for the answers of "No/Unclear" and "Yes", respectively. Therefore, the score could range from 0 until 9.

3.7 Data Analysis and Result Synthesis

The proportion outcomes and the standard error of the mean (SEM) for the proportion of every study were calculated using data extracted from the included studies prior to the insertion of the data to the RevMan version 5.4 software by Cochrane (Research Gate, 2015) (refer Appendix V for the formula and calculations of proportion and SEM). The extracted data were quantitatively analyzed using the software to identify the pooled prevalence estimates of the intermediate *Leptospira* spp. (refer Appendix VI for the step-by-step method of using RevMan software for quantitative analysis).

The overall results were recorded after adjusting the effects model which was either random- or fixed-effect models depending on the heterogeneity represented as I^2 statistic (%) calculated for the included studies. According to Higgins et al. (2019), I^2 statistic of 50 – 90% may represent substantial heterogeneity. Therefore, if the I^2 statistic was low or less than 50%, it indicates that heterogeneity was not significant and thus fixed-effect model would be used, whereas if the I^2 statistic was equal or more than 50%, it indicates that the heterogeneity was significant and therefore s random-effect model would be used.

The result of the prevalence estimates along with its 95% CI, were recorded and summarized in a table. Then, subgroup analysis based on United Nations geo-scheme regions devised by the UN Statistics Division which included the American, Asian, African, Oceania, and European regions (United Nations, 2016) were also carried out to explore and reduce the heterogeneity or variations between the included studies. This pooled data would also highlight the prevalence differences between the regions.

CHAPTER 4

RESULT

Meta-Analysis

4.1 Literature Search

The articles were extensively searched using several keywords which were (prevalence OR presence OR epidemiology) AND leptospir* AND “intermediate leptospira” AND (human* OR patient*) (Table 3.2). There were altogether 469 records identified from three databases by which 112 were from ScienceDirect, 286 were from Scopus, 68 were from PubMed, and three from the other sources. After compiling all the results into the Microsoft Excel spreadsheet document, 258 duplicated articles which have the same authors, DOI, and PMID serial number were removed. Then, the remaining 211 articles were reviewed by title and abstract according to the exclusion and inclusion criteria. After excluding 189, the remaining 22 articles were subjected for full-text screening according to the eligibility criteria. Seven studies that fit the eligibility criteria were included for meta-analysis after 15 records were removed. The flowchart of study selection and the reasons for excluding the studies were illustrated in Figure 4.1.

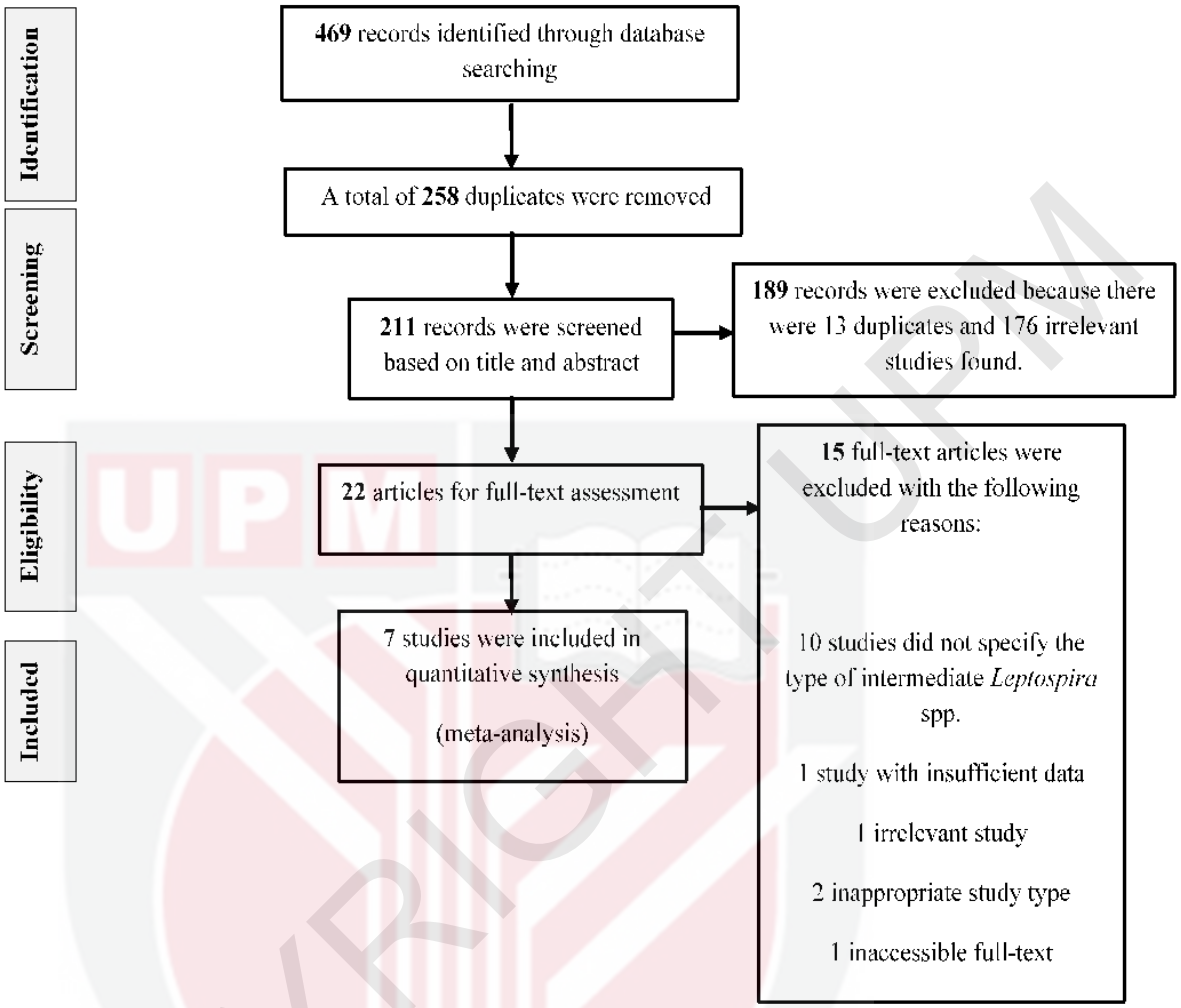

Figure 4.1: Flow diagram of literature search and selection.

4.2 Characteristics of the Included Studies

There were seven studies selected for meta-analysis which were published between 2009 to 2020 and the characteristics were detailed in Table 4.1. Most of the studies that discovered intermediate *Leptospira* spp. in human samples were from the Asian region and then followed by the American region. There were no eligible studies identified from the Oceania, European, and African regions. The total sample size

confirmed with leptospirosis was 403 while the total samples positive for intermediate and pathogenic *Leptospira* spp. were 225 and 174, respectively.

Sera and blood samples collected from humans were utilized for the characterization of the leptospires using several methods such as MAT (Microscopic Agglutination Test), IgM ELISA (Enzyme-Linked Immunosorbent Assay), PCR (Polymerase Chain Reaction) assay, partial RNA polymerase β-subunit (rpo-β) gene sequencing, MLST (Multilocus Sequence Typing), 16S rRNA gene sequencing, as well as partial 16S rDNA (rrs) gene sequencing.

Then, there were only three out of five intermediate *Leptospira* spp. found from the seven included studies. Five studies found *L. wolffii* (Balamurugan et al., 2013; Djadid et al., 2009; Philip et al., 2020; Zakeri, Khorami, et al., 2010; Zakeri, Sepahian, et al., 2010) and one study recorded the presence of both *L. wolffii* and *L. inadai* in their study subjects (Chiriboga et al., 2015). Another study reported *L. wolffii* and *L. broomii* (Chiani et al., 2016). This meta-analysis study showed that *L. wolffii* being the most predominant species (n=223/225) compared to the other two species, *L. inadai* (n=1/225) and *L. broomii* (n=1/225).

Table 4.1: Characteristics of the included studies.

Species of the intermediate (No. of samples)	State	Country	Regions	No. of intermediate positive samples/Total number of confirmed cases (% prevalence)	No. of pathogenic positive samples/Total number of confirmed cases (% prevalence)	Methodology	First Author and Year of Publication
L. wolffii (3)	Karnataka	India	Asia (Southern Asia)	3/10 (30.0%)	7/10 (70.0%)	PCR assay, partial RNA polymerase β-subunit (rpo-β) gene sequencing	Balamurugan *et al*., 2013
L. wolffii (1) and *L. broomii* (1)	Santa Fe and Buenos Aires	Argentina	Americas (South America)	2/8 (25.0%)	6/8 (75.0%)	16S rRNA gene sequencing and MLST	Chiani *et al*., 2016
L. wolffii (129)	Esmeraldas	Ecuador	Americas (South America)	129/132 (97.7%)	3/132 (2.3%)	IgM ELISA, real-time PCR, rrs sequencing	Chiriboga *et al*., 2015
L. wolffii (24) and *L. inadai* (1)	Portoviejo			25/25 (100.0%)	0/25 (0.0%)		
L. wolffii (28)	Guayaquil			28/32 (87.5%)	4/32 (12.5%)		

L. wolffii (11)	Guilan	Iran	Asia (Southern Asia)	11/42 (26.2%)	27/42 (64/3%)	PCR-RFLP assay, nested PCR-16S rRNA gene sequencing	Djadid *et al*., 2009
L. wolffii (2)	Selangor	Malaysia	Asia (South-Eastern Asia)	2/28 (7.1%)	26/28 (92.9)	MAT, PCR, partial 16S rDNA (rrs) gene sequencing	Philip *et al*., 2020
L. wolffii (18)	Mazandaran, Guilan, Ardebil, and Tehran	Iran	Asia (Southern Asia)	18/82 (21.9%)	64/82 (78.0%)	Nested PCR/RFLP analysis, 16S rRNA gene sequencing	Zakeri, Khorami, *et al*., 2010
L. wolffii (7)	Mazandaran	Iran	Asia (Southern Asia)	7/44 (15.9%)	37/44 (84.1%)	Nested PCR/RFLP analysis, 16S rRNA gene sequencing	Zakeri, Sepahian, *et al*., 2010

4.3 Quality Assessment and Risk of Bias

Quality assessment of the selected studies using JBI appraisal checklist for the prevalence study is as shown in Table 4.2. The mean score of the assessment was 5 out of 9 which ranged from 3 to 9. All of the included studies are deemed to have high risk of bias since none of them was randomized.

Table 4.2: Quality assessment of the included studies.

Author and Year	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Total
Balamurugan *et al*., 2013	U	1	0	1	0	1	U	0	U	**3**
Chiani *et al*., 2016	U	1	U	1	0	1	1	0	1	**5**
Chiriboga *et al*., 2015	1	1	1	0	0	1	1	0	U	**5**
Djadid *et al*., 2009	U	1	U	0	0	1	1	0	1	**4**
Philip *et al*., 2020	1	1	0	1	U	1	1	1	U	**6**
Zakeri, Khorami, *et al*., 2010	0	1	U	1	U	1	1	0	U	**4**
Zakeri, Sepahian, *et al*., 2010	1	1	1	1	1	1	1	1	1	**9**

Q1: Was the sample frame appropriate to address the target population? ; Q2: Were study participants sampled in an appropriate way? ; Q3: Was the sample size adequate? ; Q4: Were the study subjects and the setting described in detail? ; Q5: Was the data analysis conducted with sufficient coverage of the identified sample? ; Q6: Were valid methods used for the identification of the condition? ; Q7: Was the condition measured in a standard, reliable way for all participants? ; Q8: Was there appropriate statistical analysis? ; Q9: Was the response rate adequate, and if not, was the low response rate managed appropriately?; 0: No; 1: Yes; U: Unclear.

4.4 Pooled Prevalence of Intermediate *Leptospira* spp. in Human Samples

The analysis using RevMan software provided the pooled estimates of the input data illustrated as forest plot, as well as upper and lower bounds of 95% confidence interval (CI), p-value, and heterogeneity value (I^2 statistic). All the data obtained from the analysis were summarized in Table 4.3.

Table 4.3: Summary on the meta-analysis of the prevalence of intermediate *Leptospira* spp. in humans.

Study	Number of Articles	Heterogeneity (%)	Effect Size	95% Confidence Interval (CI)		
			Prevalence (%)	**p-value**	**Lower Value**	**Upper Value**
Prevalence of intermediate *Leptospira* spp. in humans	7	99	**32**	p = 0.12	-0.09	0.73
		Region-Wise				
UN American region	2	96	**62**	p = 0.08	-0.07	1.32
UN Asian region	5	47	**17**	p < 0.00001	0.12	0.23

4.4.1 Meta-Analysis of the Prevalence of Intermediate *Leptospira* spp. in Human Samples Worldwide

Forest plot in Figure 4.2 exhibited the overall prevalence estimates on the global prevalence of intermediate *Leptospira* spp. The diagram also showed the first author and year of the included studies. The statistical analysis revealed that the overall pooled prevalence of intermediate *Leptospira* spp. in humans was 32% (95% CI: -0.09 – 0.73; p = 0.12) after adjusting the effect model to random-effect model due to the significant heterogeneity (I^2= 99%).

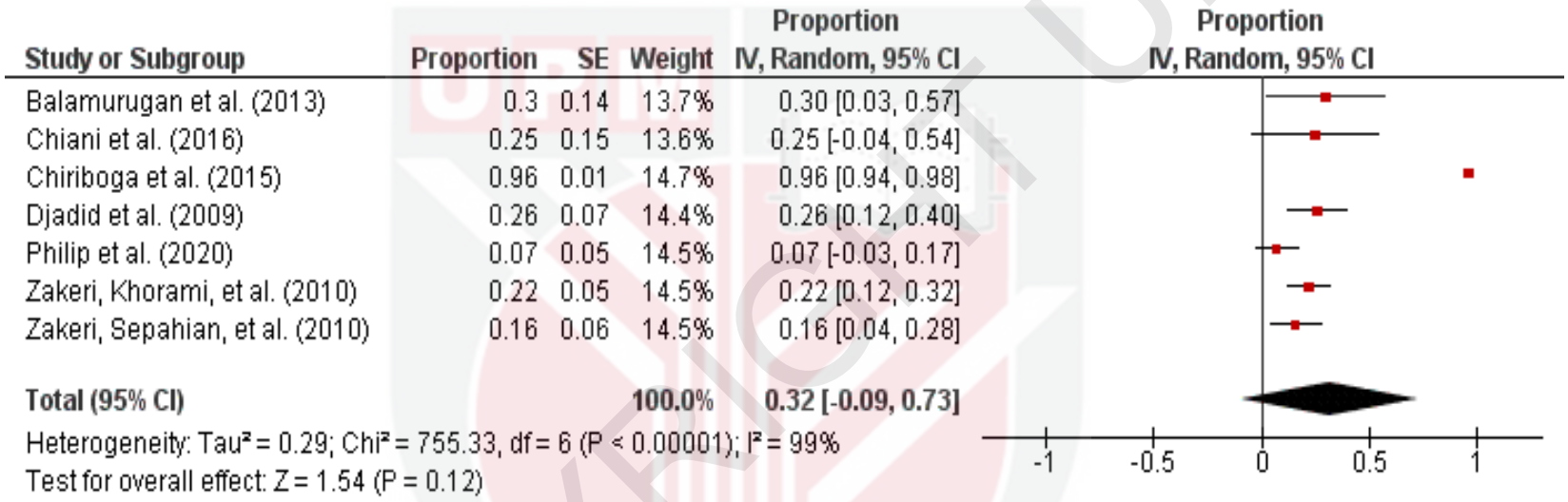

Figure 4.2: Forest plot of the meta-analysis from seven studies showed the overall prevalence estimate of intermediate *Leptospira* spp. in human samples. I^2 = 99% indicates significant heterogeneity, therefore random-effect model was used. After adjusting the effect model, the overall prevalence estimate recorded was 32% with p = 0.12 at 95% CI.

4.4.2 Sub-group Meta-Analysis of the Prevalence of Intermediate *Leptospira* spp. in Human Samples Worldwide from the American and Asian Regions

Subsequently, the studies were categorized by region-wise in accordance with the UN geo-schemes (United Nations, 2006). The pooled prevalence of intermediate *Leptospira* spp. in human samples from the American region was 62% (95% CI: -0.07 – 1.32; p = 0.08), after adjusting the effect model to random-effect model because of the presence of significant heterogeneity (I^2= 96%) (Figure 4.3). Meanwhile, pooled prevalence of the intermediate species in human samples from the Asian region using fixed-effect model was 17% (95% CI: 0.12-0.23; I^2= 47%; p < 0.00001) (Figure 4.4).

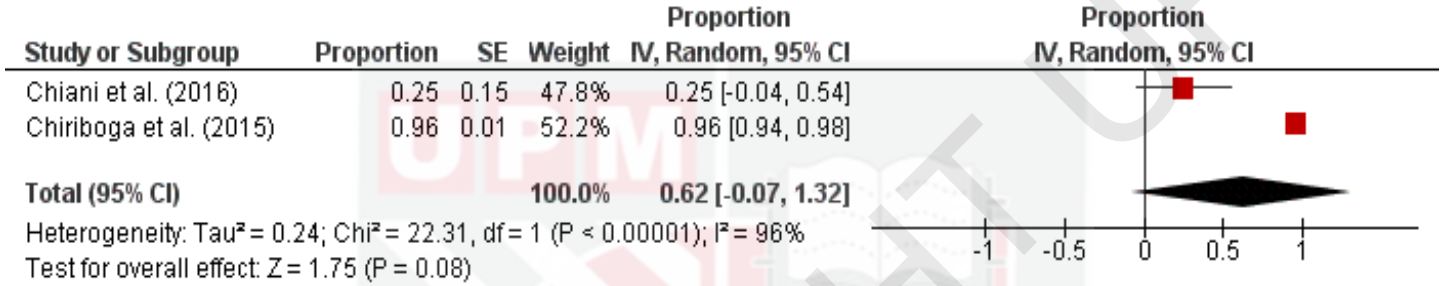

Figure 4.3: Forest plot of the subgroup meta-analysis from two studies showed the prevalence estimate of intermediate *Leptospira* spp. in human samples from the American region. I^2= 96% indicates significant heterogeneity, therefore random-effect model was used. After adjusting the effect model, the prevalence estimate for this region was 62% with p = 0.08 at 95% CI.

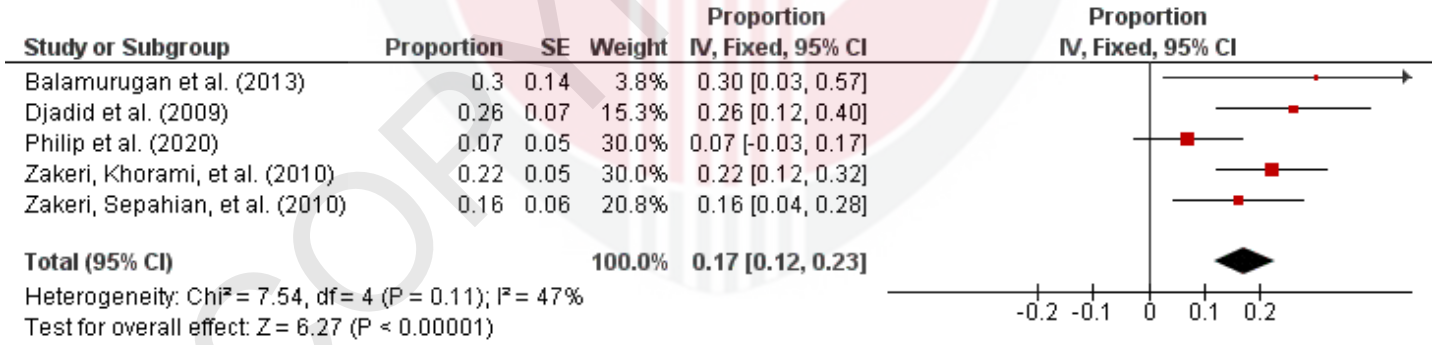

Figure 4.4: Forest plot of the subgroup meta-analysis from two studies showed the prevalence estimate of intermediate *Leptospira* spp. in human samples from the Asian region. I^2= 47% indicates insignificant heterogeneity, therefore fixed-effect model was used. After adjusting the effect model, the prevalence estimate for this region was 17% with p < 0.00001 at 95% CI.

CHAPTER 5

DISCUSSION

Intermediate *Leptospira* spp. have both pathogenic and non-pathogenic serovars, and to date, its role in human pathogenicity remains unclear which is yet to be explored. In this study, the findings from meta-analysis demonstrated that the presence of intermediate *Leptospira* spp. should be considered when making decisions for the disease control and prevention, particularly in the regions where leptospirosis is endemic.

To our knowledge, this is the first meta-analysis that summarizes the prevalence of intermediate *Leptospira* spp. in humans worldwide. There were only seven eligible studies that identified the presence of intermediate *Leptospira* spp. The reason may be due to the milder, febrile disease caused by the intermediate species which could either be undetected as some of the individuals showed no symptom or even misdiagnosed as the patients presented with a broad spectrum of symptoms. Meta-analysis of the included studies illuminates that the overall prevalence of the intermediate *Leptospira* spp. worldwide may not be high, 32% (95% CI: -0.09 – 0.73; I^2= 99%; p = 0.12), even so, it indicates that these species are indeed contributing to the burden of the disease. Moreover, out of two UN regions identified from the included studies, the region of the Americas

had the highest prevalence of intermediate *Leptospira* spp. which was at 62% (95% CI: -0.07 – 1.32; I^2= 96%; p = 0.08) as compared to the Asian region, which was at 17% (95% CI: 0.12-0.23; I^2= 47%; p < 0.00001) (Table 4.3).

However, the studies found from the European, Oceania, and African regions were excluded because they did not meet the inclusion criteria of this meta-analysis study such that the studies did not specify the intermediate *Leptospira* spp., non-English articles, and some of the studies were irrelevant for this analysis. The data from the included studies also demonstrated that there were only three out of five intermediate *Leptospira* spp. identified, which were *L. wolffii*, *L. inadai*, and *L. broomii*. The other two intermediate species, *L. licerasiae* and *L. fainei* were not found in the selected studies because both species were reported in studies related to non-human samples and were lacking essential data, which were then excluded.

5.1 Prevalence of Intermediate *Leptospira* spp. in Human Samples from the American Region

The higher prevalence of intermediate *Leptospira* spp. in the American region that then Asian region indicates that most of the countries in the region of the Americas have more access to health care facilities and better health surveillance system with accurate diagnostic and confirmation methods, which were able to detect and identify the species

that infected the patients. This was supported by a report by Schneider *et al.* (2011), in which they had pointed out that the surveillance and control strategies for leptospirosis were developed in many countries in the region of the Americas. Moreover, the improved method in detecting leptospiral DNA has also enabled the identification of intermediate clusters from patients with febrile symptoms in this region (Chiriboga et al., 2015). For instance, the utilization of the amplified leptospiral 16S rrs gene and sequencing instead of the common PCR protocols that amplify genes present only in the pathogenic species.

Furthermore, there are approximately ten million people that are affected by natural disasters such as floods (35%) and storms (41%) in the American region every year, and there have been several studies reported the outbreaks of leptospirosis associated with these events from different countries in Central and South American countries (Schneider et al., 2012, 2013). The results from local studies performed in Central America showed that leptospirosis cases were prevalent among the communities residing in the rural areas, which they depend mostly on the animals such as bovine and porcine for their income and for daily protein intake (Pan American Health Organization, 2016). All these factors might have increased the chances of the communities being exposed to the intermediate *Leptospira* spp. Apart from that, the data also revealed that *L. wolffii*, *L. inadai*, and *L. broomii* were found in the American region (Table 4.1), which signified that varieties of the intermediate species had contributed to the increased burden of leptospirosis in this region.

5.2 Prevalence of Intermediate *Leptospira* spp. in Human Samples from the Asian Region

On the other hand, the prevalence of the intermediate *Leptospira* spp. in the Asian countries, specifically in the South-Eastern Asian and Southern Asian countries, may not be high when compared to the prevalence in the American region, nonetheless it was statistically significant. The significant presence of the intermediate *Leptospira* spp. in this region, particularly in the Southern Asian countries such as in India and Iran, may be due to the poor access to safe water supplies, poor hygiene, as well as inadequate sanitation. Even though the latest estimates in 2019 showed the improvement in the access to the water supply in India, the water safety and security planning for several districts in India was still lacking and less than half of the population has the access to the safe water supply (United Nations International Children's Emergency Fund (UNICEF), n.d.). As for Iran, Zakeri, Sepahian, et al., (2010) mentioned that 18.5% of the examined cases in northern Iran had collected drinking water from wells and 52% of them had been infected with leptospirosis. The same study also revealed that *L. wolffii* was one of the species isolated from the samples tested. This suggests that unsafe water sources played a role in the transmission of the disease, which may be attributed by the indirect exposure of the intermediate *Leptospira* spp. found in the contaminated water, influencing the incidence of leptospirosis in this region.

In addition, the prevalence of intermediate *Leptospira* spp. was considerably low even though leptospirosis was endemic and causing sporadic outbreaks in most of the developing countries in the South-East Asian sub-region, particularly with humid subtropical and tropical climates such as in Malaysia (Costa et al., 2015; Garba et al., 2018; Victoriano et al., 2009). This may possibly be due to the diagnostic capabilities of the disease, of which the tools used were less sensitive in detecting the species infecting the patients. Even though culture and microscopic agglutination tests (MAT) are the gold standard for laboratory diagnostic testing and the most widely used in this region, but they require experts in handling the live pathogens, hence, these methods were offered by few hospitals and laboratories in several countries in the Asian region (Garba et al., 2018). Additionally, it was said to have little value in predicting the infecting serogroup of the patients since the screening of the serum samples is mostly based on 25 reference serovars, representing only a fraction of over 200 serovars found globally (Gamage et al., 2012; Garba et al., 2018; Katz et al., 2003; Levett, 2003; Smythe et al., 2009). There were several alternative methods to MAT in detecting the acute infection such as enzyme-linked immunosorbent assay (ELISA), IgM dipstick, lateral flow assay, and latex agglutination test, nonetheless, these assays have low sensitivity especially during the acute phase (Effler et al., 2002; Hull-Jackson et al., 2006; McBride et al., 2007; Smits et al., 2001). The accuracy of these techniques are also poor in some areas where leptospirosis is endemic (Blacksell et al., 2006; Myint et al., 2007). According to Gamage et al. (2012), the available laboratory facilities were still poor and inadequate specifically in certain South-East Asian countries, and as reported by WHO in 2009, India, Indonesia, Thailand, and Sri Lanka were the only WHO Member States that have fully or partially implemented laboratory facilities for the diagnosis of leptospirosis (WHO, 2009b).

Besides, as most of the South-East Asian countries were the major importers for the agricultural products such as Malaysia, Philippines, and Indonesia, the significant prevalence in this region may be contributed by the occupational factors such as the contact of intermediate *Leptospira* spp. in the contaminated water and soil through farming (United States Department of Agriculture, n.d.). This was corroborated with the findings of intermediate *Leptospira* spp. being isolated from the environmental and water samples in several countries in this region (Balamurugan et al., 2013; Narkkul et al., 2020; Pui et al., 2017).

5.3 Type of Intermediate *Leptospira* spp. in Human Samples Reported in the Included Studies

The data collected from the seven included studies showed that *L. wolffii* was the most predominant species (n=223/225) as compared to the other intermediate species (*L. inadai*; 1/225; *L. broomii*: 1/225). *L. wolffii* was first isolated from an individual with suspected leptospirosis in Thailand (Slack et al., 2008), and also had been found in all of the included studies which were India (Balamurugan et al., 2013), Argentina (Chiani et al., 2016), Ecuador (Chiriboga et al., 2015), Iran (Zakeri, Khorami, et al., 2010; Zakeri, Sepahian, et al., 2010), and Malaysia (Philip et al., 2020), which may suggest that *L. wolffii* was the dominant intermediate *Leptospira* circulating in most of the areas. Majority of samples were collected from the patients with acute, febrile illness with other common

symptoms for the suspected leptospirosis such as fever, myalgia, chills, rigors, gastrointestinal problems, as well as a more serious symptom like jaundice (Chiriboga et al., 2015; Djadid et al., 2009; Philip et al., 2020; Zakeri, Khorami, et al., 2010; Zakeri, Sepahian, et al., 2010). In one study, several patients with fever, jaundice, hematuria, icteric discoloration with hepatomegaly, as well as weakness on the left side were confirmed to be infected with *L. wolffii* (Balamurugan et al., 2013). In other similar studies conducted in Argentina and Malaysia, *L. wolffii* was isolated from patients with fatal cases, particularly respiratory syndrome (Chiani et al., 2016; Philip et al., 2020). In addition, as *L. wolffii* was categorized as pathogenic *Leptospira* using nested PCR-RFLP due to the absence of ApoI restriction sites, further sequencing analysis of the samples was required, by which they showed that 26% of the tested DNA belonged to *L. wolffii* (Djadid et al., 2009). All the tested samples from this study were collected from symptomatic patients manifesting fever with headache, body aches related to jaundice for several days, and headache with myalgia, which all required hospitalization. Furthermore, *L. wolffii* had also been isolated from the environments and animals such as cattle, rats, pigs, sheep, and dog from several previous reports (Chiriboga et al., 2015; Mohd Ali et al., 2018; Zakeri, Khorami, et al., 2010). This indicated that *L. wolffii* was prevalent in various environmental and animal reservoirs and that they played a significant role in the transmission cycle of leptospirosis. Therefore, all this evidence suggested that they had the highest pathogenicity than the other intermediates.

L. broomii, on the other hand, was identified from one of the human samples in Argentina (Chiani et al., 2016) while *L. inadai* was isolated from one of the human

samples in Ecuador (Chiriboga et al., 2015). Chiani et al., (2016) reported that *L. broomii* was identified from the patient with no signs of severe leptospirosis. This indicates that *L. broomii* likely caused milder disease which thus explained the smaller number of *L. broomii* being identified from human samples than *L. wolffii*. Meanwhile, *L. inadai* was mostly isolated from animal samples such as cattle, rats, dogs, and pigs (Cerqueira & Picardeau, 2009), suggesting its predominance in the animal reservoirs rather than in humans.

5.4 Limitations

There were several limitations of this study, which include the possibility of missing some of the relevant studies during the literature search procedure. Also, the information from the other countries and regions was lacking. Despite the increasing studies concerning leptospirosis disease in the African region, there were no eligible studies that reported the intermediate *Leptospira* spp. in this region which may be due to the inadequate sampling and poor access to the diagnostic facilities, leading to the under-reporting of leptospirosis. Hence, the insufficiency of the information regarding the species that infected the patients from the African region (Allan et al., 2015). Besides, there were also no eligible studies reported the presence of intermediate *Leptospira* spp. in the Oceania region, albeit the previous systematic review had revealed that the incidence of leptospirosis was notably high (150.68 cases per 100,000 per year), especially in the temperate parts of the region such as Australia and New Zealand (Berlioz-Arthaud et al., 2007; Guernier et al., 2018). However, there were too little information and

investigations regarding the specific species that infected the patients from this region. Other than that, there were also no eligible studies that reported the presence of intermediate *Leptospira* spp. in the European region. This was because leptospirosis was not a common disease with 0.2 confirmed cases per 100,000 populations which was considered as low rate in the European countries in comparison with the other regions (European CDC, 2016). This prevented us from making a thorough analysis of the prevalence data needed for species-wise stratification, reducing the accuracy of the obtained data. This also limited our study from analyzing the pathogenicity status of the intermediate *Leptospira* spp.

Furthermore, the high risk of bias of all the studies included and high I^2 statistic for the overall prevalence (99%) might influence the accuracy of prevalence estimates in this analysis. The high heterogeneity may suggest that there is high variability in the design and complexity of the included studies, as well as differences in the geographical regions, environments, and study settings. Other than that, publication bias assessment using a funnel plot was not carried out since there were only seven included studies in this study. Higgins et al. (2019) stated that the power of the tests would be too low to distinguish the chance from the real asymmetry if the studies are fewer than 10. Additionally, Debray et al., (2018) also reported that the power for the tests of the funnel plot asymmetry usually remained less than 50% even when there were ≥ 50 studies available for meta-analysis. Therefore, it is best to use a funnel plot only when there is a minimum of 10 studies. Lastly, based upon the advanced literature survey, we found only a few articles that addressed the presence of intermediate *Leptospira* spp. and identified the exact type of species in human samples.

CHAPTER 6

CONCLUSION

In conclusion, this is the first meta-analysis that summarizes the prevalence of intermediate *Leptospira* spp. from human samples worldwide. The meta-analysis obtained from this study showed that the overall prevalence of intermediate *Leptospira* spp. was not very high. On the other hand, the pooled prevalence estimates based on the UN regions showed the highest prevalence of intermediate *Leptospira* spp. in the American region which was 62% (95% CI: -0.07–1.32; I^2= 96%) and followed by the Asian region which was 17% (95% CI: 0.12–0.23; I^2= 47%). The data from the included studies also demonstrated that *L. wolffii* was the most predominant species found in the human samples as compared to the *L. inadai* and *L. broomii*. All the findings suggest that intermediate *Leptospira* spp. played an important role in the transmission of human leptospirosis. However, there were limited studies eligible for this meta-analysis study and the publication bias of the included studies was unclear.

One of the suggestions for future research is to carry out more investigations using molecular analysis to get more accurate species identification. The data can be used to further validate the outcomes of this meta-analysis, which then can be of essential information to break the chain of leptospirosis infection. It is also suggested to conduct further studies on the effect of the species on the clinical outcome of the patients to gain better understanding on the pathogenicity status of intermediate *Leptospira* spp.

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APPENDICES

APPENDIX I

Registration Form of PROSPERO

Page **1** of 5

NIHR National Institute for Health Research

PROSPERO
International prospective register of systematic reviews

The Global Prevalence of Intermediate Leptospira spp. in Humans: A Meta-Analysis

Aina Nadheera, Karuppiah Thilakavathy, Joseph Narcisse, Zhong Sun

To enable PROSPERO to focus on COVID-19 registrations during the 2020 pandemic, this registration record was automatically published exactly as submitted. The PROSPERO team has not checked eligibility.

Citation

Aina Nadheera, Karuppiah Thilakavathy, Joseph Narcisse, Zhong Sun. The Global Prevalence of Intermediate Leptospira spp. in Humans: A Meta-Analysis. PROSPERO 2021 CRD42021224163 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42021224163

Review question

What is the prevalence of the intermediate *Leptospira* spp. in humans across the globe?

Which species of intermediate *Leptospira* that are commonly found in the samples collected from patients infected with leptospirosis?

Searches

The systematic search will be performed using several databases which include PubMed, Scopus, ScienceDirect, Google Scholar, and Ovid MEDLINE. The combination of search terms such as "prevalence", "epidemiology", "leptospirosis", "intermediate leptospira", "humans", "patients" will be used using Boolean connectors "OR" within each category, "AND" between categories, as well as truncations such as * or ~ to broaden the search. This combination will be slightly altered according to the requirements of the database.

Types of study to be included

All of the studies with any research design will be included.

Inclusion criteria:
- Countries- ALL included studies
- Year of Publication: Not restricted

Exclusion criteria:
- Letter to editor
- Review papers
- Duplicate publications
- Studies using other than English language

Condition or domain being studied

Human leptospirosis infection caused by Leptospira of intermediate species.

Participants/population

Any individuals infected with leptospirosis (or co-infected with other diseases)

Inclusion criteria:
- Studies reported the number of samples positive for Intermediate Leptospira spp. and the total number of patients being investigated.
- Age and gender- ALL included

Exclusion criteria:
- Unclear or insufficient data
- Irrelevant data

Intervention(s), exposure(s)

The presence of intermediate *Leptospira* spp. detected using any recognised diagnostic methods.

Inclusion criteria:
- Studies that specified the species of the intermediate Leptospira
- Studies specified the confirmation/diagnostic methods used.

Exclusion criteria:
- Studies that do not report the methods used.

Comparator(s)/control

The absence of intermediate *Leptospira* spp. or the presence of pathogenic *Leptospira* spp.

Main outcome(s)

The raw prevalence outcome will be measured in percentage (%) by extracting the number of positive samples/the total samples taken from humans.

Measures of effect

NA

Additional outcome(s)

None

Measures of effect

None

Data extraction (selection and coding)

Data Selection:

All the articles yielded from all of three databases will be organised into a table form using Microsoft Excel Spreadsheet. Then, duplicates will be removed if they have the same authors and titles.

After that, the remaining articles will be screened by two reviewers, independently, by their titles and abstracts according to the inclusion/exclusion criteria. The articles that do not fit the criteria will be excluded.

After that, the remaining articles will be subjected to full-text screening based on the eligibility criteria. The articles that do not fit the criteria will be removed.

The results after each of the screening step will be cross-checked and any disagreement in the selection will be solved together. There will be involvement of the third reviewer if no consensus reached between the first and second reviewer. The reasons for the exclusion of the articles will be recorded.

Data extraction:
The first reviewer will extract the data and organised them in a table form using Microsoft Excel Spreadsheet which then will be checked by the second reviewer. Discussion will be done if there is any disagreement.

The data that will be extracted will consist of:
- Authors and years of publication
- Type of study, state and country
- Region
- Number of positive samples
- Total samples
- Methods used
- Species of the Intermediates

Risk of bias (quality) assessment

Risk of bias will be conducted using JBI Critical Appraisal Tool as the quality assessment tool. The risk of publication bias will also be assessed if there are more than 10 included studies, using Funnel plot, Egger's regression test, and Begg's test.

Strategy for data synthesis

The analysis will be done using RevMan (Review Manager) version 5.4 software.

The extent of heterogeneity: I^2 statistic will be used to evaluate heterogeneity between studies. If I^2 statistic is high (>75%), then random effect model will be used. If I^2 statistic is low (<75%), fixed-effect model will be used. After adjusted the effect model, p-value will be recorded.

Pooled estimates: The pooled estimate of the prevalence will be illustrated as forest plot.

Analysis of subgroups or subsets

Subgroup analysis will be conducted to identify the prevalence of the intermediates by stratifications of:

Region-wise: 6 regions according to WHO (African, Americas, South-East Asia, European, Eastern Mediterranean, Western Pacific)
Species-wise: 5 species which consist of L.wolffii, L.broomii, L.inadai, L.licerasiae, and L.fainei.

Contact details for further information

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Organisational affiliation of the review

Universiti Putra Malaysia

Review team members and their organisational affiliations

Ms Aina Nadheera. Universiti Putra Malaysia
Assistant/Associate Professor Karuppiah Thilakavathy. Universiti Putra Malaysia
Dr Joseph Narcisse. Universiti Putra Malaysia
Dr Zhong Sun. Universiti Putra Malaysia

Type and method of review

Meta-analysis, Systematic review

Anticipated or actual start date

31 October 2020

Anticipated completion date

30 June 2021

Funding sources/sponsors

None

Conflicts of interest

Language

English

Country

Malaysia

Stage of review

Review Ongoing

Subject index terms status

Subject indexing assigned by CRD

Subject index terms

MeSH headings have not been applied to this record

Date of registration in PROSPERO

06 January 2021

Date of first submission

06 December 2020

Stage of review at time of this submission

Stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	Yes	No
Formal screening of search results against eligibility criteria	Yes	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

The record owner confirms that the information they have supplied for this submission is accurate and complete and they understand that deliberate provision of inaccurate information or omission of data may be construed

as scientific misconduct.

The record owner confirms that they will update the status of the review when it is completed and will add publication details in due course.

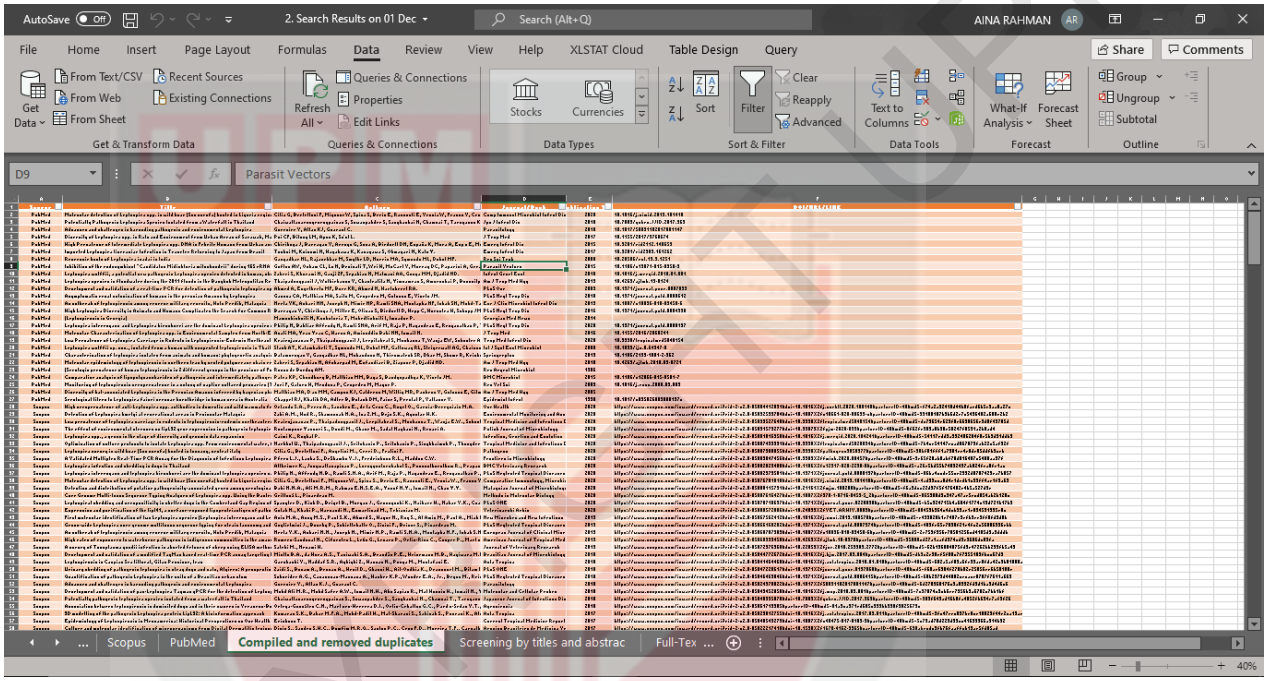
Versions

06 January 2021
06 January 2021

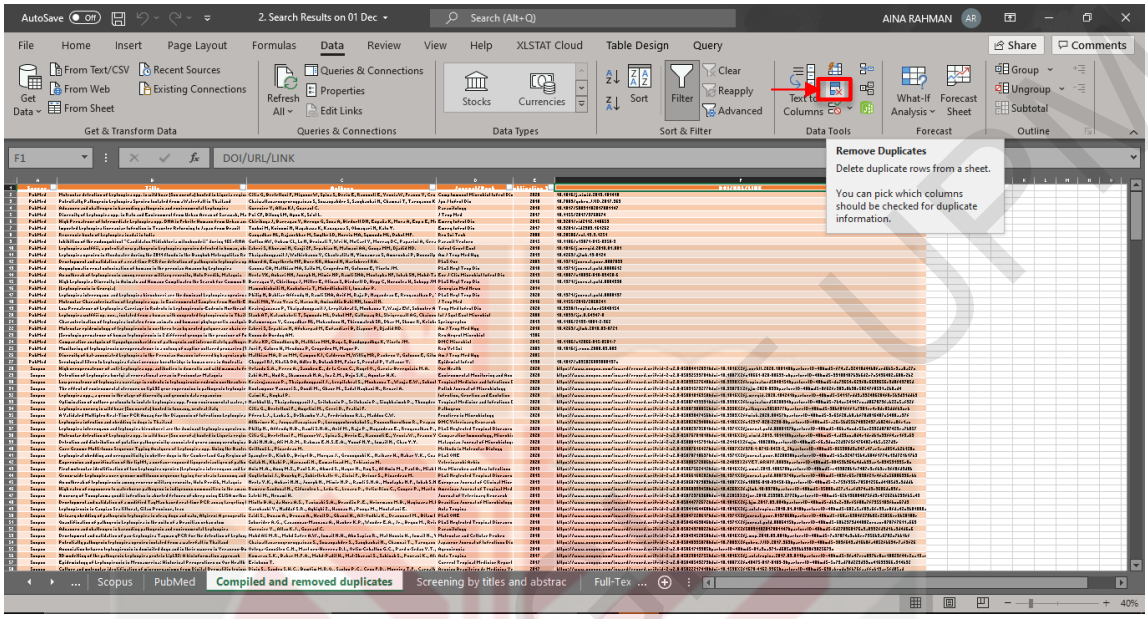
APPENDIX II

Compilation of the Studies and Removal of Duplicated Articles using Excel

1. The studies obtained from three databases including PubMed, Scopus, and ScienceDirect were compiled into one Microsoft Excel spreadsheet document.



2. After the compilation, highlight all the contents and go to ‘Data’. Then. Click the icon shown in the figure to remove the duplicated studies. The remaining articles will be then screened according to the abstract and title prior to full-text screening.



APPENDIX III

Articles for Full-Text Screening

The details of 22 articles that are eligible for full-text screening.

No.	Title	Source	Year	Type of Intermediate *Leptospira* spp.	Acceptance	Comment
1.	Leptospira interrogans and Leptospira kirschneri are the dominant Leptospira species causing human leptospirosis in Central Malaysia	PubMed	2020	*L. wolffii*	Accepted	Included in the meta-analysis
2.	High Leptospira Diversity in Animals and Humans Complicates the Search for Common Reservoirs of Human Disease in Rural Ecuador	PubMed	2016	None	Rejected	No intermediate *Leptospira* spp. identified
3.	High Prevalence of Intermediate Leptospira spp. DNA in Febrile Humans from Urban and Rural Ecuador	PubMed	2015	*L. wolffii* and *L. inadai*	Accepted	Included in the meta-analysis

4.	Characterization of leptospira isolates from animals and humans: phylogenetic analysis identifies the prevalence of intermediate species in India	PubMed	2013	*L. wolffii*	Accepted	Included in the meta-analysis
5.	Leptospira wolffii, a potential new pathogenic Leptospira species detected in human, sheep and dog	PubMed	2010	*L. wolffii*	Accepted	Included in the meta-analysis
6.	Asymptomatic renal colonization of humans in the peruvian Amazon by Leptospira	PubMed	2010	None	Rejected	No intermediate *Leptospira* spp. identified
7.	Molecular epidemiology of leptospirosis in northern Iran by nested polymerase chain reaction/restriction fragment length polymorphism and sequencing methods	PubMed	2010	*L. wolffii*	Accepted	Included in the meta-analysis
8.	Development and validation of a real-time PCR for detection of pathogenic leptospira species in clinical materials	PubMed	2009	None	Rejected	No intermediate *Leptospira* spp. identified
9.	Leptospira wolffii sp. nov., isolated from a human with suspected leptospirosis in Thailand	PubMed	2008	*L. wolffii*	Rejected	The study did not mention the

						frequency of positive and total samples.
10.	Serological titres to Leptospira fainei serovar hurstbridge in human sera in Australia	PubMed	1998	*l. fainei*	Rejected	Study on a particular serovar only
11.	Seroprevalence of Leptospirosis among High-Risk Individuals in Morocco	Scopus	2020	None	Rejected	No intermediate *Leptospira* spp. identified
12.	First molecular identification of two Leptospira species (Leptospira interrogans and Leptospira wolffii) in Bangladesh	Scopus	2019	*L. wolffii*	Rejected	Letter to the editor
13.	Prevalence of Leptospira-agglutinating antibodies in abattoir workers and slaughtered animals in selected slaughterhouses in Cavite, Philippines	Scopus	2018	None	Rejected	No intermediate *Leptospira* spp. identified

14.	Epidemiology of human leptospirosis in French Guiana (2007-2014): A retrospective study	Scopus	2018	None	Rejected	No intermediate *Leptospira* spp. identified
15.	Meta-analysis to estimate the load of Leptospira excreted in urine: Beyond rats as important sources of transmission in low-income rural communities	Scopus	2017	None	Rejected	Meta-analysis and the study was not on human subjects.
16.	Underestimation of Leptospirosis Incidence in the French West Indies	Scopus	2016	None	Rejected	No intermediate *Leptospira* spp. identified
17.	Trends in human leptospirosis in Denmark, 1980 to 2012	Scopus	2015	None	Rejected	No intermediate *Leptospira* spp. identified
18.	Leptospirosis: Indications of changes in clinical presentation in Croatia [Leptospiroza: Naznake promjene kliničke slike u Hrvatskoj]	Scopus	2006	None	Rejected	Full-text article is not available and no intermediate

						Leptospira spp. identified
19.	The importance of leptospirosis in Southeast Asia	Scopus	2002	None	Rejected	No intermediate *Leptospira* spp. identified
20.	A comparison of two molecular methods for diagnosing leptospirosis from three different sample types in patients presenting with fever in Laos	ScienceDirect	2018	None	Rejected	No intermediate *Leptospira* spp. identified
21.	Isolation and clinical sample typing of human leptospirosis cases in Argentina	ScienceDirect	2016	*L. wolffii* and *L. broomii*	Accepted	Included in the meta-analysis
22.	A simple and rapid nested polymerase chain reactionâ€“restriction fragment length polymorphism technique for differentiation of pathogenic and nonpathogenic Leptospira spp	ScienceDirect	2009	*L. wolffii*	Accepted	Included in the meta-analysis

APPENDIX IV

A Modified Critical Appraisal Checklist Recommended by JBI

The checklist for quality assessment by JBI which consisted a total of nine items.

JBI CRITICAL APPRAISAL CHECKLIST FOR STUDIES REPORTING PREVALENCE DATA

Reviewer_____Date_____

Author_____Year_____ Record Number_____

	Yes	No	Unclear	Not applicable
1. Was the sample frame appropriate to address the target population?	☐	☐	☐	☐
2. Were study participants sampled in an appropriate way?	☐	☐	☐	☐
3. Was the sample size adequate?	☐	☐	☐	☐
4. Were the study subjects and the setting described in detail?	☐	☐	☐	☐
5. Was the data analysis conducted with sufficient coverage of the identified sample?	☐	☐	☐	☐
6. Were valid methods used for the identification of the condition?	☐	☐	☐	☐
7. Was the condition measured in a standard, reliable way for all participants?	☐	☐	☐	☐
8. Was there appropriate statistical analysis?	☐	☐	☐	☐
9. Was the response rate adequate, and if not, was the low response rate managed appropriately?	☐	☐	☐	☐

Overall appraisal: Include ☐ Exclude ☐ Seek further info ☐

Comments (Including reason for exclusion)

_____

_____

Methodological Guidance for Systematic Reviews Reporting Prevalence Data by JBI

JBI CRITICAL APPRAISAL CHECKLIST FOR STUDIES REPORTING PREVALENCE DATA

How to cite: Munn Z, Moola S, Lisy K, Riitano D, Tufanaru C. Methodological guidance for systematic reviews of observational epidemiological studies reporting prevalence and incidence data. Int J Evid Based Healthc. 2015;13(3):147–153.

Answers: Yes, No, Unclear or Not/Applicable

1. **Was the sample frame appropriate to address the target population?**

   This question relies upon knowledge of the broader characteristics of the population of interest and the geographical area. If the study is of women with breast cancer, knowledge of at least the characteristics, demographics and medical history is needed. The term "target population" should not be taken to infer every individual from everywhere or with similar disease or exposure characteristics. Instead, give consideration to specific population characteristics in the study, including age range, gender, morbidities, medications, and other potentially influential factors. For example, a sample frame may not be appropriate to address the target population if a certain group has been used (such as those working for one organisation, or one profession) and the results then inferred to the target population (i.e. working adults). A sample frame may be appropriate when it includes almost all the members of the target population (i.e. a census, or a complete list of participants or complete registry data).

2. **Were study participants recruited in an appropriate way?**

   Studies may report random sampling from a population, and the methods section should report how sampling was performed. Random probabilistic sampling from a defined subset of the population (sample frame) should be employed in most cases, however, random probabilistic sampling is not needed when everyone in the sampling frame will be included/ analysed. For example, reporting on all the data from a good census is appropriate as a good census will identify everybody. When using cluster sampling, such as a random sample of villages within a region, the methods need to be clearly stated as the precision of the final prevalence estimate incorporates the clustering effect. Convenience samples, such as a street survey or interviewing lots of people at a public gatherings are not considered to provide a representative sample of the base population.

5. Was the sample size adequate?

The larger the sample, the narrower will be the confidence interval around the prevalence estimate, making the results more precise. An adequate sample size is important to ensure good precision of the final estimate. Ideally we are looking for evidence that the authors conducted a sample size calculation to determine an adequate sample size. This will estimate how many subjects are needed to produce a reliable estimate of the measure(s) of interest. For conditions with a low prevalence, a larger sample size is needed. Also consider sample sizes for subgroup (or characteristics) analyses, and whether these are appropriate. Sometimes, the study will be large enough (as in large national surveys) whereby a sample size calculation is not required. In these cases, sample size can be considered adequate.

When there is no sample size calculation and it is not a large national survey, the reviewers may consider conducting their own sample size analysis using the following formula: (Naing et al. 2006, Daniel 1999)

$n = Z^2P(1-P)$

d^2

Where:

n= sample size

Z = Z statistic for a level of confidence

P = Expected prevalence or proportion (in proportion of one; if 20%, P = 0.2)

d = precision (in proportion of one; if 5%, d=0.05)

Ref:

Naing L, Winn T, Rusli BN. Practical issues in calculating the sample size for prevalence studies Archives of Orofacial Sciences. 2006;1:9-14.

Daniel WW. Biostatistics: A Foundation for Analysis in the Health Sciences.

Edition. 7th ed. New York: John Wiley & Sons. 1999.

4. **Were the study subjects and setting described in detail?**

   Certain diseases or conditions vary in prevalence across different geographic regions and populations (e.g. Women vs. Men, sociodemographic variables between countries). The study sample should be described in sufficient detail so that other researchers can determine if it is comparable to the population of interest to them.

5. **Was data analysis conducted with sufficient coverage of the identified sample?**

   Coverage bias can occur when not all subgroups of the identified sample respond at the same rate. For instance, you may have a very high response rate overall for your study, but the response rate for a certain subgroup (i.e. older adults) may be quite low.

6. **Were valid methods used for the identification of the condition?**

   Here we are looking for measurement or classification bias. Many health problems are not easily diagnosed or defined and some measures may not be capable of including or excluding appropriate levels or stages of the health problem. If the outcomes were assessed based on existing definitions or diagnostic criteria, then the answer to this question is likely to be yes. If the outcomes were assessed using observer reported, or self-reported scales, the risk of over- or under-reporting is increased, and objectivity is compromised. Importantly, determine if the measurement tools used were validated instruments as this has a significant impact on outcome assessment validity.

7. **Was the condition measured in a standard, reliable way for all participants?**

   Considerable judgment is required to determine the presence of some health outcomes. Having established the validity of the outcome measurement instrument (see item 6 of this scale), it is important to establish how the measurement was conducted. Were those involved in collecting data trained or educated in the use of the instrument/s? If there was more than one data collector, were they similar in terms of level of education, clinical or research experience, or level of responsibility in the piece of research being appraised? When there was more than one observer or collector, was there comparison of results from across the observers? Was the condition measured in the same way for all participants?

8. **Was there appropriate statistical analysis?**

   Importantly, the numerator and denominator should be clearly reported, and percentages should be given with confidence intervals. The methods section should be detailed enough for reviewers to identify the analytical technique used and how specific variables were measured. Additionally, it is also important to assess the appropriateness of the analytical strategy in terms of the assumptions associated with the approach as differing methods of analysis are based on differing assumptions about the data and how it will respond.

9. **Was the response rate adequate, and if not, was the low response rate managed appropriately?**

   A large number of dropouts, refusals or "not founds" amongst selected subjects may diminish a study's validity, as can a low response rates for survey studies. The authors should clearly discuss the response rate and any reasons for non-response and compare persons in the study to those not in the study, particularly with regards to their socio-demographic characteristics. If reasons for non-response appear to be unrelated to the outcome measured and the characteristics of non-responders are comparable to those who do respond in the study (addressed in question 5, coverage bias), the researchers may be able to justify a more modest response rate.

APPENDIX V

Calculation for Proportion and Standard Error of Mean (SEM)

1. **Formula and Calculation of the proportion**

Proportion = The number of samples positive for intermediate *Leptospira* spp. / Total number of samples confirmed for leptospirosis

Eg.: 1. The number of samples positive for intermediate *Leptospira* spp. = 11

2. Total number of samples confirmed for leptospirosis = 42

3. Proportion = The number of samples positive for intermediate *Leptospira* spp. / Total number of samples confirmed for leptospirosis

= 11/42 = **0.26**

2. **Formula and Calculation of SEM**

SEM = SQRT (Proportion * (1-Proportion) / Total samples)

Eg.: 1. Proportion = 0.26

2. Total samples = 42

3. SEM = SQRT (Proportion * (1-Proportion) / Total samples)

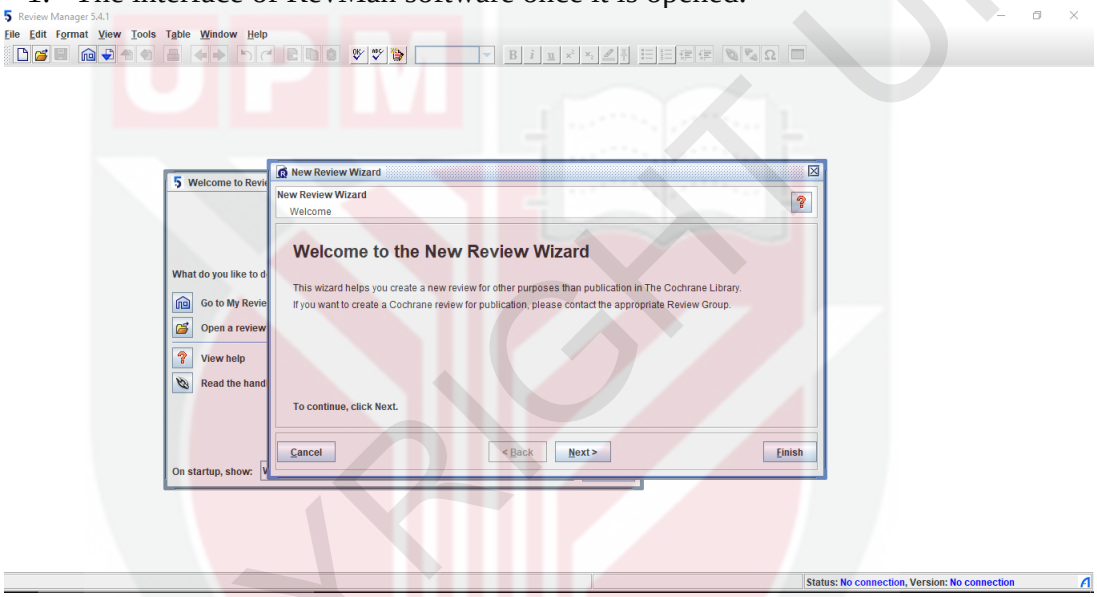
= SQRT (0.26 * (1-0.26) / 42) = **0.07**

APPENDIX VI

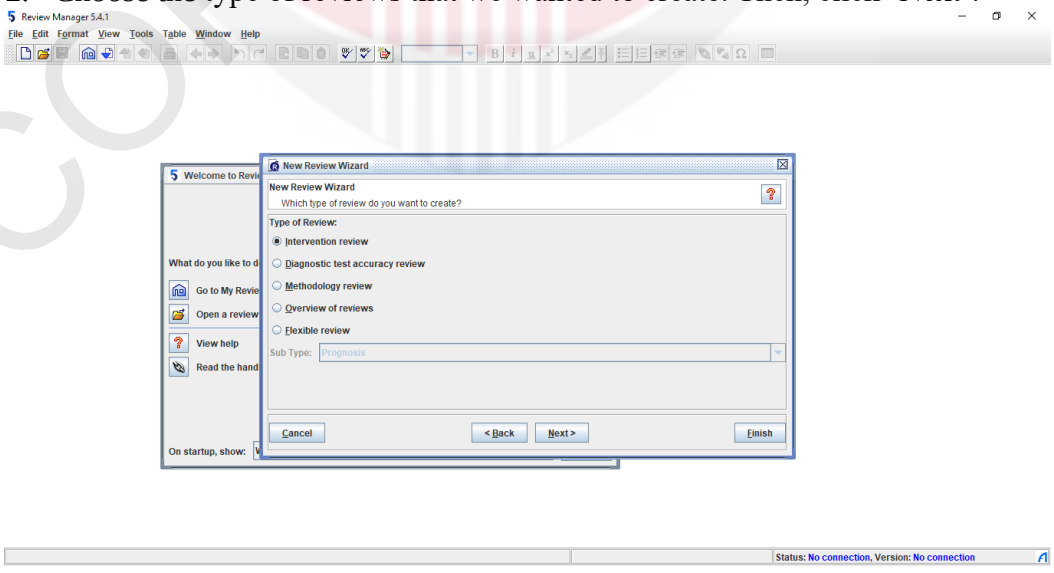
Steps to Use RevMan Software for Quantitative Analysis

- **Entering the title of the study and included studies**

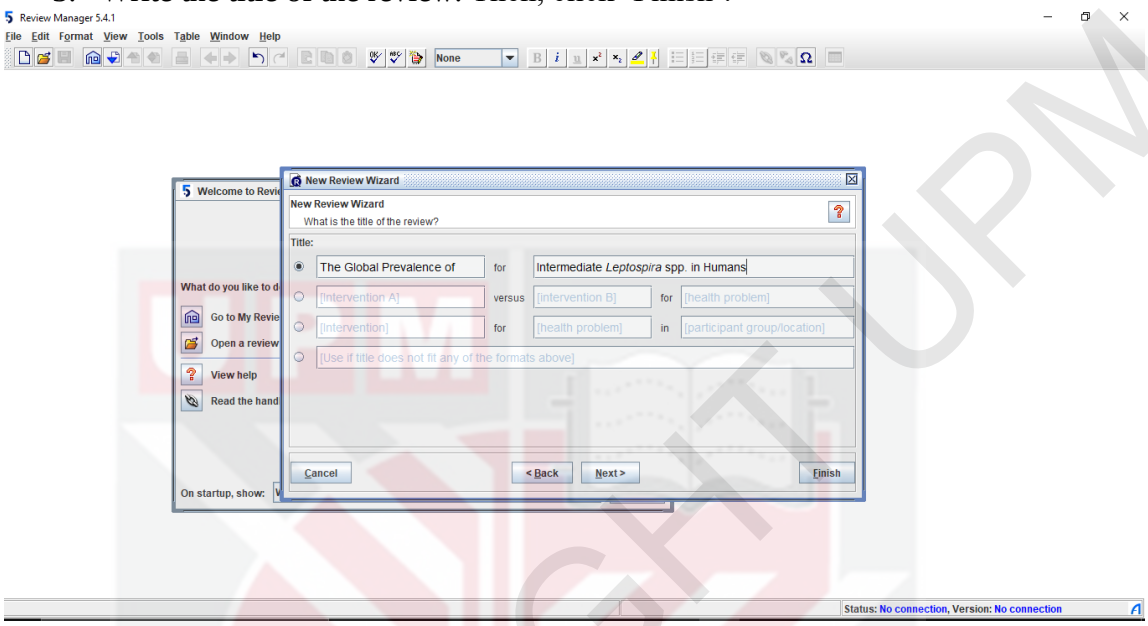
1. The interface of RevMan software once it is opened.



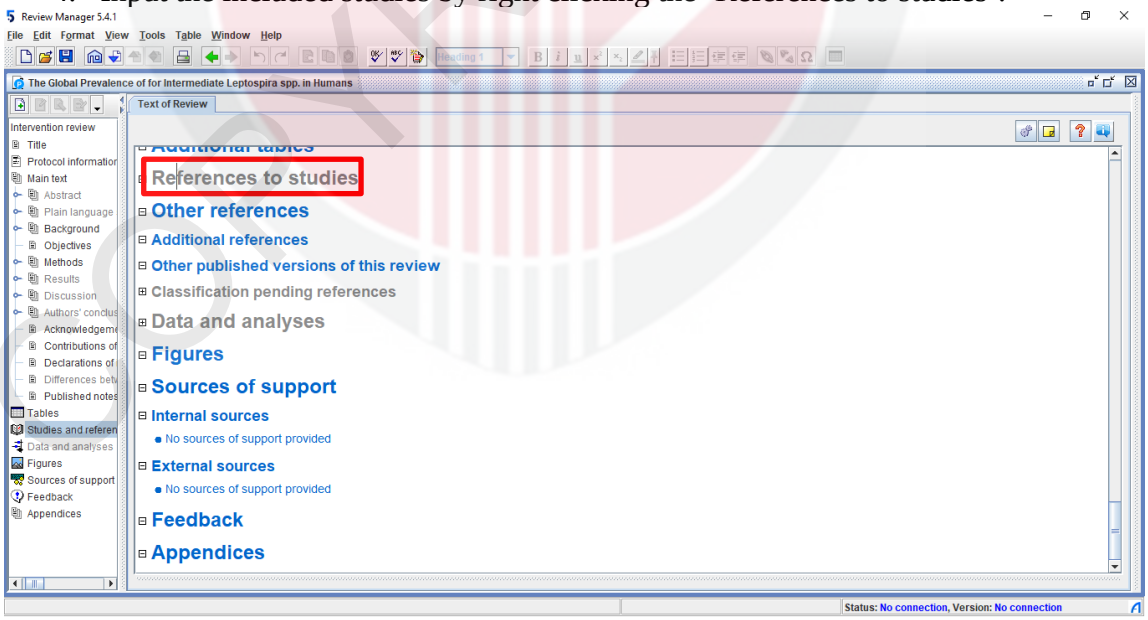
2. Choose the type of reviews that we wanted to create. Then, click 'Next'.



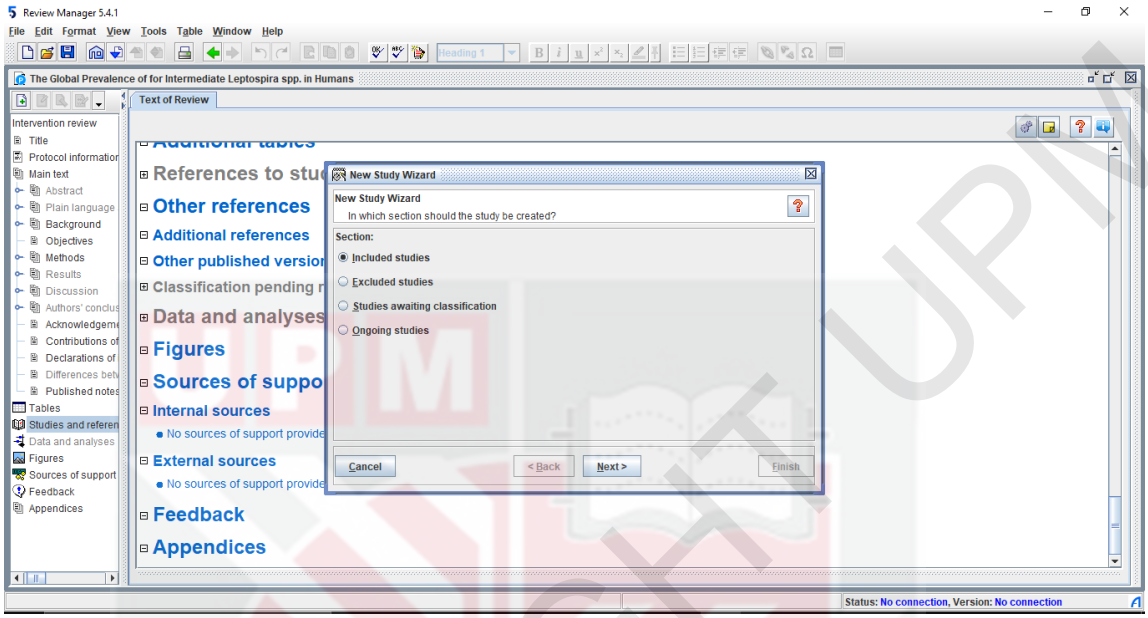
3. Write the title of the review. Then, click ‘Finish’.



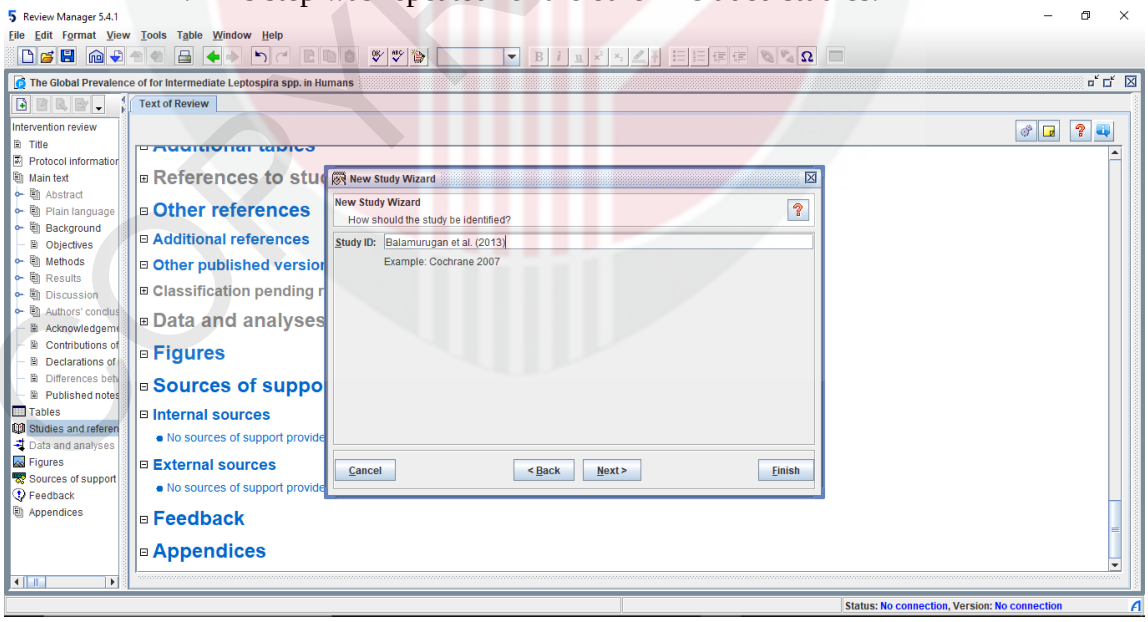
4. Input the included studies by right clicking the ‘References to studies’.



5. Choose ‘Included studies’, then click ‘Next’.

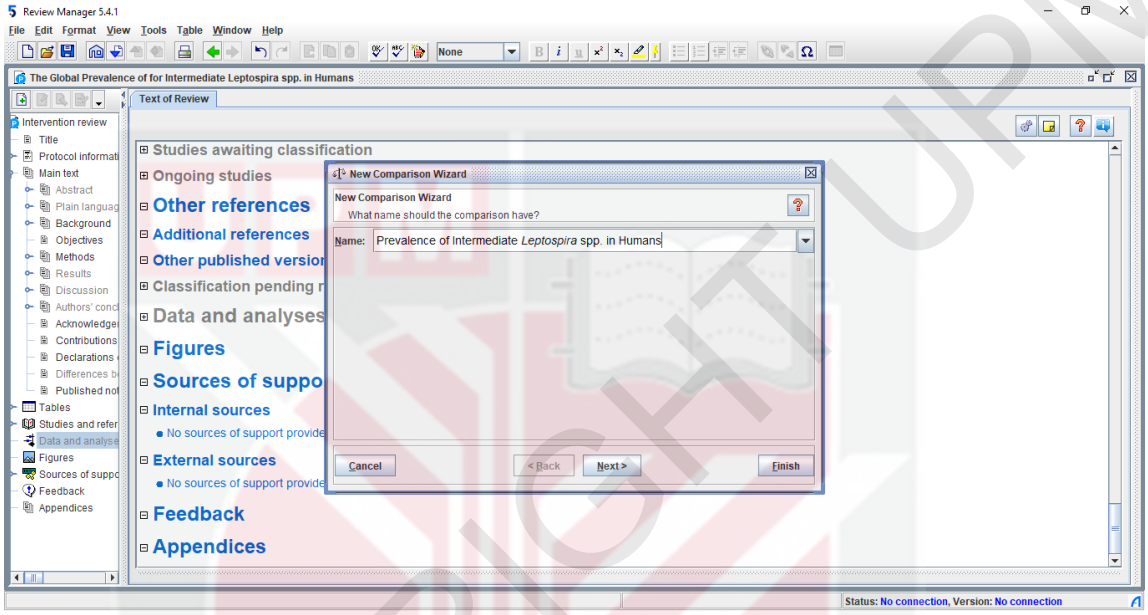


6. Next, is to write the first author and the year of the included study. Then, click ‘Next’. This step was repeated for the other included studies.

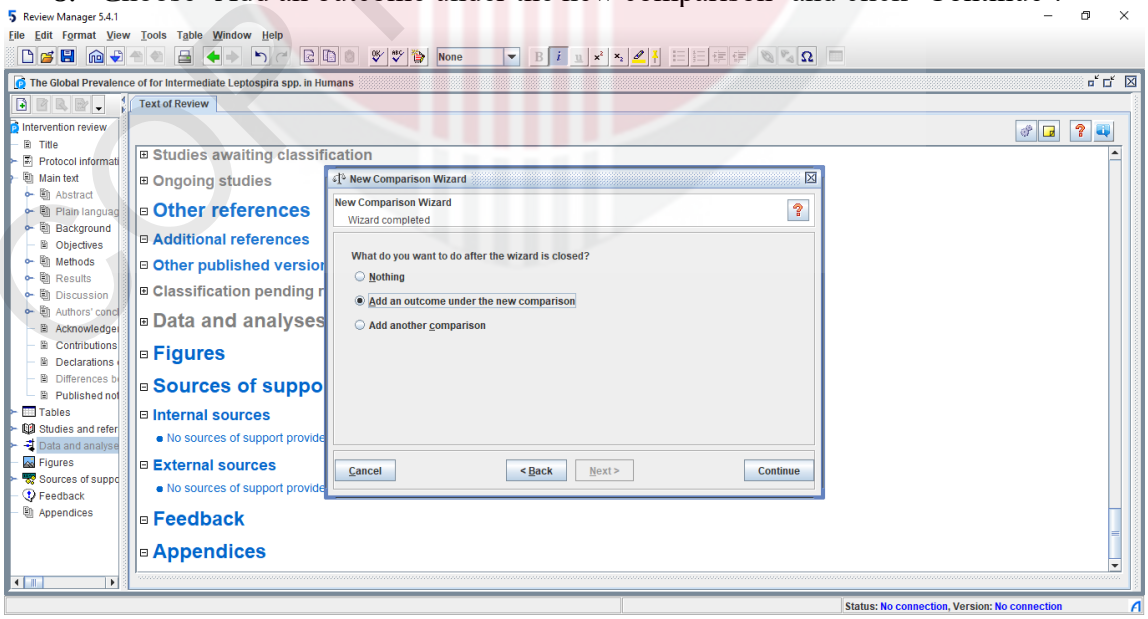


- **Inputting the data and analyses**

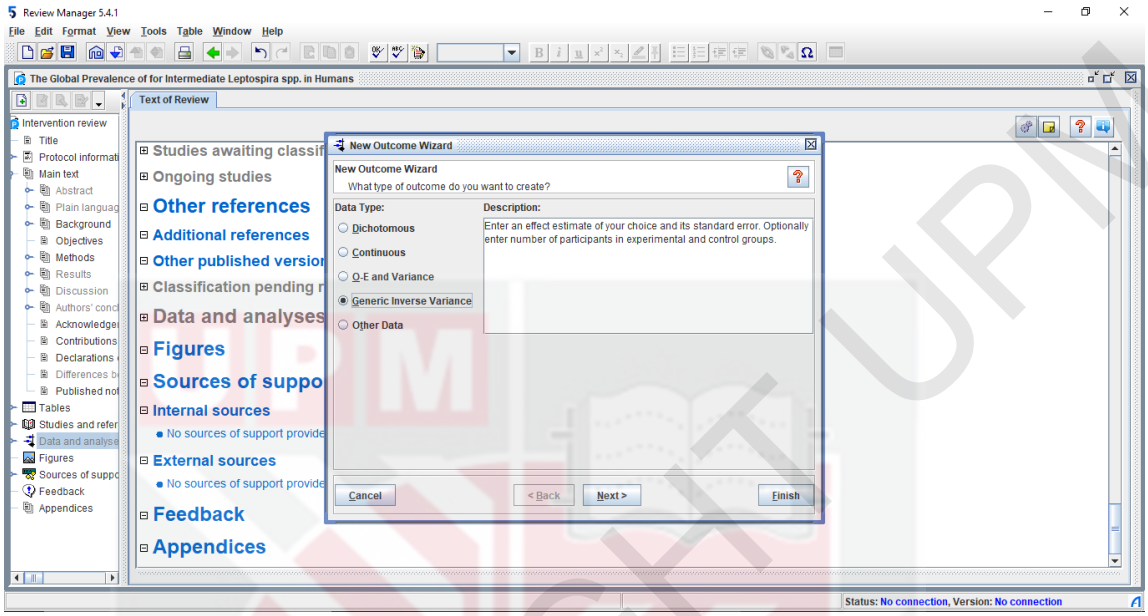
7. Input the data and analyses from the selected studies. Right click the 'Data and analyses' to add new comparison. Then, add the name of the comparison and click 'Next'.



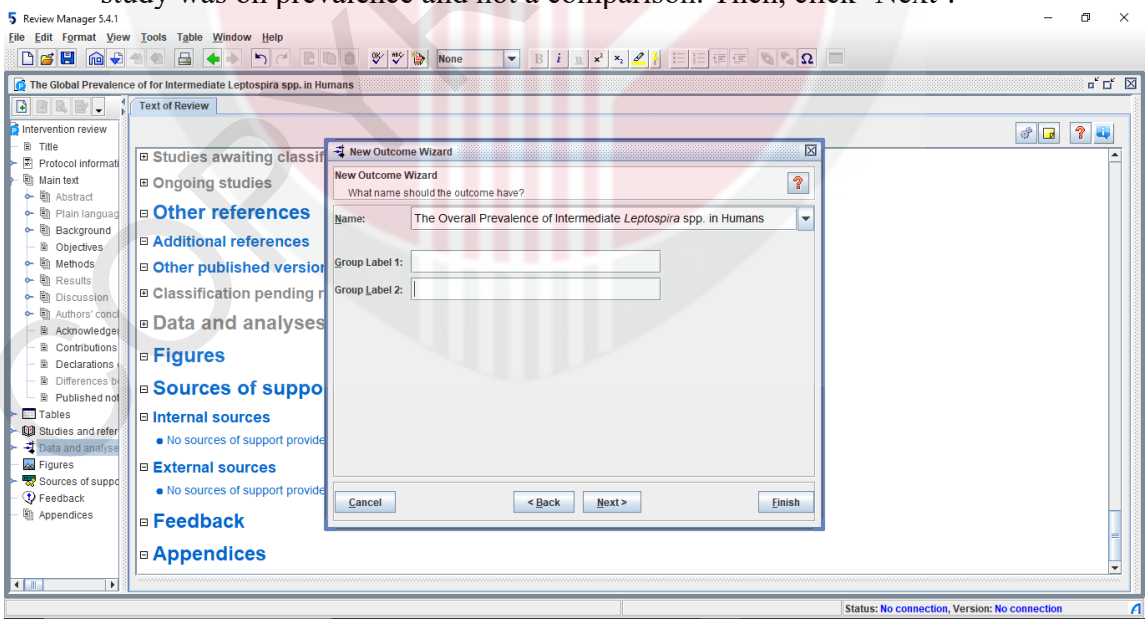
8. Choose 'Add an outcome under the new comparison' and click 'Continue'.



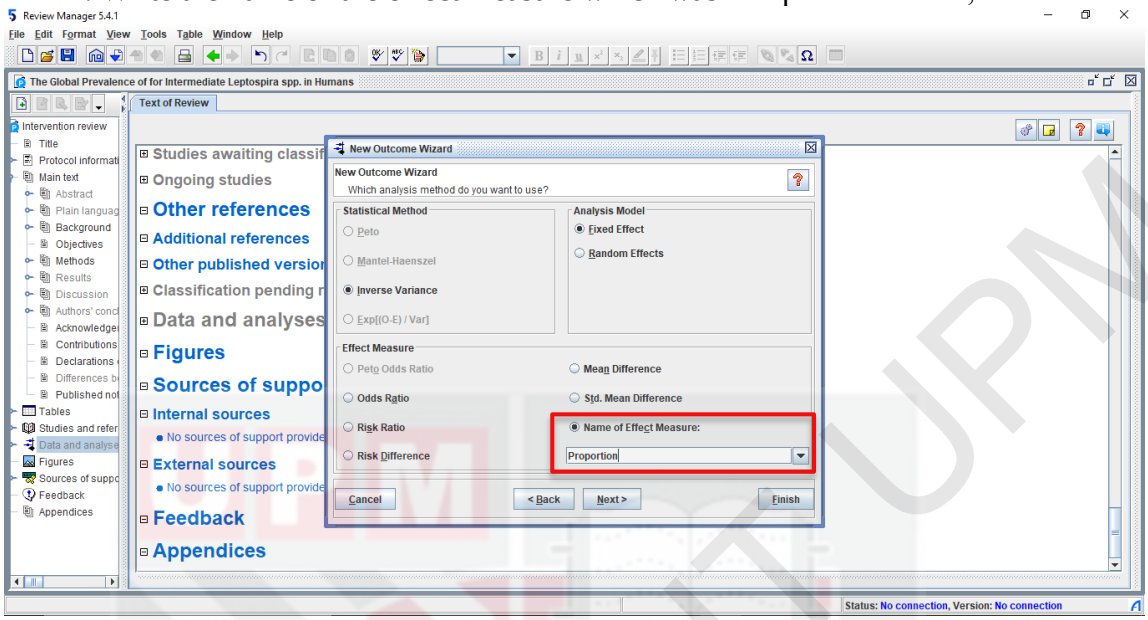
9. Choose the type of outcome. As for this study which was on prevalence, choose ‘Generic Inverse Variance’ and click ‘Next’.



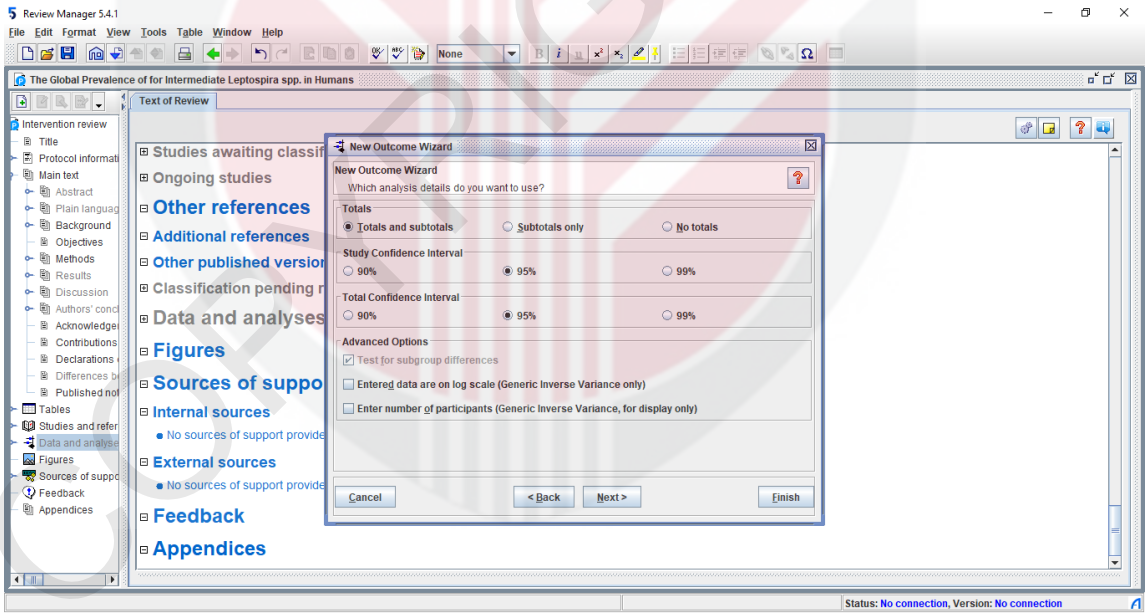
10. Write the name of the outcome. Group Label 1 and 2 were not labelled as this study was on prevalence and not a comparison. Then, click ‘Next’.



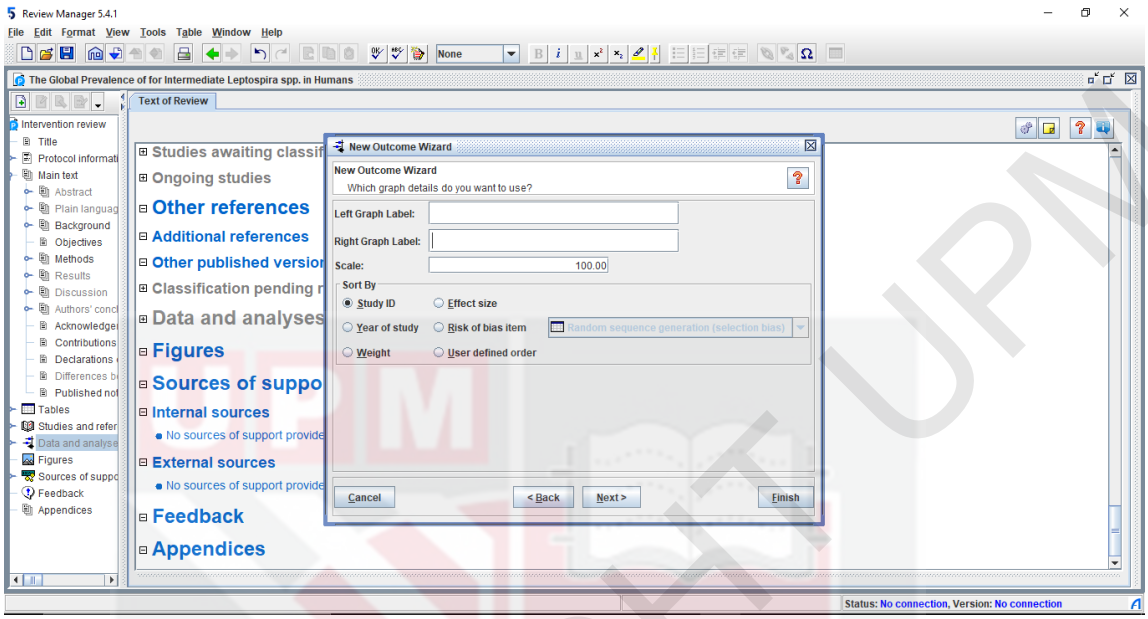
11. Write the name of the effect measure which was ‘Proportion’. Then, click ‘Next’.



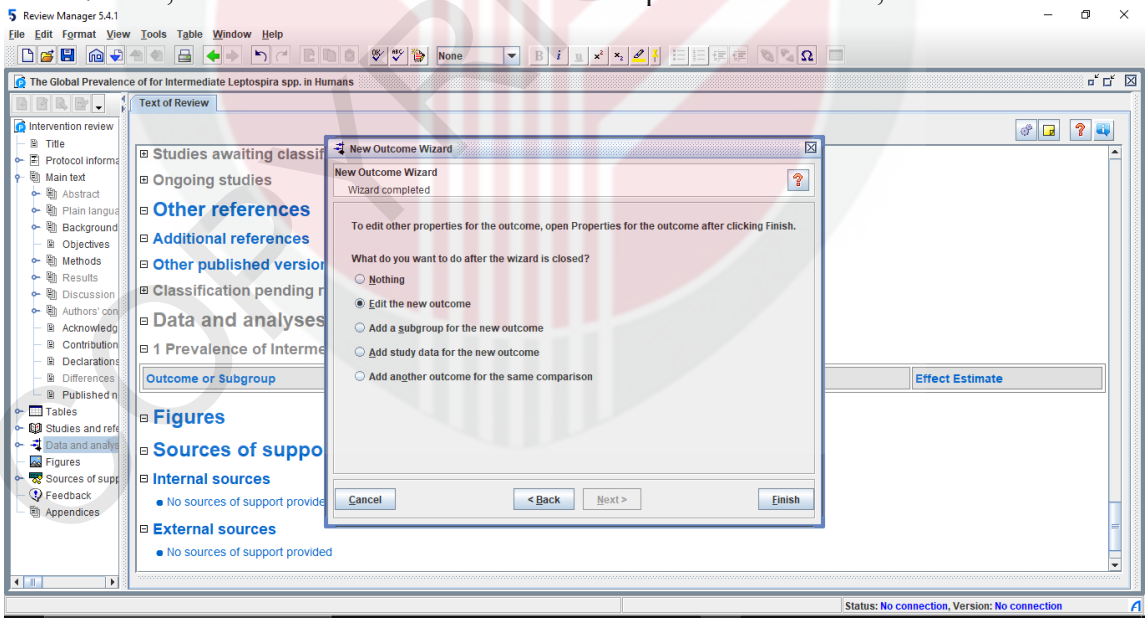
12. Click ‘Next’.



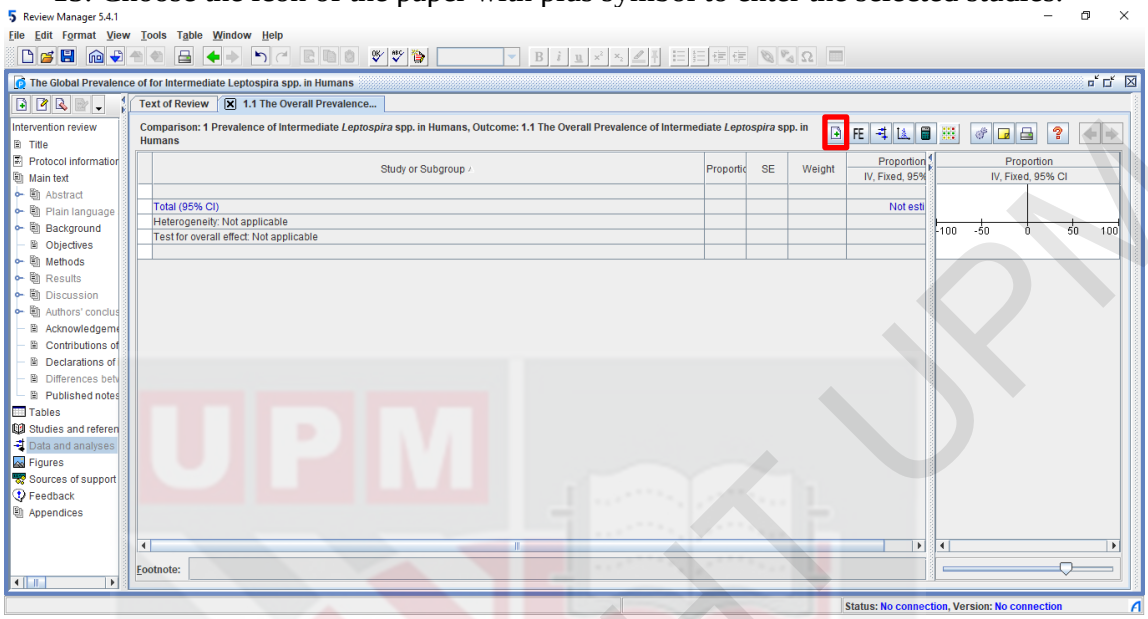
13. No labelling for left and right graph since this was a study on prevalence. Then, click ‘Next’.



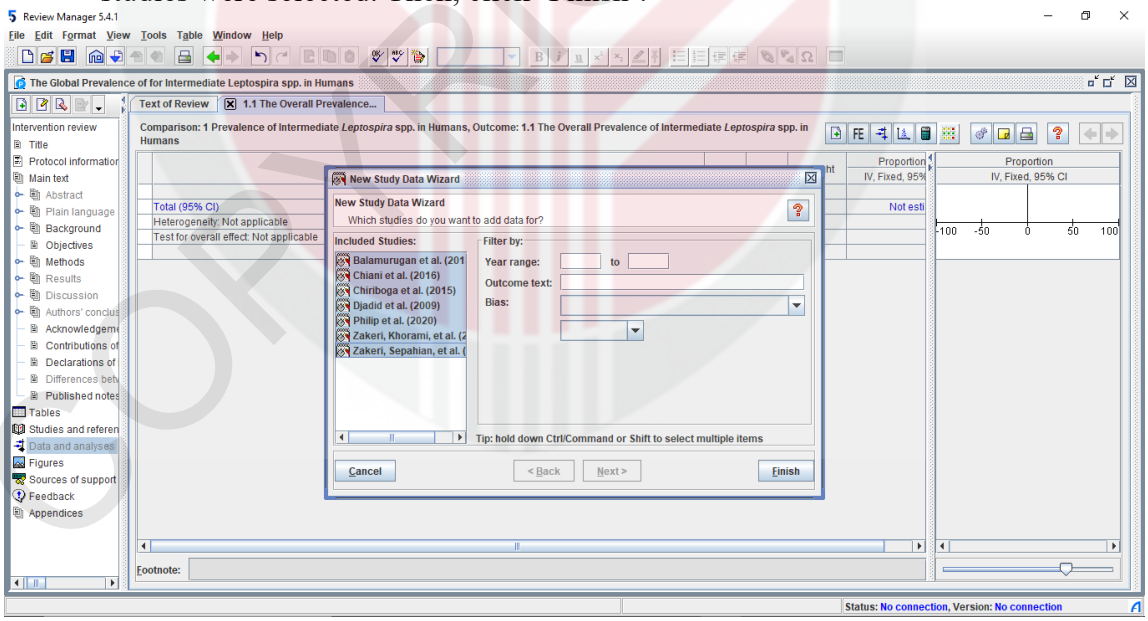
14. Then, choose ‘Edit the new outcome’ to input the data. Then, click ‘Finish’.



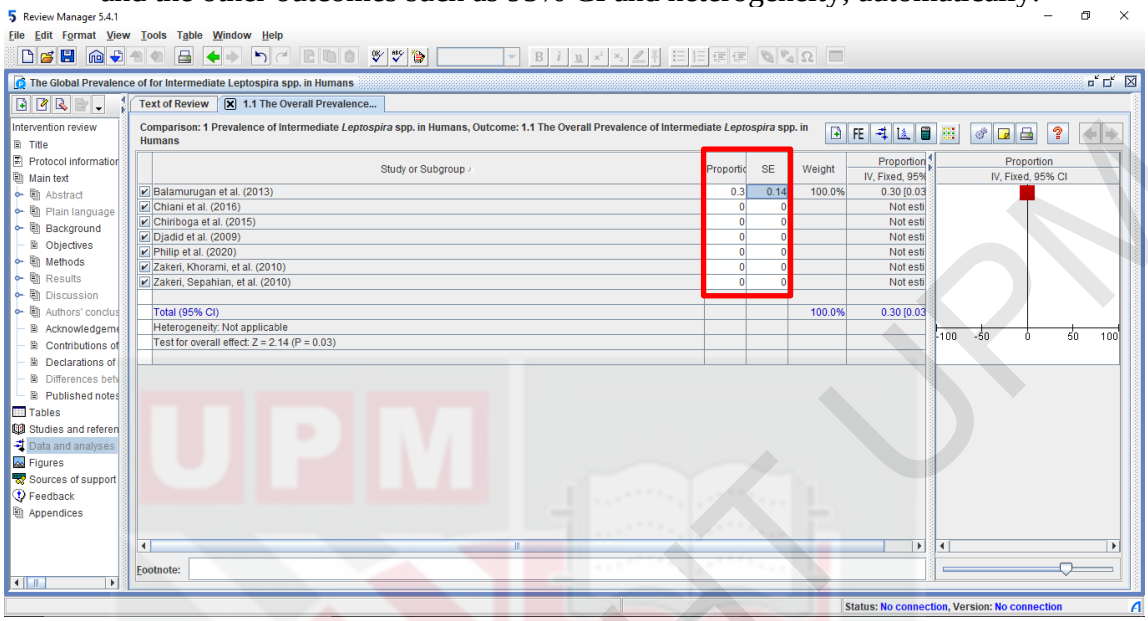
15. Choose the icon of the paper with plus symbol to enter the selected studies.



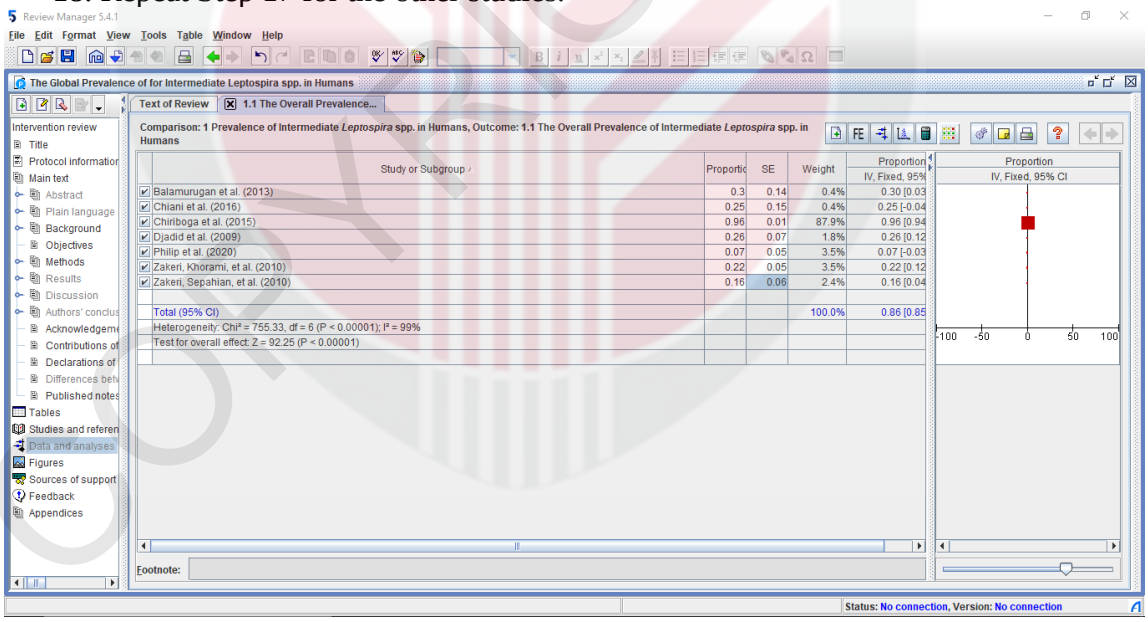
16. Choose the studies for the outcome. As for the overall prevalence, all the seven studies were selected. Then, click 'Finish'.



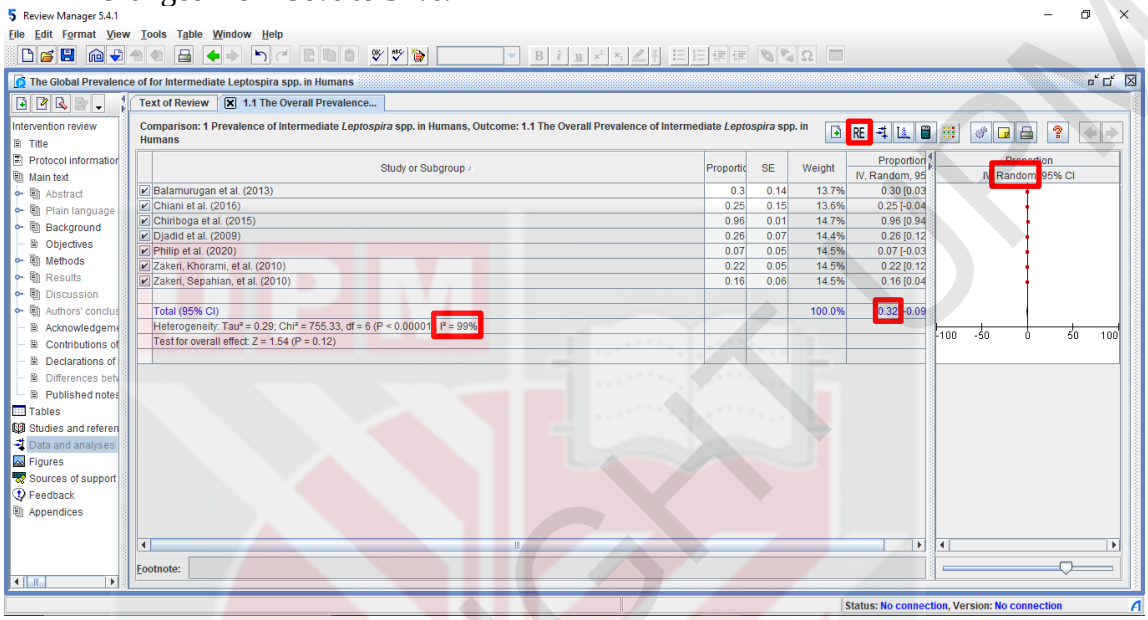
17. Enter the data of proportion and SEM. Then, the software will calculate the weight and the other outcomes such as 95% CI and heterogeneity, automatically.



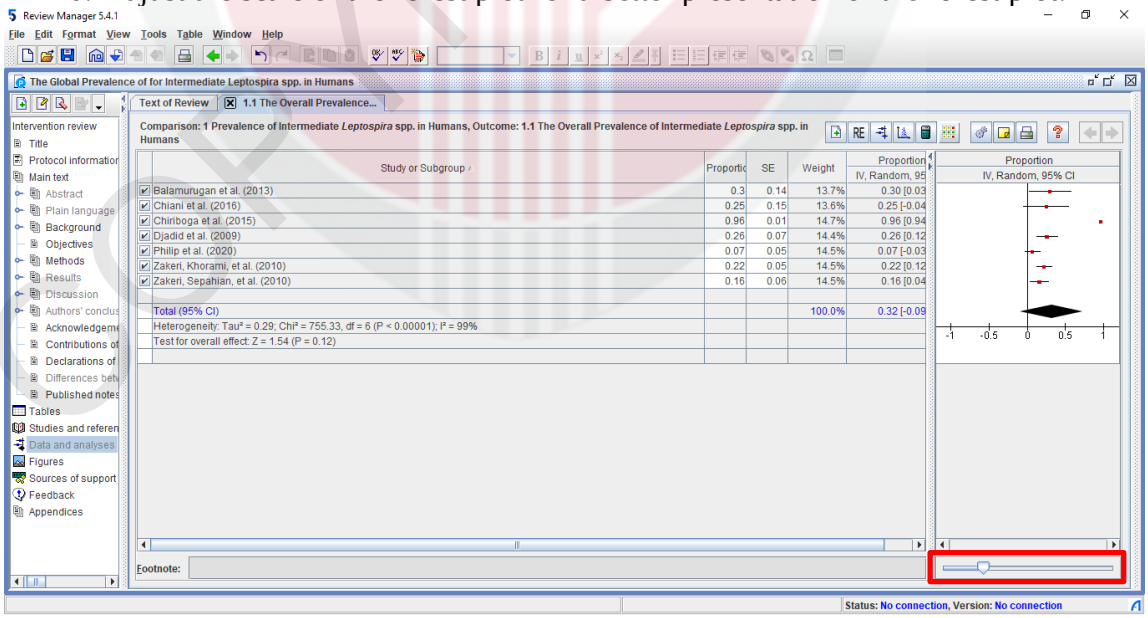
18. Repeat Step 17 for the other studies.



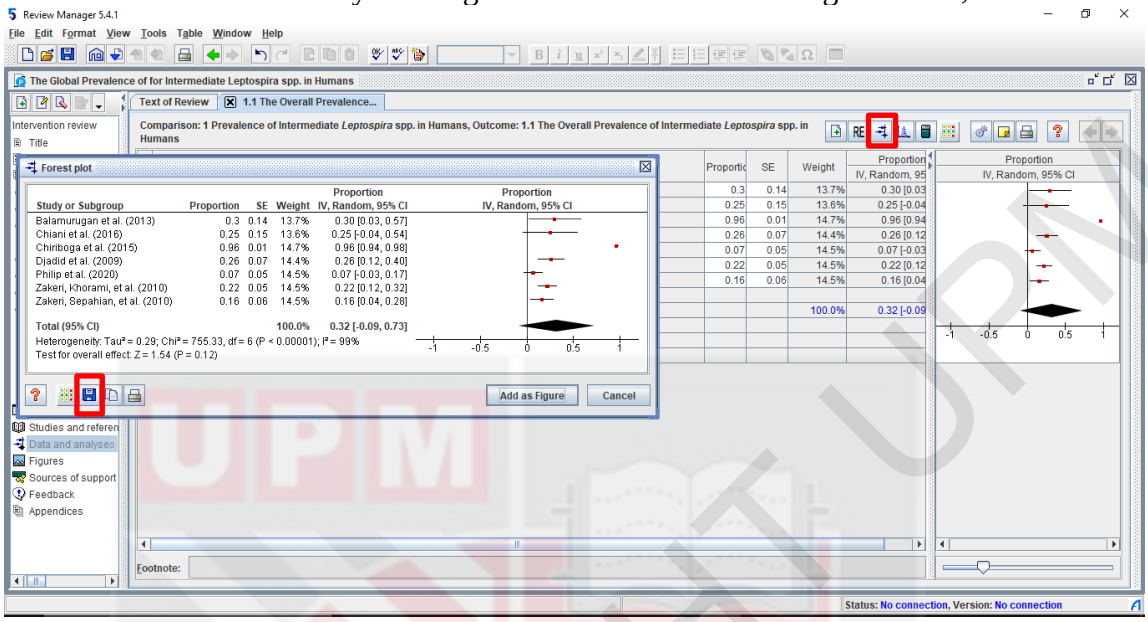
19. Adjust the outcome based on the heterogeneity (I^2 statistic) by clicking the box with 'RE'. The effect-model as shown in the figure has been changed from fixed-effect to random-effect model because I^2 statistic was more than 50% which was 99% as indicated in the figure. Therefore, the prevalence estimate was also changed from 86% to 32%.



20. Adjust the scale of the forest plot for a better presentation of the forest plot.



21. Save the outcome by clicking the icon as shown in the figure. Then, click save.



22. Next, the table of the 'Data and analyses' was right clicked to add the other outcomes. Steps 8 until 21 were repeated for the prevalence of Intermediate *Leptospira* spp. in humans from the American and Asian regions.