Hiding in plain sight: Leptospirosis and rickettsial disease

Unmasking two causes of severe imported infections

de Vries, S.G.

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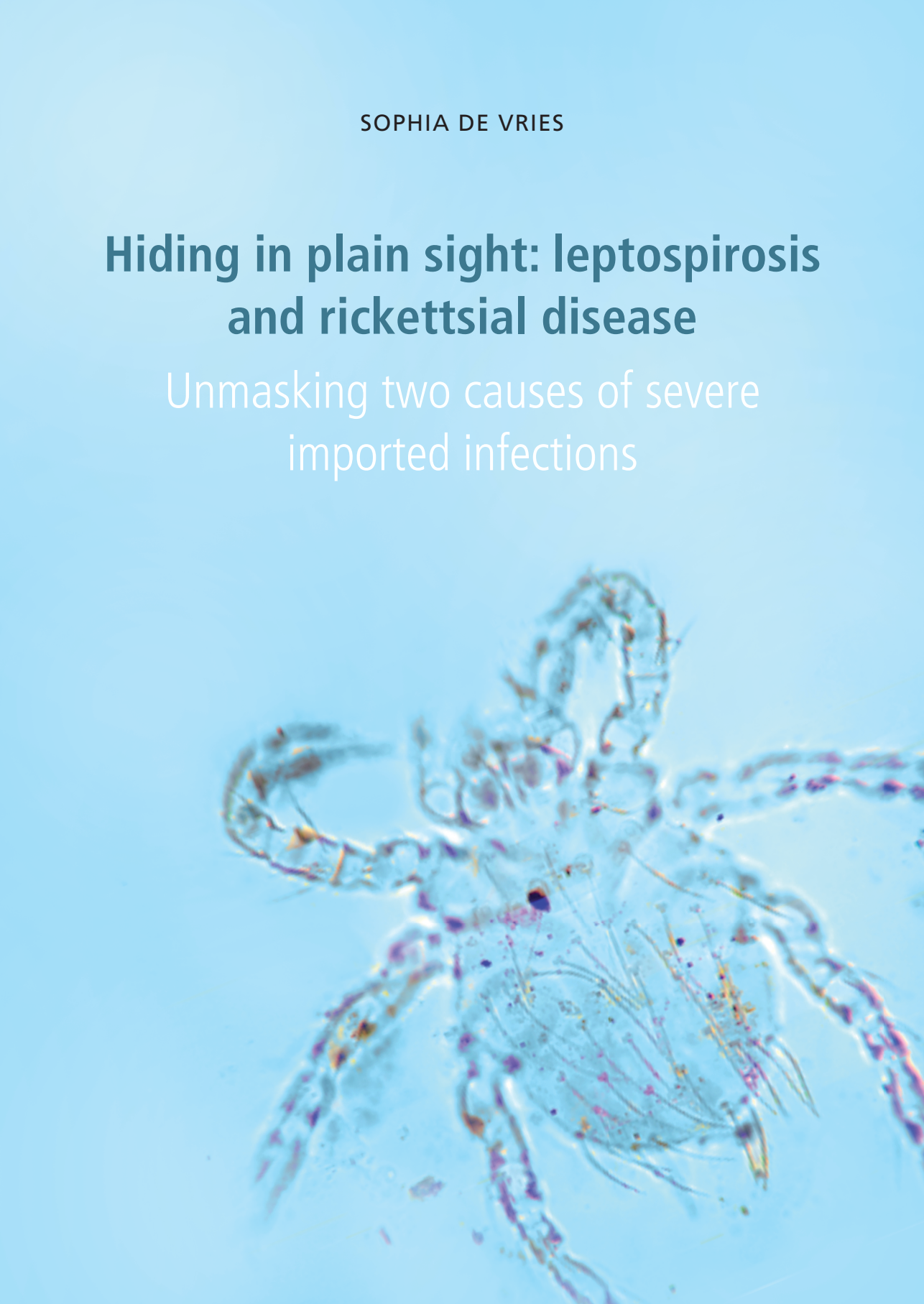

Hiding in plain sight: leptospirosis and rickettsial disease. Unmasking two causes of severe imported infections.

Sophia Geertruda de Vries

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Unmasking two causes of severe imported infections.

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Alle wriemeldiertjes
alle wiebeldiertjes
alle kruip- en kriebeldiertjes
zitten verstopt in het hoge gras.

Ik zou maar op mijn tenen lopen
als ik jou was.

- Joke van Leeuwen

Contents

Chapter 1

General introduction

Leptospirosis

Leptospirosis is a zoonotic illness, caused by spiral-shaped bacteria, called spirochetes (**Figure 1**).[1] *Leptospira* consist of pathogenic *Leptospira interrogans* sensu lato and the - non-pathogenic - saprophytic *Leptospira biflexa* sensu lato. There are 25 serogroups identified in *L. interrogans*, with over 250 serovars.

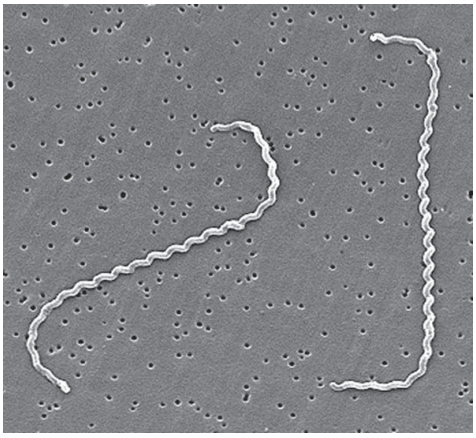

Figure 1. Scanning electron micrograph of *L. interrogans* serovar icterohaemorrhagiae.
Reprinted with permission from the publisher from: Paul N. Levett, Leptospirosis, Clin. Microbiol. Rev. 2001; doi:10.1128/CMR.14.2.296-326.2001

Epidemiology and vectors

Leptospires are omnipresent in nature. They remain in the kidneys of reservoir hosts and are shed in nature with their urine. Reservoir hosts are species with an endemic, asymptomatic, infection. The most important are small mammals such as rodents; but domestic animals such as cattle, pigs, and sheep may also be reservoir hosts. These reservoir hosts may be carrier of one serovar, but develop disease from another.[2]

Leptospires survive best in warm and wet conditions. People acquire infection either through direct or indirect contact with infected urine; the *Leptospira* enter the body through mucous membranes or abrasions of the skin. Certain professions are at greater risk of acquiring infection: those in direct contact with animals, such as livestock farmers, veterinarians, and abattoir workers, but also miners, sewer workers and fish farmers.[2-4] Other risk factors contain recreational sports (water rafting, triathlons).[2,4-6] Flooding and heavy rains are associated with outbreaks, especially in resource-poor countries.[6] Climate change is likely to increase the frequency of such outbreaks.[7]

Leptospirosis is endemic worldwide, with an estimated 1.03 million cases every year, and 58.900 deaths.[8] **Figure 2** provides an overview of global morbidity and mortality. The highest estimated burden of disease is in tropical areas in South and South-east Asia, Western Pacific, Central and South America, and Africa.[8,9]

In lower endemic countries however, there is a risk of late recognition because of the lower incidence, leading to worse disease outcomes.

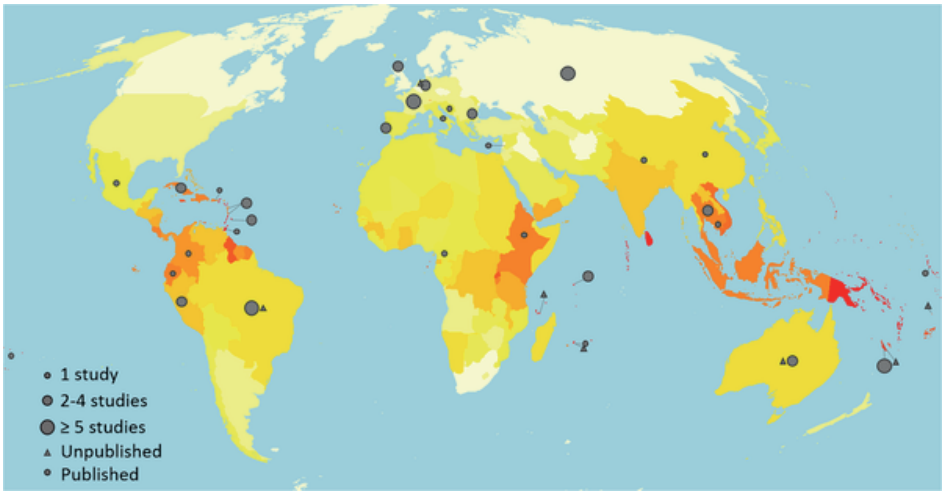

Figure 2. Annual morbidity of leptospirosis by country. Annual incidence of disease is shown as white (0–3), yellow (7–10), orange (20–25) or red (over 100), in cases per 100,000. Circles indicate the countries of origin for published studies, triangles for grey literature.
Reprinted with permission from the publisher from: Costa F et al. (2015) Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLOS Neglected Tropical Diseases 9(9): e0003898. https://doi.org/10.1371/journal.pntd.0003898

Clinical characteristics

In short, the pathology of leptospirosis is based on the development of vasculitis, endothelial damage, and inflammatory infiltrates, with liver, kidneys, heart and lungs mostly affected.[2]

There is a great variation in the clinical symptoms of leptospirosis. It usually presents with non-specific symptoms, making the list of potential differential diagnoses long: e.g. malaria, arboviral infections, rickettsioses, but also influenza and acute HIV infection.[2,3] In its mildest form, it presents as a self-limiting flu-like illness, which is hard to differentiate from other febrile illnesses. In its severest form, it is a life-threatening illness with multi-organ failure.[2-4] The incubation period from exposure to the spirochetes to clinical manifestation, is typically 7 to 12 days, with a range from 3 - 30 days.[4]

Typical symptoms at presentation include a sudden onset of fever, headache and chills. Other common symptoms include (calf) myalgia, a rash, conjunctival suffusion, a non-productive cough and gastrointestinal symptoms such as nausea, vomiting, diarrhoea and abdominal pain. Severe disease comes with organ dysfunction; kidneys, liver, lungs and the brain can be affected. The classic "Weil's disease" presents with renal failure and jaundice.[2-4] Bleeding is common in patients with severe disease, either mild or severe (such as pulmonary haemorrhage or gastrointestinal bleeding, caused by coagulation disorders that occur in severe disease.[10] There is a wide range of other symptoms leptospirosis can present with: aseptic meningitis could be found in up to 25% of cases; myocarditis, (necrotizing) pancreatitis, anterior uveitis and, rhabdomyolysis have been reported.[2,3]

Diagnostics

Diagnosis of leptospirosis has long depended on serological assays; however, over the past couple of years, molecular detection tests have gained ground.

The microscopic agglutination test (MAT) is the reference standard, often combined with an immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA), and culture. MAT detects agglutinating antibodies in serum of the patient, and requires testing of paired samples, with the second sample taken preferably at least 5-7 days after onset of the disease, when antibody production commences.[1,2] When performed 'according to the books', it has a high specificity; confirming the diagnosis. However, sensitivity is limited; thus, a negative test cannot safely rule out the illness.[11] The reported sensitivity varies among studies,[11-13] and increases when MAT is combined with IgM ELISA.[12] Moreover, MAT is a cumbersome method and confined to specialised centres, as it requires the maintenance of a full panel of live leptospires, and well-trained laboratory technicians.[1] In addition, antibodies can be detected in the blood after 5-7 days of illness,[2] rendering an early diagnosis with serological methods impossible.

A positive culture confirms the diagnosis as definite. Leptospires circulate in the blood in the first week of the illness (leptospiraemia); so samples should be taken swiftly.[2] Cultures can be obtained from blood, cerebrospinal fluid (CSF) and urine; the duration of excretion of leptospires in the urine lasts longer than the leptospiraemia. Cultures should incubate up to 13 weeks, and require weekly examination by dark-field microscopy.[1,2]

Molecular detection techniques are suited to diagnose the illness in the early stages, when leptospiraemia is present. Polymerase chain reaction (PCR) and its variants are commonly used, and can be applied on blood, CSF, urine, and aqueous humour. In blood, the diagnostic accuracy is highest when the test is performed the first ten days of illness; after that period the leptospiraemia declines.[1] The earlier the test is applied, the higher the sensitivity.[14] Molecular tests on urine get positive at a later stage: ten to fourteen days after onset of symptoms.[15] However, the true diagnostic accuracy of molecular tests is not yet elucidated, as there is a big variation between published studies.

Prevention and treatment

Socio-economic factors, sanitation and risky behaviours are consistently associated with an elevated risk for leptospirosis.[6] Floods are a major risk factor, and likely to increase with climate change; governments should prepare for this.[6,7] Rodent control is key in prevention of leptospirosis.[16] Other preventive strategies for leptospirosis in humans mainly encompass reducing risk behaviour, such as prolonged exposure to water for recreational or occupational activities, and animal (e.g., cattle, rats) contact.

For people in rural areas, who are already at higher risk, this means, for example, not walking barefoot and covering wounds. Open sewers contribute to the risk, and should be avoided.[6] People working with animals should use appropriate protective methods. In

developed countries, recreational exposure is a major risk factor. Antibiotic prophylaxis could be considered in those at high risk, though this has not been proven effective.[17] Serovar-specific vaccination is available for dogs and livestock,[3] preventing abortions - sometimes fata l- illness in those animals, but carrier state is not completely prevented, and disease can therefore still be spread.[16]

Mild cases of leptospirosis may resolve without treatment. However, early initiation of treatment may prevent severe disease and shorten the duration of illness;[18] therefore, treatment should be started when the diagnosis is considered. In mild cases, oral doxycycline, azithromycin, amoxicillin or ampicillin can be administered. In severe disease, intravenous treatment with penicillin is required, combined with supportive care measures. As it is a spirochaetal disease, clinicians should be aware of the possibility of a Jarisch-Herxheimer reaction, a febrile inflammatory reaction that occurs after initiation of treatment, which is characterised by a sudden increase of symptoms and clinical detoriation.[19]

History

Adolf Weil first described the illness in 1886, in his paper entitled 'On a strange, acute infectious disease, accompanied by swelling of the spleen, icterus, and nephritis'.[20] It is clear that the illness was already known in several continents before the aetiology was described: ancient Chinese texts describe 'rice field jaundice', in Japan 'autumn fever' and 'seven day fever' described similar syndromes. In Europe, hints at associated occupations were, for example: 'swine-herder's disease', 'Schlammfieber' (mud fever).[21,22]

Stimson was the first to observe spirochetes in kidney tissue in 1907 and called it '*Spirocheta interrogans*',[23] because of its question mark-like shape. In Japan, the illness was common in coal miners. The Japanese group of Inada et al. was the first to isolate the organism in 1908;[24] they injected guinea-pigs with blood of affected patients, which led to typical illness in the animals. In subsequent years, they defined transmissibility, routes of infection, pathological changes, tissue distribution, urinary excretion, leptospiral filterability, morphology, and motility.[21] This group also demonstrated the resilience of rats, mice, and rabbits to symptomatic disease, and proved they were renal carriers of *Leptospira*. The latter was discovered while they were researching *Orientia tsutsugamushi,* one of the common causes of rickettsial disease.

Diagnostic methods transformed over time; from 1924 to 1963, culture of patient specimens was done by inoculation of blood or urine into guinea pigs or hamsters.

The 'agglutination test' was applied from 1924 onwards, using serovars Pyrogenes and Rachmat.[25] Over the following decennia, more serovars were added;[1] the agglutination test was modified in 1954[26] and in 1973.[27]

In sub-Saharan Africa, leptospirosis was first described in Dakar, Senegal, in 1921. Spirochetes were demonstrated in the liver at autopsy, after a European died of 'blackwater

fever' (haemoglobinuria),[28] an entity at that time more commonly described in connection with quinine therapy of severe malaria.

Rickettsial disease

Rickettsioses are zoonotic diseases, caused by bacteria of the *Rickettsiaceae* family. They are gram-negative, intracellular bacteria, ordered in two genera: *Orientia* and *Rickettsia.*[29] *Orientia* consists of *Orientia tsutsugamushi*, the cause of scrub typhus, and the recently discovered *Orientia chuto*. The *Rickettsia* genus consists of four biogroups:[29,30]

1. Spotted fever group, with as most prominent members *R. rickettsii* (causing Rocky Mountain spotted fever), *R. conorrii* (Mediterranean spotted fever), *R. africae* (causing African Tick Bite Fever);
2. Typhus group, with *R. typhi* (endemic typhus) and *R. prowazekii* (epidemic typhus);
3. Translational group including *R. felis, R. australis* and *R. akari*;
4. Non-pathogenic group (which will not be discussed here).

Epidemiology and vectors

Most *Rickettsiae* require tick vectors, and also wild or domestic animals that serve as resevoirs.[31] **Table 1** displays the most common rickettsioses and their vectors. In short, ticks transmit *R. africae, R. rickettsii and R. conorii*; fleas transmit *R. typhi* and *R. felis*; lice transmit *R. prowazekii*; and mites harbour *O. tsutsugamushi* and *R. akari*.[31,32] However, recent reports have shown that *R. felis* can be found in booklice[33] and mosquitoes.[34,35] Ticks and mites transmit the bacteria during feeding; in fleas and lice, infection is caused by the entry of faeces in bite sites and cuts.[32]

The epidemiology of the rickettsial diseases depends on the occurrence of their vectors. **Figure 3** shows a map of the spread of the most important rickettsial diseases around the world. The flea-transmitted *R. typhi* and *R. felis* occur worldwide;[32] spotted fever group rickettsial diseases are spread by ixodid ticks (**Figure 4**) and have a wide distribution.[31,32] Incidence numbers are not well established for most rickettsioses. In the US, the caseload for spotted fever rickettsioses increased from 495 in 2000, to 6248 cases in 2017.[36] In Europe, surveillance of rickettsioses is mainly performed in Italy, Portugal and Spain, where Mediterranean Spotted Fever (caused by *R. conorii*) is a well-recognised disease and there is reliable reporting. In Italy, between 2001-2015, the annual hospitalisation rate was 1.36/100.000, but showed a decreasing trend. Much higher numbers were observed in Sicily and Sardinia (>20/100.000).[37] In Spain, hospitalisation rates between 1997 and 2014 also showed a decreasing trend, with an average annual rate of 0.56/100.000.[38]

Trombiculid mites, also known as Chiggers (**Figure 5**), are the vector for *O. tsutsugamushi,* the agent of scrub typhus. Scrub typhus is traditionally prevalent in the tropical Pacific triangle. The disease burden is highest in South-East Asia, with increasing yearly incidences in South Korea, Japan, Thailand and China, with the highest incidences reported in South

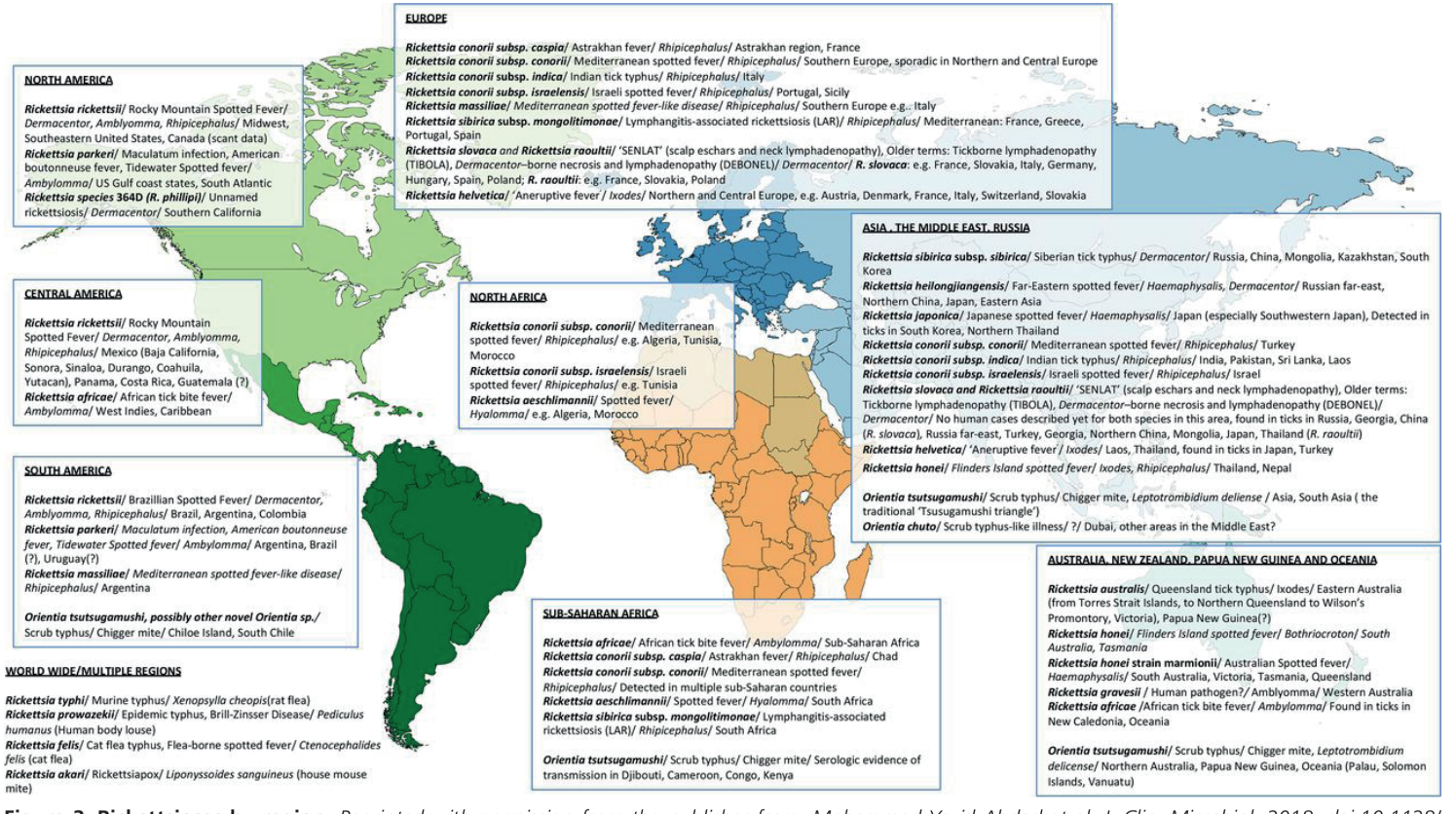

Figure 3. Rickettsioses by region. *Reprinted with permission from the publisher from: Mohammad Yazid Abdad et al. J. Clin. Microbiol. 2018; doi:10.1128/JCM.01728-17*

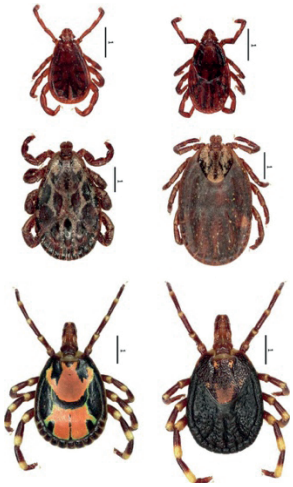

Figure 4. Tick vectors of spotted fever group rickettsioses. Three tick vectors of spotted fever group rickettsioses. Top: *Rhipicephalus sanguineus*, vector of *R. conorii* (Mediterranean spotted fever). Middle: *Dermacentor marginatus*, vector of *R. slovaca* and *R. raoultii*. Bottom: *Amblyomma variegatum*, vector of *R. africae* (African Tick Bite Fever). Males are shown left, females are shown right. The scale of the bar is 1 mm.
Reprinted with permission from the publisher from: Philippe Parola et al. Clin. Microbiol. Rev. 2013; doi:10.1128/CMR.00032-13.

Figure 5. Chigger mite (*Leptotrombidium delicense*, vector of scrub typhus. The top picture shows a complete picture, the bottom the scutum and dorsal setae
Reprinted with permission from the publisher from: Peng PY et al. (2018) Landscapes with different biodiversity influence distribution of small mammals and their ectoparasitic chigger mites: A comparative study from southwest China. PLOS ONE 13(1):e0189987. https://doi.org/10.1371/journal.pone.0189987

Korea (59.7/100.000 in 2013) and Thailand (17.1/100.000 in 2013).[39] There is an average case mortality rate of around 6.0%.[40]

More recent studies suggest that it may be endemic in Africa, and there are reports from South America, confirming its presence there.[32,41] *Orientia chuto*, a new *Orientia* species, was isolated from a patient returning from the United Arab Emirates,[42] and was recently identified in chigger mites from Kenya.[43]

Clinical characteristics

Generally speaking, rickettsial diseases present as acute febrile illness, initially with general symptoms that are hard to distinguish from other febrile illnesses, such as dengue, malaria, and leptospirosis. Patients may, or may not, have an inoculation eschar (**Figure 6**). Murine typhus (*R. Typhi*) and Rocky Mountain Spotted Fever (*R. rickettsii*) rarely present with an eschar; and for the other types, reported rates vary widely (**Table 1**).

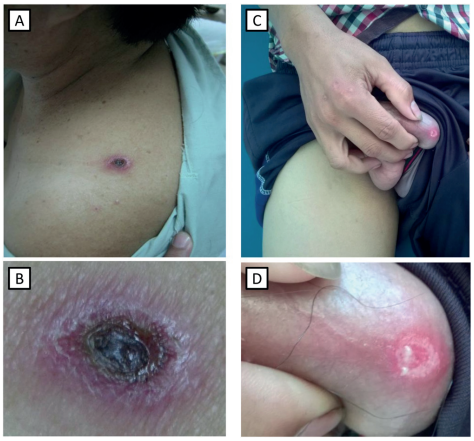

Figure 6. **Eschars in scrub typhus patients.** Eschar on the shoulder (a, b) of a female and on the base of the penis (c, d) of a male scrub typhus patient.
Reprinted with permission from the publisher from: Le Viet N. et al. (2017) Use of eschar swabbing for the molecular diagnosis and genotyping of Orientia tsutsugamushi causing scrub typhus in Quang Nam province, Vietnam. PLOS Neglected Tropical Diseases 11(2): e0005397. https://doi.org/10.1371/journal.pntd.0005397

Table 1. Most common rickettsioses and their characteristics.

Group	Bacteria	Disease	Vector	Animal reservoir	Geographic	Eschar occurrence
Spotted fever group	*Rickettsia rickettsii*	Rocky Mountain spotted fever	Tick	Rodents	Americas	0%
	Rickettsia conorii	Mediterranean spotted fever	Tick	Dogs, rodents	Meditterranean and Kaspic sea sub-Saharan Africa India	29-71%*
	Rickettsia africae	African tick bite fever	Tick	Ruminants	sub-Saharan Africa West Indies, Caribbean	52-87%*
	Rickettsia akari	Rickettsiapox	Mite	House mice, wild rodents	Worldwide	100%
	Rickettsia felis	Flea-borne spotted fever / cat flea typhus	Flea	Domestic cats, rodents, oppossums	Worldwide	~20%
Typhus group	*Rickettsia typhi*	Murine / endemic typhus	Flea	Rodents	Worldwide	0%
	Rickettsia prowazeki	Epidemic typhus	Lice	Humans, flying squirrels	Worldwide	0%
Scrub typhus group	*Orientia tsutsugamushi*	Scrub typhus	Mite	Rodents	Asia Australia, Oceania sub-Saharan Africa South America	20-73%*
	Orientia chuto		?	?	United Arab Emirates, Chile, Kenya	?

Adapted from A. Goorhuis 2014[58] and the CDC Yellow Book[44]
** in this thesis,* ***Chapter 6***

Clinical symptoms typically develop after 1-2 weeks of infection. Symptoms vary per agent, but common symptoms include fever, myalgia, headache, rash, nausea and vomiting. African tick-bite fever is typically mild; Mediterranean spotted fever and Rocky Mountain spotted fever can be life threatening.[44-46]

Diagnostics

To date, diagnostic methods are mainly based on the detection of antibodies in the patient serum. Commonly-applied test are immunofluorescence, western blotting, and IgM ELISA. Antibodies can be detected only at a later stage of the infection, after 15 days or later;[47-49] rendering these test unsuitable for an early diagnosis. Also, in endemic areas, the background antibody titres are high, making the interpretation of the tests even more difficult.[50] Moreover, cross-reactivity between different species is shown.[51] Molecular detection tests can provide a specific and early diagnosis,[52-55] but availability is still a problem. Also, the diagnostic accuracy of the tests varies highly between reports, influenced by sample type, technique and target.[50]

Prevention and treatment

No vaccines are available. Key in prevention is the avoidance of exposure to the vectors (ticks, mites, flea, and lice). Tick-born rickettsioses can be prevented by preventing tick bites. Adjusting behaviour (avoiding wooded and bushy areas), wearing protective clothing and insect repellents, and a careful examination after exposure are important measures.

For scrub typhus, prevention is based on mite control and chemoprophylaxis. Applying insect repellents on clothing and skin is effective. Prophylactic use of tetracyclines (such as 200 mg doxycycline weekly) could be effective for non-immune individuals in endemic areas.[56] Rodent control can have an adverse effect, as the mites will refer to humans in the absence of their preferred hosts. Flea-borne rickettsioses can be prevented by keeping pet animals flea free, and rodent control.[57] Epidemic typhus is transmitted by body lice, and can be prevented by basic measures such as avoiding overcrowded areas and personal and bedding hygiene.[44]

Treatment should be initiated as soon as the illness is suspected. It is not advised to wait for diagnostic test outcomes, as these can take time, and are often unreliable in the early stages of illness. Doxycycline is the most effective treatment (100mg twice daily for 7 days). In case of intolerance, pregnancy or for children, azithromycin and clarithromycin are good alternatives.[58-60]

History

Epidemic typhus (caused by *R. prowazekii*) has been the most infamous rickettsial illness throughout history. In 1546, a Florentine doctor, Girolamo Fracastoro, described the illness in his book '*De contagione et contagiosis morbis',* but may have been recognized as early as

1082 in Spain, where patients were described with fever, rashes and parotid tumefaction.[61] Napoleon's army is thought to have been significantly reduced through epidemic typhus.[62]

As typhus spreads in unhygienic and crowded conditions, described historical epidemics often followed war, natural disasters, famine, and social unrest.[61,63] As Hirsch described it in 1883:[63,64]

> "*The history of typhus is written in those dark pages of the world's story which tell of the grievous visitations of mankind by war, famine, and misery of every kind. In every age, as far back as the historical inquirer can follow the disease at all, typhus is met in association with the saddest misfortunes of the populace; and it is, therefore, a well-grounded surmise that the numerous pestilences of war and famine in ancient times and in the Middle Ages, which are known to us, not from medical sources but merely from the chronicles, had included typhus fever as a prominent figure among them.*"'

Later, it was known to be prevalent in armies throughout Europe, and caused many fatalities in Wold War I in Russia, Poland and Romania. In World War II, many American soldiers succumbed to the illness, which they are thought to have contracted from native populations in overcrowded European cities.[63]

In 1909, transmission by the human body louse (*Pediculus humanus corporis*) was discovered by Charles Nicolle.[61] The bacterium was first described in 1916, and named after the scientists who contracted the illness and died of it, Dr. H.T. Ricketts and Dr. S. von Prowazek. Dr. Ricketts also identified *Rickettsia rickettsii* in 1906. In 1916, the Weil-Felix test was developed, to demonstrate antibodies to *Rickettsiae*.[61,63]

Scrub typhus was described first in 313 AD in a Chinese clinical manual.[65] 'Shashitsu', the Chinese name for scrub typhus, occurred throughout old Chinese writings. It was recognized as an illness, caused by the bite of a mite in summer, especially after floods. Red mites were associated with a febrile illness and a pustule.[65] Later on, the disease was recognised throughout the 'Tsutsugamushi triangle' (Asia-Pacific region). The first report from the illness outside of the Tsutsugamushi triangle was published in 1951, describing a febrile illness outbreak in autochthonous Africans in Belgian Congo.[66]

1.3. Leptospirosis and Rickettsial disease: why in one thesis?

As we will demonstrate in **Chapter 7**, in cases of returning travelers with febrile illness, suspected but testing negative for leptospirosis, a large proportion of cases of imported disease turn out to be rickettsial disease in the end, thus demonstrating the clinical-epidemiological overlap between both conditions.

Leptospirosis and rickettsial disease are emerging zoonotic illnesses with a wide geographical occurrence. They have an overlapping geographical distribution and clinical presentation, that can be hard – or even impossible – to distinguish on clinical grounds alone.

Although the illnesses have been recognised for a long time, in the recent years, there is an increased interest and identification of the illnesses. This is due to many factors: improved diagnostic approaches facilitate an easier diagnosis; increased tourism leads to increased imported illness, and therefore more recognition in the western world; and last, but not least, climate change leads to changes in the environment that are very beneficial to both pathogens and their vectors (e.g., floods, warmer weather, increased rodent populations, etc.), expanding their endemicity in both tropical, and more temperate, climates.

1.4. Aim and outline of this thesis

This thesis intends to contribute to the knowledge of the epidemiology of leptospirosis and rickettsial disease; we hypothesized that both illnesses are vastly under diagnosed, in native populations, as well as in travellers. The research in this thesis aimed to increase awareness of both illnesses among clinicians, which will lead to increased recognition of the diseases, ultimately reducing morbidity and mortality.

Part 1: Leptospirosis

The first step was to generate an overview of leptospirosis in sub-Saharan Africa. This because the extent of the prevalence of the illness on the continent was not well established. **Chapter 2** is a systematic review, summarising all studies on leptospirosis in both animals and humans performed in sub-Saharan Africa after 1970. **Chapter 3** is a study on leptospirosis in returning Dutch travellers, describing epidemiological and clinical characteristics, including case reports of a few special cases. **Chapter 4** also describes leptospirosis in returned travellers, reported through the worldwide network of travel clinics GeoSentintel. There is also a survey among the clinicians of those clinics to assess clinical and diagnostic practices. **Chapter 5** is an extensive systematic review, and meta-analysis on nucleic acid and antigen detection tests for leptospirosis. Those tests have the ability to diagnose the illness in the early stages, and are therefore highly solicited.

Part 2: Rickettsial disease

Rickettsial disease is a common cause of fever. Many disease episodes are thought to commonly present with an eschar, which might not be the case. In **Chapter 6**, we performed a systematic review in order to make an estimation of the under-diagnosis of rickettsial disease (Scrub Typhus and Mediterranean Spotted Fever) not presenting with an eschar (non-eschar rickettsial disease, NERD) in native populations. **Chapter 7** analyses the occurrence of this non-eschar rickettsial disease in returned Dutch travellers who presented with a febrile illness.

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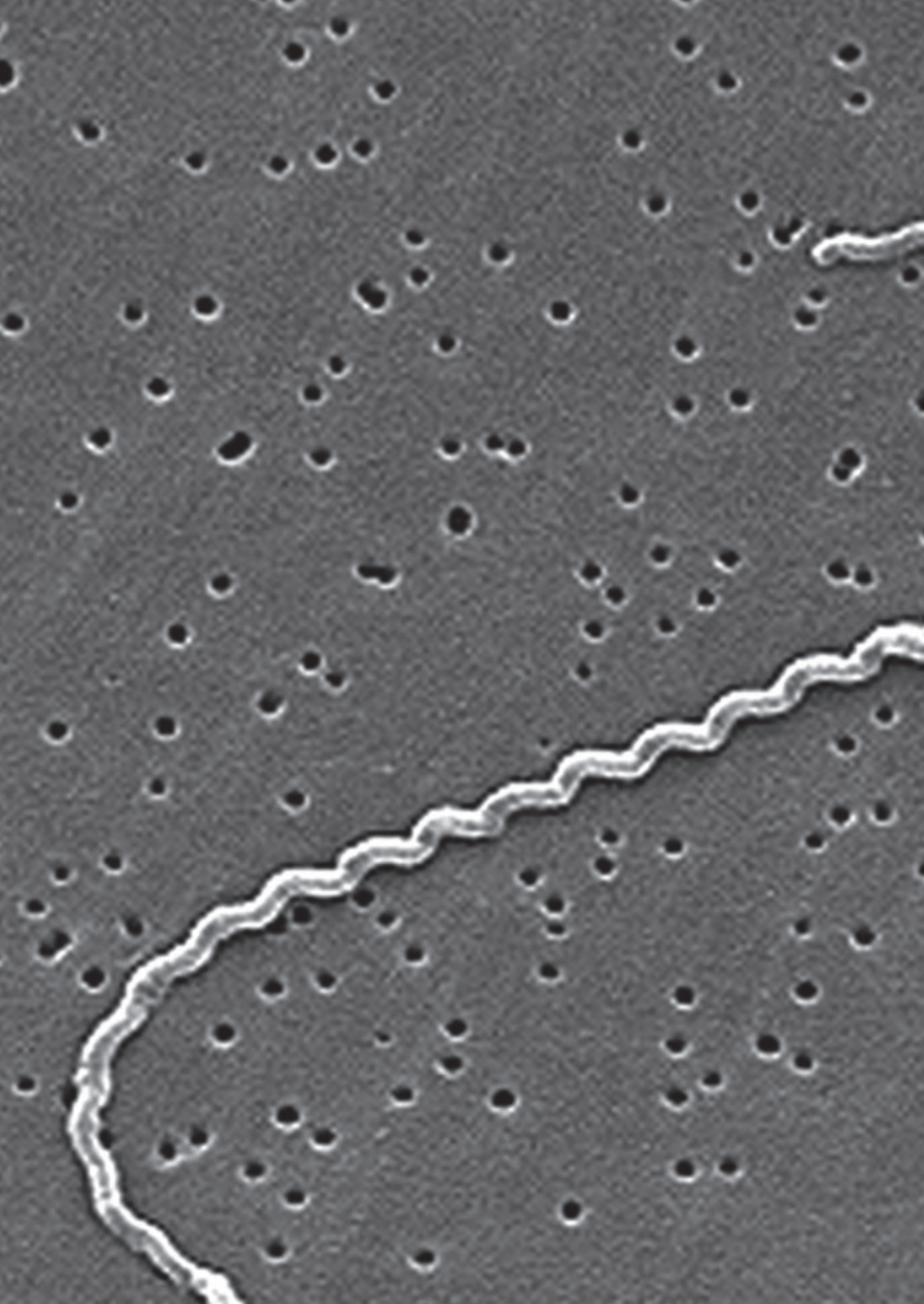
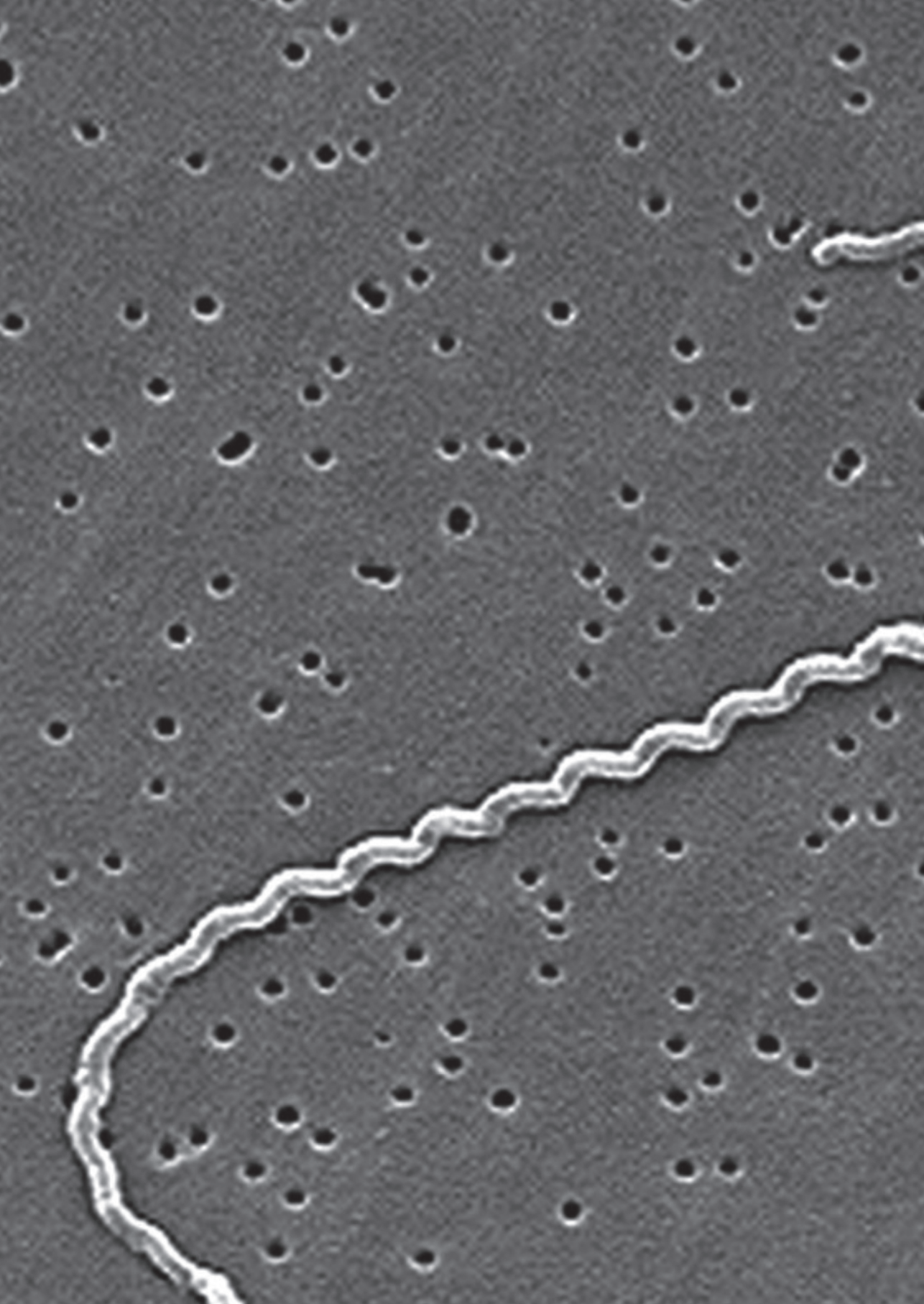


PART 1

Leptospirosis



Chapter 2

Leptospirosis in Sub-Saharan Africa: a systematic review

Sophia G. de Vries, Benjamin J. Visser, Ingeborg M. Nagel, Marga G.A. Goris, Rudy A. Hartskeerl, Martin P. Grobusch

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ABSTRACT

Background

Leptospirosis is an emerging zoonotic infection worldwide, possibly due to climate change and demographic shifts. It is regarded as endemic in sub-Saharan Africa; however, for most countries scarce epidemiological data, if any, exists. The primary objective was to describe the prevalence of leptospirosis in countries in sub-Saharan Africa, and to develop options for prevention and control in the future.

Methods

A systematic review to determine the prevalence of leptospirosis in sub-Saharan Africa was conducted, following PRISMA guidelines. Medline/PubMed, Embase, The Cochrane Library, Web of Science, Biosis Previews, the African Index Medicus, AJOL and Google Scholar were searched.

Results

Information about the prevalence and incidence of leptospirosis in humans is available, but remains scarce for many countries. Data is unavailable or outdated for many countries, particularly in Central Africa. Most data is available from animals, probably due to the economic losses caused by leptospirosis in livestock. In humans, leptospirosis is an important cause of febrile illness in sub-Saharan Africa. It concerns numerous serogroups, harboured by many different animal carriers.

Discussion

A wide variety of data was identified. Prevalence rates vary throughout the continent and more research, especially in humans, is needed to reliably gauge the extent of the problem. Preventive measures need to be reconsidered to control outbreaks in the future.

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Article Highlights

- Leptospirosis constitutes a neglected tropical disease in sub-Saharan Africa.
- It is probable that the combination of climate change, increased risk of flooding, population growth, and urbanisation will lead to an increase in the burden of leptospirosis in sub-Saharan Africa.
- With an ever-growing proportion of febrile disease episodes being recognized as non-malarial, leptospirosis moves into the light as an important differential diagnosis to malaria.

- More research has been conducted in animals compared to humans, probably because of the larger economic losses caused by leptospirosis in livestock (e.g. abortion) compared to economic losses due to human morbidity and mortality.
- Future leptospirosis research should be a collaborative effort of multiple disciplines, mainly human and animal medicine, to attain the optimal health for individuals and animals.

INTRODUCTION

Leptospirosis is a globally important zoonotic disease caused by host-dependent spirochaetes of the genus *Leptospira* (order *Spirochaetales*).[1] Humans are usually infected by contact with urine of an infected host, contaminated drinking water or soil, or infected animal tissue. Notorious reservoirs are rodents, but reservoirs include a variety of wild and domestic animals, livestock, and insectivores. Pathogenic leptospires enter the body through mucous membranes, conjunctivae, small cuts, abrasions, and possibly wet skin.[2] Leptospires survive longest in warm, humid tropical and sub-tropical conditions but also persist in temperate regions.[3] The known endemicity of human disease is focused in the Caribbean and Latin America, the Indian subcontinent, Southeast Asia, Oceania, and Eastern Europe.[4,5] To a lesser extent, it is endemic in other European countries, like Denmark, Greece, Portugal, France, Germany[4], and the Netherlands.[6]

The disease was first described by Adolf Weil in 1886, but leptospires were identified as the causative organism of Weil's disease in Japan in 1908, where it affected coal miners.[7] They are conventionally divided into two species, the pathogenic *Leptospira interrogans* sensu lato and the saprophytic *Leptospira biflexa* sensu lato. In the latter, more than 60 serovars have been described, and over 250 serovars in 25 serogroups were recognized in *L. interrogans*. Different hosts carry distinct serovars.[3] Although this classification has been supplemented by a genotypic classification,[8] the serological classification is commonly used.

Human infection is associated with highly variable clinical manifestations, ranging from asymptomatic or undifferentiated fever to complex illness with high morbidity and mortality rates, like Weil's disease.[2,3] The diagnosis is often, missed, particularly in mild cases; due to unawareness amongst clinicians and its broad spectrum of clinical appearances, often mimicking other infectious diseases. A diagnosis on clinical grounds alone may be difficult.[3,9] A laboratory-based diagnosis is therefore necessary, but current diagnostic methods are cumbersome.[10] Clinical signs, diagnosis and case management, and prevention and control of leptospirosis, including in sub-Saharan Africa, are not in the focus of this review and have been, or are being covered, in detail elsewhere.[2,3,11-14] Although researchers accept the ubiquity of this zoonosis in sub-Saharan Africa, the epidemiological pattern of leptospirosis remains unclear. The aim of this review is to summarise the data for leptospirosis available from sub-Saharan Africa, to gain insight into the burden of the disease in the region, and to develop options for prevention and control in the future.

Leptospirosis as a human disease is a result of a complex interaction between humans, animal reservoirs and the environment. A study from Vietnam showed that in rural endemic areas exposure begins at a young age with a substantial asymptomatic rate of seroconversion.[15] Transmission in rural areas is related to increased rainfall, livestock holding, and farming. In urban areas the transmission is usually rodent-borne and associated with poor hygiene, inadequate waste disposal, and overcrowding; circumstances typical in urban slums

in the developing world.[16] Heavy rainfall and flooding are often, but not always associated with outbreaks of leptospirosis worldwide.[17,18] The epidemiology of leptospirosis throughout the world has been reviewed.[4,5] However, data from sub-Saharan Africa remains scarce. Africa has the second largest urban growth rates (0.23 million people per week in the last decade) and subsequently, high slum growth rates;[19] a large proportion of those slums are situated in flood-prone areas near the Atlantic coastlines.[20] It is probable that the combination of climate change, increased risk of flooding, population growth, and urbanisation will lead to an increase in the burden of leptospirosis in sub-Saharan Africa.[21]

Diagnosis

Clinical and laboratory findings are non-specific, and a high index of suspicion is required based on epidemiological exposure associated with clinical signs and symptoms. Box 1 provides a concise overview on diagnostic methods. Box 2 details the current case definitions.[5]

Prevention and treatment

Vaccines tested in humans to date are of limited, if any, value due to an unfavourable adverse events profile, insufficient protection levels, and the variety in patterns of circulating serovars.[2] Serovar-specific vaccination is widely used in livestock and dogs[2] but yields variable levels of protection, possibly preventing illness but not leptospiruria,[22-25] and therefore not the transmission to humans. Doxycycline as a prophylactic could not be proven effective in humans.[13]

Measures for preventing human leptospirosis include avoiding possible sources of infection, antibiotic prophylaxis in those at high risk, and possibly animal vaccination. Preventive measures depend on the epidemiological pattern. In tropical areas, where more serovars circulate, large numbers of maintenance hosts exist and infection results from environmental contamination, with peaks after rainy and harvest seasons; and outbreaks potentially following floods or other natural disasters. Therefore, rodent and wet area control are important measures. In urban environments, when infrastructure is disrupted or underdeveloped, rodent control is also of use.

Severe leptospirosis should be treated with intravenous penicillin immediately after the diagnosis is considered.[26] Aggressive supportive care is essential and potentially life-saving.[27] In mild cases, oral treatment with doxycycline, azithromycin, ampicillin, or amoxicillin is recommended. However, the benefit of antibiotic treatment for leptospirosis could neither be proven nor refuted[12] due to insufficient evidence. Further clinical research is needed.

METHODS

This systematic review was registered in advance in PROSPERO, an international database of prospectively registered systematic reviews in health and social care (Registration number:

CRD42013006545).[179] We followed recommendations made by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) group.[180] We searched the electronic databases MEDLINE/PubMed (1946 to January 2014), Embase (via Ovid, 1947 to January 2014), The Cochrane Library (January 2014), Web of Science (1975 to January 2014), Biosis Previews (1993 to January 2014), The Cumulative Index to Nursing and Allied Health Literature (CINAHL) (1937 to January 2014), the African Index Medicus (1993 to January 2014), African Journals Online (AJOL) (January 2014), Google Scholar (January 2014) for studies published up to 13 January 2014 without date, publication status or language restrictions. The search strategy consisted of free-text words and subject headings related to leptospirosis and countries in sub-Saharan Africa according to the United Nations. The full search strategies for every searched database are reported in supplementary file II: Search strategies. Data on leptospirosis in each member country were requested from official national organizations. All primary trial registries that participate in the WHO's International Clinical Trial Registry were searched to identify ongoing, terminated, or planned trials (see supplementary file III: Trial registries). The archive at the KIT Biomedical Research in Amsterdam was searched for additional papers. An experienced clinical librarian (IMN) conducted the actual searches on 24 to 26 October 2013 and an update search on 13 January 2014. Bibliographies of relevant studies retrieved from the studies were checked for additional publications. We used Reference Manager 12.0.3 (Thomson Reuters) to manage, deduplicate, and screen the references for eligibility. Selection criteria for inclusion of retrieved studies were as follows: the study population consisted of any group of people or mammals in sub-Saharan Africa who had been tested for leptospirosis or leptospiral antibodies, of all age groups. Descriptive, cross-sectional studies, prospective, or retrospective studies and case reports and series in which the prevalence rate of leptospirosis in any country in sub-Saharan Africa was reported were included. Conference abstracts were also included. Only studies published after 1970 were included in the present systematic review. An overview of the historic articles published before 1970 is given in additional file 5: Historic leptospirosis. Studies performed on the Western Indian Ocean islands and Madagascar were excluded because an extensive systematic review was published recently on this topic.[181] Eligibility assessment of studies found was performed independently in an unblinded standardized manner by 2 authors (SGV & BJV). Titles and abstracts were screened first; next, SGV screened and selected relevant full-text articles. Disagreement in the selection process between reviewers were resolved by consensus or on consultation with the senior review author (MPG). We summarized the study selection process in the PRISMA flow diagram (Figure 1). Risk-of-bias assessments were performed independently by two review authors (SGV & BJV) for all included human studies, using an unique assessment tool, extracted and modified from an evidence based tool[182] (see supplementary file VIII). The tool was pilot-tested on five randomly selected studies by both reviewers. No studies were excluded on the basis of quality. We developed a data extraction sheet (modified version of the Cochrane Consumers and Communication Review Group's data extraction template), pilot-tested it and refined it ac-

cordingly. SGV extracted the following study characteristics: first author, year of publication, PubMed ID if available, language, study site & setting, study design, characteristics of trial participants, objectives / measure of primary outcome, target population and selection criteria, total enrolment, attrition rate (if applicable), sample size, diagnostic methods and cut-off values and, if applicable, prevalence of leptospiral antibodies, leptospiral serovars/serogroups/strains, characteristics of leptospirosis cases, risk factors, seasonal influences, demographics, co-infections, treatment, and mortality. Leptospiral serovars and strains were placed in serogroups according to the "Leptospira Library" from KIT Biomedical Research.[183] 95% confidence intervals (CI) of prevalence rates were calculated using the modified Wald method. The following equations were used. p'= (S+0.5z2)/(n+z2) (S= numerator, n= denominator, z= 1.96, as for a 95% confidence interval) and concomitantly to compute the margin of error of the CI: W= z H((p'(1-p'))/(n+z2)). Data was plotted in forest plots using Prism version 6.0 (GraphPad Software, Inc., CA, USA). The primary outcome in the present review is the prevalence of leptospirosis in countries in sub-Saharan Africa. Secondary outcomes include risk factors, circulating serogroups, serovars and/or strains, clinical manifestations, treatment, prevention measures, seasonal influences, and mortality. Extracted data was double checked by BJV for all the included articles (n=140) using the original records. Regional and national WHO offices were contacted for additional data on leptospirosis in the region, but this did not result in additional data. We did not contact authors for further information or confirm the accuracy of information included in our review with the original researchers, since for the majority of papers adequate contact information was missing. A meta-analysis could not be performed due to the clinical heterogeneity, and the non-uniformity of the diagnostic tools and case definitions. We did not investigate publication bias.

RESULTS

Literature search results

The initial search yielded 910 records, of which 398 remained after the removal of duplicates (see PRISMA flow diagram, **Figure 1**). Another additional 31 references from bibliographies of screened studies were identified and screened. Of the 429 screened records, 140 records met the inclusion criteria for this review. Of the excluded studies, 40 were published before 1970 and are not discussed in the present review; an overview of these historic records can be found in Supplementary file VII. Of the 140 included studies, four were conference abstracts of studies never published and five studies were mentioned in theses or other articles, but never published in an online database. The majority of records were published in English: eight were published in another language (French, n = 6; German, n = 1; South African, n = 1). For a general overview of characteristics of included studies, see Table 1. Studies were found from 27 of 44 sub-Saharan African countries. Overall quality assessment scores for risk

of bias in studies included in the review ranged from three to ten, out of a maximum of ten. Of the total 35 studies assessed, 23 were scored >7, of which 15 were scored >8. All studies were observational studies. **Table 2** and **3** provide overviews of surveys of human and animal leptospirosis reported from sub-Saharan Africa. There were no outbreak reports in animals. **Table 4** details a miscellaneous study. Supplementary files IV and V provide detailed information about individual case reports and case series of leptospirosis in people in sub-Saharan Africa and surveys of leptospirosis in humans and animals from this region; supplementary file VI an overview of circulating serogroups and an overview of research published before 1970 can be found in supplementary file VII.

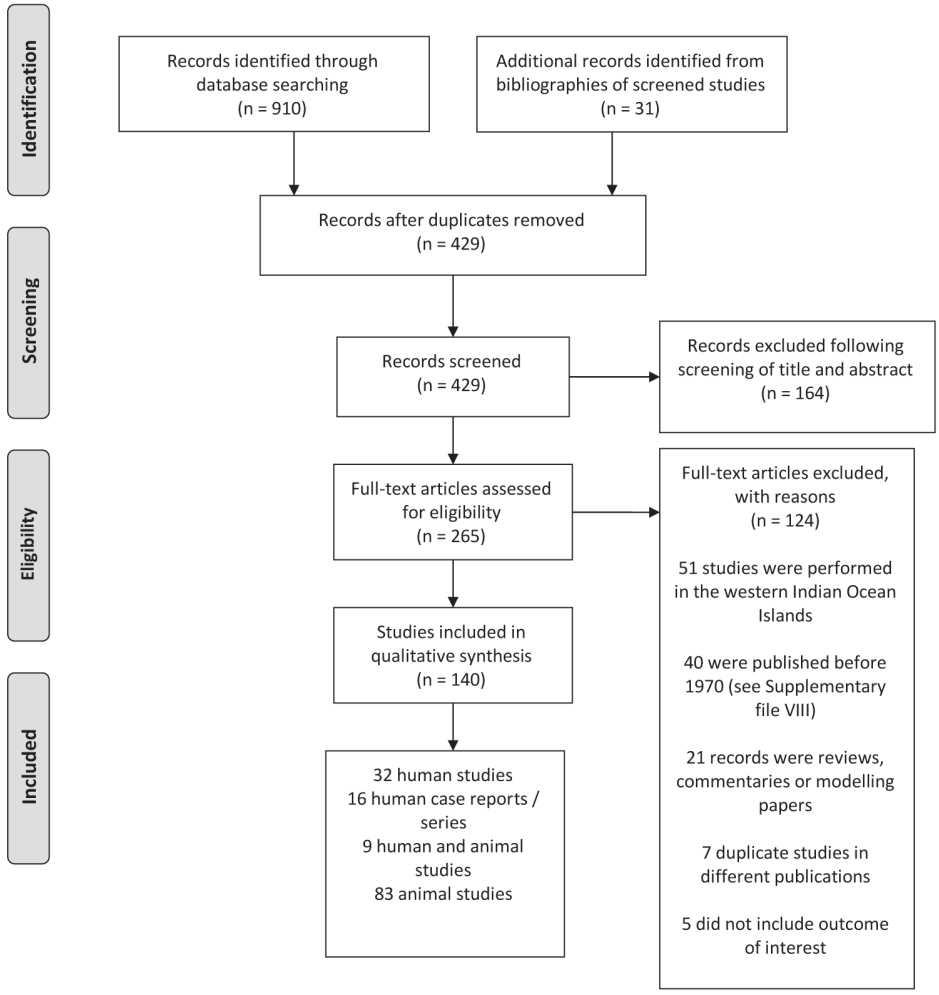

Figure 1. Study selection (Prisma Flow Diagram)

Leptospirosis epidemiology in sub-Saharan Africa by region

Table 1 Overview characteristics included studies

	Human surveys	Human + animal surveys	Case reports	Animal	Total	Percentage
West Africa	12	3	3	15	33	23.6%
Central Africa	3	0	4	4	11	7.9%
East Africa	12	2	1	30	45	32.1%
Southern Africa	2	4	5	34	45	32.1%
Travellers	3	0	3	0	6	4.3%
Total	32	9	16	83	140	100.0%
Percentage	22.9%	6.4%	11.4%	59.3%	100.0%	

West Africa

Characterized by diverse climates, ranging from the Sahara desert and semi-arid Sahel in the North to a belt of tropical forests on the Southern coast, the region harbours numerous large, overcrowded cities. These are often situated in more tropical Southern areas where heavy rainy seasons and slums with poor sanitation are present. Data is available from seven of 16 West African countries; with the majority of data from Nigeria (n=18)[28-44] and Ghana (n=7).[45-51]

In Nigeria, three background serosurveys in healthy people showed prevalences of leptospiral antibodies ranging from 13·5% in Eastern State[30] to 18·0% in Plateau State,[31] and 20·4% in a countrywide survey,[29] with significantly higher prevalence in coal mine workers,[30] abattoir workers,[30,31] and farmers.[29-31] A recent risk analysis among abattoir workers revealed low levels of awareness and high levels of risk practices.[52] No surveys on leptospirosis as a cause of febrile illness have been conducted in Nigeria; however, a recent case report from Plateau State proved it to be a diagnosis to consider in febrile, icteric illness.[53] Livestock has been studied extensively in Nigeria.[28-44] Leptospirosis affecting livestock can cause reproductive problems (e.g. abortion) and consequently, huge economic losses. Several surveys in cattle have been done in Zaria in the central northern Kaduna State, displaying varying prevalences: 3·5% (5/142),[40] 6·8% (5/74),[41] 8·4% (20/237),[38] and 11·0% (18/164).[40] Other studies demonstrated leptospirosis in cows in Plateau state[42,43] and Ibidan,[33] and in dogs,[35,36] sheep, goats, and pigs.[34] Two rodent studies in Plateau State showed seroprevalences between 0·0% and 44·8%.[41]

In Ghana, only humans have been studied.[45-51] Hogerzeil and colleagues showed a seroprevalence of 33·3% in 460 healthy inhabitants from the Ashanti district, with a significantly higher prevalence in bush farmers and cocoa plantation workers.[50] Concomitantly, they demonstrated confirmed leptospirosis in 3·2% of 190 patients with jaundice and/or fever of unknown origin (FUO) in the same region. In 1973, a 21·2% seroprevalence was demonstrated in ill people from the Ashanti and Volta regions.[51] This millennium, four surveys were conducted in febrile patients; numbers varied from 0·0%[45] and 1·7%[48] to 7·8%[45] in the Accra region. In the Northern Region, leptospirosis was diagnosed in one of 263 patients.[47]

Table 2 Leptospirosis in sub-Saharan Africa in humans published after 1970
*In **bold** studies performed on diseased subjects.*

Country	Region	Method	Number of individuals	Prevalence / Incidence	Quality (1-10)
West Africa					
Cape Verde Islands	Santo Antao & Santiago[58]	Cross-sectional serosurvey healthy people; MAT (12 serovars), positive ≥ 1:100	611	7.2% (95% CI: 5.7-9.1%)	8
Ghana	**Accra[46]**	**Cross-sectional study in patients with FUO; IgM ELISA**	**166**	**7.8% (95% CI: 4.5-13.0%)**	**8**
Ghana	**Accra + Northern Region[45]**	**Cross-sectional study in patients with FUO; IgM ELISA and PCR**	**180 (ELISA)** **40 (PCR)**	**7.8% (95% CI: 4.6-12.7%) (ELISA)** **0.0% (95% CI: 0.0-7.6%) (PCR)**	**7**
Ghana	Northern Region[47]	Cross-sectional survey (inclusion criteria not reported (NR)); method NR	263	0.4% (95% CI: 0.0-2.3%)	3
Ghana	**Greater Accra Region[48]**	**Cross-sectional study in patients with FUO "Serology with 4x titre rise"**	**172**	**1.7% (95% CI: 0.4-5.2%) "confirmed diagnosis"**	**6**
Ghana	Ashanti-Akim district[50]	Cross-sectional serosurvey healthy people; MAT (24 serogroups), positive ≥ 1:20	460	33.0% (95% CI: 28.9-37.4%)	7
Ghana	**Ashanti-Akim district[49]**	**Prospective cohort study** **Group A: FUO + jaundice with high degree of suspicion of acute LS** **Group B: Any patient presenting with jaundice** **MAT (23 serogroups), 4x titre rise**	**88 (group A)** **102 (group B)**	**4.5% (95% CI: 1.4-11.4%) (group A)** **2.0% (95% CI: 0.1-7.3%) (group B)**	**10**
Ghana	**Ashanti region + Volta Region[51]**	**Cross-sectional study in suspected cases of LS & jaundice; MAT (16 serogroups), positive ≥ 1:300**	**99**	**21.2%** (95% CI: 14.2-30.3%)	**5**
Guinea	Conakry[55]	Cross-sectional survey healthy people ELISA IgM + IgG, MAT	1200	7.0% (95% CI: 5.7-8.6%)	6
Nigeria	**Abuja[28]**	**Case control study in kennel workers after LS outbreak dogs: ELISA**	**10 (cases)** **10 (controls)**	**100.0% (cases)** **0.0% (controls)**	**7**
Nigeria	Countrywide[29]	Cross-sectional serosurvey healthy people; MAT (12 serovars), positive ≥ 1:100	538	20.5% (95% CI: 17.3-24.1%)	9
Nigeria	Enugu + other eastern states[30]	Cross-sectional serosurvey healthy people; MAT (11 serovars), positive ≥ 1:100	661	13.5% (95% CI: 11.1-16.3%)	8

Table 2 Leptospirosis in sub-Saharan Africa in humans published after 1970
*In **bold** studies performed on diseased subjects. (continued)*

Country	Region	Method	Number of individuals	Prevalence / Incidence	Quality (1-10)
Nigeria	Plateau State[31]	Cross-sectional serosurvey healthy people MAT (13 serovars), positive ≥ 1:100	710	18.0% (95% CI: 15.4-21.0%)	8
Senegal	**Dakar[32]**	**Cross-sectional study in patients with LS suspicion; MAT (16 serogroups), positive ≥ 1:100**	**109**	**6.4%(95% CI: 2.9-12.9%)**	**8**
Central Africa					
DRC (*Zaire*)	**Kivu Mountains[63] (Katana Hospital)**	**Prospective cohort study in patients with haemoglobinuria; IgM ELISA, 4fold titre rise in sera**	**38**	**5.3% (95% CI: 0.5-18.2%)**	**8**
Gabon	Northeast[65]	Cross-sectional serosurvey healthy people; Macroscopic Agglutination Test	235	15.7% (95% CI: 11.6-21.0%)	6
Gabon	**Libreville[66]**	**Cross-sectional study in military Frenchmen with FUO (T≥39°C, no malarial parasites, no other explanation) Screening with MaAt, then IgM ELISA, then MAT (19 serovars), positive ≥ 1:100**	**55**	**5.5% (95% CI: 1.3-15.4%)**	**6**
East Africa					
Ethiopia	**Wonji[97]**	**Cross-sectional field study, malaria negative febrile patients; LeptoTek Dri-Dot**	**59**	**47.5% (95% CI: 35.4-60.0%)**	**5**
Kenya	**Malindi, Coast Province[70]**	**Outbreak investigation of malaria negative acute fever; PCR + ELISA**	**21**	**0.0% (95% CI: 0.0-13.5%)**	**5**
Kenya	**North Eastern Province[71]**	**Cross-sectional survey among patients with non-malarial fever; RT-qPCR**	**304**	**0.0% (95% CI: 0.0-1.1%)**	**8**
Kenya	**Damajale, North Eastern Province[72]**	**Retrospective outbreak investigation, patients with new-onset fever / arthralgia; IgM ELISA**	**12**	**25.0% (95% CI: 8.3-53.9%)**	**6**
Kenya	Nyandarua + Turkana districts[73]	Cross-sectional serosurvey healthy people; MAT (11 serovars), suspicious ≥ 1:50; positive ≥ 1:200	681 (Nyandaru 315, Turkana 366)	Nyandaru: 0.0% (95% CI: 0.0-1.5%) (positive); 7.6% (suspicious) Turkana: 4.6% (95% CI: 2.9-7.4%) (positive); 9.3% (suspicious)	8

Table 2 Leptospirosis in sub-Saharan Africa in humans published after 1970
*In **bold** studies performed on diseased subjects. (continued)*

Country	Region	Method	Number of individuals	Prevalence / Incidence	Quality (1-10)
Kenya	Nairobi, Miwani + Ramisi[74]	Cross-sectional serosurvey healthy people; IgM ELISA + MAT, positive ≥ 1:80	690	7.0% (95% CI: 5.3-9.1%) (Miwani: 7.4%, Ramisi: 16.9%, Nairobi: 0.0%)	8
Kenya	**Coast Province[76]**	**Prospective cohort study, patients FUO / jaundice; MAT, positive > 1:3000 or 10x titre rise in convalescent sera**	**91**	**11.0% (95% CI: 5.9-19.2%)**	**8**
Kenya	**Nyanza province[75]**	**Prospective cohort study, patients FUO / jaundice; MAT, positive > 1:3000 or 10x titre rise in convalescent sera**	**281**	**3.2% (95% CI: 1.6-6.1%)**	**8**
Tanzania	**Moshi[87]**	**Incidence calculation**	**Based on Biggs 2011[9]**	**Incidence: Overall estimated 75 – 102 per 100.000 persons 0 to < 5y: 175-288 cases 5 to < 15y: 149-161 cases ≥ 15 years: 33 – 59 cases**	**9**
Tanzania	**Moshi[9]**	**Prospective cohort study febrile patients; MAT (17 serogroups), confirmed ≥ 4x titre rise: probable ≥ 1:800: exposure ≥ 1:100**	**831 (total sera) 453 (paired sera)**	**8.4% (95% CI: 6.7-10.5 %) confirmed / probable 36.4% (95% CI: 33.1-40.0%) evidence of exposure.**	**9**
Tanzania	**Tanga City[88]**	**Cross-sectional serosurvey healthy people; MAT (6 serovars), positive ≥ 1:160**	**199**	**15.1% (95% CI: 10.7-20.8%)**	**7**
Tanzania	**Countrywide[89]**	**Cross-sectional serosurvey healthy people; MAT (3 serovars), positive ≥ 1:160**	**375**	**0.3% (95% CI:0.0-1.7%)**	**5**
Somalia	Mogadishu area + Shabeele river[101]	Cross-sectional serosurvey in: 1. healthy people 2. **suspected viral hepatitis patients** MAT (20 serovars), positive ≥ 1:100	372	33.6% (95% CI: 29.0-38.6%) (Mogadishu 37.0%, Shabeele 64.0%, ill Mogadishu patients 56.2%)	8
Southern Africa					
Angola	**Huanda + Luambo[144]**	**Cross-sectional survey in febrile patients; MAT, positive ≥ 1:100**	**650**	**8.0% (95% CI: 6.1-10.4%)**	**7**

Table 2 Leptospirosis in sub-Saharan Africa in humans published after 1970
*In **bold** studies performed on diseased subjects. (continued)*

Country	Region	Method	Number of individuals	Prevalence / Incidence	Quality (1-10)
Mozambique	**Maputo[150]**	**Cross-sectional study in febrile patients; MAT (20 serovars), positive ≥ 1:100**	**160**	**8.8% (95% CI: 5.2-14.3%)**	**8**
Namibia	Kavango[160]	Cross-sectional study, healthy adults + patients immune thrombocytopenic purpura; MAT (case definition NR)	211	2.8% (95% CI: 1.2-6.2%)	6
South Africa	**Johannesburg[106]**	**Cross-sectional survey in febrile patients; IgM ELISA+ Nested PCR**	**332 (ELISA) 247 (PCR)**	**7.8% (95% CI: 5.4-11.3%) (ELISA) 0.0% (95% CI: 0.0-1.3%) (PCR)**	**7**
South Africa	Durban[107]	Cross-sectional serosurvey in healthy people: MAT (case definition NR)	217	18.9% (95% CI: 14.2-24.7%)	7
Zimbabwe	Harare, two farms[121]	Cross-sectional survey healthy farm workers & families; MAT (8 serogroups), positive ≥ 1:100	182	83.5% (95% CI: 77.4-88.3%)	7

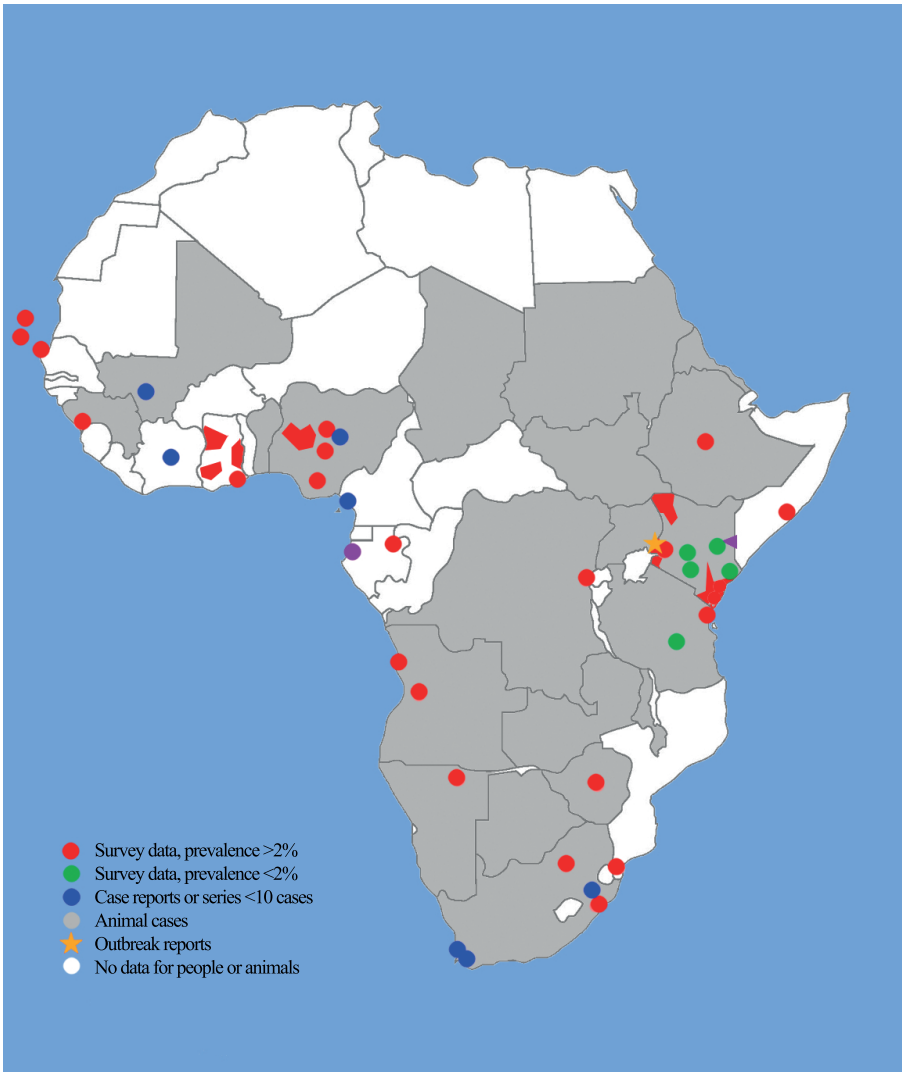

Figure 2. Availability and nature of reported epidemiological data. Prevalences and numbers refer to human cases unless specified otherwise.

Recent work from Cotonou, Benin, showed a prevalence of 18·9% in 90 rodents.[54] Rodents from Conakry, Guinea, showed lower figures:1·5% of 330 rodents were PCR-positive just after the rainy season. The same study demonstrated leptospirosis in 3/172 individuals (1·7%) with FUO.[55]

In Senegal[32,56,57] and the Cape Verde islands,[58] four surveys were performed in the seventies and eighties. In the Dakar region, 6·4% of 109 ill patients had positive titres, predominantly against the Icterohaemorrhagiae serogroup.[32] In a Dakar abattoir, with animals from all over the country, seroprevalence in cattle was 20·8% and 5·0% in sheep, goats,

and pigs.[57] On Santo Antão and Santiago, a serosurvey showed a prevalence of 7·2% in 611 healthy inhabitants, and lower rates in cattle, donkeys, sheep and goats.[58-60]

No data were retrievable from other West African countries, except for Mali. A Bamako survey showed 44·9% of 642 dairy cows to be seropositive.[61] No human data exist from Mali except for a case report.[62]

Central Africa

Data from the Central African region is very limited. Even though van Riel and colleagues performed extensive research on the disease in the Democratic Republic of Congo from 1939 onwards (see Supplementary file VII), no data was published after the country's independence in 1960. In 1995, a survey was performed in the Katana hospital in the Kivu region, before the arrival of longstanding, violent conflict. In a prospective cohort study on the aetiology of haemoglobinuria[63], the diagnosis of acute leptospirosis was established in one of 65 patients. This is probably an underestimation since the study was not designed to detect leptospirosis. Subsequently, a serosurvey in 61 antelopes from the Ituri rainforest showed leptospiral antibodies in 26·2%.[64]

Most Central African data comes from Gabon where in the 1990s, two surveys were performed in people.[65,66] In five villages in the northeast, a seroprevalence of 15·7% in healthy people was demonstrated.[65] No risk factors could be determined; members from the Bakoule tribe, traditionally fisherman, were found to have a higher risk of being seropositive. All villages were located in a gold panning area; this was not found to be a risk factor. Of 52 French military personnel presenting with FUO in Libreville, two had high single antibody titres against pathogenic serovars.[66] They had been dispatched in the field and exposed to freshwater. Recently, a case series was published of four men from poorer areas in Libreville with proven acute leptospirosis.[67]

Scolamaccia and colleagues[68] designed a prevalence model for cattle from the Adamwa region in Cameroon, adjusting for the diagnostic test performance and study design. Based on banked sera of 1.377 cows from 146 herds, they estimated the region-wide animal prevalence to be 30·4%, and 93·0% of herds to be positive.

The only other report originating from this region is a case report of a military dog from Chad with leptospirosis-induced renal insufficiency.[69]

East Africa

Most epidemiological data on leptospirosis in East Africa originates from Kenya[70-86] and Tanzania.[9,87-89] Since the fifties, Kenya has been key in establishing knowledge on the epidemiology of leptospirosis on the African continent. Since 1997, Tanzania, has evolved as the focus of many leptospirosis studies.

The only well established incidence calculation for leptospirosis in sub-Saharan Africa was done in 2013, for the rural and urban area of Moshi, in the Kilimanjaro Region of Tan-

zania.[87] Based on a prospective cohort study,[9] the overall estimated incidence was 75–102 per 100.000 persons per year, with the highest incidence in children under five, i.e. 175–288 cases per 100.000 per year (table 2). This is probably an underestimation because it only represents hospitalized cases. The higher incidence in younger children can be explained by their lower admission threshold.[87] In the aforementioned study[9] among febrile adults and children, 8·4% (70/831) serologically tested participants met the definition of confirmed or (highly) probable leptospirosis; another 277 patients (36·4%) exhibited evidence of exposure. Patients with confirmed or probable leptospirosis were older than those without (P<0·001), and serological evidence of exposure increased with age (P<0·001). In adults, thrombocytopenia was associated with confirmed (OR 3·5, P=0·005) or probable disease (OR 2·2, P=0·017). In children, lymphadenopathy was associated with leptospirosis (25·9% vs. 9·2%; P=0·14). The study demonstrates clinicians' unawareness of leptospirosis: provisional diagnoses in confirmed and probable cases were mainly malaria and pneumonia; leptospirosis was never included into the differential diagnosis. 40% of confirmed cases suffered from a co-infection; HIV (15%), rickettsiosis, brucellosis, and community acquired blood stream infections (12·5% each) were most prevalent. No association between HIV and leptospirosis could be demonstrated, in contrast to the later re-analysis of this study[9], where leptospirosis was found to be less prevalent in a group of HIV infected patients (OR 0·43, P=0·019).[90] The only risk factor identified was living in a rural area (OR 3·4, P<0·001). No distinguished link was found between chest X-ray abnormalities and leptospirosis.[91]

In the Tanga region, bordered by the Indian Ocean and Kilimanjaro mountains, a 2009 survey showed a seroprevalence of 15·1% among 199 healthy inhabitants; milking of cows constituted a risk (OR 3·44, P<0·001).[88] Serosurveys in cattle demonstrated prevalences varying from 11·0%,[92] and 30·0%[93] in larger surveys, with upto 51·0% in a smaller abattoir survey.[94] A countrywide serosurvey among healthy blood donors (n=375) showed a low prevalence of 0·3%;[89] however, the sera were only screened against three serovars.

First reports of leptospirosis in Kenya date from 1944 in cattle. De Geus and colleagues later conducted 2 studies in Coast[76] and Nyanza provinces,[75] in adults and children with FUO or jaundice. Applying strict case definitions, 11·0% from 91 patients from Coast and 3·2% of 281 patients from Nyanza province were found to suffer from acute leptospirosis. A significant higher prevalence in males, and in hospitals close to sugar-estates was found.[95,96] In 1987, 7·4% of 353 healthy people in Nyanza province, and 16.9% of 130 in Coast province were found to have leptospiral antibodies;[74] recent outbreak investigation of malaria-negative fever in 21 patients in Malindi in Coast province could not prove leptospirosis.[70]

In Kibera, a densely populated part of Nairobi with poor sanitary conditions, leptospiral DNA was recently demonstrated in 41/224 (18·3%) of rodents.[77] Human data available from Nairobi is from 1987; none of 207 healthy subjects had leptospiral antibodies.[74] Recent studies in patients with FUO in North East province did not yield positive *Leptospira* PCRs;[71] yet no serological tests were performed. However, a case series after an outbreak of acute febrile

illness amongst pastoralists showed leptospiral antibodies in 3/12 patients.[72] In the eighties, a seroprevalence study was performed amongst people from Nyandaru (0·0%) and Turkana (4·6%) districts; moreover, it showed high seroprevalences in Nyandaru sheep, goats, and cattle from (~34% each); lower prevalences were reported in Turkana.[73] A 1982 countrywide study in 2.864 cows showed a seroprevalence of 25·0%.[81] Ten years later, 898 sheep and goats from all over the country were found to have an antibody seroprevalence of 16·0%.[79]

In Ethiopia, three serosurveys,[97-100] performed in several animal species in the seventies, demonstrated leptospirosis endemicity.[99,100] In 2004, 47·5% of 59 febrile patients in Wonji tested positive for leptospiral antibodies with the Lepto Tek Dri-Dot test.[97] However, these results could not be reproduced or confirmed in a reference laboratory (Royal Tropical Institute, The Netherlands) (MGAG, personal communication).

In Somalia, from 1975 to 1976,[101] of 105 healthy adults from (dry) Mogadishu, 37·1% were found to be seropositive, with a significant higher seropositivity in people from wetter areas (63·5%; 68/107) near the Shabeele river; close contact with cattle was a risk factor.

Data from other East African countries is scarce. Recently, leptospirosis was demonstrated in Uganda; 26·7% of 116 owned dogs from in and around three national parks were seropositive.[102] An earlier study in buffalos from the same park showed no signs of leptospirosis.[103] A study in Djibouti[104] revealed remarkably high seroprevalence in a small group of horses, camels, and cattle. Figures from Sudan date back to the eighties, when 15·3% from 1.142 cows[105] from all over the country were seropositive; 9·8% of a group of 717 wild mammals from Melut district,[60] and infection was demonstrated in rodents.[105]

Figure 3. Malawian cattle, typical probable host for leptospirosis.

Southern Africa

Forty-six studies from Southern Africa were identified (see table 1). Most studies came from South Africa (n=15)[80,106-120] and Zimbabwe (n=17).[121-136] No data was found from Swaziland and Lesotho.

Feresu and colleagues established knowledge on leptospirosis in Zimbabwe. In 1995/6, they demonstrated that 83·5% of 182 workers and their families from two farms in and around Harare carried leptospiral antibodies in one of the samples after following them for 1·5 years; they could not identify any seasonal influences.[121] Of 437 rodents from the same farms, 62·5% were seropositive.[121] In the early 80s, 26·8% of a cattle herd (n=2382) were seropositive,[127] and later leptospires were isolated in 10.4% of 480 cattle in a Harare abattoir.[129] These isolates revealed several new serovars and strains.[130-134,136] In Harare, rural dogs (n=250) were found to have a positive ELISA in 15·6%.[122] Similar numbers in dogs were found in 1979.[137] Zimbabwean wildlife has been studied: in rhinoceroses numbers varied from 63·0% (38/60)[125] to 4·9% in a group of 102.[124] Buffalos, elands, wildebeests, and zebras were also found to carry antibodies.[124] No other human data from Zimbabwe was retrieved except two case reports.[138,139]

South Africa, human data is relatively scarce: two surveys were published.[107,140] In 2010, 7·8% of 332 febrile patients from Johannesburg were seropositive;[140] a survey in Durban among healthy volunteers showed a seroprevalence of 18·9%, with a significantly higher percentage among people from 18-22 years.[107] The latter study found seropositivity in 22 of 221 rodents trapped in the same area, and 14·5% of 69 kidneys to be PCR positive. Other sporadic data on human leptospirosis dates from the eighties.[141-143]

Concerning livestock, a survey in 33 districts of KwaZulu-Natal proved 19·4% of 2.021 cows to be seropositive.[109] Other surveys showed numbers varying from 3·0% (of 860 cows) in Mpumalanga Province[114] to 52·4% in Transvaal.[116] Cows and pigs showed high prevalence rates after abortion-clusters in pigs in farms in Mpumalanga, Gauteng Province and Free State.[110] Earlier outbreak investigation in pigs showed similarly high rates.[120] An abattoir sero-survey demonstrated a 22·2% prevalence in 5.041 pigs from several regions.[111] In Transvaal, 1.3% of 152 sheep had antibodies.[116]

Seropositivity was shown in rhinoceroses (n=182) from four National Parks (26·4%);[80] buffaloes from Kruger National Park (7/406);[114] in a small group of wild animals from Northern Natal (12%);[115] and in 1978 in 8·0% of 50 vervet monkeys.[119]

In South African dogs, rates were equally varied: 86.5% in 37 ill dogs in the early seventies;[120] 1.5% (6/400) in healthy dogs in Pretoria;[112] a recent study in four coastal regions demonstrated a seroprevalence of 4·7% in 530 dogs. Stray dogs were 4·4 times more likely to have antibodies compared to owned dogs (7·6% vs. 1·9%, P=0·0017), with dogs in KwaZulu Natal and Eastern Cape more likely to be positive.[108]

In Angola, in a group of 650 febrile patients, 8·0% were probable cases of leptospirosis. 13·0% of 77 rodents showed PCR positive kidney samples.[144] Zambian rodents, recently

showed a low seroprevalence (1/466).[145] Of note, reservoir rodents often do not have antibodies against the serovar they are carrying.[146] A survey of piggeries around Lusaka showed positive serology in 15% of 121 pigs.[126] No data for human infection is available from Zambia, Malawi, or Botswana. The only available countrywide Malawian survey showed a 21·5% (59/275) seroprevalence in cattle in 1989.[147] Recently, 43·0% of 42 Botswanan mongooses had PCR positive kidney tissue.[148] After a 1983 abortion cluster in cattle in the Chobe district (Botswana), 27/40 cows were seropositive.[149]

In Mozambique, 8·8% of 160 febrile patients in Maputo carried leptospiral antibodies. In a subgroup of 43 patients with non-specific febrile illness, four (9·3%) showed high MAT titres, which could direct towards acute leptospirosis. No risk factors were identified.[150]

Travellers ex Africa

Leptospirosis is sporadically reported in travellers from Africa. The GeoSentinel surveillance network reported five cases of leptospirosis in travellers coming from sub-Saharan Africa between 1996 and 2011.[152] EuroTravNet reported one case each from Cameroon, the Central African Republic, and Réunion.[153] Returning from a water sports holiday in South Africa, a 49-year old man presented with acute leptospirosis and a Jarisch-Herxheimer reaction after treatment initiation. He recovered completely.[154] A young Australian female returning from Nigeria died due to myocarditis and a complete AV-block due to haemorrhages, caused by *L. icterohaemorrhagiae*.[155] A French female returning from Ivory Coast was reported ill in 1997, having lived in a rural area with extensive livestock contact.[156] In the Netherlands alone, seven travellers returning from sub-Saharan Africa were diagnosed with leptospirosis in the period 1985-2008.[6]

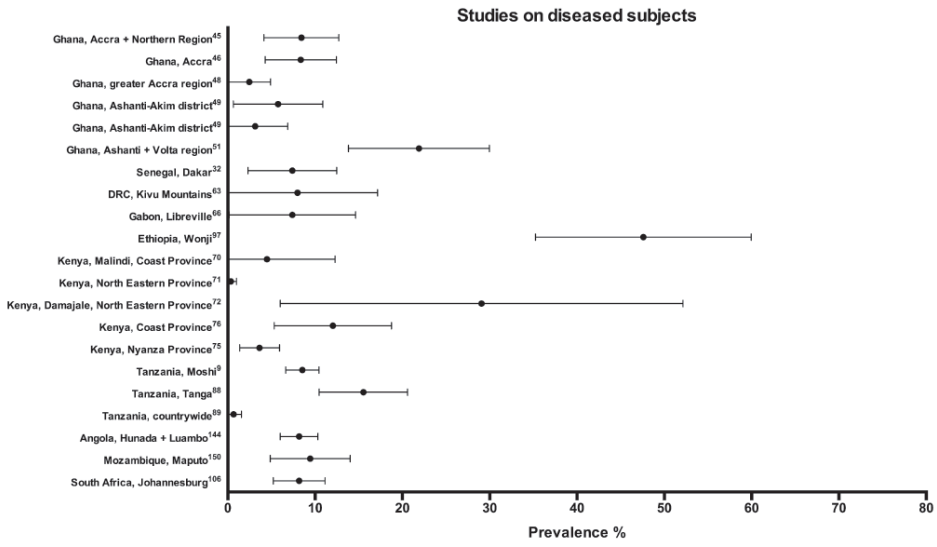

Figure 4. Prevalence of leptospirosis in studies on diseased subjects, presented with their 95% confidence intervals.

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970

Country	Region	Type of animal	Method	No of animals	Prevalence
West Africa					
Benin	Cotonou[54]	Rodents	Organ survey PCR kidney samples	90	18.9%
Cape Verde Islands	Santo Antao & Santiago[58]	Cattle	Serosurvey (MAT 1:100)	150	2.0%
Cape Verde Islands	Santo Antao & Santiago[58]	Goats	Serosurvey (MAT 1:100)	640	5.3%
Cape Verde Islands	Santo Antao & Santiago[58]	Sheep	Serosurvey (MAT 1:100)	39	0.0%
Cape Verde Islands	Santo Antao & Santiago[58]	Donkeys	Serosurvey (MAT 1:100)	61	3.3%
Cape Verde Islands	Santo Antao & Santiago[58]	Pigs	Serosurvey (MAT 1:100)	316	0.0%
Cape Verde Islands	Santo Antao & Santiago[58]	Dogs	Serosurvey (MAT 1:100)	89	0.0%
Cape Verde Islands	Santo Antao & Santiago[58]	Rodents	Serosurvey (MAT 1:100)	211	0.0%
Cape Verde Islands	Santo Antao & Santiago[58]	Horses	Serosurvey (MAT 1:100)	3	0.0%
Guinea	Conakry[55]	Rodents	Organ survey (PCR + culture kidney tissue)	330	1.5%
Nigeria	Ibadan + Abeokuta[33]	Cattle	Abattoir survey (kidney culture)	108	82.4% (Ibidan: 95.2%, Abeokuta: 79.3%)
Nigeria	Ibadan[34]	Sheep	Abattoir serosurvey (MAT 1:100)	255	23.5%
Nigeria	Ibadan[34]	Goats	Abattoir serosurvey (MAT 1:100)	320	13.1%

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Nigeria	Ibadan, Lagos + Ilorin[35]	Dogs	Prospective serological survey in sick dogs with positive LS serology MAT positive ≥ 1:100 (vaccinal serovars) + a 4-fould rise in 10-14 days or a single titre between 1:800 to 1:3200 (non-vaccinal serovars). Vaccinal serovars: Canicola & Icterohaemorrhagiae Treatment: ampicillin/amoxicillin/ enrofloxacin, rehydration, diuretics.	52 (98% were (51/52) primarily vaccinated)	100.0% (see inclusion criteria) - Clinical signs: sudden illness, anorexia, vomiting, depression, dehydration, oliguria, subnormal rectal temp, anuria, rnomegaly, uremia - Urinalysis: occult blood (61.5%), bilirubin (51.9%), protein (11.5%)
Nigeria	Ibadan[36]	Dogs	Serosurvey (MAT)	NR	16.7%
Nigeria	Ibadan[36]	Pigs	Abattoir serosurvey (MAT)	423	NR
Nigeria	Kaduna state, Zaria[40]	Cattle	Serosurvey (ELISA)	164	11.0%
Nigeria	Kaduna state, Zaria[38]	Cattle	Serosurvey (ELISA)	237	8.4%
Nigeria	Kaduna state, Zaria[39]	Cattle	Abattoir serosurvey (ELISA)	142	3.5%
Nigeria	Kaduna State, Zaria[41]	Cattle	Organ survey (culture)	74	6.8%
Nigeria	Kaduna State, Zaria[41]	Rodents	Serosurvey (NR)	252	4.0%
Nigeria	Plateau State[42]	Cattle	Serosurvey (MAT 1:100)	1537	14.4%
Nigeria	Plateau State[43]	Cattle	Isolation from kidney	NR	5 strains isolated, 1 new serovar and strain: serovar Nigeria, strain Vom
Nigeria	Plateau State[44]	Rodents	Organ survey (culture)	503	0.0%
Nigeria	Plateau State[44]	Rodents	Serosurvey (MAT 1:100)	105	44.8%
Mali	Bamako[61]	Cattle (dairy)	Serosurvey (MAT 1:100)	642	44.9%
Senegal	Cap-Vert region[56]	Rodents	Sero-+ organ survey	170	0.0%

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Senegal	Dakar[57]	Cattle	Abattoir serosurvey (MAT 1:100)	433	20.8%
Senegal	Dakar[57]	Sheep	Abattoir serosurvey (MAT 1:100)	200	9.0%
Senegal	Dakar[57]	Goats	Abattoir serosurvey (MAT 1:100)	150	0.6%
Senegal	Dakar[57]	Pigs	Abattoir serosurvey (MAT 1:100)	66	4.5%
Cameroon	Adamwa[80]	Cattle	Serosurvey (ELISA): estimation true seroprevalence adjusting for diagnostic test performance and study design.	1.377 from 146 herds	P_{animal}=0.304 (95% CI 0.276–0.332) (30.4%) P_{herd}=0.933 (95% CI 0.894–0.972) $P_{withinherd}$=0.334 (95% CI 0.304–0.364)
Cameroon	Adamwa[161]	Cattle	Risk-factor analysis (based on (Scolamacchia et al. 2010[80])	1.377 from 146 herds	Risk Factors: Cattle > 2 y OR 2.76 (95% CI 2.15-3.55). Mixing herds at night (OR 1.48 (95% CI 1.05-2.07)
Chad	NR[69]	Dog	Case report MAT + ELISA	1	
DRC	Ituri Forest[64]	Duikers	Serosurvey (MAT 1:100)	61	26.2%
Djibouti	NR[104]	Cattle, horses, camels	Serosurvey (MAT)	31	84.0%
Ethiopia	NR[98]	Dogs	Serosurvey (RMAT)	NR	0.0%
Ethiopia	West & Southern[99]	Rodents	Organ + serosurvey (MAT + culture)	376	0.0%
Ethiopia	West & Southern[99]	Cattle	Serosurvey (MaAT)	137	26.3%
Ethiopia	West & Southern[99]	Sheep	Serosurvey (MaAT)	31	0.0%
Ethiopia	West & Southern[99]	Goats	Serosurvey (MaAT)	36	16.7%
Ethiopia	NR[100]	Cattle	Serosurvey (MAT 1:100)	307	70/7%
Ethiopia	NR[100]	Sheep	Serosurvey (MAT 1:100)	159	43.3%
Ethiopia	NR[100]	Goat	Serosurvey (MAT 1:100)	93	47.3%
Ethiopia	NR[100]	Camels	Serosurvey (MAT 1:100)	78	15.4%
Ethiopia	NR[100]	Pigs	Serosurvey (MAT 1:100)	21	57.1%

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Ethiopia	NR[100]	Dogs	Serosurvey (MAT 1:100)	12	8.3%
Ethiopia	NR[100]	Rodent	Serosurvey (MAT 1:100)	6	0.0%
Ethiopia	NR[100]	Horses	Serosurvey (MAT 1:100)	34	91.2%
Ethiopia	NR[100]	Monkeys	Serosurvey (MAT 1:100)	24	0.0%
Ethiopia	NR[100]	Birds	Serosurvey (MAT 1:100)	24	0.0%
Kenya	Nairobi, Kibera[77]	Rodents	Organ survey (PCR)	237	18.3% (95% CI 13.5–24.0%)
Kenya	NR[78]	Cattle	Outbreak report (NR)	34	70.6% (24/34) affected, fed on a fresh batch of hay.
Kenya	Countrywide[79]	Goats	Serosurvey (NR)	898	16.2%
Kenya	Nyandaru district[73]	Cattle	Serosurvey (MAT 1:200)	326	34.0% (MAT 1:50: 49.0%)
Kenya	Turkana district[73]	Cattle	Serosurvey (MAT 1:200)	439	22.3% (MAT 1:50: 44.2%)
Kenya	Nyandaru district[73]	Sheep & goats	Serosurvey (MAT 1:200)	357	34.2% (MAT 1:50: 55.0%)
Kenya	Turkana district[73]	Sheep & goats	Serosurvey (MAT 1:200)	369	3.8% (MAT 1:50: 24.0%)
Kenya	Countrywide[80]	Rhinoceroses	Serosurvey (MAT 1:50)	58	15.5%
Kenya	Countrywide[81]	Cattle	Serosurvey (MAT 1:200)	2.864	25.0%
Kenya	Coast Province, Nyaza Province[82]	Rodent	Isolation & typing of new serovars	3	1. Tarassovi, serovar Kanana 2. Autumnalis, serovar Lambwe 3. Ballum, serovar Njenga
Kenya	NR[84]	Bovine	Serological survey	NR	“high incidence “ (NR)
Kenya	Kiambu area[85]	Bovine	Outbreak report	NR	NR
Kenya	Countrywide[86]	Cattle	Outbreak report	NR	Yearly outbreak reports concerning hundreds of cattle

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Kenya	Nakuru district, Rift valley province[86]	Cattle	Outbreak report	NR	Outbreak on a farm – disease was later spread to Nakuru, Nyandarua, Baringo, Rumuruti, Thika + Naivasha
Kenya	Kabete[83]	Cattle	NR	NR	NR
Sudan	Melut district[60]	Cattle	Serosurvey (MAT 1:100)	195	54.0%
Sudan	Melut district[60]	Wild mammals (22 species)	Serosurvey (MAT 1:100)	717	9.8%
Sudan	Melut district[60]	Wild birds (14 species)	Serosurvey (MAT 1:100)	54	1.9%
Sudan	Countrywide[105]	Cattle	Serosurvey (MAT)	1.142	15.3% (higher rates wetter areas)
Sudan	Countrywide[105]	Goats	Serosurvey (MAT)	30	13.3% (higher rates wetter areas)
Sudan	Countrywide[105]	Rats	Serosurvey (MAT)	56	23.3% (higher rates wetter areas)
Sudan	Omdurman[105]	Cattle	Organ survey (kidney immunofluorescence)	235	2.5%
Sudan	NR[162]	Animal	NR	NR	NR
Tanzania	Central region[163]	Rodent	Serosurvey (MAT 1:20)	350	17.7%
Tanzania	Tanga city[94]	Cattle	Serosurvey (MAT 1:160)	51	51.0% (95% CI 44.1 – 57.9)
Tanzania	Tanga region[93]	Cattle	Serosurvey (MAT 1:160)	655 from 130 herds	Cattle: 30.3% (95% CI = 26.7-33.9) Herds: 58.5% (95% CI = 49.5-67.1)
Tanzania	Morogoro[164]	Pigs	Serosurvey (MAT 1:160)	385	4.42%
Tanzania	Morogoro[164]	Pigs	Urine survey (culture)	236	0.8%
Tanzania	Countrywide[89]	Rodents	Serosurvey (MAT 1:160)	537	1.9%
Tanzania	Countrywide[89]	Cattle	Serosurvey (MAT 1:160)	374	7.5%
Tanzania	Countrywide[89]	Dogs	Serosurvey (MAT 1:160)	208	37.5%
Tanzania	Countrywide[89]	Cattle	Urine survey (culture)	1.021	0.7%

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Tanzania	East Usambara Mountains[165]	Cattle	Serosurvey (MAT 1:160)	80	21.3%
Tanzania	Morogoro[166]	Rodents	Organ survey (PCR + culture + MAT)	27	15.0% (PCR), 7.4% (isolation), 0% (MAT)
Tanzania	Tanga region[92]	Cattle	Serosurvey (MAT)	230	10.8% (95%CI = 7.15, 15.62)
Tanzania	Morogoro[167]	Rodents	Isolation + characterization isolates (Sh9 + Sh25), Cross-agglutination absorption test (CAAT) and DNA fingerprinting	2	N.A.
Tanzania	Morogoro[168]	Cattle	Isolation + typing L kirschneri CAAT + monoclonal antibodies, PCR	1	N.A.
Tanzania	Central region[169]	Rodents	Model of LS infection dynamics in rodents	N.A.	- strong seasonality in the force of infection of humans with a peak between January and April in agricultural environments - in urban areas the dynamics are more stable, with a period of high number of infected animals from February to July - removal of animals by trapping will have a greater impact on human LS (instead of reducing suitability environment)
Uganda	NR[102]	Dogs	Serosurvey (MAT 1:200)	116	26.7% (95% CI 19.0 – 36.1)
Uganda	Queen Elizabeth + Bwindi Impenetrable National Park[170]	Cattle African buffalo	Cross-sectional serosurvey ELISA IgG + IgM for L.Hardjo	Cattle 92 Buffalo 92	Cattle: 29.4% Buffalo: 42.4%
Uganda	Queen Elizabeth + Bwindi Impenetrable National Park[103]	African Buffalo	Serosurvey (MAT)	42	0.0%
Angola	NR[144]	Rodents	Organ survey (PCR + culture)	77	13.0% (PCR), 10.8% (culture, from 37)
Botswana	Chobe district[148]	Banded mongooses	Organ survey (PCR)	41	41.5% (95% CI 27.7 – 56.7%)

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Botswana	Chobe district[148]	Selouse mongoose	Organ survey (PCR)	1	100.0%
Botswan	Tropic of Capricorn[149]	Cattle	Abortion outbreak investigation, urine + serosurvey (culture + MAT 1:80)	47	67.5% MAT (27/40), 1/7 isolation of Pomona
Namibia	Damaraland[125]	Rhinoceroses	Serosurvey (MAT 1:100)	3	0.0%
Namibia	Waterberg Plateau[80]	Rhinoceroses	Serosurvey (MAT 1:50)	32	9.4%
Namibia	Etosha National Park[171]	Impala	Serosurvey (MAT 1:100)	27	0.0%
Malawi	Countrywide[147]	Cattle	Abattoir serosurvey (MAT 1:100)	275	21.4%
Mozambique	Maputo[151]	Pigs	Abortion outbreak investigation (MAT serum fetus)	80	0.0%
South Africa	Johannesburg[106]	Rodents	Organ serosurvey	193	0.0%
South Africa	Coastal regions[108]	Dogs	Serosurvey (MAT 1:100)	530	4.7% (4.347 times more in stray dogs, more risk in Eastern Cape + KwaZulu-Natal)
South Africa	KwaZulu-Natal[109]	Cattle	Serosurvey (MAT)	2.021	19.4% (95% CI, 14.8-24.1%)
South Africa	Durban, Cato Crest[107]	Rodents	Serosurvey (LeptoTek Dri-Dot)	221	10.0%
South Africa	Durban, Cato Crest[107]	Rodents	Serosurvey (PCR)	69	14.5%
South Africa	Four National Parks[80]	Rhinoceroses	Serosurvey (MAT 1:50)	182	26.4%
South Africa	Mpumalanga Province[110]	Pigs	Abortion outbreak investigation farm Organ + serosurvey (kidney + fetal culture + MAT 1:160)	52 serology 9 organs	17.0% 9/9 isolation of Pomona
South Africa	Mpumalanga Province[110]	Cattle	Serosurvey piggery (see above) (MAT 1:160)	170	52.0%

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
South Africa	Mpumalanga Province[110]	Sheep	Serosurvey piggery (see above) (MAT 1:160)	153	1.3%
South Africa	Mpumalanga Province[110]	Horses	Serosurvey piggery (see above) (MAT 1:160)	13	39.0%
South Africa	Gauteng Province[110]	Pigs	Abortion outbreak investigation farm Organ + serosurvey (kidney + foetal culture + MAT 1:160)	64	36.8% during outbreak, 0% after outbreak 9/9 isolation of Pomona
South Africa	Gauteng Province[110]	Cattle	Serosurvey after abortion outbreak (MAT 1:160)	19	58.0%
South Africa	Gauteng Province[110]	Sheep	Serosurvey after abortion outbreak (MAT 1:160)	24	12.5%
South Africa	Gauteng Province[110]	Dogs	Serosurvey after abortion outbreak (MAT 1:160)	6	50.0%
South Africa	Gauteng Province[110]	Horses	Serosurvey after abortion outbreak (MAT 1:160)	6	0.0%
South Africa	Gauteng Province[110]	Rodent	Organ survey after abortion outbreak (culture)	11	0.0%
South Africa	Free State[110]	Cattle	Outbreak investigation after death cows, serosurvey (MAT 1:100)	222	49.8% of herd, 54.0% of recently ill animals
South Africa	Free State[110]	Cattle	Outbreak investigation after death cows, organ + urine survey (culture)	13	3/12 urine serovar Pomona 1/1 kidney serovar Pomona
South Africa	Free State[110]	Pigs	Outbreak investigation after death cows, organ + serosurvey (culture + MAT 1:100)	36	45.0% (9/20) positive MAT 6/16 kidneys serovars Pomona
South Africa	Free State[110]	Dogs	Outbreak investigation after death cows, serosurvey (MAT 1:100)	8	0.0%
South Africa	Free State[110]	Rodents	Outbreak investigation after death cows, serosurvey (MAT 1:100)	8	0.0%
South Africa	Countrywide[111]	Pigs	Abattoir serosurvey (MAT 1:80)	5.041	22.2%

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
South Africa	Greater Pretoria area[112]	Dogs	Serosurvey (MAT)	400	1.5%
South Africa	Volksrust district[113]	Cattle	Serosurvey (MAT 1:80)	860	3.0%
South Africa	Kruger National Park[114]	Buffalo	Serosurvey (MAT)	406	1.7% (6.6% inconclusive)
South Africa	Northern Natal[115]	Wild mammals (12 species)	Serosurvey (MAT 1:40)	50	12.0%
South Africa	Transvaal[116]	Cattle	Serosurvey (MAT)	170	52.0%
South Africa	Transvaal[116]	Sheep	Serosurvey (MAT)	152	1.0%
South Africa	Transvaal[116]	Pigs	Serosurvey (MAT)	52	17.0%
South Africa	Transvaal[116]	Horses	Serosurvey (MAT)	13	38.0%
South Africa	Transvaal[116]	Dogs	Serosurvey (MAT)	2	100.0%
South Africa	Onderstepoort[117]	Cattle	Serovar isolation and typing from cow-urine	1	N.A.
South Africa	Transvaal[118]	Cattle	Serovar isolation and typing from cow-urine after abortion storm	1	N.A.
South Africa	Countrywide[119]	Vervet monkeys	Serosurvey (Complement Fixation Test)	50	8.0%
South Africa	NR[120]	Pigs	Outbreak investigation abortion outbreak, organ + serosurvey (culture + MAT 1:100)	250	62.1% MAT positive 6/10 isolation canicola
South Africa	NR[120]	Dogs	Outbreak investigation illness outbreak, organ + serosurvey (culture + MAT 1:100)	38	86.5% MAT positive, 1/1 isolation of Canicola
Zambia	NR[145]	Rodents	Serosurvey (ELISA *Leptospira* rLipL32 antigen)	466	0.2%
Zimbabwe	Harare + rural communities[122]	Dogs	Serosurvey (ELISA IgG)	250	15.6% (95% CI: 11.0% - 20.2%) (significant higher prevalence in urban dogs)
Zimbabwe	Mashonaland East Province[123]	Pigs	Abstract + full text not available		

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Zimbabwe	NR[124]	Wildlife	Serosurvey (MAT)	992	4.6 % (Buffalo 21/347 (6.0%), eland 8/74 (10.8%), wildebeest 9/54 (16.7%), zebra 2/11 (18.2%), white rhinoceroses 1/72 (1.4%), black rhinoceroses 5/102 (4.9%))
Zimbabwe	Harare[121]	Rodents	Organ, urine + survey (culture + MAT 1:100)	437	62.5% MAT, 52 isolates (40 from kidneys, 12 from urine)
Zimbabwe	Zambezi Valley + Escarpment[125]	Rhinoceroses	Serosurvey (MAT 1:100)	60	63.0% (88.0% in river side animals, 18.0% in escarpments animals)
Zambia	Lusaka[126]	Pigs	Serosurvey (NR)	121	14.9%
Zimbabwe	Urban areas[127]	Cattle	Serosurvey (MAT 1:100)	2.382	27.0% (significant higher in commercial farming and purchase areas)
Zimbabwe	Harare (animals from Mashonaland + Manicaland)[129]	Cattle	Abattoir organ survey (culture)	480	10.4%
Zimbabwe	Harare[130]	Cattle	Isolation + typing by CAAT, monoclonal antibody + restriction endonuclease DNA analyses of new Serovar found in study Feresu et al. 1992[129]	1	N.A.
Zimbabwe	Harare[131]	Bovine	Isolation + typing by CAAT, monoclonal antibody + restriction endonuclease DNA analyses of new Serovar found in study Feresu et al. 1992[129]. CAA + RFLPA	5	N.A.
Zimbabwe	Harare[132]	Bovine	Identification of strains (Feresu 1992[129], 1993[130], 1994[131]) by CAAT mAbs, RFLP and PCR	2	N.A.

Table 3 Leptospirosis in sub-Saharan Africa in animals published after 1970 (continued)

Country	Region	Type of animal	Method	No of animals	Prevalence
Zimbabwe	Harare[133]	Bovine	Isolation + typing by CAAT, RFLP, pulsed-field gel electrophoresis of genomic DNA of new isolates found in study Feresu et al. 1992[129]	4	N.A.
Zimbabwe	Harare[134]	Bovine	Isolation + typing by CAAT and RFLP of new isolates found in study Feresu et al. 1992[129]	2	N.A.
Zimbabwe	Harare[136]	Bovine	Isolation + typing by CAAT, RFLP, PCR of new isolates found in study Feresu et al. 1992[129]	1	N.A.
Zimbabwe	NR[137]	Dogs	NR	146	13.7% (19/146)

MAT = Microscopic agglutination test, MaAT = Macroscopic agglutination Test, ELISA = Enzyme-linked immunosorbent assay, CAAT = Cross-Agglutinin Absorption Test, mAbs = mouse monoclonal Antibodies, RFLP = Restriction Fragment Length Polymorphism, PCR = Polymerase Chain Reaction

Table 4 Miscellaneous – environmental study

Country	Study site	Type of sample	Method	No samples	Prevalence	*Serogroup* / Serovar	Other findings
South Africa	Bruma, Rhodes Park, Gilloolys Farm, Modderfontein, Alexandra (part Juksie Rive catchment) [106]	Environmental samples: water & soil	Cross-sectional environmental study from 10 different sites along Juksie river in 2 years time. Nested PCR (*Leptospira* 16S rDNA	Water 77 Soil 79	Soil: 23.8% soil Rhodes Park + Bruma 9.5% soil Dobsonville, Gilloolys + Modderfontein 4.8% soil Alexandra Extension Water: 14.3% water Bruma Lake 4.8% water Modderfontein Dam	NR	Soil vs water P = 0.0018 (chi-square 7.5 1 degree freedom)

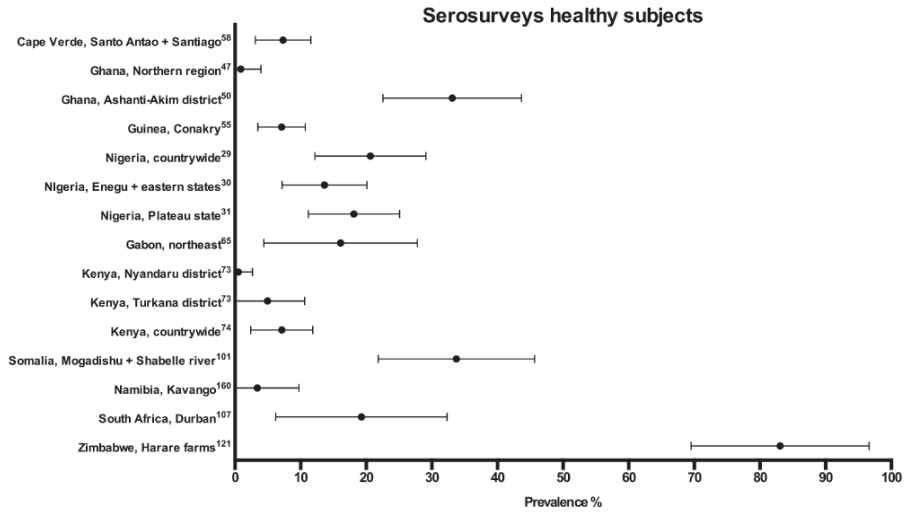

Figure 5. Prevalence of leptospirosis in studies on healthy subjects, presented with their 95% confidence intervals.

DISCUSSION

Leptospirosis is a zoonosis of global importance that remains under-recognized in some parts of the world. This applies to much of sub-Saharan Africa, reflected in the fact that only observational studies were identified, while interventional studies were lacking. With an ever-growing proportion of febrile disease episodes being recognized as non-malarial (due to the recent scaling-up of rapid diagnostic malaria testing before treatment initiation), leptospirosis is highlighted as one important differential diagnosis. Additionally, adequate diagnostic facilities across Africa are increasing. We depict leptospirosis as a highly prevalent, ubiquitous disease in sub-Saharan Africa and confirm The Leptospirosis Aphorism, "*Wherever leptospires and leptospirosis is searched for, they are invariably discovered*".[157] However, our data compilation is not conclusive, due to substantial between-study variability. Large regional differences in reporting exist, and recent epidemiological data on the prevalence of leptospirosis is scarce for many African countries (particularly Central Africa). Limited efficient surveillance systems and data collection methods result in incomplete information regarding the epidemiology of disease. Remarkably, more research is conducted in animals compared to humans. This is probably due to the larger economic losses caused by leptospirosis in livestock (e.g. abortion) compared to losses due to human morbidity and mortality. Undoubtedly, animal leptospirosis climbs higher on the political agenda compared to its neglected human counterpart.

Epidemiological data derived from animal studies as described in this review should be interpreted with caution. First, the slaughter of animals above certain ages creates an un-

avoidable selection bias in livestock surveys (often abattoirs). This gives a distorted prevalence because higher seroprevalence of leptospirosis is related to older host ages. Second, prevalence and incidence of leptospirosis fluctuates; there is usually more leptospirosis during, and just after the rainy and harvest season. Often, studies did not report the season and time frame of the study and are therefore not easily comparable to other studies. Finally, large proportions of reservoir hosts have no antibodies against the serovar they carry. Thus, while seroprevalence studies in healthy animal populations indicate levels of local infection exposure, these are less suited to define their role as infection reservoirs. Therefore, assumptions that the seroprevalence in animals corresponds with leptospirosis prevalence or incidence in humans cannot easily be made. Animals with low seroprevalence may be a substantial cause of infection in humans; high seroprevalence may indicate exposure pressure from different animal populations, hence a high infection risk in humans as well.

In human studies, two types of survey are distinguished; studies establishing leptospirosis prevalence as a cause of (mostly febrile) illness and background seroprevalence studies. The background studies showed variable, but generally high seroprevalences, which were elevated in risk groups. This does not necessarily mean a high disease burden; asymptomatic seroconversion in young children in rural areas occurs,15 which may apply to most leptospiral infections in general. Patient surveys show varying data. An equivalent exposure level to leptospirosis does not necessarily result in the same prevalence of clinical disease, possibly due to different immunity patterns and circulating serovars. Variations in inclusion criteria, case definitions, and diagnostic tools make outcome data comparison between different studies and regions challenging. In some of the original articles, the terms fever of unknown origin (FUO) or pyrexia of unknown origin (PUO) have been used. With some degree of uncertainty as to whether these classifications would match with the precise definition of what constitutes FUO according to the current accepted definition, we decided to refer to those conditions consistently as 'undifferentiated febrile disease' (UFD) in order to avoid confusion.

Further studies are required to elucidate the exact epidemiology of leptospirosis in sub-Saharan Africa. Even in regions where leptospirosis research has been conducted, such as East Africa, researchers believe that it remains under-diagnosed because of lack of awareness, facilities, and surveillance systems. Many questions remain unanswered, and new questions continue to arise (see Panel). Moreover, countries are confronted with larger scale epidemics (HIV, tuberculosis, malaria), which consume already limited resources. Thus, leptospirosis can be considered a neglected tropical disease in sub-Saharan Africa. Knowledge of the human leptospirosis burden has not yet translated into increased or strengthened control and prevention programmes, nor is it regularly considered routinely as an important differential diagnosis in non-malarial fevers.

In most countries, an adult predominance in clinical disease has been noted, which is not reflected across all clinical surveys. The only incidence calculation, from Tanzania, showed a higher (serological) incidence in younger age groups,[87] which can only partly be explained

by a lower hospital admission threshold for children. Immunological and genetic host factors may play a particular role in the development of active disease. However, the same study showed that patients with confirmed or probable leptospirosis were older than those without leptospirosis;[9] or there was no difference in age groups at all.[158] Almost certainly, exposure to leptospirosis starts at a young age and infection often remains unnoticed or undiagnosed.

The findings of this review are subject to methodological limitations. Heterogeneity between studies was high. Few studies reported sufficient outcome data to allow for population comparisons. Novel diagnostic tests have shed more light on the prevalence of leptospirosis and require further investigation. Although we assessed the quality of included studies, we were unable to control for differences in study design and quality of microbiological data. For many surveys, the prevalence of leptospirosis was not the primary outcome, making a formal meta-analysis potentially misleading. Unpublished data was included in this review (conference abstracts), although controversy on this policy exists.[159] We minimized bias by searching clinical trial registries for ongoing or unpublished studies. Whilst no restrictions were placed on the publication language, the focus of the majority of the search engines (Medline/PubMed being the primary source of studies) is predominantly English. Other unpublished data were not included; therefore, our findings may have a publication bias towards studies that report high levels of concordance.

CONCLUSION

Leptospirosis is an important zoonosis in sub-Saharan Africa. The combination of climate change, massive urbanisation, and the interdependence of humans and animals may be critical for the burden of leptospirosis on Africa's people. The nature of leptospirosis highlights the need for a fresh approach to control and prevention, incorporating the links between veterinary and human medicine. Future leptospirosis research should be a collaborative effort between the fields of human and animal medicine, to attain optimal health outcomes.

PANEL: PRESENT KNOWLEDGE, UNANSWERED QUESTIONS AND RESEARCH GAPS

Leptospirosis is common in sub-Saharan Africa, as shown by numerous observational studies

Which populations are most at risk? To what extent are leptospirosis cases misclassified as malaria? Do more infections occur in poorly sanitized urban settings or in rural areas as a result of occupational or environmental exposure? How prevalent is the disease in the Central African region?

The increasing urbanisation and climate change are thought to be risk factors for future outbreaks of leptospirosis in sub-Saharan Africa

What public health or control measures should be taken? Will livestock vaccination, sanitation measures and rodent control be enough to prevent larger outbreaks?

Data on the risk of acquiring leptospirosis related to age vary and remain unclear

Which age-groups are most at risk? Is disease vastly under recognized in children due to their milder symptoms? What is the influence of acquired immunity?

Antibiotic treatment is recommended but there are no clear cut clinical trials to prove or refute the use of antibiotical treatment for leptospirosis,

An almost certain cause of some of the inconclusive studies is that patients in resource-poor countries arrive for treatment with advanced disease: antibiotics are then unlikely to be of benefit because organ damage has already occurred. How can this problem be avoided in future clinical trials?

Contributors

MPG, SGV, and BJV conceived the paper. SGV wrote the first draft. BJV double-checked the data. IMN conducted the searches and contributed to the methods section. All authors contributed to the concept of the paper and all have been involved in drafting and revising the manuscript; all have agreed to be fully accountable for all aspects of this work. All authors read and approved the final manuscript.

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Box 1. Diagnostic methods

In daily practice, diagnosis is predominantly based on serological testing. There is a leptospiremia during the first 10 days of the disease,[3] during which the leptospires can be cultured from blood or CSF. However, blood culture is insensitive and isolation of the fastidious leptospires can take weeks to months.[172] Polymerase chain reaction (PCR) is used as standard molecular diagnostic test in the first week of illness.[3] After about 5 to 10 days leptospires may appear in the urine and concomitantly, antibodies become detectable in blood and CSF.[3] The most frequently used tests for antibody detection are the Microscopic Agglutination Test (MAT),[173] which is the reference standard assay, and enzyme-linked immunosorbent assay (ELISA), introduced in 1984[174] (see Box 2). MAT serology is insensitive, especially in acute-phase specimens and seroconversion in fulminantly ill people might not happen before the patient dies.[175] The most likely infective serogroup can be deduced from the highest titre against one or more serovars,[176] but interpretation is complicated by the high degree of cross-reactions between different serogroups, especially in acute-phase serum samples[3] IgM detection by ELISA has been shown to be more sensitive in the acute phase.[173,177] MAT, ELISA, and PCR are expensive, technically demanding and not widely available and applicable in most settings in sub- Saharan Africa. Rapid diagnostic tests (RDTs) for antibody detection are available.[177] Three RDTs were recently evaluated.[178] All three had low sensitivity rates based on a single sample, which substantially increased when the results of paired subsequent samples were combined, although yielding lower specificity.

MAT can provide a general impression about which serogroups circulate within a population,[3] but conclusions about infecting serovars cannot be drawn without isolates. Animals can be divided in accidental and maintenance hosts: the latter occurs by persistent colonization of the proximal renal tubules of carrier hosts. These can remain symptom-free and may present as seronegatives while excreting leptospires in the urine for the entire life.[2]

Box 2. Case definitions for human leptospirosis (Adapted and modified from WHO[5]/CDC)

A. Clinical description:

A common presentation is an acute febrile illness with head- ache, myalgia (particularly calf muscle) and prostration associated with any of the following symptoms/signs:

- Conjunctival suffusion
- Anuria or oliguria
- Jaundice
- Cough, haemoptysis and breathlessness
- Haemorrhages (from the intestines; lung bleeding is notorious in some areas)
- Meningeal irritation
- Cardiac arrhythmia or failure
- Skin rash
- Aseptic meningitis*

Note. Other common symptoms include nausea, vomiting, abdominal pain, diarrhoea and arthralgia. The clinical diagnosis is difficult where diseases with symptoms similar to those of leptospirosis occur frequently. *Only mentioned in CDC Case Definition of leptospirosis in 2013.

B. Laboratory criteria:

Laboratory-confirmed cases of leptospirosis: Clinical signs and symptoms consistent with leptospirosis and any one of the following:

- Fourfold increase in microscopic agglutination test (MAT) titre in acute and convalescent serum samples;
- MAT titre ≥ 1:400 in single or paired serum samples;
- Isolation of pathogenic *Leptospira* species from normally sterile site;
- Detection of *Leptospira* species in clinical samples by histological, histochemical or immunostaining technique;
- Pathogenic *Leptospira* species DNA detected by PCR

Probable cases of leptospirosis: Clinical signs and symptoms consistent with leptospirosis and one of the following:

- Presence of IgM or a fourfold increase in indirect immunofluorescence assay (IFA) antibody titre in acute and convalescent serum samples;
- Presence of IgM antibodies by enzyme-linked immunosorbent assay (ELISA) or dipstick;
- MAT titre ≥ 1:100 in single acute-phase

Seroprevalence studies of healthy people, any of the following:

- MAT titre ≥ 1:80
- Presence of IgM antibodies by ELISA or IFA

Seroprevalence studies of healthy animals, any of the following:

- MAT titre ≥ 1:80
- Presence of IgM antibodies by ELISA or IFA
- Isolation of pathogenic *Leptospira* species from normally sterile site;
- Detection of *Leptospira* species in clinical samples by histological, histochemical or immunostaining technique;
- Pathogenic *Leptospira* species DNA detected by PCR

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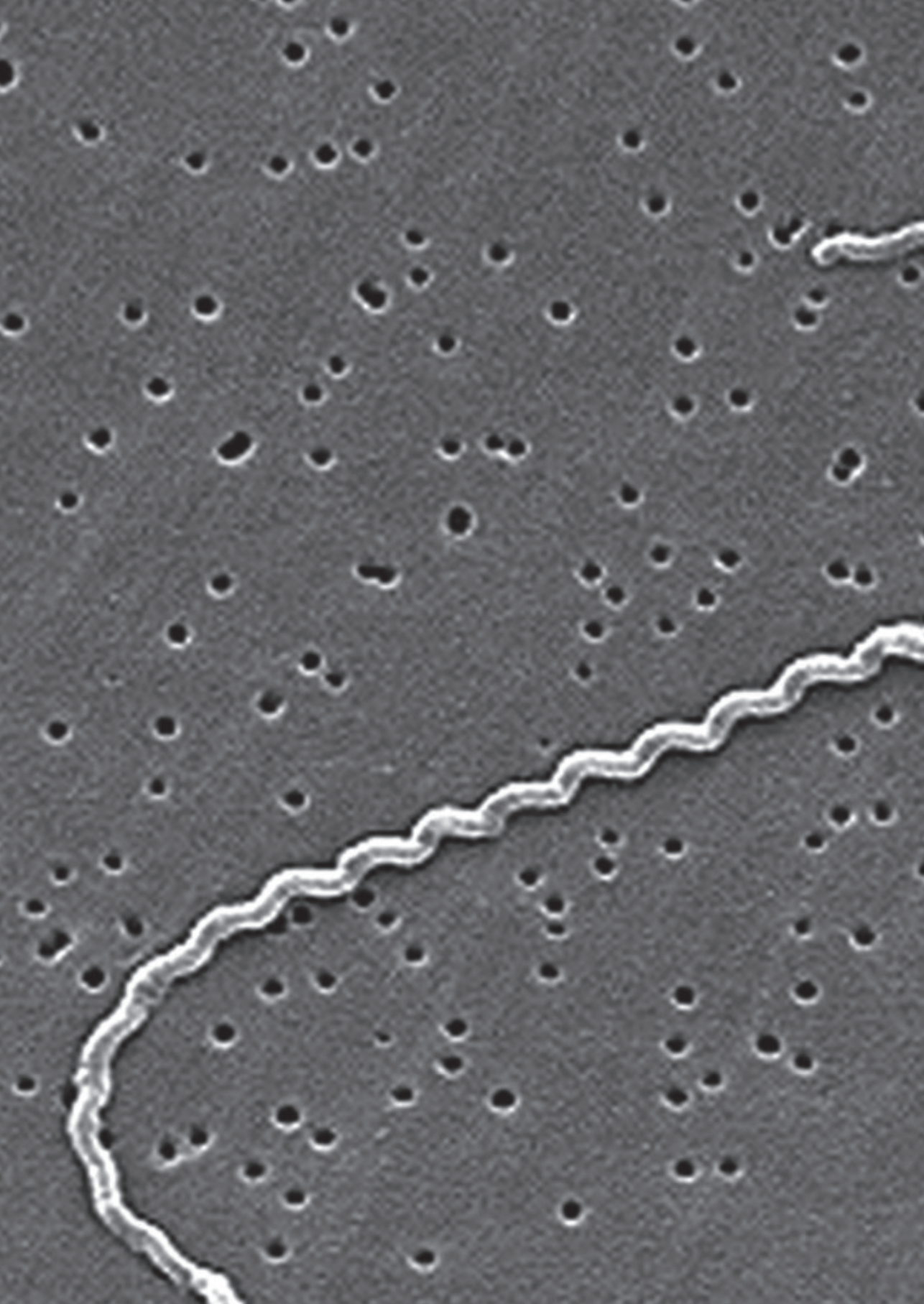
SUPPLEMENTARY MATERIALS

The supplementary materials provide details on the systematic review methodology, extensive tables, historic leptospirosis in sub-Saharan Africa, and risk of bias assessments. This data can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2014.06.013

Supplementary Material I: Protocol
Supplementary Material II: Search Strategy
Supplementary Material III: Trial registries
Supplementary Material IV: Table human leptospirosis after 1970 – extended version
Supplementary Material V: Table animal leptospirosis after 1970 – extended version.
Supplementary Material VI: Trends in Leptospirosis Serogroups
Supplementary Material VII: Historic leptospirosis before 1970
Supplementary Material VIII: Assessment Risk of Bias.



Chapter 3

Travel-related leptospirosis in the Netherlands 2009-2016: An epidemiological report and case series

Sophia G. de Vries, Maud M.I. Bekedam, Benjamin J. Visser, Cornelis Stijnis, Pieter P.A.M. van Thiel, Michèle van Vugt, Abraham Goorhuis, Jiri F.P. Wagenaar, Martin P. Grobusch, Marga G.A. Goris

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ABSTRACT

Background

Leptospirosis is a potentially fatal zoonotic disease that is prevalent in travellers. Here, we describe epidemiological and diagnostic characteristics of all returning travellers diagnosed with leptospirosis in the Netherlands between 2009 and 2016. Furthermore, we present a detailed clinical case series of all travellers with leptospirosis who presented at the Academic Medical Center (AMC) in the same period.

Method

We extracted data from the records of the Dutch Leptospirosis Reference Center (NRL) of all cases of leptospirosis in travellers in the Netherlands from 2009-2016. Patients who presented at the AMC were identified and clinical data were extracted from the hospital records.

Results

224 cases of travel-related leptospirosis were included. An increase of cases was observed from 2014 onwards. The majority of cases were male (78.1%), and had travelled to South-East Asia (62.1%). Of 41 AMC cases, 53.7% were hospitalised, but most patients had a relatively mild disease course, with no fatalities. A longer delay in diagnosis and treatment initiation existed in hospitalised compared to non-hospitalised patients, suggesting a benefit of early recognition and treatment.

Conclusions

Leptospirosis was increasingly observed in returning travellers in the Netherlands, and is a diagnosis that should be considered in any returning febrile traveller.

INTRODUCTION

Leptospirosis is a zoonotic disease,[1] caused by pathogenic *Leptospira* that are shed in the environment via the urine of host animals, such as rodents and livestock. In warm and wet conditions, they can survive for several months.[2] Transmission patterns are complex; risk factors largely encompass water-related exposures, such as flooding, heavy rain, and recreational water activities.[3-5] Other risk factors include open wounds or abrasions, animal contact, and contact with soil, for example through gardening or walking barefoot.[3] Outbreaks may become more common due to climate change.[6]

Leptospirosis is responsible for over a million severe cases and 60,000 deaths worldwide.[7] These numbers are likely underestimated due vague clinical symptoms and difficult laboratory diagnostic technique. Classic severe disease (also known as Weil's disease) presents with acute renal failure, jaundice and (pulmonary) haemorrhages, but most commonly the disease presents as a mild acute febrile illness. The list of differential diagnostic considerations is long, including malaria, (arthropod borne) viral infections, rickettsial disease, and typhoid fever; these cannot be excluded on the grounds of clinical presentation only.[1,8] Early treatment is thought to prevent disease complications,[8-10] and therefore establishing an early diagnosis is crucial. The present reference tests (Microscopic Agglutination Test (MAT) and culture) are cumbersome methods requiring sophisticated laboratories, and cannot provide early diagnosis. MAT is based on detection of antibodies (which appear in the blood after only 5 to 10 days of illness), and can determine a presumptive infecting serogroup.[11] Culturing *Leptospira* can take months.[1,2] Immunoglobulin M Enzyme-Linked Immunosorbent Assay (IgM ELISA) is more widely used but meets similar problems with establishing an early diagnosis, as it is based on antibody detection. Polymerase chain reaction (PCR) is based on DNA/RNA detection, and is therefore applicable in the first week of illness when the bacteria are circulating in the blood. There is an increase in the routine use of this method; however, diagnostic accuracy may vary among settings and laboratories.[12,13]

International travel is a major independent risk factor for leptospirosis.[14] Numbers of international tourists are increasing annually,[15] and tourists are increasingly visiting high-endemic regions and engaging in high-risk activities involving leisure freshwater exposure such as river rafting, canoeing, and other adventure sports.[5,16] Consequently, reported proportions of travel-related leptospirosis worldwide are increasing.[14,16-18] A recent systematic review showed an association between leptospirosis and whitewater sports, spelunking (exploring caves), and adventure races and trekking, and recommends that prophylactic doxycycline should be considered in those participating in such activities in endemic areas.[5]

In 2015, there were 18.1 million holidays abroad among Dutch people (with a total population of 16.9 million in 2015).[19] In the Netherlands, in the period from 1924 – 2008, a gradual increase of imported leptospirosis was observed, along with an increase of imported infections in general: in the period 2005 – 2008, 53% of all infections in the Netherlands

were imported; 80% of these cases were associated with water-related activities.[16] In 2014, a four-fold increase in autochthonous leptospirosis, and a 1.6-fold increase in cases of imported leptospirosis were observed in the Netherlands.[20]

Here, we describe the epidemiological and demographic characteristics of confirmed acute leptospirosis in returned travellers in the Netherlands in the period from 2009 – 2016. Furthermore, we describe detailed clinical presentations of all leptospirosis cases presenting in the Academic Medical Center (AMC) of the University of Amsterdam (UvA), the Netherlands. Finally, we demonstrate the clinical spectrum of acute leptospirosis, by detailing four cases of returned travellers who presented at the AMC.

METHODS

Leptospirosis is a notifiable disease in the Netherlands since 1928,[21] and ~99% of cases are confirmed by the World Organisation for Animal Health and the National Collaborating Centre for Reference and Research on Leptospirosis (NRL). For definitions of cases, national guidelines are followed.[22] When clinicians and general practitioners across the Netherlands suspect leptospirosis, clinical samples are submitted to the NRL, where serology (MAT and IgM ELISA) is done for the detection of antibodies. If blood is collected before the 11th day of illness, culture is performed as well, and, from September 2012 onwards, also PCR; PCR is performed on urine in all disease stages). MAT can determine the presumptive infecting serogroup. A confirmed case of leptospirosis is defined by: a positive culture and/or PCR and/or serology (MAT or IgM ELISA) and fever or at least two of the following signs and symptoms: rigors, headache, myalgia, conjunctival injection, skin or mucosal bleeding, rash, jaundice, myocarditis, meningitis, renal failure or pulmonary haemorrhages.[11,16]

Confirmed imported cases of leptospirosis diagnosed at the NRL from the 1st of January 2009 to the 31st of December 2016 were selected, as all cases of leptospirosis in the Netherlands up to 2008 have been described elsewhere [16]. Epidemiological and diagnostic data were extracted, including presumptive infecting serogroups. For all patients who had presented at the AMC, clinical data were extracted from patient files. From those, four case vignettes were selected, representing the broad spectrum of clinical presentation and disease course. Diagnostic delay was defined as the number of days between the first visit to any healthcare professional in the Netherlands, and the first request for leptospirosis diagnostics. Data were organized and analysed using Microsoft Excel (Microsoft Corporation, 2010). The vector map was created using an open source vector map (https://commons.wikimedia.org/wiki/Atlas_of_the_world), and further edited using Adobe® Illustrator® CS6 (Adobe Systems Incorporated).

Ethical issues

This study was exempted from further ethical review of human subject research by the Medical Ethical Review Committee of the Academic Medical Centre, University of Amsterdam (protocol W16_311#16.366). All data were de-identified and not attributable to individual patients. For the individuals described in the case reports, consent was obtained.

RESULTS

Imported leptospirosis in the Netherlands

During 2009 – 2016, patient materials from 4,642 Dutch patients suspected for leptospirosis were submitted to NRL for diagnostic testing, of which 428 were confirmed as leptospirosis (positivity rate 9.2%), comprising 224 (52.3%) imported cases. From 2014 onwards, an increase in the total number of confirmed cases was observed, with the proportion of imported cases remaining more or less the same (see Figure 1). The increase of autochthonous cases in 2014 has been described elsewhere.[20] Figure 2 displays the total number of traveller-samples submitted per year, the percentage of positive samples, and the percentage of PCR positive samples (the latter from 2012 onwards, when PCR was used routinely).

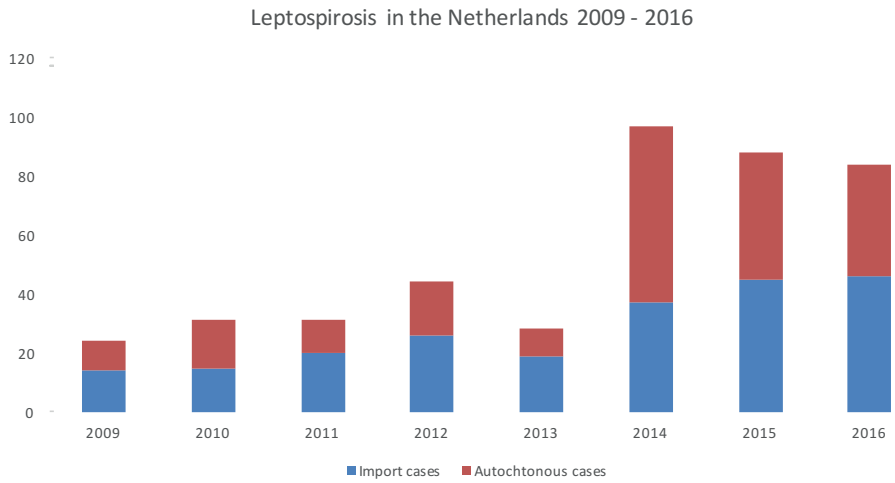

Figure 1. Total number of patients diagnosed with leptospirosis in the Netherlands, autochthonous and imported, 1 January 2009–31 December 2016.

The majority of imported cases concerned male patients (175/224; 78.1%), who had mainly travelled to South-East Asia (139/224; 62.1%). Thailand was the most frequently reported country of infection (92/224; 41.1%). Exposure to fresh water was common (90/224; 40.2%) and the Sejroe/Hebdomadis/Mini complex was the most commonly found infecting

serogroup (38/224; 17.0%). Characteristics of the imported leptospirosis cases are described in Table 1; exposure countries are shown in Figure 3.

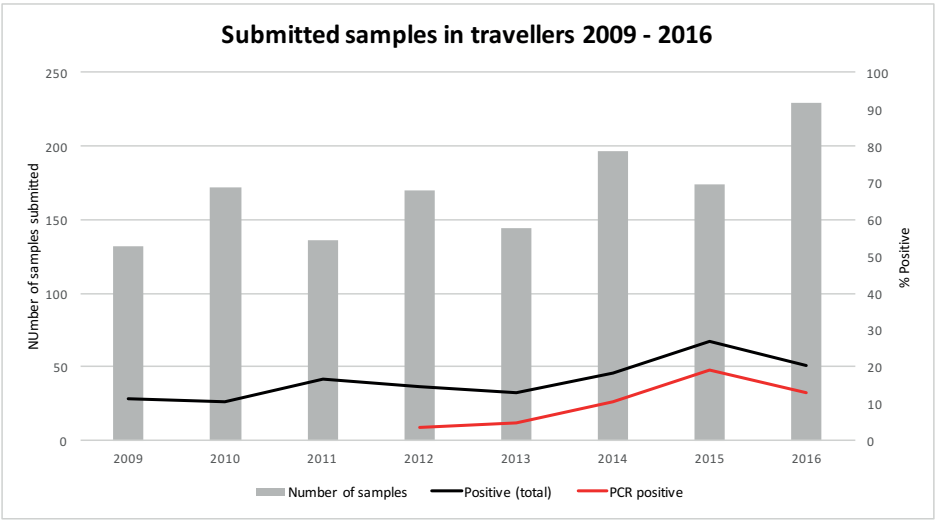

Figure 2. Total number of samples of cases suspected for imported leptospirosis submitted from 1 January 2009–31 December 2016, and the percentage of positive samples, and PCR positive samples.

Cases of imported leptospirosis in the Academic Medical Center

From 1 January 2009 to 31 December 2016, 41 cases of leptospirosis were diagnosed and/or treated at the AMC. Details of four cases of leptospirosis meningitis, and a case of pulmonary haemorrhage have been described elsewhere.[23,24] Data of five of those 41 patients were also included in a recent GeoSentinel report on global data on leptospirosis in travellers (submitted for publication). Here, data of all 41 patients in our database from 2009-2016 are described.

The majority of patients presenting at the AMC was young (median age 27.8 years), male (33/41; 80.5%), and had visited South-East Asia (29/41; 70.7%) (Table 1). Thailand was the most frequently reported country of exposure (n=19; 46.3%), followed by Indonesia (n=5; 12.2%), Costa Rica (n=4; 9.8%), Colombia, Laos, Malaysia (n=2; 4.9% for all three countries), and the Dominican Republic, French Guiana, Ghana, Haiti, Sri Lanka, Trinidad and Tobago, Uganda (n=1; 2.4% for all 7 countries). Exposure to fresh water was reported in 90.3% of all patients.

Table 2 summarizes general characteristics of the clinical disease courses of the patients. The majority of patients was hospitalised (22/41; 53.7%). One patient, with pulmonary haemorrhages,[24] was admitted to the ICU; none required kidney dialysis. Of note is the observation that only one patient presented with jaundice, as part of the classic Weil's triad. The laboratory parameters of the 41 AMC patients are shown in Table 3. Renal impairment was observed in 17 (41.5%) patients, of which six (14.6%) had a glomerular filtration rate

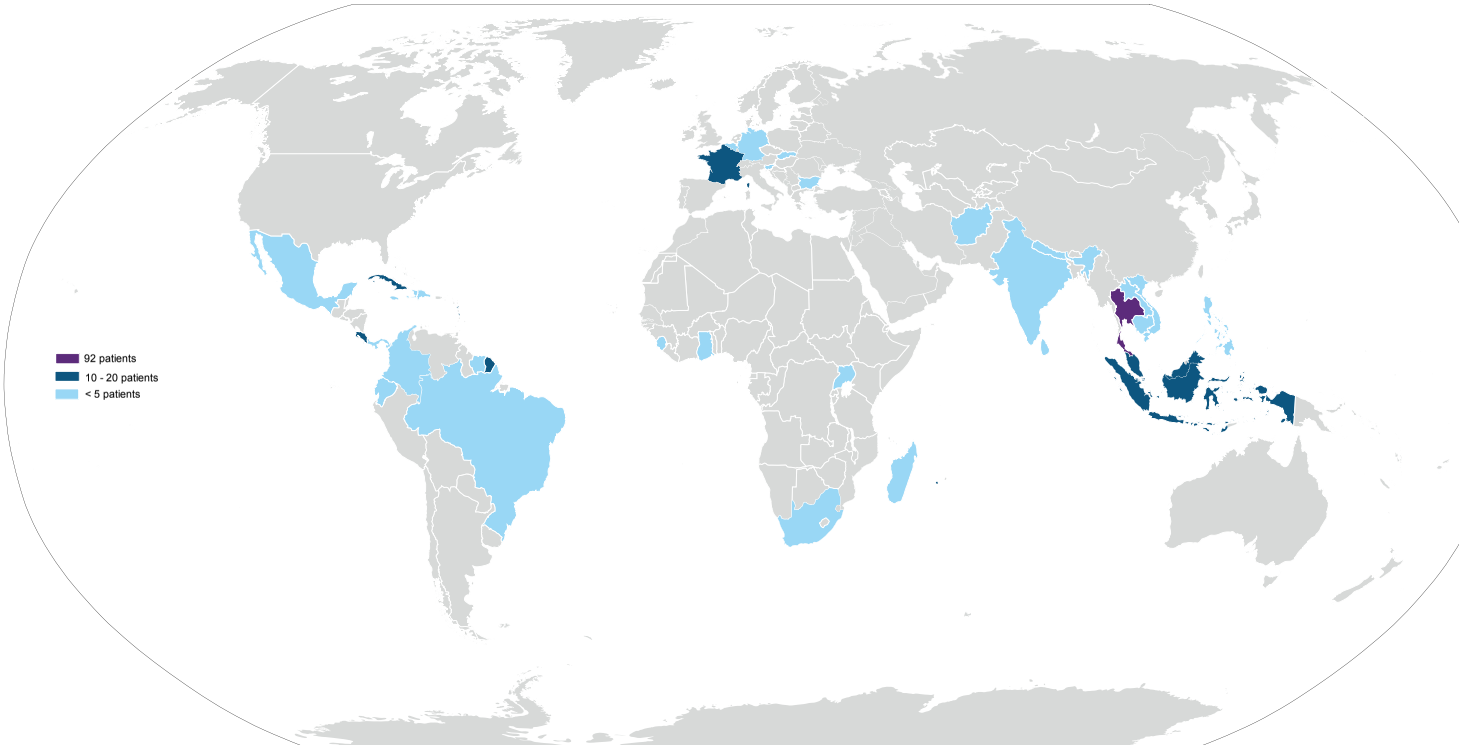

Figure 3. Exposure countries among imported cases of confirmed leptospirosis in the Netherlands, 1 January 2009–31 December 2016 (n=222) *†. (For interpretationof the references to colour in this figure legend, the reader is referred to the web version of this article.)

* Includes only patients for whom country of exposure data were available.

†Purple represents 92 patients exposed in Thailand. Dark blue represents countries with 10–20 exposed patients (19 for Malaysia; 16 for Indonesia; 13 for Cuba; 12 for Costa Rica; and 10 for France). Light blue represents countries with<5 patients (4 each for Belgium, Colombia, Laos, Suriname, and Vietnam; 3 each for Germany, Panama, and Sri Lanka; 2 each for Bulgaria, Cambodia, Dominican Republic, Jamaica, Philippines, India, Nepal, South Africa; and 1 each for Afghanistan, Brazil, Ecuador, French Guiana, Ghana, Haiti, Luxembourg, Madagascar, Mauritius, Mexico, Sierra Leone, Slovakia, Slovenia, Trinidad & Tobago, and Uganda).

(GFR) of <30ml/min. Two patients presented with spontaneous bleeding, one with pulmonary haemorrhages[24] and one with rectal blood loss. Fifteen patients (36.6%) presented with low platelets.

Table 1. Characteristics of imported cases of leptospirosis in the Netherlands, 1 January 2009 – 31 December 2016

Characteristic	*Total imported cases, n=224*	*AMC cases, n=41*
Male sex (%)	175 (78.1)	33 (80.5)
Median age (range)	30 (8-75)	27.8 (10-63)
Mean age (SD)	34.3 (14.4)	32.2 (11.9)
Region of exposure (%)		
South-Eastern Asia	139 (62.1)	29 (70.7)
Europe	22 (9.8)	0 (0.0)
Caribbean	19 (8.5)	3 (7.3)
Central America	18 (8.0)	3 (7.3)
South America	11 (4.9)	3 (7.3)
Southern Asia	8 (3.6)	1 (2.4)
Sub-Saharan Africa	7 (3.1)	2 (4.9)
Likely route of infection (%)		
Water	90 (40.2)	30 (73.2)
Water and animals	6 (2.7)	7 (17.1)
Animals	3 (1.3)	1 (2.4)
Unknown	125 (55.8)	3 (7.3)
Serogroup		
Sejroe-Hebdomadis-Mini Complex	38 (17.0)	8 (19.5)
Icterohaemorrhagiae	21 (9.4)	4 (9.8)
Australis	10 (4.5)	4 (9.8)
Grippotyphosa	8 (3.6)	1 (2.4)
Celledoni	6 (2.7)	5 (12.2)
Bataviae	5 (2.2)	1 (2.4)
Pyrogenes	5 (2.2)	-
Autumnalis	2 (0.9)	2 (4.9)
Canicola	2 (0.9)	-
Cynopteri	2 (0.9)	2 (4.9)
Tarassovi	1 (0.4)	2 (4.9)
Ballum	1 (0.4)	-
Javanica	1 (0.4)	1 (2.4)
Shermani	1 (0.4)	-
Not identified	121 (54.0)	9 (22.0)

Table 2. Clinical characteristics of patients with travel-related leptospirosis in the AMC

	All (n=41)
Male sex, n (%)	33 (80.5)
Median age (range)	27.8 (10-63)
Hospital admission, n (%)	22 (53.7)
ICU admission, n (%)	1 (2.4)
Dialysis, n (%)	0 (0.0)
Deaths, n (%)	0 (0)
Treatment with antibiotics, n (%)	
Yes	40 (97.6)
Oral	22 (53.7)
IV	2 (4.9)
IV then oral	16 (39.0)
Median number of days in the hospital, days (range)	5 (1-9)
Median time from return to presentation, days (range)	5 (0-13)
Median duration of illness at first visit to any clinic (range)	3 (0-32)
Median number of days between start of symptoms and start antibiotics (range)	4 (1-34)
Median day of illness at confirmed diagnosis (range)	8 (1-41)
Median diagnostic delay* (range)	1 (0-40)
Symptoms at presentation, n (%)	
Fever/sweats	41 (100.0)
Headache	39 (95.1)
Myalgia	30 (73.2)
Nausea	25 (61.0)
Arthralgia	20 (48.9)
Acute diarrhoea	18 (43.9)
Conjunctival injection	17 (41.5)
Skin rash	13 (31.7)
Vomiting	12 (29.3)
Cough	12 (29.3)
Abdominal pain	8 (19.5)
Tachycardia	7 (17.1)
Nuchal rigidity/meningism	5 (12.2)
Anuria/oliguria	4 (9.8)
Dyspnoea	4 (9.8)
Hepatosplenomegaly	4 (9.8)
Dysuria/haematuria	2 (4.9)
*Spontaneous bleeding***	2 (4.9)
Weight loss	2 (4.9)
Lymphadenopathy	1 (2.4)
Jaundice	1 (2.4)
Petechial bleeding	1 (2.4)

ICU = Intensive Care Unit; IV = intravenous

** defined as: number of days between first visit to any clinic in the Netherlands and first leptospirosis diagnostics ordered,* **One case of pulmonary haemorrhage, and one case of rectal blood loss.

Table 3. Laboratory parameters of patients at presentation with travel-related leptospirosis in the AMC (n = 41)

Value	*Number of cases (%)*	*Median value (range)*	*Reference ranges*
Blood			
Elevated CRP	31 (75.6)	165 (26 - 368)	0 - 5 mg/L
Elevated creatinine	17 (41.5)	179 (111 - 668)	75 - 110 µmol/L
Low platelets	15 (36.6)	131 (88 - 147)	150 – 400 *10^9/L
Elevated ALAT	15 (36.6)	88 (46 - 305)	0 - 45 U/L
Elevated ASAT	15 (36.6)	65 (43 - 354)	0 - 40 U/L
Leucocytosis	10 (24.4)	12.8 (11.0 - 18.9)	4 - 10.5*10^9/L
Elevated urea	9 (22.0)	9 (7.5 - 21.6)	2.1 - 7.1 mmol/L
Elevated bilirubin	8 (19.5)	22 (18 - 75)	0 - 17 µmol/L
Low haemoglobin	8 (19.5)	8 (7.3 - 8.4) (*all male*)	M 8,5 – 10,5 / F < 7,5 - 10 mmol/L
Hypokalaemia	7 (17.1)	3.3 (3.0 - 3.4)	3.5 – 4.5 mmol/L
Leukocytopenia	3 (7.3)	4 (0.4 - 4.4)	4 – 10.5 *10^9/L
Urine			
Haematuria	13 (31.7)	NR	0 - 17 /µL
Proteinuria	11 (26.8)	NA	(yes/no)
Leukocyturia	3 (7.3)	NR	0 - 28 /µL

CRP = C-reactive protein; ALAT = Alanine-aminotransferase; ASAT = Aspartate aminotransferase

Most patients were diagnosed by serological methods (MAT or IgM ELISA, n =25; 61.0%). As expected, given the late occurrence of antibodies in the blood, in most of these cases the diagnosis was only made after a convalescent sample had been tested, with a median duration of illness of 12 days (range 6 – 41 days). PCR provided the diagnosis for 16 (39.0%) patients, all on the first submitted sample, at a median disease duration of 3 days (range 1 – 17 days). Nine patients (22.0%) had a positive culture, of which seven also had a positive PCR; two (4.9%) had a negative PCR result (their cultures became positive after 4 and 6 weeks of incubation, respectively).

Clinical description of cases

Case 1

A 37-year old previously healthy male presented to the emergency department, 12 days after his return from a two-week trip to Thailand, where he had visited Chiang Mai and the surrounding forests. He was exposed to fresh water during an elephant ride. Five days before presentation, he developed a severe headache (starting in the neck, later retro-orbital) with fever up to 40°C, anorexia, and dark urine. The general practitioner had initially prescribed diazepam and oxycodone to treat his headache. From three days before presentation, he stopped urinating and did not pass any stools; one day later he started vomiting. On physical

examination, his vital signs were normal, with modest enlargement of the liver. Laboratory investigation revealed a normal haemogram, creatinine 668 µmol/l, C-reactive protein (CRP) 342 mg/l, and normal pH and oxygen saturation levels. Urine examination showed albuminuria and leukocyturia. A chest radiograph revealed no abnormalities; an ultrasound of the abdomen showed hepatomegaly, without signs of kidney abnormalities. Leptospirosis was clinically suspected, and he was admitted and treated with intravenous ceftriaxone and fluid resuscitation. During admission, because of the fluid resuscitation, he developed a cardiac decompensation with pulmonary oedema (without signs of pulmonary bleeding) and mild liver enzyme abnormalities. Urine production recovered rapidly, and from the fourth day after admission the creatinine levels started normalizing, and the patient was discharged 6 days after admission. The initial tests for leptospirosis (PCR, MAT and IgM ELISA) were negative, but the IgM ELISA turned positive on the 7th day of illness. Blood cultures and specific laboratory tests for malaria, dengue, chikungunya and typhoid fever were all negative.

Case 2

A 63-year old male, with a history of angina pectoris, for which he had a stent placed in the left anterior descending artery two years earlier, presented at the emergency department three days after returning from a 2-week trip to Malaysia, where he had been in contact with freshwater during a jungle trekking in Taman Negara National Park. He had presented to the general practitioner 11 days earlier with an itching confluent erythema rash on his arms, chest and lower legs, for which antihistaminic drugs and skin creams had been prescribed, with no effect. Two days later, he had developed a fever up to 40°C with chills, and the next day a painful skin and arthralgia, mainly in the knees. He had nausea and vomiting, but no respiratory or urogenital complaints. On presentation at the emergency department, vital signs and physical examination were normal, besides erythema on his lower arms and lower legs. Laboratory tests revealed a thrombocytopenia (135*10^9/l, renal insufficiency (creatinine 285 µmol/l) and increased liver enzymes (ASAT 58 U/l; ALAT 88 U/l; AF 120 U/l; Gamma-GT 87 U/l). Urinalysis showed proteinuria and leukocyturia. *Leptospira* DNA was detected in EDTA blood by PCR on the day of admission and intravenous ceftriaxone was started. Thick blood smear for malaria, dengue and rickettsia serology and cultures of urine and blood were negative. The fever and renal insufficiency subsided within two days after the start of antibiotics. The patient was released from the hospital and finished a seven days course of doxycycline at home. Two weeks after the initial presentation, a convalescent blood sample showed antibody titres in the MAT (highest titre 1:1280) in the Sejroe-Hebdomadis-Mini complex serogroup).

Case 3

A 27-year old male presented at the outpatient department, ten days after his return from a three-week trip to Colombia, where he had rafted and walked through mud with bare feet.

Seven days before presentation, he developed fever up to 39.7°C, with chills and arthralgia in the ankles, knees and lower back, and myalgia in the calves during the first two days of disease. Two days later, he became nauseated and vomited, and developed watery diarrhoea one day later. The next day, he developed pain in both testes. He finally presented at the AMC two days later. On physical examination, there was a conjunctival injection, and a light exanthema on the abdomen and lower back, and both testes were swollen and painful. Laboratory tests revealed no abnormalities other than an increased CRP (196 mg/L). The thick blood smear for malaria, stool and urine cultures, and serology for syphilis and mumps were all negative. As the patient started to feel better, no treatment was initiated. Two days later, at follow-up visit he had improved clinically. In the meantime, the PCR for leptospiral DNA on serum became positive. As complaints of orchitis continued, doxycyline was administered for seven days, with good result. Two weeks later, a follow-up sample showed a more than four-fold increase in MAT titre against strains in the Sejroe-Hebdomadis-Mini complex serogroup.

Case 4

A 31-year old male was admitted with fever, headache, nausea, and malaise. Symptoms had started the during a 2.5-week journey to Costa Rica, and at presentation, symptoms had existed for five days. In Costa Rica, there had been floods after heavy rainfall, and he had been exposed to prolonged water contact because the hotel was flooded. He presented with retro-orbital headache, photophobia, myalgia in the calves of his legs, nausea, vomiting, and rectal blood loss. Physical examination showed red, injected conjunctivae, mild jaundice, a tachycardia of 102 bpm, fever (39.9°C), and a normal blood pressure. Laboratory investigations revealed a haemoglobin of 7.3 mmol/l; thrombocytes of $107*10^9$/l, elevated liver enzymes (bilirubin 75 µmol/l, ASAT 111 U/l; ALAT 104 U/l; AF 180 U/l; gamma-GT 98U/l), and a normal renal function. Leptospirosis, typhoid fever, or an arthropod borne viral infection were suspected, and the patient was admitted and treated with intravenous ceftriaxone. The PCR for *Leptospira* was positive, after which treatment was continued with oral doxycycline. The patient improved substantially, but after two days he developed a second-degree atrioventricular block, for which he was observed for 48 hours in the cardiac observation unit. The atrioventricular block disappeared spontaneously, but an incomplete right bundle branch block remained, for which he still receives cardiologic follow-up. Despite initial slow resolution of general fatigue, he had recovered one month after discharge.

DISCUSSION

During the time period 2009 – 2016, leptospirosis was increasingly observed in the Netherlands among returned travellers. In addition, a marked increase of the total number of leptospirosis cases, including autochthonous infections, was observed from 2014 onwards.

The increase in autochthonous cases in 2014 was thought to be due to a warm winter, followed by the warmest year in centuries.[20] This trend continued over 2015 and 2016, possibly for the same reason. For the imported cases however, the explanation is less obvious. The number of Dutch tourists travelling abroad and their destinations have been more or less stable since 2008.[19,25] Possibly, travellers are increasingly participating in high-risk activities, such as rafting and jungle trekking. Another explanation could be that physicians in the Netherlands have become more aware of leptospirosis, and thus request diagnostic tests more often. This is supported by the fact that the NRL has received increasing numbers of samples over the past years. Additionally, PCR was implemented in September 2012 in the NRL. PCR can identify leptospirosis cases in the early disease stages, which would have needed a convalescent sample for diagnosis in the period before September 2012, which is often not submitted to the laboratory. However, an increase of positivity rate was only observed from 2014 onwards. This increase was mainly attributable to PCR positive cases.

The majority of cases comprised relatively young males, who had travelled to Southeast Asia, consistent with other reports on leptospirosis in travellers.[16,26,27] Thailand, Malaysia, and Indonesia were the most frequently reported countries of exposure. Remarkably, Europe was the second most common region of exposure, with France contributing ten cases, and nine more cases from Belgium, Bulgaria, Luxembourg, Slovakia, and Slovenia. France is the number one destination for Dutch holidays, which could explain the relatively high number of cases. It does indicate however, that leptospirosis should be considered in all travellers presenting with a febrile illness, independent of the region they have visited, in particular also because delayed diagnosis leads to serious complications, shown in our cases.

Clinicians are usually well aware of the risk of leptospirosis when a typical exposure history is present, such as floods and contact with freshwater. In travellers, clear exposure histories have been reported,[26,28] but in the general population, the mechanism of infection often remains uncertain.[3] In our data, exposure histories were not known in almost 56% of all travel-related leptospirosis cases in the Netherlands; among the patients that presented at the AMC, where leptospirosis is a frequently diagnosed travel related disease, a clear exposure history was registered in more than 90% of the patients. It is possible that the lower percentage in the national group is due to incompleteness of the data, or due to unfamiliarity with the disease among physicians who rarely encounter leptospirosis.

It is likely that the cases reported here merely represent the more severe cases, as mild cases are more likely to remain unrecognized.[1,29] It has been described that the disease presentation in Dutch imported cases is less severe than in autochthonous cases, which has been postulated to be associated with a lower number of imported infections with serovars from the Icterohaemorrhagiae serogroup, linked to severe disease.[16,20] Indeed, the most common infecting serogroup in imported cases was the Sejroe-Hebdomadis-Mini complex (17% of cases), for which milder disease courses have been described,[16] whereas only around 10% of the described cases were infected with the Icterohaemorrhagiae serogroup.

We did not collect detailed clinical data of the overall group of 224 travellers with leptospirosis in the Netherlands. We do report, however, detailed data on a subset of 41 cases (18.3% of all confirmed cases of leptospirosis in the Netherlands) who were diagnosed and treated in the AMC. Of note, no single patient of those 41 succumbed to leptospirosis. The diagnostic delay in this group was generally short, with most patients being tested for leptospirosis relatively quickly (median of one day).

The AMC is a tertiary hospital with a specialized travel clinic (the Center of Tropical Medicine and Travel Medicine), and the Leptospirosis Reference Center is located at the premises building, which likely explains the relatively short delay, and possibly the relatively high caseload at the AMC. Another explanation is that there is a low threshold for consideration of the disease. Diagnostic delay most frequently occurred before presentation at the AMC. In addition, several patients had received inadequate treatments before presentation, such as very short courses of oral antibiotics.

None of the patients presented with a classic Weil's syndrome. Hospital admission was required in only 22 cases (53.7%), which is lower than in the previous report.[16] There was only one ICU admission,[24] and no need for renal replacement therapy in any patient. All patients survived. Further symptoms at presentation were similar to those described in other case series,[16,30] except for lower rates of jaundice.

Case #4, a young male, developed a second-degree atrioventricular block during admission, which resolved spontaneously, but a right bundle-branch block remained. Electrocardiographic alterations have been described in case series on leptospirosis, with ventricular repolarization disorders, atrial fibrillation and first-degree atrioventricular blocks most common, also in younger patients.[31,32] Different theories on the aetiology of cardiac involvement in leptospirosis have been postulated.[31-33] In severe and fatal cases of leptospirosis, myocardial involvement has been described.[33] The more commonly observed electrocardiographic abnormalities could be an effect of the leptospiraemia, or a general occurrence in febrile disease, also through metabolic and electrolytic disturbances.

Case #3 developed an orchitis four days after the acute febrile episode started. Orchitis has been described as a complication of leptospirosis in the older literature.[34-38] Most of those cases had been infected with the Ballum serogroup, contracted from laboratory[34-36] or pet mice,[37] and developed the orchitis at a later stage in the disease, after about 10 to 20 days. Our patient was PCR positive and later developed high MAT titres against serovars from the Sejroe-Hebdomadis-Mini complex serogroup. A similar case has been described in a 25-year old dairy farm worker who presented with a fever and epididymitis, who was also found to be infected with a serovar from the Sejroe-Hebdomadis-Mini complex serogroup.[38]

In the group of 41 AMC patients, the diagnosis of leptospirosis was most often obtained through serology, and for most patients, only the follow up sample was positive (median 12 days after onset of symptoms). These patients were either tested too late to perform PCR, or the PCR was negative. In confirmed cases with a negative PCR, the test was performed

later in the illness compared to the confirmed cases with a positive PCR, with a median of 5 days after the onset of symptoms in PCR negatives vs. a median of 2 days in PCR positive cases. The latter suggests that the PCR is more sensitive in the earliest stages of the disease, which is in line with previous studies.[12,39] These findings show that it is important to request diagnostic testing as early as possible in the disease course; when the time-frame for PCR is missed, it can take over a week for the serology to become positive. However, a negative PCR does not rule out the disease.

It is likely that leptospirosis is often missed, because many returning travellers with an acute febrile illness are treated with antibiotics empirically. If they improve, a diagnosis is not always sought. However, because of the potentially severe disease course when diagnosis is delayed or missed, there is an urgent need for an easy-to-use and simple diagnostic test in the acute phase of the disease.

Limitations to this study are that, even though ~99% of all leptospirosis cases in the Netherlands are diagnosed in the NRL, the data presented here may be incomplete. Clinical data were only available in the subset of patients who had presented at the AMC. Data on the serogroups were mostly based on the MAT, which can only determine a presumptive infecting serogroup.[11] Furthermore, the cases presented in detail here, were diagnosed and treated at the AMC, which is a tertiary hospital with a specialized travel clinic, which possibly resulted in selection bias; this could may implicate that the clinical picture of diagnosed leptospirosis patients the total population is not accurately reflected.

We conclude that leptospirosis is an increasing and likely underestimated cause of febrile illness and hospitalisation in returned travellers in the Netherlands. The disease has different and often surprising clinical manifestations in travellers. Mild outcome is associated with early diagnosis after the start of symptoms. Therefore, diagnostic testing should therefore be performed with a low threshold of suspicion in any febrile returning traveller.

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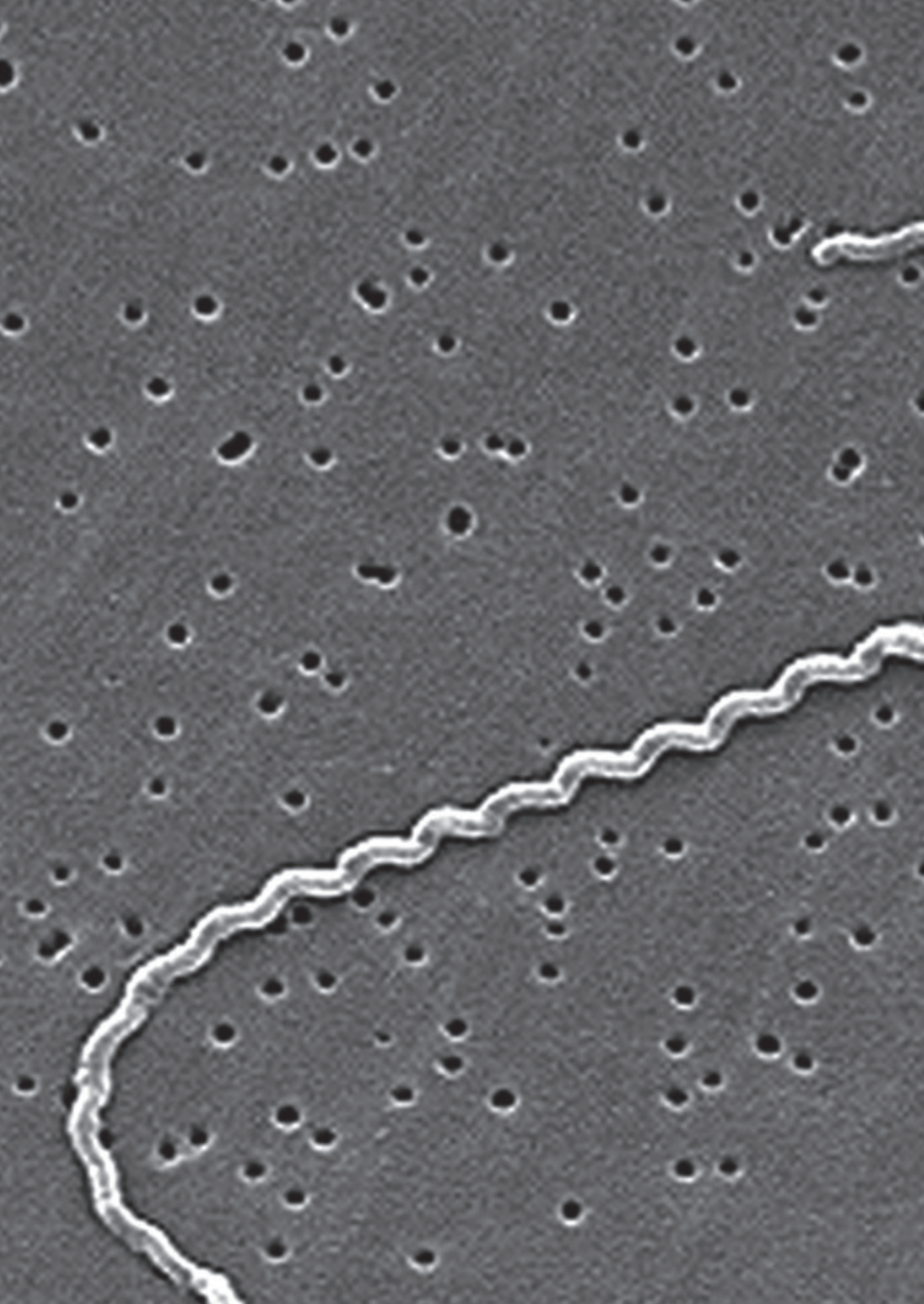

Chapter 4

Leptospirosis among Returned Travelers: a GeoSentinel Site Survey and Multicenter Analysis – 1997-2016

Sophia G. de Vries; Benjamin J. Visser; Rhett J. Stoney; Jiri F.P. Wagenaar; Emmanuel Bottieau; Lin H. Chen; Annelies Wilder-Smith, Mary Wilson; Christophe Rapp; Karin Leder; Eric Caumes; Eli Schwartz, Noreen A. Hynes; Abraham Goorhuis; Douglas H. Esposito; Davidson H. Hamer; Martin P. Grobusch, for the GeoSentinel Surveillance Network¶

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ABSTRACT

Leptospirosis is a potentially fatal emerging zoonosis with worldwide distribution and a broad range of clinical presentations and exposure risks. It typically affects vulnerable populations in (sub)tropical countries, but is increasingly reported in travelers as well. Diagnostic methods are cumbersome and require further improvement. Here, we describe leptospirosis among travelers presenting to the GeoSentinel Global Surveillance Network.

We performed a descriptive analysis of leptospirosis cases reported in GeoSentinel from January 1997 through December 2016. We included 180 travelers with leptospirosis (mostly male; 74%; mostly tourists; 81%). The most frequent region of infection was Southeast Asia (52%); the most common source countries were Thailand (n=52), Costa Rica (n=13), Indonesia and Laos (n=11 each). Fifty-nine percent were hospitalized; one fatality was reported.

We also distributed a supplemental survey to GeoSentinel sites to assess clinical and diagnostic practices. Of 56 GeoSentinel sites, three-quarters responded to the survey. Leptospirosis was reported to have been most frequently considered in febrile travelers with hepatic and renal abnormalities and a history of freshwater exposure. Serology was the most commonly used diagnostic method, although convalescent samples were reported to have been collected infrequently.

Within GeoSentinel, leptospirosis was diagnosed mostly among international tourists and caused serious illness. Clinical suspicion and diagnostic workup among surveyed GeoSentinel clinicians were mainly triggered by a classical presentation and exposure history, possibly resulting in under-diagnosis. Suboptimal usage of available diagnostic methods may have resulted in additional missed, or misdiagnosed, cases.

INTRODUCTION

Leptospirosis is an emerging zoonotic disease, causing more than a million severe cases worldwide and around 60,000 deaths annually.[1] The disease-causing spirochetes of the *Leptospira* genus[2] display complex transmission patterns.[3] A wide range of infecting serovars exist in a broad range of host animals, most notoriously in rats.[2] Well-known major risk factors are water-associated, such as recreational water activities; water exposure following floods and heavy rain; contact with animal or animal urine such as rodents and livestock; and poor sanitation.[3] Less common risk factors include exposure through open skin wounds and soil contact,[3] which might occur while gardening[4, 5] or walking barefoot.[3] Moreover, leptospirosis has emerged as an important problem in urban slums in the developing world, where rat-borne transmission increasingly triggers outbreaks.[6, 7, 8, 9, 10, 11] Most cases are sporadic; however, outbreaks do occur, and may be increasingly frequent due to climate change.[12]

Clinical manifestations can vary from a mild self-limiting infection to life-threatening illness. However, patients seeking care typically present with a mild acute febrile illness including chills, headache (often with retro-orbital pain), conjunctival suffusion, photophobia, myalgia, abdominal pain, nausea, vomiting, and sometimes transient exanthema.[2, 13] About 10% of patients progress to severe disease, including Weil's disease, characterized by jaundice, acute kidney failure, or (pulmonary) hemorrhage.[2, 13, 14, 15, 16] Pulmonary hemorrhage can also be a stand-alone manifestation. Aseptic meningitis may be seen in up to 25% of leptospirosis cases.[13] Cardiac involvement is likely more common than recognized.[13] The list of differential diagnoses for the evaluating clinician to consider is long, encompassing, among others, malaria, arboviral infections, rickettsial diseases, and typhoid fever,[13, 17] and misdiagnosis is common.[6, 18, 19, 20] Early recognition and treatment may be essential to improve patient outcomes[13, 21, 22] and minimize hospitalization costs.

Establishing an early, rapid and accurate diagnosis remains a complex matter. Widely used serological tests that detect antibodies, including the microscopic agglutination test (MAT, the current reference test), and immunoglobulin M enzyme-linked immunosorbent assay (IgM ELISA), are not suitable for early diagnosis, as antibodies only appear in the blood about 5 to 10 days after onset of symptoms.[2] Leptospirae can be cultured from blood in the early stages of disease, but this process can take weeks or months.[2, 17] Molecular detection tests, such as polymerase chain reaction (PCR), are increasingly used for routine diagnosis of the disease in the first week of illness,[23, 24, 25] but are not yet available in all countries.[26, 27] Commercially available multiplex panels, testing for multiple pathogens, could be a more accessible alternative.[28] For early presentations, PCR is recommended; if possible in combination with culture. For later presentations, MAT and IgM ELISA are suggested. Convalescent samples should be collected, and a combination of the above tests is always preferable.[24]

The disease burden of leptospirosis is highest in resource-poor, tropical countries; the highest incidence rates occur in Oceania, Southeast Asia, the Caribbean, and East Africa.[1, 29]

Notably, international travel to these regions now constitutes a major independent risk factor for acquisition of leptospirosis.[30, 31] In 2015, almost 1.2 billion international tourist arrivals were recorded worldwide, with an ever-growing number of travelers visiting tropical and subtropical regions.[32] With travelers being increasingly engaged in high-risk recreational activities, such as white-water rafting,[33] growing numbers of travelers with leptospirosis returning from tropical countries have been reported.[4, 31, 33, 34, 35, 36, 37] In a GeoSentinel analysis (1996-2011) of acute and potentially life-threatening tropical disease among 3,666 ill travelers from higher-resource areas, leptospirosis was the fourth most common diagnosis.[38]

Here, we describe the epidemiology of leptospirosis among travelers reported to the GeoSentinel Surveillance Network since its inception more than 20 years ago (January 1997 until 31 December 2016). Additionally, we report our analysis of the reported diagnostic approaches utilized by current GeoSentinel Surveillance Network members, through a supplemental survey among GeoSentinel sites.

METHODS

Data source – GeoSentinel surveillance system

GeoSentinel is a global surveillance network designed to monitor travel-related morbidity. It was established in 1995, with systematic data collection beginning in 1997.[39, 40] Currently, 70 GeoSentinel travel and tropical medicine clinics, located in 31 countries on six continents, contribute anonymous clinician-based surveillance information on ill travelers with a focus on infections acquired during travel (see http://www.istm.org/geosentinel for an up-to-date site distribution map and other information). In brief, for inclusion in the GeoSentinel database, patients must have crossed an international border within 10 years of presentation, and sought medical care from a GeoSentinel site for a presumed travel-associated condition. Standard data collection forms capture patient demographic characteristics, detailed recent travel itinerary, countries visited within 5 years, reason for recent travel, symptom-based grouping by affected organ system, and whether there was a reported encounter with a health care provider before travel.[40] Final diagnoses are assigned by the attending clinician and chosen from a list of standard diagnosis codes, guided by GeoSentinel diagnostic definitions. Each patient may have more than one travel-related diagnosis, and each diagnosis is reported as confirmed, probable, or suspected, based on the strength of the diagnosis.[40] All GeoSentinel sites use the best reference diagnostic methods available in their own country. Clinical treatments and outcomes are not routinely reported.

GeoSentinel's data-collection protocol is for public health surveillance, and has received a determination of non-research by a CDC human subjects' advisor.

GeoSentinel surveillance data inclusion criteria and definitions

GeoSentinel records for patients with a post-travel diagnosis of 'confirmed' leptospirosis and a clinic visit date from 1 January 1997 to 31 December 2016 were included. GeoSentinel defined criteria for 'confirmed' leptospirosis diagnosis state: 'a compatible clinical history (e.g. fever with associated symptoms) plus positive microscopy, culture, histopathology, nucleic acid amplification test, antigen detection, or seroconversion with a ≥4x titer rise on serology.' Furthermore, only patients presenting within 30 days after return from travel were included, based on the incubation period of leptospirosis of 2-30 days (average 7-10 days).[17] Patients seen during travel, missing travel itinerary data, or having a non-ascertainable region of exposure were excluded. The reason for travel was stratified into four categories: tourism, business, visiting friends and relatives (VFR), and other (combining small numbers of foreign aid and missionary workers or military deployment).

Data source – Supplemental GeoSentinel network survey

A 21-question multiple-choice survey (Supplementary Material 1) aimed to assess clinical and diagnostic practices among the GeoSentinel sites. After piloting among selected sites, the survey was distributed by email between December 2015 and March 2016 to 56 GeoSentinel sites active at the time of the survey, excluding the site of survey origin (Amsterdam). Reminders to complete the survey were sent twice via separate emails. Each site was permitted one response. In case of multiple responses, incomplete surveys or the last submitted survey were discarded.

Data management and analysis

A descriptive analysis of data from the GeoSentinel surveillance system, including demographics, travel characteristics, and symptoms, was performed. Simple frequency statistics were calculated for categorical variables. Analysis of symptoms only included data from October 2015 onward, the point where revised methods for collection of symptom data were implemented. Data from before this date are known to be incomplete. Data on the method of diagnosis have been only been captured in GeoSentinel since October 2015; similar data are not available for patients seen prior to that time. Data were analyzed using Microsoft Excel (2010) and SAS Enterprise Guide v5.1 (SAS Institute, Cary, NC). The GeoSentinel network survey was distributed in Survey Monkey® (www.surveymonkey.com). Results were directly exported to, and descriptively analyzed in, Microsoft Excel (2010). The vector map was created using an open source vector map (https://commons.wikimedia.org/wiki/Atlas_of_the_world), and further edited using Adobe® Illustrator® CS6 (Adobe Systems Incorporated).

Further statistical analyses of GeoSentinel data, which is not population-based, are not appropriate or advised. Such methods are limited by the biases that are introduced by each

site and by their distribution, and may be misleading. These data are most appropriately analysed descriptively, except in specific circumstances, none of which apply here.

RESULTS

Between 1 January 1997 and 31 December 2016, 227 patients with 'confirmed' leptospirosis were entered into GeoSentinel; 180 met the inclusion criteria. Reasons for exclusion were: seen during travel (22), clinic visit date >30 days after return (14), missing travel itineraries (5), or other incomplete information (6). The first patient was reported in 1999; few were entered during 1999-2007 (<5 per year), then increased to 7 per year in 2008-2009, rising to 14 to 29 cases yearly thereafter (Figure 1). During the same period, the number of GeoSentinel sites increased.

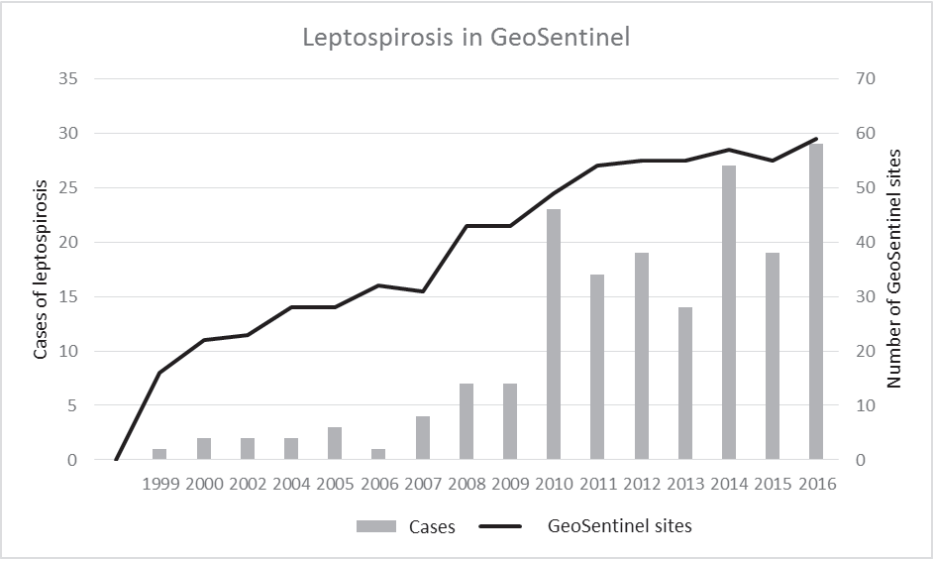

Figure 1. Travelers diagnosed with leptospirosis (N = 180), and the number of reporting sites in the GeoSentinel Network, 1 January 1999 – 31 December 2016
The number of patients with leptospirosis meeting inclusion criteria in the GeoSentinel Network during 1999-2016 (grey columns), and the number of sites reporting to GeoSentinel (black line).

Table 1 provides an overview of demographic and travel characteristics. The majority of patients were male, and the median age was 32 years (range 14 – 72 years). The most common reason for travel was tourism (81%). Many of the reported infections were acquired in Southeast Asia (n=93, 52%). Common source countries of infection were Thailand (n=52), Costa Rica (n=13), Indonesia (n=11), and Laos (n=11) (Figure 2). Fifty-nine percent of patients were hospitalized; there was one death. Female patients with leptospirosis were hospitalized

less frequently than male patients, and travelers to Southeast Asia were hospitalized slightly more frequently than patients infected in other regions (Table 2). For 22 patients, more than one diagnosis was registered (Supplementary Material 2).

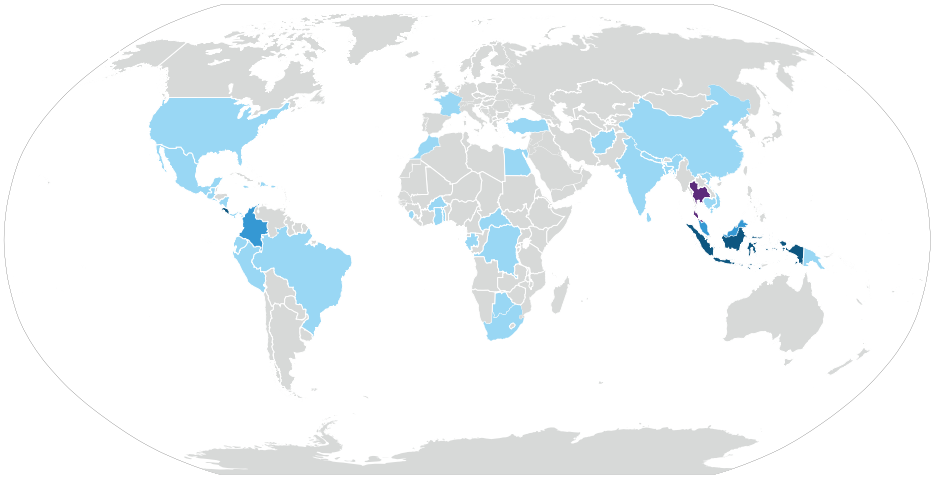

Figure 2. Exposure countries among travelers diagnosed with leptospirosis in the GeoSentinel Network, 1 January 1999 – 31 December 2016 (n = 167) *†

* Includes only patients for whom country of exposure data were available.

†Purple represents 52 patients exposed in Thailand. Dark blue represents countries with 10-20 exposed patients (13 for Costa Rica and 11 each for Laos and Indonesia). Medium blue represents countries with 5-9 exposed patients (9 for Colombia and 5 for Malaysia). Light blue represents countries with <5 patients (4 for Philippines; 3 each for Cambodia, Gabon, Jamaica, Mexico, and Panama; 2 each for Brazil, Dominican Republic, Ecuador, Guadeloupe, India, Martinique, Papua New Guinea, Peru, Sri Lanka, and Trinidad and Tobago; and 1 each for Afghanistan, Bangladesh, Botswana, Burkina Faso, Central African Republic, China, Democratic Republic of the Congo, Egypt, El Salvador, France, Ghana, Guatemala, Mauritius, Morocco, Nepal, New Zealand, Nicaragua, Palau, Puerto Rico, Reunion, Samoa, Sierra Leone, South Africa, Togo, Turkey, United States, and Vietnam.

Analysis of symptoms only included data from October 2015 onward. Figure 3 shows signs and symptoms among the 30 travelers with leptospirosis as the only diagnosis. Fever, headache, fatigue, and myalgia were reported frequently.

Data on the method of diagnosis were only available for 44 records. Twenty-two (50%) patients were diagnosed by serology (MAT, IgM ELISA, and/or a rapid test) alone, twelve (27%) by a nucleic acid amplification test (e.g., real time PCR or loop-mediated isothermal amplification [LAMP]) alone, five by both methods (11%), two by microscopy (5%), one by a nucleic acid amplification test and culture (2%) and two by other methods (not specified).

Table 1. Demographic and travel characteristics of travelers diagnosed with leptospirosis in the GeoSentinel Network, 1 January 1999 – 31 December 2016, n = 180*

Characteristic	Number	%
Male	132	74%
Median age in years (range)	32 (14-72)	
Age groups		
≤ 17 years	4	2%
18 – 34 years	94	53%
35 – 49 years	46	26%
50 – 64 years	30	17%
≥ 65 years	3	2%
Pre-travel advice obtained		
Yes	64	37%
No	83	47%
Unknown	28	16%
Travel reason		
Tourism	145	81%
Business	15	8%
Missionary / volunteer / researcher / aid work	9	5%
Visiting friends and relatives	9	5%
Military	1	1%
Travel duration in days (range)†	21 (5 -165)	
Time from return to presentation in days (range)	9 (1 – 28)	
Hospitalization	96	63%
Death	1	0.7%
Region of exposure		
Southeast Asia	93	52%
Central America	24	13%
South America	15	8%
Sub-Saharan Africa	14	8%
Caribbean	12	7%
South Central Asia	8	4%
Oceania	4	2%
North Africa	2	1%
Australia & New Zealand	1	1%
Middle east	1	1%
Northeast Asia	1	1%
North America	1	1%
Western Europe	1	1%
Not Ascertainable	1	1%

*Not all cells add to 180 due to missing data
†Among travelers who traveled to only one country (N = 121)

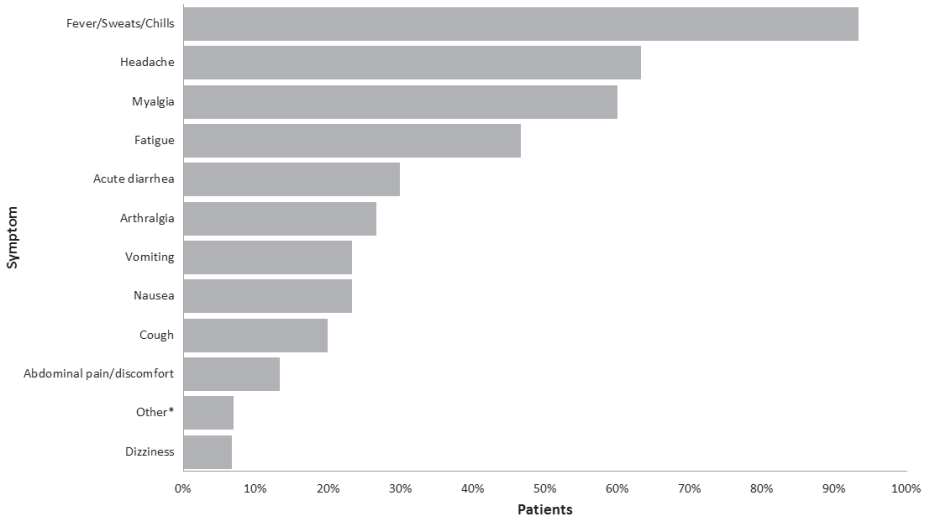

Figure 3. Proportion of reported signs or symptoms among patients diagnosed with leptospirosis in the GeoSentinel Network, 31 October 2015 – 31 December 2016, (n=30, patients with one diagnosis only)

Clinical symptoms reported in recognized disease in patients with one diagnosis, from 31st October 2015 onwards, when a revised coding system of symptoms in GeoSentinel was started. Reporting may be incomplete in GeoSentinel and may not reflect the complete disease course, but rather the initial symptoms.

* Other symptoms were not specified

† Less frequent reported symptoms (<5%, or 1 person each) included: constipation, diffuse rash, focal rash, itch, neck stiffness/photophobia, skin lesion/superficial infection, and weight loss.

Survey results

Of 56 sites solicited, 42 (75%) completed the survey. Eighty-eight percent of the responding sites were academic institutions; 81% of respondents diagnosed 1-10 travelers with leptospirosis per year in the past 5 years; 19% had not yet diagnosed any. Most sites (88%) were able to obtain leptospirosis diagnostic test results within 2 weeks. Key survey questions and responses, information on availability and usage of diagnostic methods, as well as key suspicion-raising exposures and clinical symptoms, are shown in Table 3. Supplementary Material 1 shows an overview of all questions and responses.

Half of the respondents (52%) considered leptospirosis in "non-specific febrile illness"; only 7% performed diagnostics for leptospirosis in the majority of those cases. A majority of respondents (66%) reported not testing for leptospirosis without a compelling exposure history, equating to testing of <10% of travelers with unspecified febrile illness returning from endemic areas. The decision to request diagnostic testing was influenced by suitable exposure histories (76%), exclusion of alternative established causes such as arboviral infections (48%), and certain laboratory abnormalities (60%). Clinical severity was reported to

Table 2. Distribution of gender and exposure regions with hospitalization status among travelers diagnosed with leptospirosis in the GeoSentinel Network, 1 January 1999 – 31 December 2016*

	Inpatient n (%)	Outpatient n (%)
Female	21 (46)	25 (54)
Median age (range)	29 (18 - 56)	28 (14 - 72)
Male	86 (66)	44 (34)
Median age (range)	35.5 (16 - 66)	33 (19 - 59)
Median age, overall (range)	33 (16-66)	31 (14-72)
Southeast Asia	52 (63)	31 (37)
Central America	14 (58)	10 (42)
South America	9 (60)	6 (40)
Sub-Saharan Africa	6 (43)	8 (57)
Caribbean	6 (50)	6 (50)
South Central Asia	5 (63)	3 (37)
Oceania	2 (50)	2 (50)
North Africa	1 (50)	1 (50)
Australia & New Zealand	0 (0)	1 (100)
North America	1 (100)	0 (0)
Northeast Asia	1 (100)	0 (0)
Western Europe	1 (100)	0 (0)

*N = 180; 110 inpatients, 70 outpatients. Not all cells add to 180 due to missing data.

Table 3. Selected results of a GeoSentinel supplemental site survey regarding clinical and diagnostic practices for leptospirosis

What exposures raise your suspicion of leptospirosis? (multiple answers possible)	n	% (of 42)
Freshwater contact	42	100
Natural disasters (e.g. floods)	36	86
Any travel to endemic areas	29	69
Animal contact	28	67
Soil contact	18	43
Use of freshwater for cleaning and other activities	12	29
Gardening	11	26
Which clinical presentation makes you suspect leptospirosis (multiple answers possible)		
Febrile illness with liver enzyme or renal function abnormalities	31	74
Febrile illness and jaundice	29	69
Febrile illness and conjunctival suffusion	26	62
Febrile illness and myalgia	23	55
Febrile illness with hemorrhagic manifestations	23	55
Non-specific febrile illness	22	52
Febrile illness with headache	17	41

Febrile illness with clinical signs of meningitis	13	31
Febrile illness with exanthema	12	29
Febrile illness and respiratory symptoms	12	29
All of the above	17	41
How often do you perform diagnostics for leptospirosis in the first diagnostics work-up in patients with unspecified febrile illness from endemic areas?		**% (of 41)**
<10% of cases	25	61
10 – 30% of cases	9	22
31 – 50% of cases	4	10
>50% of cases	3	7
How often do you test for leptospirosis in patients without a fitting exposure history?		
Never	11	27
In <10% of cases	16	39
In 10 - 30% of cases	10	24
In 31 - 50% of cases	0	0
In >50% of cases	4	10
What diagnostics for leptospirosis are available in your clinical setting? (Multiple answers possible)		
ELISA	30	73
PCR	19	46
MAT	14	34
Serological Rapid Test (RDT)	13	32
Culture	9	22
Do not know	4	10
Which diagnostic test for leptospirosis do you usually request? (Multiple answers possible)		
ELISA	24	59
PCR	12	29
MAT	12	29
Serological rapid test (RDT)	12	29
Determined by the laboratory	9	22
Determined by duration of clinical illness	6	15
Culture	3	7
Do not know	1	2
Approximately how often are convalescent samples collected for leptospirosis diagnostics after a negative first test?		**% of 40**
<20%	19	48
20 – 40%	9	23
41 – 60%	4	10
61 – 80%	3	8
>80%	5	13

ELISA = enzyme-linked immunosorbent assay; MAT = microscopic agglutination test; PCR = polymerase chain reaction; RDT = rapid diagnostic test.

have no influence on testing by 69% of respondents. Most sites (63%) continued to search for a definitive diagnosis (in >50% of patients), even when the patient was improving after empiric antibiotic therapy. Sixty percent of respondents would prescribe empiric doxycycline after exclusion of key differential diagnoses such as malaria, typhoid fever, and arboviral infections (Supplementary Material 1).

DISCUSSION

GeoSentinel surveillance data

Leptospirosis was an infrequent diagnosis among febrile returning travelers in GeoSentinel, but was identified mostly among those who had traveled for tourism purposes. With increasing popularity of tourism in tropical areas,[32] including active, environmental exposures like rafting, canoeing, and triathlons, the risk of leptospirosis acquisition among travelers may be increasing, as illustrated in previous case reports.[37, 41, 42] Known high-risk areas, such as Southeast Asia and Central America, were the most common regions of exposure in our database. The top countries where leptospirosis was acquired, Thailand, Costa Rica and Laos, are not necessarily known to be most endemic,[1, 43] but rather have higher numbers of travelers who are potentially engaged in freshwater recreational activities. The epidemiology of leptospirosis worldwide[1, 43] should be considered when evaluating the ill traveler presenting with fever.

Increased risk of leptospirosis has recently been observed in more temperate regions,[34, 44, 45] possibly influenced by climate change or different types of risk activities.[12, 45] Case series have reported significant proportions of travelers infected within Europe as well.[4, 33, 35, 46] In the Netherlands, 318 imported cases of leptospirosis were diagnosed between 1925 and 2008; 134 (42.1%) were acquired in Asia and 132 (41.5%) in Europe. France is a major tourist destination, with one of the highest reported leptospirosis incidences in Europe.[47] Because travelers returning ill from developed temperate-zone countries may be less likely to visit GeoSentinel clinics, patients with leptospirosis acquired in those regions may be underrepresented here.

An overall increase in patients with leptospirosis reported within the GeoSentinel network was observed over time; however, this was likely associated with an increase in the number of sites reporting in the network in the same period. Alternatively, it is possible that clinicians have become more aware of the illness, and that the wider introduction of PCR provided an option for more accurate diagnosis.

Only one fatality was reported to GeoSentinel, despite the potential risk of mortality associated with leptospirosis;[1] however, the GeoSentinel surveillance approach may not capture deaths efficiently.[38]

Most patients in this series were in the 18- to 49-year age group and reported non-specific symptoms, consistent with other published reports.[33, 46, 48] Fever was present in almost all patients; neurologic and respiratory symptoms were present in lower proportions than found in other reports,[13] but similar to a small case series in travelers.[37] It is also possible that the disease presentation was different in these travelers than is typically seen in leptospirosis in endemic areas. Other possibilities are that leptospirosis was not recognized in patients presenting with those rarer reported symptoms, that they were treated with empiric doxycycline, or that patients with these more severe forms of disease presented elsewhere. A prospective study would be needed to better address these uncertainties.

Most patients were male, although the proportion of female patients (26%) was higher than in some other large case series (reported proportions of <10%).[33, 37, 46, 48] A higher proportion of the reported hospitalized cases were male compared with female patients, akin to other reports.[33, 49] Some possible factors that can contribute to male predominance in leptospirosis include a greater tendency to participate in high-risk outdoor activities, later presentation for medical evaluation, androgenic steroids and other biologic factors,[49, 50] or a higher infectious inoculum. The relatively high percentage of female patients in our analysis could signal a shift in behavior of female travelers, increasing their risk of contracting the disease. Female travelers should be considered equally at risk as those who are male with respect to leisure exposure, and leptospirosis should be routinely considered when evaluating the febrile returned female traveler.

Site survey

Leptospirosis is an infection that is frequently under-diagnosed, especially in mild disease presentations.[17] In our survey among GeoSentinel clinicians, all reported seeing relatively few leptospirosis patients each year (<10). The majority of respondents (75% of all sites) worked at an academic institution, where leptospirosis diagnostics are expected to be readily available. Molecular detection techniques are the cornerstone of diagnosis of leptospirosis in the acute phase,[26] but were available in only half of the clinics, and were actually used in less than a third of the clinics. In addition, convalescent samples were reported to have been collected in a minority of cases after a negative acute serology, despite the fact that antibodies only appear in the blood 5-7 days or sometimes after 10 days or longer following disease onset.[2] Possibly, patients with mild disease improved on treatment and abandoned follow-up, forgoing further testing.

Half of the clinicians considered leptospirosis in a case of "non-specified febrile illness", but very few actually performed diagnostics for it. Few clinicians reported testing patients without a suggestive exposure history. Classical exposures, such as freshwater contact and floods, were well known among clinicians. In the general population the infection mechanism often remains unclear, and often there is no classical exposure history.[3] Data on travelers are scarce, but the case series that exist do report clear exposure histories.[35, 37] Nevertheless,

pre-selecting patients based only on a well-defined exposure history may result in under-diagnosis of leptospirosis among patients without a clear-cut exposure, especially when they returned from highly endemic areas.

Although the classic Weil's triad of fever, jaundice, renal failure, with or without accompanying hemorrhagic features, was well recognized, other severe disease manifestations were less known among GeoSentinel Surveillance Network respondents. Aseptic meningitis can occur in up to 25% of cases,[13] but this manifestation was recognized by relatively few respondents (31%). Pulmonary hemorrhage is an important but under-recognized form of the disease, and outbreaks with only this manifestation have been described.[14, 16] Almost half of the respondents did not include leptospirosis in the differential diagnosis of the febrile traveler presenting with hemorrhagic disease manifestations in the absence of the classic Weil's triad. In this analysis of returned travelers, the lesser-known disease manifestations were not frequently reported, possibly due to the nature of symptom data collection in GeoSentinel, which combines specific symptoms into broad systemic categories.[40]

The results of our survey on clinical practices in specialized travel medicine settings suggest that the number of leptospirosis cases in travelers reported in the GeoSentinel Surveillance system is likely to be an underestimate. This may be due to suboptimal access to diagnostic testing at many sites, especially in the setting where acute disease is encountered. Furthermore, we found that the diagnosis of leptospirosis may not be routinely considered when clear-cut exposure histories are absent and when some rather typical clinical features are missing. Based on our site survey, there is a need for improved awareness among clinicians about the spectrum of exposures, clinical presentations, and diagnostic considerations.

Strengths and limitations

In addition to those already discussed, the GeoSentinel surveillance data have several other limitations and may not be generalizable to the traveler population as a whole.[38, 51, 52, 53] GeoSentinel reporting may be biased towards capture of more complicated or more severe disease, since most GeoSentinel sites are also academic institutions. Overall, hospitalization may be underestimated in GeoSentinel, but there are some sites that almost exclusively capture data from the inpatient setting. Therefore, the high proportion of hospitalizations should be interpreted carefully. Furthermore, leptospirosis may have been missed among travelers presenting with atypical exposures or symptoms, or presentations of milder, self-limited disease, and may have had an influence on the results. GeoSentinel preferentially captures travelers returning from tropical regions due to the nature of the clinics that make up the network; so, travelers with disease acquired in temperate regions are likely under-represented. GeoSentinel criteria for diagnosing patients with leptospirosis may differ from the definitions used by national reference laboratories and other reporting systems, limiting direct comparison with data from those sources. Despite these limitations, this is one of the

largest series of leptospirosis in travelers published to date and provides valuable information about the epidemiology of leptospirosis in international travelers.

A limitation of the network survey is that the answers were captured from clinicians specialized in travel medicine and may not be representative of all clinical practices in a particular institution. Furthermore, the data are self-reported and may therefore not be reflective of actual practice. Some questions allowed multiple answers, leading to respondents choosing incompatible answers, making the interpretation more complex. However, the relatively high response rate of 75% of GeoSentinel sites in our view accounts for a representative survey among our global network of mostly academic travel clinics.

Conclusions

Leptospirosis may be an infrequently encountered cause of substantial morbidity among international travelers that may not be clinically suspected. Although leptospirosis was most frequently diagnosed among persons visiting highly endemic countries, it may occur elsewhere and warrants broader consideration in the differential of the ill traveler. Given the laboratory diagnostic challenges and non-specific presentation of many clinically evident cases, the burden of reported disease is likely underestimated, as supported by other published data.[1, 18, 19, 20] We recommend enhancement of awareness about leptospirosis and heightened clinical suspicion when evaluating the ill traveler. Laboratories need to have up-to-date diagnostic methods available; molecular detection techniques are key to early diagnosis, which is helpful for the early initiation of treatment that may substantially reduce morbidity and improve outcomes. Empiric treatment in cases of high suspicion is recommended. Efforts to improve knowledge among clinicians regarding (the often unclear) exposures and clinical presentations are needed.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Supplementary Materials

The Supplementary Materials provide data on the site survey, and on additional diagnoses of leptospirosis patients.

Supplementary Material 1. Questionnaire used for the GeoSentinel supplemental site survey regarding clinical and diagnostic practices for leptospirosis

1. Are you working in an academic institution?		
Answer Options	**Response Percent**	**Response Count**
Yes	88%	37
No	12%	5
	answered question	**42**
	skipped question	**0**
2. What is the (estimated) number of cases of leptospirosis you have seen annually in the past 5 years?		
Answer Options	**Response Percent**	**Response Count**
None	19%	8
1 - 10 / year	81%	34
11 - 20 / year	0%	0
> 20 / year	0%	0
	answered question	**42**
	skipped question	**0**
3. What is the average return time for leptospirosis diagnostic test results in your institution?		
Answer Options	**Response Percent**	**Response Count**
1 week or less	41%	17
1 - 2 weeks	48%	20
2 - 3 weeks	5%	2
> 3 weeks	7%	3
	answered question	**42**
	skipped question	**0**
4. What exposures raise your suspicion of leptospirosis? (Multiple answers possible)		
Answer Options	**Response Percent**	**Response Count**
Gardening	26%	11
Animal contact	67%	28
Fresh water contact	100%	42
Use of fresh water for cleaning and other activities	29%	12
Soil contact	43%	18
Any travel to endemic areas	69%	29
Natural disasters (floodings)	86%	36
Not sure	0%	0
Other (please specify)	12%	5
	answered question	**42**
	skipped question	**0**

5. How often do you test for leptospirosis in a patient without a fitting exposure history?

Answer Options	Response Percent	Response Count
Never	27%	11
In 10% of cases	39%	16
In 10 - 30% of cases	24%	10
In 30 - 50% of cases	0%	0
In > 50% of cases	10%	4
	answered question	**41**
	skipped question	**1**

6. I do not test for leptospirosis if there is no history of fresh water contact.

Answer Options	Response Percent	Response Count
True	5%	2
False	95%	39
	answered question	**41**
	skipped question	**1**

7. I do not test for leptospirosis if there is no history of animal contact.

Answer Options	Response Percent	Response Count
True	5%	2
False	95%	38
	answered question	**40**
	skipped question	**2**

8. Which clinical presentation makes you suspect leptospirosis? (Multiple answers possible)

Answer Options	Response Percent	Response Count
Unspecific febrile illness	53%	22
Febrile illness with: liver enzyme or renal function abnormalities	74%	31
Febrile illness with exanthema	29%	12
Febrile illness with haemorrhagic manifestations	55%	23
Febrile illness with clinical signs of meningitis	31%	13
Febrile illness with headache	41%	17
Febrile illness and myalgia	55%	23
Febrile illness and conjunctival suffusion	62%	26
Febrile illness and jaundice	69%	29
Febrile illness and respiratory symptoms	29%	12
All of the above	41%	17
Other (please specify)	12%	5
	answered question	**42**
	skipped question	**0**

9. When do you request leptospirosis diagnostics in a patient with febrile illness from an endemic area? (Multiple answers possible)

Answer Options	Response Percent	Response Count
As soon as it is considered in the differential diagnosis	71%	30

After exclusion of other causes (dengue, chikungunya, malaria, typhoid fever)	48%	20
If there is a suitable exposure history	76%	32
If there are particular laboratory abnormalities (liver enzyme- AND/OR renal function disorders)	60%	25
Only in a very sick patient without diagnosis	5%	2
Other (please specify)	0%	0
	answered question	**42**
	skipped question	**0**

10. How often do you perform diagnostics for leptospirosis in the first diagnostics work-up in patients with unspecified febrile illness from endemic areas?

Answer Options	**Response Percent**	**Response Count**
< 10% of cases	61%	25
10 - 30% of cases	22%	9
30 - 50% of cases	10%	4
> 50% of cases	7%	3
	answered question	**41**
	skipped question	**1**

11. Does the clinical severity of a febrile illness lower the threshold to request leptospirosis diagnostics?

Answer Options	**Response Percent**	**Response Count**
No. Diagnostic testing is performed upon clinical suspicion, regardless of disease severity.	69%	29
Yes. No testing if disease is mild/self limiting.	19%	8
Yes. Only testing in case of moderate to severe illness.	12%	5
Yes. Only testing in case of severe illness (requiring hospitalization).	0%	0
	answered question	**42**
	skipped question	**0**

12. If a patient with a differential diagnosis of leptospirosis is improving (spontaneously or following empirical antibiotic treatment), do you still request diagnostics for leptospirosis to confirm your diagnosis?

Answer Options	**Response Percent**	**Response Count**
Yes	61%	25
No	5%	2
Sometimes	34%	14
	answered question	**41**
	skipped question	**1**

13. When do you prescribe empiric doxycycline in a returning traveler with unspecified febrile illness?

Answer Options	**Response Percent**	**Response Count**
When illness persists, after exclusion of other causes such as dengue, chikungunya, malaria and typhoid fever	60%	24
When illness persists, after exclusion of other causes such as dengue, chikungunya, malaria and typhoid fever, plus exclusion of leptosopirosis	8%	3
When illness persists, after exclusion of other causes such as dengue, chikungunya, malaria and typhoid fever, plus exclusion of rickettsial disease	5%	2

When illness persists, after exclusion of other causes such as dengue, chikungunya, malaria and typhoid fever, plus exclusion of leptosirosis and rickettsial disease	5%	2
In any traveler with unspecified febrile illness	2%	1
Other (please specify)	20%	8
	answered question	**40**
	skipped question	**2**

14. If a patient with unspecified febrile illness is improving, following empirical treatment with doxycycline, how often do you still look for a definitive diagnosis?

Answer Options	**Response Percent**	**Response Count**
Never	2%	1
< 20% of cases	22%	9
20 - 50% of cases	12%	5
> 50% of cases	63%	26
	answered question	**41**
	skipped question	**1**

15. Are diagnostics for rickettsial infections easily available in your institution?

Answer Options	**Response Percent**	**Response Count**
Yes	61%	25
No	7%	3
Equally easy/difficult as leptospirosis diagnostics	32%	13
Don't know	0%	0
	answered question	**41**
	skipped question	**1**

16. Which one do you request earlier in the diagnostic process: diagnostics for leptospirosis or for rickettsial disease?

Answer Options	**Response Percent**	**Response Count**
Leptospirosis	10%	4
Rickettsial disease	39%	16
No difference	51%	21
	answered question	**41**
	skipped question	**1**

17. What diagnostics for leptospirosis are available in your clinical setting? (Multiple answers possible)

Answer Options	**Response Percent**	**Response Count**
Serological rapid test	32%	13
ELISA	73%	30
MAT (Microscopic Agglutination Test)	34%	14
PCR	46%	19
Culture	22%	9
I don't know	10%	4
	answered question	**41**
	skipped question	**1**

18. Which diagnostic test for leptospirosis do you usually request? (Multiple answers possible)

Answer Options	Response Percent	Response Count
Serological rapid test	29%	12
ELISA	59%	24
MAT (Microscopic Agglutination Test)	29%	12
PCR	29%	12
Culture	7%	3
I don't know	2%	1
Determined by the laboratory	22%	9
Determined by the duration of clinical illness	15%	6
	answered question	**41**
	skipped question	**1**

19. What specimen(s) do you use for the diagnostic tests? (Multiple answers possible)

Answer Options	Response Percent	Response Count
Blood	100%	40
Urine	38%	15
CSF	15%	6
Other (please specify)	10%	4
	answered question	**40**
	skipped question	**2**

20. Approximately how often are convalescent samples collected for leptospirosis diagnostics after a negative first test?

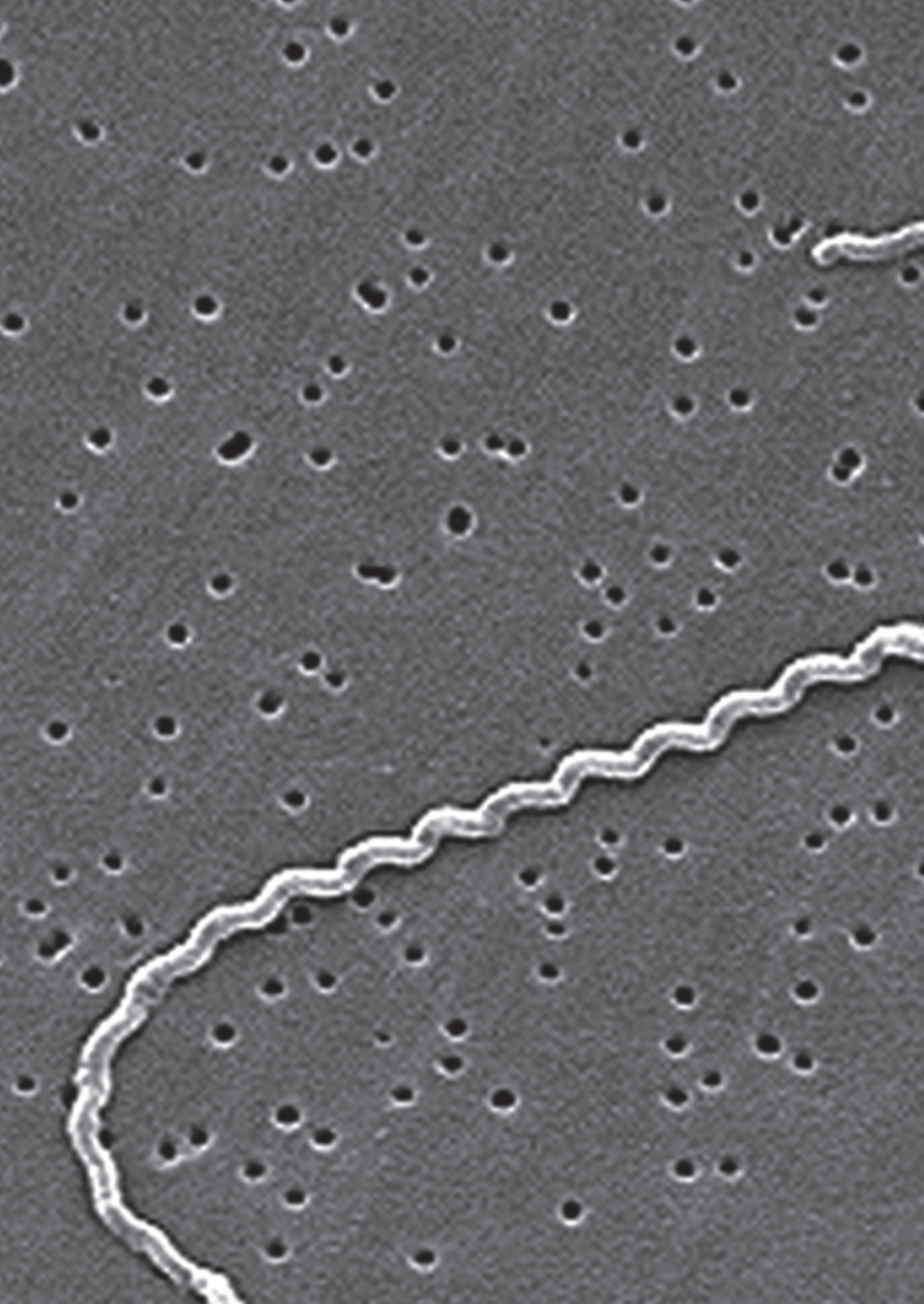
Answer Options	Response Percent	Response Count
< 20%	48%	19
21 - 40%	23%	9
41 - 60%	10%	4
61 - 80%	8%	3
> 81%	13%	5
	answered question	**40**
	skipped question	**2**

Supplementary Material 2. Additional diagnoses among those patients with leptospirosis and at least one other diagnosis, GeoSentinel Network, 1 January 1999 – 31 December 2016

	Diagnosis 1*	Diagnosis 2	Diagnosis 3
D1	Leptospirosis	Acute gastroenteritis > 12 hours	-
D2	Leptospirosis	Febrile illness unspecified (<3 weeks)	Rickettsia typhi (flea-borne murine typhus)
D3	Leptospirosis	Weight loss	-
D4	Leptospirosis	Influenza-like illness	Diarrhea, acute unspecified
D5	Leptospirosis	Giardia	-
D6	Leptospirosis	Acute renal failure	-
D7	Leptospirosis	Acute renal failure	-
D8	Leptospirosis	Schistosomiasis, human species unknown	-
D9	Leptospirosis	Rickettsia (now Orientia) tsutsugamushi	-
D10	Leptospirosis	Dengue, uncomplicated	-
D11	Leptospirosis	Fatigue < 1 month (not febrile)	Diarrhea, chronic, unspecified
D12	Leptospirosis	Dengue, uncomplicated	-
D13	Leptospirosis	Other*	-
D14	Leptospirosis	Cytomegalovirus	Blastocystis sp.
D15	Leptospirosis	Pyelonephritis	-
D16	Leptospirosis	Conjuctivitis	Fatigue >= 1 month (not febrile)
D17	Leptospirosis	Zika virus	-
D18	Leptospirosis	Cutaneous larva migrans, hookworm-related	-
D19	Leptospirosis	Anemia	Gastrointestinal or rectal bleeding, unspecified
D20	Leptospirosis	Acute gastroenteristis >12 hours	
D21	Leptospirosis	Acute renal failure	Upper respiratory tract infection
D22	Leptospirosis	Febrile illness, unspecified	

*Not necessarily reflecting the patient's primary or most important diagnosis

** Other reported as "Hepatomegaly, papules and swelling over feet"



Chapter 5

Nucleic acid and antigen detection tests for leptospirosis

Bada Yang, Sophia G. de Vries, Ahmed Ahmed, Benjamin J. Visser, Ingeborg M. Nagel, René Spijker, Martin P. Grobusch, Rudy A. Hartskeerl, Marga G.A. Goris, Marika M.G. Leeflang

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ABSTRACT

Background

Early diagnosis of leptospirosis may contribute to the effectiveness of antimicrobial therapy and early outbreak recognition. Nucleic acid and antigen detection tests have the potential for early diagnosis of leptospirosis. With this systematic review, we assessed the sensitivity and specificity of nucleic acid and antigen detection tests.

Objectives

To determine the diagnostic test accuracy of nucleic acid and antigen detection tests for the diagnosis of human symptomatic leptospirosis.

Search methods

We searched electronic databases including MEDLINE, Embase, the Cochrane Library, and regional databases from inception to 6 July 2018. We did not apply restrictions to language or time of publication.

Selection criteria

We included diagnostic cross-sectional studies and case-control studies of tests that made use of nucleic acid and antigen detection methods in people suspected of systemic leptospirosis. As reference standards, we considered the microscopic agglutination test alone (which detects antibodies against leptospirosis) or in a composite reference standard with culturing or other serological tests. Studies were excluded when the controls were healthy individuals or when there were insufficient data to calculate sensitivity and specificity.

Data collection and analysis

At least two review authors independently extracted data from each study. We used the revised Quality Assessment of Diagnostic Accuracy Studies tool (QUADAS-2) to assess risk of bias. We calculated study-specific values for sensitivity and specificity with 95% confidence intervals (CI) and pooled the results in a meta-analysis when appropriate. We used the bivariate model for index tests with one positivity threshold, and we used the hierarchical summary receiver operating characteristic model for index tests with multiple positivity thresholds. As possible sources of heterogeneity, we explored: timing of index test, disease prevalence, blood sample type, primers or target genes, and the real-time polymerase chain reaction (PCR) visualisation method. These were added as covariates to the meta-regression models.

Main results

We included 41 studies evaluating nine index tests (conventional PCR (in short: PCR), real-time PCR, nested PCR, PCR performed twice, loop-mediated isothermal amplification,

enzyme-linked immunosorbent assay (ELISA), dot-ELISA, immunochromatography-based lateral flow assay, and dipstick assay) with 5981 participants (1834 with and 4147 without leptospirosis). Methodological quality criteria were often not reported, and the risk of bias of the reference standard was generally considered high. The applicability of findings was limited by the frequent use of frozen samples. We conducted meta-analyses for the PCR and the real-time PCR on blood products.

The pooled sensitivity of the PCR was 70% (95% CI 37% to 90%) and the pooled specificity was 95% (95% CI 75% to 99%). When studies with a high risk of bias in the reference standard domain were excluded, the pooled sensitivity was 87% (95% CI 44% to 98%) and the pooled specificity was 97% (95% CI 60% to 100%). For the real-time PCR, we estimated a summary receiver operating characteristic curve. To illustrate, a point on the curve with 85% specificity had a sensitivity of 49% (95% CI 30% to 68%). Likewise, at 90% specificity, sensitivity was 40% (95% CI 24% to 59%) and at 95% specificity, sensitivity was 29% (95% CI 15% to 49%). The median specificity of real-time PCR on blood products was 92%. We did not formally compare the diagnostic test accuracy of PCR and real-time PCR, as direct comparison studies were lacking. Three of 15 studies analysing PCR on blood products reported the timing of sample collection in the studies included in the meta-analyses (range 1 to 7 days postonset of symptoms), and nine out of 16 studies analysing real-time PCR on blood products (range 1 to 19 days postonset of symptoms). In PCR studies, specificity was lower in settings with high leptospirosis prevalence. Other investigations of heterogeneity did not identify statistically significant associations. Two studies suggested that PCR and real-time PCR may be more sensitive on blood samples collected early in the disease stage. Results of other index tests were described narratively.

Authors' conclusions

The validity of review findings are limited and should be interpreted with caution. There is a substantial between-study variability in the accuracy of PCR and real-time PCR, as well as a substantial variability in the prevalence of leptospirosis. Consequently, the position of PCR and real-time PCR in the clinical pathway depends on regional considerations such as disease prevalence, factors that are likely to influence accuracy, and downstream consequences of test results. There is insufficient evidence to conclude which of the nucleic acid and antigen detection tests is the most accurate. There is preliminary evidence that PCR and real-time PCR are more sensitive on blood samples collected early in the disease stage, but this needs to be confirmed in future studies.

PLAIN LANGUAGE SUMMARY

How accurate are nucleic and antigen detection tests in diagnosing leptospirosis?

What was studied in this review?

Leptospirosis is an infectious disease, caused by bacteria called Leptospira that can be found in soil, freshwater, or in the infected urine of certain animals. It is mainly a problem in humid, tropical countries in Southeast Asia, and Central and South America, but it can also occur in temperate regions.

Leptospirosis causes fever and headache, and in some cases kidney, lung, or heart problems. Often, the symptoms are not unique for the disease, which makes it difficult to diagnose, and is therefore frequently missed.

Laboratory tests confirm diagnosis. These tests are based on demonstration of the presence of Leptospira, its DNA, or antibodies against Leptospira. Nucleic acid and antigen detection tests, such as conventional polymerase chain reaction (PCR) and real-time PCR, identify the bacterium or its DNA directly in blood or urine. Nucleic acid and antigen detection tests may detect Leptospira better in the early days of an infection, so that people can be treated earlier with antibiotics – resulting in better outcomes – and can provide useful information in outbreak situations. In outbreak situations, nucleic acid and antigen detection tests could serve as early warning systems.

What was the aim of this review?

The aim was to assess how well nucleic acid and antigen tests perform in detecting leptospirosis. In other words, to assess how many mistakes these tests make by either missing people with leptospirosis or misidentifying people without leptospirosis (healthy people or people with another disease).

What were the main results in this review?

The review included information from 41 studies with 5981 participants. We identified nine nucleic acid and antigen detection tests, of which PCR and real-time PCR were most often investigated.

An important finding was that the accuracy of both PCR and real-time PCR varied strongly between studies. We presented average accuracies for both tests, but there was great uncertainty around these averages. PCR often correctly identified people without leptospirosis (averaging 95 in 100 people), but frequently missed people with leptospirosis (averaging 30 in 100 people). The accuracy of the real-time PCR depended on the cut-off value for a positive test result. At a cut-off value where real-time PCR often correctly identified people without leptospirosis (averaging 95 in 100 people), it also frequently missed

people with leptospirosis (averaging 71 in 100 people). If a person tests positive or negative for PCR or real-time PCR, the chance of the person actually having the disease depends on whether the suspicion of leptospirosis in that person was already high before taking the test. So, when interpreting the results of any of these tests, one must consider the strength of suspicion of leptospirosis in an individual, and how often leptospirosis occurs in the setting in which the test will be used.

It was uncertain whether PCR or real-time PCR performed better in detecting leptospirosis, since studies directly comparing these two tests were lacking. The results of other nucleic and antigen detection tests are described in the main text of the review.

How reliable were the results of the studies in this review?

Not all studies were conducted according to the highest scientific standards. This means that the results of some studies may have been overestimated or underestimated. Furthermore, the tests used to verify whether a person truly had leptospirosis or not (called the reference standard) may not accurately distinguish people with or without leptospirosis. For these reasons, more high-quality studies are needed to confirm the reliability of these results.

Who do the results of this review apply to?

The results may apply to people who may have leptospirosis. However, the performance of the PCR and real-time PCR vary considerably among studies and it is yet unclear what causes this difference in performances. It is probable that the test performs better or worse depending on how prevalent leptospirosis is in the region, and depending on the time between the onset of symptoms and time of testing. Therefore, it is difficult to generalise the results of this review to all settings.

How up-to-date is this review?

The review authors searched for and used studies published up to 6 July 2018.

SUMMARY OF FINDINGS

Summary of findings 1. Conventional polymerase chain reaction (PCR)

Conventional polymerase chain reaction (PCR)				
Population: people suspected of leptospirosis in different stages of disease (early to late), excluding those with solely ocular problems or aseptic meningitis				
Setting: worldwide, primary to tertiary care facilities, outbreak as well as non-outbreak settings				
Index test: conventional PCR on blood samples (whole blood, serum), all inhouse tests				
Reference standard: MAT on serum alone, or MAT on serum alongside culturing, or MAT on serum alongside IgM ELISA, or MAT on serum alongside culturing and IgM ELISA				
Number of cases/non-cases (studies): 660/1224 (15)	**Pooled sensitivity:** 70% (95% CI 37 to 90)	**Pooled specificity:** 95% (95% CI 75 to 99)	**Consequences in a cohort of 1000**	
Prevalence:	**Positive post-test probability:**	**Negative post-test probability:**	**Missed diseased:**	**Falsely diagnosed:**
32.5% (median of all studies)	87 (95% CI 53 to 97)	87 (95% CI 71 to 95)	98 (95% CI 32 to 205)	35 (95% CI 6 to 168)
9.7%	59 (95% CI 20 to 89)	97 (95% CI 92 to 99)	29 (95% CI 9 to 61)	47 (95% CI 8 to 225)
Positive likelihood ratio: 13.56 (95% CI 2.61 to 70.29)		**Negative likelihood ratio:** 0.32 (95% CI 0.12 to 0.82)		
Quality of evidence: none of the studies scored 'low risk of bias' on all domains. 6/15 studies used an unreliable reference standard. Risk of spectrum bias was unclear to high.				
Investigations of heterogeneity: readers should note that the results are **very heterogeneous** between studies. Specificity declined with increasing leptospirosis prevalence. The choice of PCR blood sample type was not associated with test accuracy.				
Sensitivity analysis: sensitivity increased to 87% (95% CI 44% to 98%) when studies at high risk of bias for the 'reference standard' domain were excluded. However, the CIs were extremely wide with substantial overlap with the results of the overall meta-analysis.				

CI: confidence intervals; IgM ELISA: immunoglobulin M enzyme-linked immunosorbent assay; MAT: microscopic agglutination test

Summary of findings 2. Conventional polymerase chain reaction (PCR) sensitivity analysis, excluding high risk of bias (reference standard domain)

Conventional polymerase chain reaction (PCR) sensitivity analysis, excluding studies at high risk of bias (reference standard domain)				
Population: people suspected of leptospirosis in different stages of disease (early to late), excluding those with solely ocular problems or aseptic meningitis				
Setting: worldwide, primary to tertiary care facilities, outbreak as well as non-outbreak settings				
Index test: conventional PCR on blood samples (whole blood, serum), all inhouse tests				
Reference standard: MAT on serum alone (2-gate studies), or MAT on serum alongside culturing, or MAT on serum alongside IgM ELISA, or MAT on serum alongside culturing and IgM ELISA (single-gate studies)				
Number of cases/non-cases (studies): 538/487 (9)	**Pooled sensitivity:** 87% (95% CI 44% to 98%)	**Pooled specificity:** 97% (95% CI 60% to 100%)	**Consequences in a cohort of 1000**	
Prevalence:	**Positive post-test probability:**	**Negative post-test probability:**	**Missed diseased**	**Falsely diagnosed**
32.5% (median of all studies)	94% (95% CI 41% to 100%)	94% (95% CI 70% to 99%)	42 (95% CI 5 to 183)	17 (95% CI 1 to 272)
9.7%	78% (95% CI 13% to 99%)	99% (95% CI 91% to 100%)	13 (95% CI 2 to 55)	23 (95% CI 1 to 363)
Positive likelihood ratio: 33.86 (95% CI 1.59 to 719.39)		**Negative likelihood ratio:** 0.13 (95% 0.02 to 0.85)		
Quality of evidence: none of the studies scored 'low risk of bias' on all domains. Risk of bias for the 'reference standard' domain was unclear to low. Risk of bias for the 'patient selection' domain was unclear to high.				

CI: confidence intervals; IgM ELISA: immunoglobulin M enzyme-linked immunosorbent assay; MAT: microscopic agglutination test; NA: not applicable.

Summary of findings 3. Real-time polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction (PCR)		
Population: people suspected of leptospirosis in different stages of disease (early to late), excluding those with solely ocular problems or aseptic meningitis		
Setting: worldwide, primary to tertiary care facilities, outbreak as well as non-outbreak settings		
Index test: real-time PCR on blood samples (whole blood, plasma, serum), all inhouse tests, using unknown thresholds		
Reference standard: MAT on serum alone, or MAT on serum alongside culturing, or MAT on serum alongside IgM ELISA, or MAT on serum alongside culturing and IgM ELISA		
Number of cases/non-cases (studies): 826/2384 (16)	**Sensitivity at fixed value:** 49% (95% CI 30% to 68%)	**Specificity at fixed value:** 85% (fixed, unknown threshold)
	Sensitivity at fixed value: 40% (95% CI 24% to 59%)	**Specificity at fixed value:** 90% (fixed, unknown threshold)
	Sensitivity at fixed value: 29% (95% CI 15% to 49%)	**Specificity at fixed value:** 95% (fixed, unknown threshold)
Prevalence:	**Positive post-test probability:**	**Negative post-test probability:**
32.5% (median of all studies)	NA	NA
9.7%	NA	NA
Positive likelihood ratio: NA		**Negative likelihood ratio:** NA
Quality of evidence: only 2 studies scored 'low risk of bias' on all domains. 8/16 studies used an unreliable reference standard. Risk of bias for the 'patient selection' domain was generally unclear, with 9/16 studies not reporting clear selection processes.		
Investigations of heterogeneity: readers should note that the results are **very heterogeneous** between studies. The choice of blood sample type, real-time PCR visualisation method and prevalence were not associated with test accuracy.		
Sensitivity analysis: when low-quality studies were excluded, there was no important change in test accuracy.		

We refrained from estimating post-test probabilities and likelihood ratios because the thresholds for the pooled sensitivities and specificities were unknown.
CI: confidence intervals; IgM ELISA: immunoglobulin M enzyme-linked immunosorbent assay; MAT: microscopic agglutination test; NA: not applicable.

INTRODUCTION

Target condition being diagnosed

Leptospirosis is a worldwide prevalent zoonosis caused by the pathogenic spirochaetes of the bacterial genus Leptospira.[2] Humans acquire the infection through direct contact with the infected urine of carrier animals, or by contact with the environment contaminated with pathogenic leptospires. In recent years, leptospirosis has been identified as a common public health problem, illustrated by outbreaks in Southeast Asia, and Central and South America. Furthermore, the incidence of leptospirosis in both low-income and middle- to high-income countries appears to be increasing,[3-7] causing substantial morbidity and mortality.[8] The disease is most frequently found in tropical and subtropical climates with incidences ranging from 10 to 100 per 100,000 people in endemic regions. Pathogenic leptospires also persist in more temperate regions, such as Denmark, Greece, Portugal, France, Germany, and

the Netherlands, where it is an important cause of illness in returning travellers.[9,10] Factors contributing to higher levels of prevalence are local agricultural practices, close proximity to mammalian reservoirs, poor sanitation, soil contact, and high rainfall.[11] Flooding associated with heavy seasonal rainfall and natural disasters may increase incidence to epidemic proportions, to more than 100 per 100,000 people.[12] It is thought that the emergence of leptospirosis is aggravated by global climate change, increasing contact between humans and wild animal populations, and the exponential expansion of urban slums.[13,14]

The clinical manifestations of leptospirosis are diverse; symptoms range from a mild undifferentiated fever syndrome including myalgia and headaches, to the severest form that may involve renal failure and jaundice (classically known as Weil's disease), and other complications such as pulmonary haemorrhages, aseptic meningitis, and myocarditis.[15] Fatality rates for severe forms range from 5% to 50%.[12,13] The non-specific clinical presentation of leptospirosis makes it challenging to distinguish from infections such as malaria, dengue, influenza, hepatitis, and yellow fever.[15] Consequently, laboratory tests are essential to confirm the diagnosis. These tests are based on either demonstration of leptospires, antibodies against leptospires, or their DNA.

The current reference standard for the diagnosis of leptospirosis is based on antibody detection by the microscopic agglutination test (MAT), with or without culture. Since anti-Leptospira antibodies appear only in the later stage of the disease, MAT and other serological tests, such as the immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA), are impractical in establishing an early diagnosis.[16] In addition, the culture of leptospires does not contribute to an early diagnosis, due to their slow growth.[12] Nucleic acid tests, such as the polymerase chain reaction (PCR) and antigen detection tests, can detect leptospiral DNA or antigens directly in blood in the first days of the disease and are thus capable of yielding an early diagnosis.[12] This type of early detection test may facilitate early outbreak warnings and make the administration of early microbial treatment possible. Additionally, leptospires appear in the urine after a few days, on which nucleic acid detection methods can be applied as well.[12] Early administration of treatment is generally considered to improve a person's outcome compared to treatment at a later disease stage, although more studies are needed to confirm this.[17]

Index test(s)

This review evaluated nucleic acid and antigen detection tests for pathogenic leptospires. Commonly used nucleic acid tests for the diagnosis of human leptospirosis are the PCR, its variants, and isothermal amplification tests such as loop-mediated isothermal amplification (LAMP). Nucleic acid tests can be used to test blood, cerebrospinal fluid (CSF), aqueous humour, and urine samples. Other antigen detection tests include ELISA and fluorescent antibody testing (FAT) for the detection of Leptospira antigens, silver staining, and immunohistochemistry.

Summary of findings 4. Nested polymerase chain reaction (PCR), conventional PCR performed twice, loop-mediated isothermal amplification (LAMP), immunochromatography-based lateral flow assay (ICG-based LFA) enzyme-linked immunosorbent assay (ELISA), dot-ELISA, and dip-stick Assay

Nested polymerase chain reaction (PCR), conventional PCR performed twice, loop-mediated isothermal amplification (LAMP), immunochromatography-based lateral flow assay (ICG-based LFA) enzyme-linked immunosorbent assay (ELISA), dot-ELISA, and dipstick assay			
Population: people suspected of leptospirosis in unknown stages of disease, excluding those with solely ocular problems or aseptic meningitis			
Setting: worldwide, primary to tertiary care facilities, outbreak as well as non-outbreak settings			
Index test: nested PCR (on serum samples, all inhouse tests), conventional PCR performed twice (on serum samples, all inhouse tests), LAMP (on whole blood, plasma, or urine samples, all inhouse tests), ICG-based LFA (on urine samples, inhouse test), ELISA (on urine samples, inhouse test), dot-ELISA (on urine samples, inhouse test), dipstick assay (on urine samples, inhouse test)			
Reference standard: MAT on serum alone or MAT on serum alongside culturing			
Quality of evidence: none of the studies scored 'low risk of bias' on all domains. 8/11 studies were rated 'high risk of bias' for the 'reference standard' domain. Risk of bias for the 'patient selection' domain was generally unclear, with 7/11 studies not reporting clear selection processes.			
Study ID	**Number of cases/non-cases**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
Nested PCR (4 studies)[a]			
Blanco 2014	28/493	86% (67% to 96%)	100% (99% to 100%)
Gokmen 2016 (*lipL32*)[c]	21/26	90% (70% to 99%)	42% (23% to 63%)
Koizumi 2009	26/81	0% (0% to 13%)	96% (90% to 99%)
Merien 2005	17/34	71% (44% to 90%)	62% (44% to 78%)
Conventional PCR performed twice (2 studies)[a]			
Seng 2007	4/117	75% (19% to 99%)	94% (88% to 98%)
Yersin 1998	60/52	47% (34% to 60%)	96% (87% to 100%)
LAMP (2 studies)[a]			
Thaipadungpanit 2011 (*lipL41)*[c]	133/133	38% (29% to 46%)	90% (84% to 95%)
Kitashoji 2015 (plasma)[c]	132/155	14% (9% to 22%)	83% (76% to 89%)
ICG-based LFA (1 study)[a]			
Widiyanti 2013	28/16	96% (82% to 100%)	56% (30% to 80%)
ELISA (1 study)[a]			
Chaurasia 2018 (LipL32)[c]	23/6	100% (85% to 100%)	67% (22% to 96%)
Dot-ELISA (1 study)[a]			
Saengjaruk 2002	25/18	64% (43% to 82%)	100% (81% to 100%)
Dipstick assay (1 study)[a]			
Widiyanti 2013	28/16	89% (72% to 98%)	63% (35% to 85%)

[a]*No meta-analyses were conducted for these index tests.*
[b]*Zero cell correction by applying 0.5 to each cell*
[c]*Randomly chosen dataset out of multiple two-by-two tables*
CI: confidence intervals; MAT: microscopic agglutination test.

Positive post-test probability (95% CI)	Negative post-test probability (95% CI)	Positive likelihood ratio (95% CI)	Negative likelihood ratio (95% CI):
100% (100% to 100%)	99% (98% to 100%)	834.69 (52.05 to 13,384.42)[b]	0.14 (0.06 to 0.35)
56% (47% to 64%)	85% (58% to 96%)	1.57 (1.10 to 2.24)	0.23 (0.06 to 0.91)
13% (0% to 70%)[b]	75% (74% to 76%)	0.43 (0.02 to 8.13)[b]	1.04 (1.00 to 1.08)
48% (35% to 61%)	81% (66% to 90%)	1.85 (1.09 to 3.12)	0.48 (0.22 to 1.04)
30% (15% to 52%)	99% (95% to 100%)	12.54 (5.02 to 31.28)	0.27 (0.05 to 1.45)
93% (78% to 98%)	61% (55% to 67%)	12.13 (3.04 to 48.50)	0.55 (0.44 to 0.71)
79% (69% to 87%)	59% (56% to 63%)	3.85 (2.20 to 6.74)	0.69 (0.60 to 0.80)
42% (30% to 56%)	53% (51% to 56%)	0.86 (0.50 to 1.48)	1.03 (0.93 to 1.14)
79% (69% to 87%)	90% (56% to 98%)	2.20 (1.26 to 3.86)	0.06 (0.01 to 0.46)
92% (79% to 97%)	90% (38% to 100%)[b]	3.00 (0.97 to 9.30)	0.03 (0.00 to 0.58)**
97% (73% to 100%)[b]	67% (54% to 77%)	24.12 (1.54 to 377.45)[b]	0.36 (0.21 to 0.61)
81% (69% to 89%)	77% (52% to 91%)	2.38 (1.25 to 4.54)	0.17 (0.06 to 0.53)

Substantial variation can be expected between laboratories on how the index tests are performed with regard to the timing of sample collection and threshold values. The timing of sample collection may greatly affect the test's accuracy, as leptospires are known to (dis) appear in different sample types as the disease progresses. For example, it is recommended that nucleic acid and antigen detection tests are performed on blood between one and 10 days postonset (DPO) of symptoms, as leptospiraemia declines rapidly until below detection after 10 DPO.[12] Tests performed on blood samples collected after 10 DPO may lead to false-negative findings. Tests in urine are expected to be positive after 10 to 14 DPO.[16]

Reference standard

MAT is the most widely used serological test for leptospirosis. It is considered to be the reference standard, often used in combination with other serological tests (such as IgM ELISA), and with or without culture of leptospires from blood or urine.

MAT is considered an imperfect reference standard. It has a high diagnostic specificity, as the observation of seroconversion or a titre rise confirms current leptospirosis, but a negative MAT does not rule out the possibility of leptospirosis. Limmathurotsakul and colleagues used a Bayesian latent class analysis (LCA) to estimate the accuracy of MAT, which was 49.8% sensitive and 98.8% specific.[18] The LCA assumes that there is no reference standard, and estimates disease prevalence by taking the results of multiple tests into account.[19]

In another study to estimate the accuracy of MAT, Goris and colleagues selected culture-positive people as being infected (proof of leptospirosis) and people with other known diseases and unknown disease as controls, and performed MAT on both groups.[1] In this study, the sensitivity of MAT was estimated at 81.7% and specificity of MAT was estimated at 100%.

Using a reference standard with low sensitivity to compare against the index test may result in biased estimates of specificity. However, when the case definition in the Goris 2012 study was changed to include people who were IgM ELISA positive, the sensitivity increased to 93.3% without sacrificing the specificity.[1] This indicates that combining multiple tests with high specificity as a composite reference standard can yield increased sensitivity. Therefore, we decided to include studies with only MAT as the reference standard, and studies that used other serological tests, or culturing, or both, alongside MAT as the reference standard.

Variability in MAT performance between laboratories exists and may affect test accuracy. MAT requires a panel of live Leptospira serovars (group of micro-organisms characterised by specific set of antigens) that occur in the region, supplemented with a panel of globally standardised serovars when people present with a travelling history.[1] Determining and maintaining such panels are major, but essential, tasks; inadequate panels may lead to false-negative results. The timing of sample collection may also influence sensitivity or specificity; antibodies are usually detectable from five to seven DPO onwards. MAT-case definitions may vary between laboratories; a four-fold rise in titre in paired sera or seroconversion is indicative

of current infection, but some laboratories may use a high titre in a single serum sample (seropositivity) as a case definition for people who do not return for follow-up. Seropositivity is not necessarily evidential of a current infection, since antibodies may persist after a previous infection, or cross-reactivity with other diseases may occur (such as legionellosis, hepatitis, and autoimmune diseases).[12] Therefore, the desirable cut-off titres for the single-sample MAT are higher in regions where leptospirosis and similar infectious diseases are highly prevalent.

Leptospires can be cultured from blood, CSF, dialysate fluid, and (postmortem) tissue, often within 10 DPO. Culture of urine is useful after 10 DPO. Leptospires are slow-growing, fastidious bacteria. Cultures have to be maintained for at least four months before being regarded negative. Culturing provides evidence for leptospirosis but lacks sensitivity and does not contribute to an early diagnosis. The sensitivity of culture is estimated not to exceed 23%, according to an analysis of people with leptospirosis from 1925 to 2008 in the Netherlands.[20]

Clinical pathway

Figure 1 shows a diagnostic pathway, as suggested by Goris and colleagues.[1] A person with symptoms compatible with leptospirosis (such as fever, headaches, myalgia, conjunctival effusion, and vomiting) is evaluated for likelihood by assessing risk factors, and consequently classified as an 'early presentation' (DPO 10 or fewer) or a 'late presentation' (DPO greater than 10). Real-time PCR is recommended as the test of choice for early presentations as it can detect leptospiral DNA in blood. Blood culture is conducted alongside real-time PCR to confirm leptospirosis as well as to provide insight in locally occurring serovars. MAT and IgM ELISA are recommended for later presentations since antibodies are expected to appear in serum after five to seven DPO. The person is considered to have leptospirosis if any of the test results is positive.

If a person tests positive with either an antibody or an antigen test, this person will be treated with antibiotics. If the tests return negative, then the recommendation is to test again in two weeks' time. However, if the person is very ill, clinicians will in some cases decide to treat with antibiotics anyway.

Rationale

The aim of this systematic review was to assess the diagnostic test accuracy of nucleic acid and antigen detection tests for human symptomatic leptospirosis. A similar diagnostic accuracy review on serology tests (antibody detection tests) for leptospirosis is being conducted by Goris and colleagues.[21]

Nucleic acid and antigen detection tests may serve several purposes based on their ability for early detection. First, and most important, an accurate test in the early stage of the disease may improve patient outcomes by facilitating timely administration of effective antibiotics. Although the limited available evidence presented by the latest Cochrane Review on antimicrobial therapy was inconclusive,[17] one study reported a shortened duration of

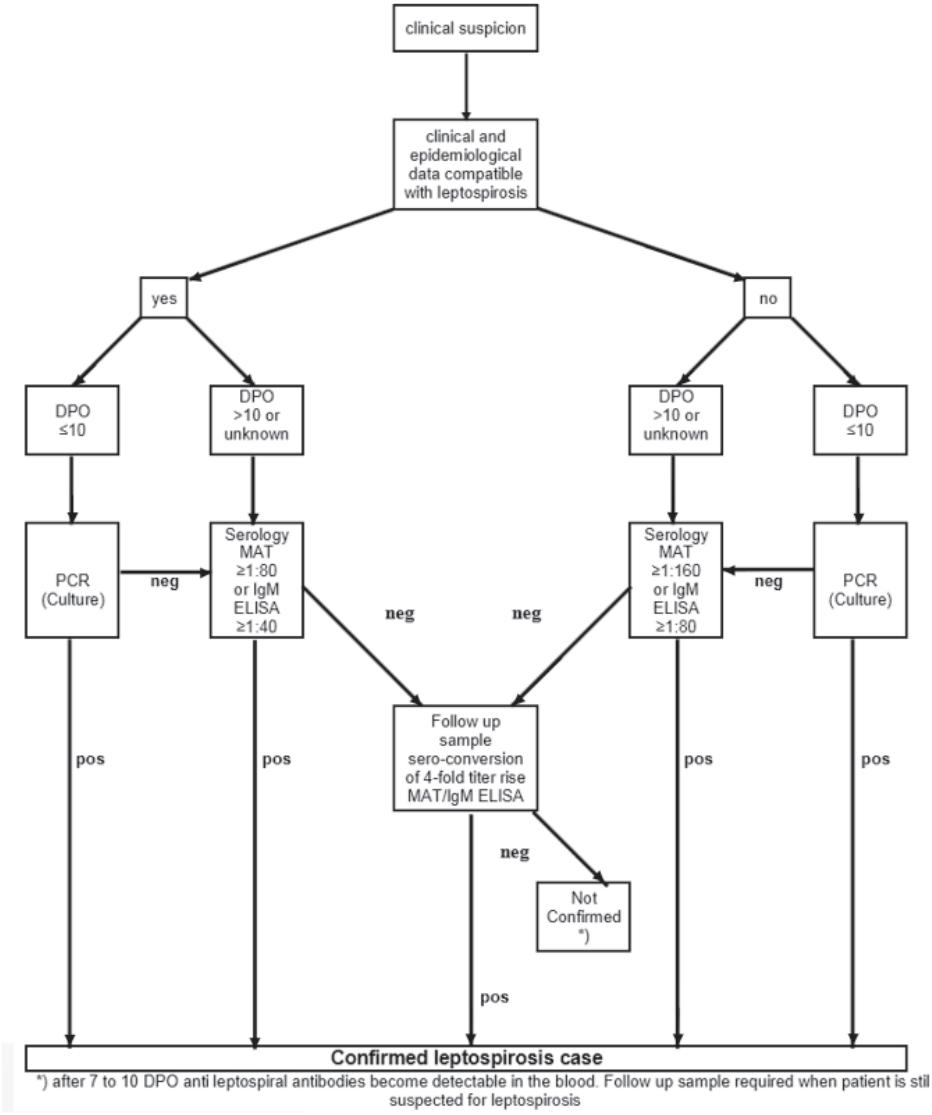

Figure 1. Algorithm, assisting with interpretations and conclusions on the outcome of laboratory testing (adapted from Goris 2012[1]). Antibody titres shown in this figure are optimised for leptospirosis cases in the Netherlands.

DPO: days postonset of symptoms; IgM ELISA: immunoglobulin M enzyme-linked immunosorbent assay; MAT: microscopic agglutination test; neg: negative; PCR: real-time polymerase chain reaction; pos: positive.

illness in early-stage leptospirosis,[22] while three studies that studied advanced leptospirosis yielded conflicting results.[23-25] This raises the possibility that antibiotic therapy may have a greater effect when delivered earlier. Second, an early test may be useful in participant re-

cruitment for studies evaluating antibiotics in early-stage leptospirosis. Third, it may facilitate early warning of leptospirosis outbreaks and yield more reliable estimates of leptospirosis incidence in the affected region. Not all antigen tests may be applicable as early detection tests, but they are nevertheless good candidates for assessment since accurate, low-cost, simple, and convenient point-of-care tests are urgently needed.

OBJECTIVES

To determine the diagnostic test accuracy of nucleic acid and antigen detection tests for the diagnosis of human symptomatic leptospirosis.

Secondary objectives

To investigate the comparative accuracy of nucleic acid and antigen detection tests.

To assess the influence of potential sources of heterogeneity on the diagnostic test accuracy of nucleic acid and antigen detection tests, namely:

- timing of sample collection for the index test;
- disease prevalence in the study population;
- blood sample type for the index test (whole blood, plasma, or serum);
- primers or target genes for the PCR and other nucleic acid tests;
- threshold of the index test;
- real-time PCR visualisation method;
- brand of the test.

METHODS

Criteria for considering studies for this review

Types of studies

We included diagnostic test accuracy studies, that is, any study that evaluated the sensitivity and specificity of a nucleic acid or antigen detection test in comparison with a reference standard. In this review, we discerned three types of eligible diagnostic test accuracy studies based on their method of participant selection: the cross-sectional study, the single-gate case-control study, and the two-gate case-control study. Their respective characteristics are summarised in Table 1 and illustrated in Figure 2.

In cross-sectional studies, people with clinical suspicion of leptospirosis are consecutively enrolled and undergo both the index test and reference standard. In the similar single-gate

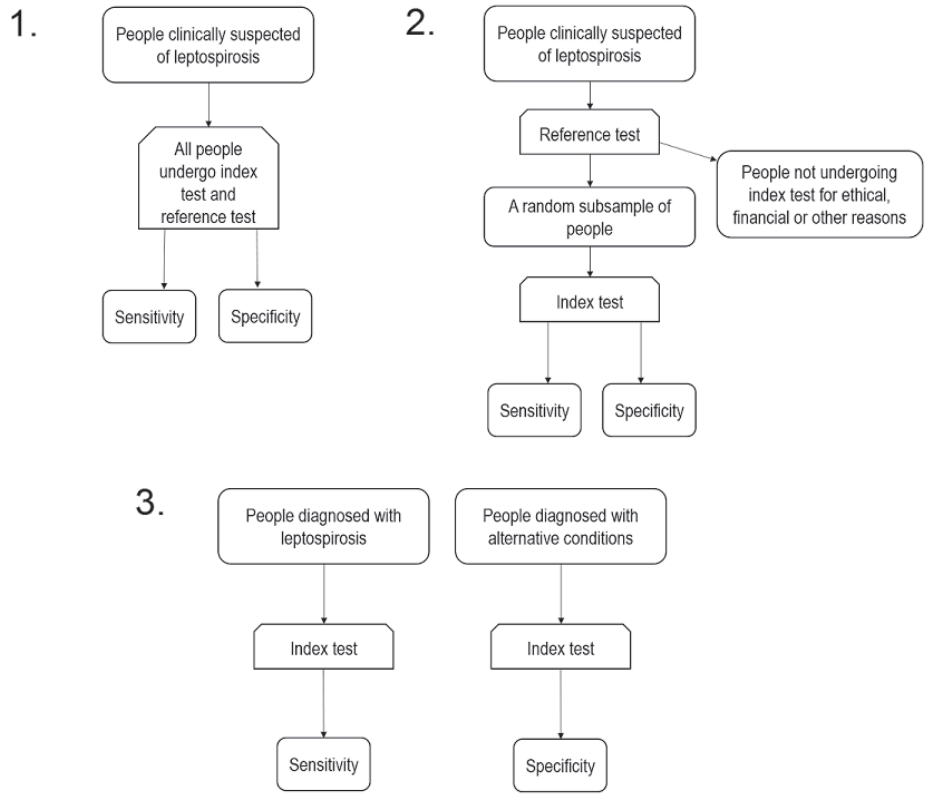

Figure 2. Eligible study designs. 1. Cross-sectional study; 2. single-gate case-control study; 3. two-gate case-control study

case-control study, usually all people with positive reference standard results and a subsample of people with negative reference standard results from an original clinically suspected cohort are subsequently tested with the index test. We referred to these two study designs simply as 'single-gate' designs (i.e. having a single inclusion criteria for clinical presentation).[26] The main difference between these two designs is that the prevalence of the target condition in the single-gate case-control study is artificial, whereas in a cross-sectional study, a true prevalence can be estimated.

In a two-gate case-control design, people with positive reference standard results and people who do not have leptospirosis are enrolled to subsequently undergo the index test. Since the participants with and without the target condition are selected from two separate cohorts, this study design is at a higher risk of bias in comparison to the single-gate designs. The two-gate case-control designs can be further separated into studies in which the controls have an alternative condition resembling leptospirosis (two-gate with alternative diagnoses controls), and studies in which the controls are healthy (two-gate with healthy controls).

Table 1. Study designs

Design type	Design name	Description
Single-gate	Cross-sectional study	Recruitment of a consecutive series of participants in whom leptospirosis is suspected. The index test and the reference standard is done on all participants and the results of the 2 tests are compared with each other.
	Case-control study	Recruitment of participants with a positive reference standard result and participants with a negative reference standard result who are randomly selected from the same cohort of participants with the suspicion of leptospirosis. The index test is subsequently applied to all participants.
2-gate	Case-control study	Recruitment of participants with a positive reference standard result and participants who are diagnosed with alternative conditions that resemble the clinical presentation of leptospirosis. The index test is subsequently applied to all participants.

We excluded two-gate case-control designs with healthy controls because these studies are known to produce inflated estimates of diagnostic accuracy.[26]

We placed no restrictions on language and publication date. When studies met our eligibility criteria but reported insufficient data for the construction of two-by-two tables, we excluded them. In cases where the full-text article was not retrievable or in case of meeting abstracts, we included the study if the abstract reported data for the construction of two-by-two tables. We excluded studies when both abstract and the full-text article were not retrievable. We contacted study authors to obtain the full-text article or study data prior to exclusion, but we excluded studies when no answer was obtained after a lengthy interval. Finally, we excluded studies with fewer than 10 participants, as they would add little value to the review.

Participants

Eligibility of participants depended on the study design.

Cross-sectional studies: people with clinical suspicion of leptospirosis were eligible. Compatible symptoms were, but were not limited to, fever, myalgia, headaches, malaise, conjunctival suffusion, rash, nausea/vomiting, anorexia, and cough. Single-gate case-control studies: eligible were cases with a positive reference standard result and controls derived from the same clinically suspected group as the cases, but with a negative reference standard result. Two-gate case-control studies: eligible were cases with a positive reference standard result and controls with a different known disease that resembled the clinical presentation of leptospirosis.

We excluded studies that screened asymptomatic people for leptospirosis.

Index tests

All diagnostic tests that used nucleic acid and antigen detection methods were included. Tests eligible for inclusion were, but were not limited to, PCR and its variants, that is, LAMP,

ELISA, FAT, silver staining, or immunohistochemistry. We included index tests with any sample type (e.g. blood products, urine, CSF), any timing of sample collection (recorded as DPO), any variation in laboratory processing, and any threshold for tests on a continuous scale. We excluded studies that did not analyse different sample types separately, as it would be unclear which sample should be tested by the clinician in order to obtain a similar test accuracy.

Target conditions

This review was restricted to human symptomatic leptospirosis. We excluded studies of ocular and neurological manifestations of leptospirosis, as it was unclear whether MAT was a valid reference standard for these target conditions.

Reference standards

We considered several types of reference standards, which are summarised in Table 2. We elaborate the inclusion criteria separately for single-gate and two-gate designs.

For single-gate designs, we considered studies that used MAT, with or without culture or other serological tests such as IgM ELISA. We included these tests alongside MAT in order to compensate for the imperfect sensitivity of MAT as a reference standard. Since these tests have high specificity, we considered any positive result from this composite reference standard as a leptospirosis case. If a study used MAT as a sole reference standard, we considered the risk of bias to be high. We excluded single-gate designs with culture as the sole reference standard, since culture has a very low sensitivity.[20]

In two-gate designs, the people without leptospirosis are not necessarily reference standard negatives, but they are diagnosed with an alternative condition. Hence, we only required a reference standard that ruled in leptospirosis in the case of a positive result. Reference standards considered eligible for studies with this design were those with a high specificity: MAT used alone, or culture used alone.

Table 2. Reference Standards.
All reference standards eligible for inclusion. Tests 2, 3, and 4, which are composite reference standards, are intended to increase sensitivity, provided that each reference standard has been applied to all participants. In two-gate designs, the sensitivity of the reference standard is irrelevant, as controls are not reference standard negatives.
ELISA: enzyme-linked immunosorbent assay; MAT: microscopic agglutination test.

Study design	Eligible reference standards	Case definition
Single gate	1. MAT only	MAT positive
	2. MAT and culture	≥ 1 of the tests positive
	3. MAT and ELISA (or other serological tests)	≥ 1 of the tests positive
	4. MAT and culture and ELISA	≥ 1 of the tests positive
2 gate	5. MAT only	MAT positive
	6. Culture only	Culture positive

In order to avoid incorporation bias (the reference standard uses or incorporates the index test), we excluded studies which contained a nucleic acid or antigen detection test in the reference standard.

Search methods for identification of studies

Electronic searches

We searched the following 16 electronic databases: the Cochrane Library (6 July 2018), MEDLINE Ovid (1946 to 6 July 2018), Embase Ovid (1974 to 6 July 2018), Web of Science (1975 to 6 July 2018), CINAHL (1937 to 6 July 2018), BIOSIS Previews (1993 to 8 February 2015 due to terminated institutional subscription), PubMed (for publications not yet included in MEDLINE; 1946 to 8 February 2015), Google Scholar, African Index Medicus (1993 to 6 July 2018), African Journals Online (from inception to 8 February 2015), LILACS (Literature in the Health Sciences in Latin America and the Caribbean, 1982 to 6 July 2018), KoreaMed (from inception to 8 February 2015), IMSEAR (Index Medicus for the South-East Asian Region, from inception to 6 July 2018), IMEMR (Index Medicus for the Eastern Mediterranean Region, from inception to 8 February 2015), WPRIM (Western Pacific Region Index Medicus, from inception to 6 July 2018), and IndMed (from inception to 8 February 2015). For each database, we identified subject headings or free-text terms and synonyms (or both) related to: leptospirosis, antigen, nucleic acids, PCR, LAMP, hybridisation, immunohistochemistry, silver staining, and dot blot. Appendix 1 shows the search strategies for each database.

Searching other resources

Additionally, we scanned the reference lists of included articles and we searched the World Health Organization's (WHO) International Clinical Trial Registry Platform (www.who.int/ictrp) for ongoing or unpublished trials.

Data collection and analysis

Selection of studies

Two review authors (BY, MG) independently screened the titles and abstracts of all records, and excluded records with no relevance to the review question (first sift). We retrieved the full-text of the remaining records, and three review authors (BY, MG, SdV) independently checked the full-text articles for eligibility, using a full-text assessment checklist, with each record being assessed by at least two review authors (second sift). Studies that were excluded during data extraction, excluded meeting abstracts and studies with irretrievable full-texts, are listed in the Characteristics of excluded studies tables. We resolved disagreements between review authors by consensus or by consulting a senior author (ML).

Data extraction and management

From each study, two out of three review authors (BY, MG, SdV) independently extracted data by using a specially designed data collection form. The data collection form contained the following items.

- Study ID.
- Study design.
- Study region.
- Regional prevalence.
- Participants: selection methods, sex and age distribution, symptoms, risk factors.
- Index tests: threshold values, timing of sample collection (defined as DPO of symptoms where 1 DPO was 0 to 24 hours after onset of symptoms); type of sample.
- Reference standards: threshold values, timing of sample collection.
- Two-by-two contingency table for sensitivity and specificity calculations.

Each of the three review authors first piloted the form on two included studies to check for applicability. We resolved discrepancies between the authors by discussion and consensus. We contacted study authors for missing information.

Assessment of methodological quality

We assessed the quality of included studies using the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool.[27] The QUADAS-2 tool helps quality assessment by assessment of risk of bias and applicability of results across four domains: participant selection, index test, reference standard, and flow and timing. We custom-tailored QUADAS-2 to the needs of our review by adding additional signalling questions where needed. We also piloted the tool on two included studies and refined it accordingly. See Appendix 2 for the signalling questions and review-specific guidance.

Statistical analysis and data synthesis

We arranged results from each study in two-by-two contingency tables in which we compared people with confirmed leptospirosis (as defined by a positive MAT or other serological test or culture result) and people without leptospirosis (none of the reference standard tests were positive, or people having another disease than leptospirosis) to the binary test results from the index tests. From these tables, we calculated sensitivity and specificity for each study. As previously described, we excluded studies reporting insufficient data for the construction of two-by-two tables.

Some studies reported two thresholds for MAT, where the higher threshold was considered 'confirmed leptospirosis', and the lower threshold was considered 'probable leptospirosis'. In these studies, we chose the higher threshold dataset for the primary analysis. This was

because we considered the specificity of the reference standard to be more important than its sensitivity. The lower threshold dataset was analysed in a sensitivity analysis.

We presented individual study results graphically by plotting estimates of sensitivity and specificity in forest plots and the summary receiver operating characteristic (SROC) space. We conducted a random-effects meta-analysis using the bivariate model to estimate summary values for sensitivity and specificity when little variation in threshold values was presumed. If studies used multiple thresholds for the index test, we constructed a SROC curve using the hierarchical summary receiver operating characteristic (HSROC) model. All analyses were done in SAS 9.4 (Cary Inc.).

We separately described studies that reported head-to-head comparisons of index tests (or index test characteristics) in the same study population, but did not perform meta-analyses to formally compare these index tests due to the lack of a sufficient number of studies.

Investigations of heterogeneity

We assessed heterogeneity initially by visually inspecting the forest plots and the ROC plot. The following covariates were investigated as potential sources of heterogeneity.

- Timing of sample collection for the index test. We planned to analyse this based on how study authors reported the timing: as a continuous variable using medians or means, or as a categorical variable using timing intervals (e.g. 1 DPO to 4 DPO versus 5 DPO to 10 DPO).
- Prevalence in the study population (continuous variable). This was computed using two-by-two table data from cross-sectional studies. If a case-control study reported prevalence data of the original cohort, we also used these data.
- Blood sample type for the index test (categorical variable; whole blood, plasma, or serum).
- Primers or target genes for the PCR and other nucleic acid tests (categorical variable). Since two PCRs with the same target gene could use different primers, we also specified the original reference of the technique.
- Threshold of the index test, if applicable (continuous variable; e.g. threshold cycles (Ct) for the real-time PCR).
- Real-time PCR visualisation method (categorical variable; TaqMan probe; or SYBR green).
- Brand of the test, if applicable (categorical variable).

Sensitivity analyses

To examine the robustness of the results to the decisions we made in the review process, we conducted analyses with the following alternative decisions.

- Exclusion of studies with only abstracts.
- Exclusion of studies with high risk of bias for the 'patients' domain.
- Exclusion of studies with high risk of bias for the 'reference standard' domain.

- Exclusion of studies that used antibiotics before the index test.
- The choice of the lower MAT threshold dataset for the analysis, in studies that reported two thresholds for MAT.

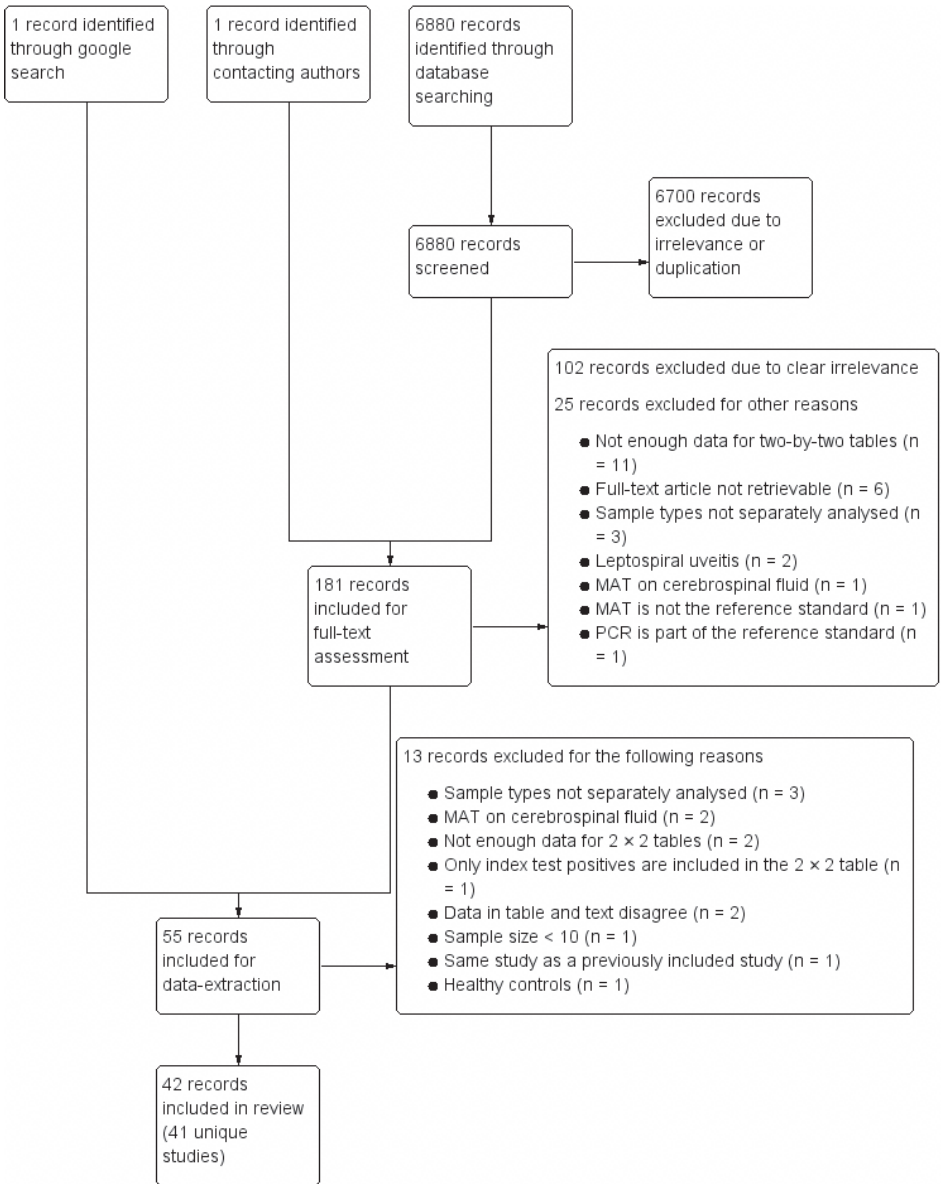

Figure 3. Study flow diagram. *MAT: microscopic agglutination test; n: number of records; PCR: polymerase chain reaction.*

RESULTS

Results of the search

We conducted the final electronic search on 6 July 2018 and identified 6880 records (see Figure 3). After title and abstract screening, and after inclusion of one additional record, which we identified by contacting one of the authors (Destura 2007), we included 181 records for full-text assessment. We excluded 127 records; 102 records due to clear irrelevance and 25 records for other reasons (see Characteristics of excluded studies table, Supplementary Materials). At this stage, we identified and included one full-text publication of a meeting abstract (Denipitiya 2016).[28]

We included the remaining 55 records for data collection, of which 13 were excluded for various reasons.

The review included 42 records,[28-69] corresponding to 41 unique studies. We regarded four publications as two studies because they included the same population (Thaipadungpanit 2011[58] and Sonthayanon 2011[56] are grouped under Thaipadungpanit 2011; Waggoner 2014[61] and Waggoner 2014[62] are grouped under Waggoner 2014). We considered one publication as two studies because two different populations were included (Villumsen 2012 BC; Villumsen 2012 U).[60] Searching the WHO International Clinical Trial Registry Platform yielded no relevant records.

Description of studies

Included studies

The Characteristics of included studies table and Table 3 give an overview of all included studies. Forty-one studies included 5981 participants, of whom 1834 were classified as having leptospirosis, and 4147 as not having leptospirosis. Thirty studies were cross-sectional, five were single-gate case-control, four were two-gate case-control studies, and study design was dubious in two studies (Zhang 1992;[69] Gravekamp 1993[44]), with Zhang 1992 being most likely either a cross-sectional or single-gate case-control study, but not a two-gate study.

The index tests evaluated were conventional PCR (henceforth PCR; 17 studies), real-time PCR (18 studies), nested PCR (four studies), PCR performed twice (performed twice on each participant at different DPO and regarded as positive if at least one result was positive; two studies), LAMP (two studies), ELISA (one study), dot-ELISA (one study), immunochromatography-based lateral flow assay (ICG-based LFA; one study), and dipstick assay (one study) (see Table 4). Five studies directly compared tests in the same population: PCR versus real-time PCR (Vanasco 2016[59]), PCR versus nested PCR (Blanco 2014[35]), nested PCR versus real-time PCR (Merien 2005[47]), real-time PCR versus LAMP (Thaipadungpanit 2011[56,58]), and ICG-based IFA versus dipstick assay versus PCR (Widiyanti 2013[65]).

We observed high heterogeneity regarding the characteristics of the participants, the execution of the index tests, and the choice of the reference standards. Most of the

Table 3. Overview of characteristics of included studies

Summary table of included studies. 95% confidence intervals are not shown. Timing of sample collection (DPO of symptoms) is presented as median numbers or range or interquartile range.

* Underlined are the direct comparisons of index tests.

Study ID	Study design	Region	Sample size	Prevalence	Sensitivity	Specificity	Index test*
Agampodi 2012	CC1	Sri Lanka	105	21.7%	51.0% 18.4%	98.2% 98.2%	qPCR qPCR
Agampodi 2016	CS	Sri Lanka	96	43.8%	27.3%	25.0%	qPCR
Ahmed 2009	CS	Nether-lands	75 62 133	19.5%	100% 68.8% 88.5%	100% 100% 100%	qPCR qPCR qPCR
Ananyina 2000	CC2	Russia & China	158	?	68.0%	100%	cPCR
Backstedt 2015	CS	Brazil	25	72.0%	27.8% 55.6%	71.4% 14.3%	qPCR qPCR
Blanco 2014	CS	Brazil	521	5.4%	14.3% 85.7%	100% 100%	cPCR N PCR
Cardona 2008	CS	Venezuela	73	27.4%	20.0% 45.0%	77.4% 71.7%	cPCR cPCR
Cespedes 2007	CS	Peru	118	22.0%	55.4%	100%	cPCR
Chandrasiri 2010	CS	Sri Lanka	59	11.8%	14.3%	86.5%	cPCR
Chaurasia 2018	CS	India	29	79.3&	100% 91.3% 78.3% 91.3% 100% 91.3% 39.1%	66.7% 50.0% 83.3% 66.7% 66.7% 66.7% 83.3%	ELISA (*lipL32*) ELISA (*Fla1*) ELISA (*lipL41*) ELISA (*HbpA*) ELISA (*SphCD210*) ELISA (*Sph2*) ELISA (*Sph4*)
De Abreu Fonseca 2006	CC2	Brazil	80	?	38.3% 36.7%	100% 100%	cPCR cPCR
Denipitiya 2016	CS	Sri Lanka	111	58.6%	67.7%	91.3%	qPCR
Fan 1999	CS	China	15	33.3%	100%	80.0%	cPCR
Gokmen 2016	CS	Turkey	47	44.7%	90.5% 95.2%	42.3% 42.3%	N PCR N PCR
Gonzalez 2013	CS	Uruguay	183	46.4%	30.6%	100%	qPCR
Gravekamp 1993	?	NL & Barbados	119	?	49.4%	100%	cPCR
Kitashoji 2015	CS	Philippines	287	46.0%	14.4% 14.1%	83.2% 90.6%	LAMP LAMP

Original reference of index test method	Target gene/ primer*	Threshold	Timing (DPO)*	Sample type*	Reference test	Sample for MAT
Smythe 2002 Smythe 2002	*rrs* *rrs*	? ?	1-10 1-10	Whole blood Serum	MAT	Paired only
Smythe 2002	*rrs*	?	3-7	Blood/serum	MAT	Single + paired
Ahmed 2009 Ahmed 2009 Ahmed 2009	*secY* *secY* *secY*	Ct 35 Ct 35 Ct 35	1-4 5-10 1-10	Blood/serum Blood/serum Blood/serum	MAT OR IgM ELISA OR Culture	Single + Paired
Gravekamp 1993	G1/G2 and B64-I/ B64-II primers	NA	?	Serum	MAT	Single + Paired
Stoddard 2009 Backstedt 2015	*lipL32* *rrs*	? ?	? ?	Whole blood Whole blood	MAT OR Culture	Single + Paired
Merien 1992 Merien 1992	*rrs* *rrs*	NA NA	? ?	Serum Serum	MAT	Single + Paired
Gravekamp 1993 Gravekamp 1993	G1/G2 and B64-I/ B64-II primers G1/G2 and B64-I/ B64-II primers	NA NA	? ?	Serum Urine	MAT	Single + Paired
Merien 1992	*rrs*	NA	1-7	Whole blood	MAT OR IgM ELISA OR Culture	Single + Paired
?	G1/G2 primers	NA	?	Whole blood	MAT	Single
Chaurasia 2018 Chaurasia 2018 Chaurasia 2018 Chaurasia 2018 Chaurasia 2018 Chaurasia 2018 Chaurasia 2018	NA NA NA NA NA NA NA	? ? ? ? ? ? ?	? ? ? ? ? ? ?	Urine Urine Urine Urine Urine Urine Urine	MAT	Single
Gravekamp 1993/Kee 1994 Gravekamp 1993/Kee 1994	G1/G2 and LP1/LP2 primers G1/G2 and LP1/LP2 primers	NA NA	? ?	Whole blood Urine	MAT OR Culture	Single + Paired
Ahmed 2009	*secY*	35 Ct	1-5	Whole blood	MAT	Single + paired
Fan 1999	*rrs*	NA	?	Serum	MAT	?
Bomfim 2008 Merien 1992	*lipL32* *rrs*	NA NA	? ?	Serum Serum	MAT	Single
Stoddard 2009 and Bourhahy 2001	*lipL32*	?	?	Serum	MAT	Paired only
Gravekamp 1993	G1/G2 and LP1/LP2 primers	NA	?	Serum	MAT OR IgM ELISA	?
Koizumi 2012 Koizumi 2012	*rrs* *rrs*	NA NA	6.5 ?	Plasma Urine	MAT	Single + Paired

Koizumi 2009	CS	Sri Lanka	107	24.3%	0.0%	96.3%	N PCR
Merien 2005	CS	Oceania	51	33.3%	70.6% 70.6%	61.8% 61.8%	N PCR qPCR
Ooteman 2006	CS	Brazil	125	37.6%	36.2%	70.5%	cPCR
Pakoa 2018	CS	Vanuatu	130	11.5%	0%	97.4%	qPCR
Riediger 2007	CS	?	66	22.7%	46.7% 40.0%	76.5% 80.4%	cPCR cPCR
Riediger 2017	CS	Brazil	150	84.7%	60.6% 29.1%	56.2% 87.0%	qPCR qPCR
Saengjaruk 2002	CC2	Thailand	43	?	64.0%	100%	dot-ELISA
Samsonova 1997	CC2	China & Russia	75	?	66.0%	96.4%	cPCR
Seng 2007	CS	Cambodia	121	3.3%	75.0%	94.0%	cPCR 2x
Sonthayanon 2013	CC1	Thailand	250	31.8%	59.0%	92.0%	qPCR
Thaipadunpanit/ Sonthayanon 2011	CC1	Thailand	266	31.8%	55.6% 42.9% 43.6% 37.6%	89.5% 93.2% 83.5% 90.2%	qPCR qPCR LAMP LAMP
Vanasco 2016	CC1	Argentina	188	35.5%	29.9% 13.4%	81.0% 88.4%	qPCR cPCR
Villumsen 2012-BC	CS	Denmark	29	24.1	85.7% 100%	100% 95.5%	qPCR qPCR
Villumsen 2012-U	CS	Denmark	54	5.6%	100% 100%	98.0% 98.0%	qPCR qPCR
Waggoner 2014	CS	Brazil	55	10.9%	100% 100%	4.1% 0.0%	qPCR qPCR (UFI Assay)
Waggoner 2015	CC1	Brazil	478	NA	9.1%	92.8%	qPCR (UFI Assay)
Wangroongsarb 2005	CS	Thailand	93	16.1%	80.0%	96.2%	cPCR
Widiyanti 2013	CS	Philippines	44	63.6%	57.1% 89.3% 96.4%	56.3% 62.5% 56.3%	cPCR Dipstick ICG- LFA
Woods 2018	CS	Laos	766	4.4%	9.4% 3.0% 17.2% 9.4% 12.1% 13.8%	98.5% 99.0% 90.1% 98.8% 99.0% 99.0%	qPCR qPCR qPCR qPCR qPCR qPCR
Wu 1996	CS	China	19	47.4%	100%	0.0%	cPCR

Kawabata 2001/ mod:Koizumi 2008	*flaB*	NA	7	Serum	MAT	Single only
Merien 1992 Merien 2005	*rrs* LFB1-F/LFB1-R primers	NA ?	5 5	Serum Serum	MAT	Single + Paired
Gravekamp 1993	G1/G2 primers	NA	?	Serum	MAT	Single + Paired
Stoddard 2009	*lipL32*	?	?	Serum	MAT	Single
Gravekamp 1993 Gravekamp 1993	G1/G2 and B64-I/ B64-II primers G1/G2 and B64-I/ B64-II primers	NA NA	? ?	Whole blood Urine	MAT	Single + Paired
Stoddard 2009 Stoddard 2009	*lipL32* *lipL32*	40 Ct 40 Ct	? ?	Whole blood Serum	MAT OR culture	Single + paired
Saengjaruk 2002	NA	NA	5	Urine	Culture	NA
Gravekamp 1993	G1/G2 and B64-I/ B64-II primers	NA	?	Serum	MAT	?
?	*rrl* (23S)	NA	(1) 14, (2) 35	Serum	MAT OR Culture	Single + Paired
Slack 2007	*rrs*	?	?	Whole blood	MAT OR Culture	Single + Paired
Slack 2007 Stoddard 2009 Sonthayanon 2011 Lin 2009	*rrs* *lipL32* *rrs* *lipL41*	? ? NA NA	5 5 5 5	Whole blood Whole blood Whole blood Whole blood	MAT OR Culture	Paired only
Stoddard 2009 Stoddard 2009	*lipL32* *lipL32*	40 Ct NA	5 5	Serum/blood Serum/blood	MAT OR IgM ELISA	Single + paired
Villumsen 2012 Smythe 2002	*lipL32* *rrs*	? ?	? ?	Blood culture Blood culture	MAT	Single + paired
Villumsen 2012 Smythe 2002	*lipL32* *rrs*	? ?	? ?	Urine Urine	MAT	Single + paired
Waggoner 2014 Waggoner 2014	*rrs* *rrs*	45 Ct 45 Ct	8 Range 1-19	Plasma/serum Plasma/serum	MAT	Single only
Waggoner 2014	*rrs*	45 Ct	?	Serum	MAT	Single only
? / Kawabata 2001	*rrs* / *flaB*	NA	?	Whole blood	MAT OR Culture	Paired
Kawabata 2001 Widyanti 2013 Widiyanti 2013	*flaB* NA NA	NA NA NA	5.5 5.5 5.5	Urine Urine Urine	MAT	Single only
Slack 2007 Slack 2007 Slack 2007 Woods 2018 Woods 2018 Woods 2018	*rrs* *rrs* *rrs* *rrs/lipL32* *rrs/lipL32* *rrs/lipL32*	40 Ct 40 Ct 40 Ct 45 Ct 45 Ct 45 Ct	5 5 5 5 5 5	Serum Buffy coat Urine Serum Buffy coat Urine	MAT OR Culture	Single + paired
Wu 1993	*rrs*	NA	?	Serum	MAT OR Culture	?

Yersin 1998	CS	Seychelles	112	53.6%	46.7%	96.2%	cPCR 2x
Zhang 1992	?	China	175	75.4%	100%	32.6%	cPCR

CC1: single-gate case-control study; CC2: two-gate case-control study; CS: cross-sectional study; Ct: threshold cycle; DPO: days post onset; EDTA: ; ICG-LFA: immunochromatography-based lateral flow assay; IgM ELISA: immunoglobulin G enzyme-linked immunosorbent assay; LAMP: loop-mediated isothermal amplification; MAT: microscopic agglutination test; MAT OR … OR …: a positive result of any one of these tests is considered a leptospirosis case; PCR: polymerase chain reaction; cPCR: conventional PCR; N PCR: nested PCR; NA: not applicable; cPCR 2x: conventional PCR performed twice at different moments in time; qPCR: real-time PCR; ?: unknown.

participants were from (sub)tropical countries, and prevalence of leptospirosis in the study population ranged from 3.3% (Seng 2007;[54] Cambodia) to 84.7% (Riediger 2017;[51] Brazil) (median 32.5%; interquartile range (IQR) 18.7 to 46.7; computed from only cross-sectional studies or with data from the original cohort studies). All participants were reported to be suspect of having leptospirosis, but symptoms were often not reported. Most commonly reported symptoms consisted of fever, myalgia, headaches, malaise, and jaundice. Some studies were reportedly conducted in an outbreak setting (Samsonova 1997;[53] Ananyina 2000;[32] Céspedes 2007;[37] Agampodi 2012;[30] Kitashoji 2015;[45] Agampodi 2016[29]). Antibiotic use was often unreported, but eight studies gave antibiotics to some participants before the index test (Yersin 1998;[68] Ananyina 2000;[32] Seng 2007;[54] Koizumi 2009;[46] Thaipadungpanit 2011;[56,58] Sonthayanon 2013;[55] Kitashoji 2015;[45] Woods 2018[66]).

Regarding the index test, timing of sample collection was often not reported, and reported DPOs differed substantially between studies (Table 4). We also identified a large variety of primers or target genes used in the PCR, real-time PCR, and LAMP. None of the nucleic acid or antigen detection tests included in this review were commercially available. The variation in the choice of reference standard and its methodological significance will be discussed in methodological quality of included studies.

Excluded studies

We excluded 127 records after full-text assessment. One hundred and two records were excluded for one of five main reasons: not a diagnostic test accuracy study, animal studies, inclusion of healthy controls, use of only culture as reference standard, and no distinction between different sample types for the index test. Twenty-five records were considered potentially eligible but were excluded for the following main reasons: no two-by-two table data (11 records), full-text article not retrievable (six records), sample types were not separately analysed (three records), target condition being leptospiral uveitis (two records), MAT was tested with CSF (one record; we were uncertain whether this was an appropriate reference standard), MAT was not the reference standard (one record), and PCR was part of the reference standard (one record).

Merien 1995	*rrs*	NA	(1) 3.9-4.5 (2) ≥14	Serum	MAT	Paired only
Zhang 1992	*rrl* (23S)	NA	1-5	Serum	MAT OR Culture	Paired only

We excluded an additional 13 studies after data collection for one of the following reasons: sample types were not separately analysed (three studies), MAT was tested with CSF (one study), no two-by-two table data (two studies), only index test positives being included in the two-by-two table (one study), data in table and text disagree (two studies), sample size fewer than 10 (one study), same study as a previously included study (one study), and healthy controls (one study) (see Characteristics of excluded studies table).

Methodological quality of included studies

We assessed methodological quality using the QUADAS-2 tool. See Figure 4 and Figure 5 for quality assessment results of PCR, Figure 6 and Figure 7 for real-time PCR, and Figure 8 and Figure 9 for all other tests. Overall, the reporting of quality items was poor; therefore, it remained difficult to quantify the risk of bias in included studies.

Table 4. Overview of index tests included in the review

[a] Numbers between parentheses indicate the number of studies.

[b] For whole blood, EDTA blood was used in all studies.

Ct: threshold cycle; ELISA: enzyme-linked immunosorbent assay; ICG-based LFA: immunochromatography-based lateral flow assay; LAMP: loop-mediated isothermal amplification; NA: not applicable; PCR: polymerase chain reaction; PCR 2×: PCR performed twice at different moments in time;

Index test	Studies	Samples[a]	Target genes/primers (original reference)[a]	Timing of sample collection, # days post onset of symptoms[a]	Threshold
Conventional PCR	17	Whole blood[b] or serum (1) Serum (9) Whole blood[b] (4) Blood product, unspecified (1) Urine (5)	Testing on blood products • G1/G2 and B64-I/B64-II primers (Gravekamp 1993) (4) • *rrs* (Merien 1992) (2) • G1/G2 primers (Unknown) (1) • G1/G2 primers (Gravekamp 1993) (1) • G1/G2 and LP1/LP2 primers (Gravekamp 1993/Kee 1994) (1) • G1/G1 and A + B64-I/B64-II primers (Gravekamp 1993/Merien 1992) (1) • *rrs* (Fan 1999) (1) • *rrs* and *flaB* (Unknown/Kawabata 2001) (1) • *rrs* (Wu 1993) (1) • *lipL32* (Stoddard 2009) (1) • *rrl* (23S) (Zhang 1992) (1) Testing on urine • G1/G2 and B64-I/B64-II primers (Gravekamp 1993) (1) • G1/G2 and LP1/LP2 primers (Gramekamp 1993/Kee 1994) (1) • G1/G1 and A + B64-I/B64-II primers (Gravekamp 1993/Merien 1992) (1) • *lipL32* (Stoddard 2009) (1) • *flaB* (Kawabata 2001) (1)	Testing on blood products • Unknown (12) • Range 1–7 (1) • Range 1–5 (1) • Median 5 (1) Testing on urine • Cases: median 3, controls: median 4 (1) • Median 5.5 (1) • Unknown (3)	NA

Real-time PCR	18	Blood or serum (3) Serum or plasma (9) Whole blood[b] (6) Blood culture (1) Buffy coat (1) Urine (2)	Testing on blood products • *rrs* (Smythe 2002) (3) • *rrs* (Waggoner 2014) (2) • *rrs* (Slack 2007) (3) • *rrs* (Backstedt 2015) (1) • *secY* (Ahmed 2009) (2) • LFB1-F/LFB1-R primers (Merien 2005) (1) • *rrs*/*lipL32* (Woods 2018) (1) • *lipL32* (Stoddard 2009) (6) Testing on urine • *lipL32* (Villumsen 2012) (2) • *rrs* (Smythe 2002) (1) • *rrs* (Slack 2007) (1) • *rrs*/*lipL32* (Woods 2018) (1) Testing on blood culture • *lipL32* (Villumsen 2012) (1) • *rrs* (Smythe 2002) (1) Testing on buffy coat • *rrs* (Slack 2007) (1) • *rrs*/*lipL32* (Woods 2018) (1)	Testing on blood products • Unknown (7) • Range 1–4 (1) • Range 1–5 (1) • Range 5–10 (1) • Range 1–10 (2) • Range 1–19 (1) • Median 5 (3) • Median 4 (1) • Median 8 (1) • Cases: median 4, controls: median 6 (1) Testing on urine • Median 5 (1) • Unknown (1) Testing on blood culture • Unknown (1) Testing on buffy coat • Median 5 (1)	Testing on blood products: • Unknown (9) • 35 Ct (3) • 40 Ct (3) • 45 Ct (3) Testing on urine: • Unknown (1) • 40 Ct (1) • 45 Ct (1) Testing on blood culture: • Unknown (1) Testing on buffy coat: • 40 Ct (1) • 45 Ct (1)
Nested PCR	4	Serum (4)	*rrs* (Merien 1992) (3) *flaB* (Kawabata 2001 /Koizumi 2008) (1) *lipL32* (Bomfim 2008) (1)	All samples 7 (1) Median 5 (1) Not reported (2)	NA
PCR 2×	2	Serum (2)	*rrl* (23S) (unknown) (1) *rrs* (Merien 1995) (1)	Median of 1st: 14, 2nd: 35 (1) 1st: 4, 2nd: 18 (1)	NA
LAMP	2	Whole blood[b] (1) Plasma (1) Urine (1)	*rrs* (Sonthayanon 2011) (1) *rrs* (Koizumi 2012) (1) *lipL41* (Lin 2009) (1)	Cases: median 4, controls: median 6 (1) Median 6.5 (1)	NA
ELISA	1	Urine (1)	NA	Not reported (1)	Not reported (1)
Dot-ELISA	1	Urine (1)	NA	Not reported (1)	NA
ICG-based LFA	1	Urine (1)	NA	Median 5.5 (1)	NA
Dipstick	1	Urine (1)	NA	Median 5.5 (1)	NA

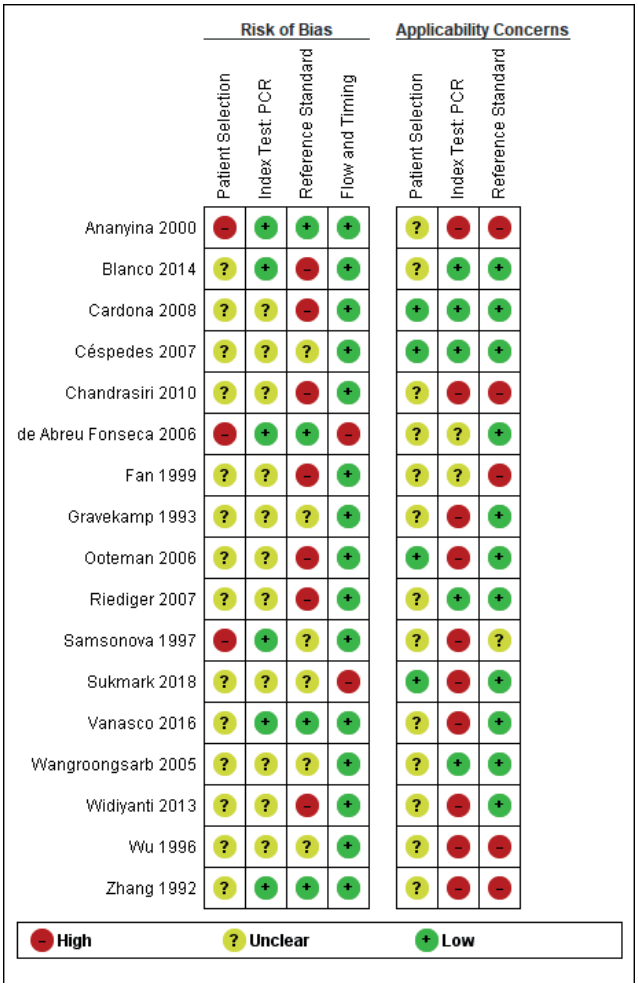

Figure 4. All conventional polymerase chain reaction (PCR) studies: risk of bias and applicability concerns. Sukmark 2018[57] and Widiyanti 2013[65] were not part of the PCR (blood products) meta-analysis.

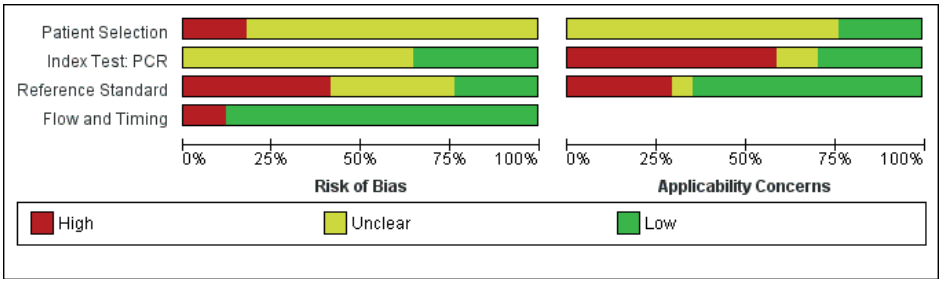

Figure 5. All conventional polymerase chain reaction (PCR) studies: risk of bias and applicability concerns graph.

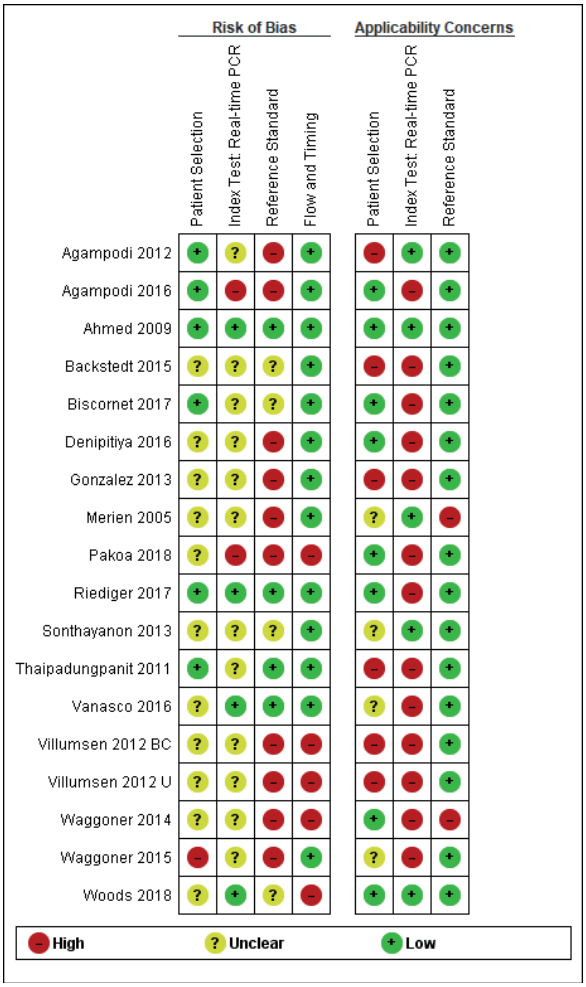

Figure 6. All real-time polymerase chain reaction (PCR) studies: risk of bias and applicability concerns. Villumsen 2012 BC[60] and Villumsen 2012 U[60] were not part of the real-time PCR (blood products) meta-analysis.

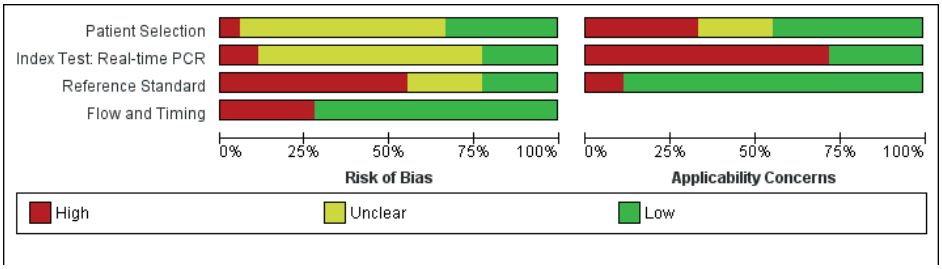

Figure 7. All real-time polymerase chain reaction (PCR) studies: risk of bias and applicability concerns graph.

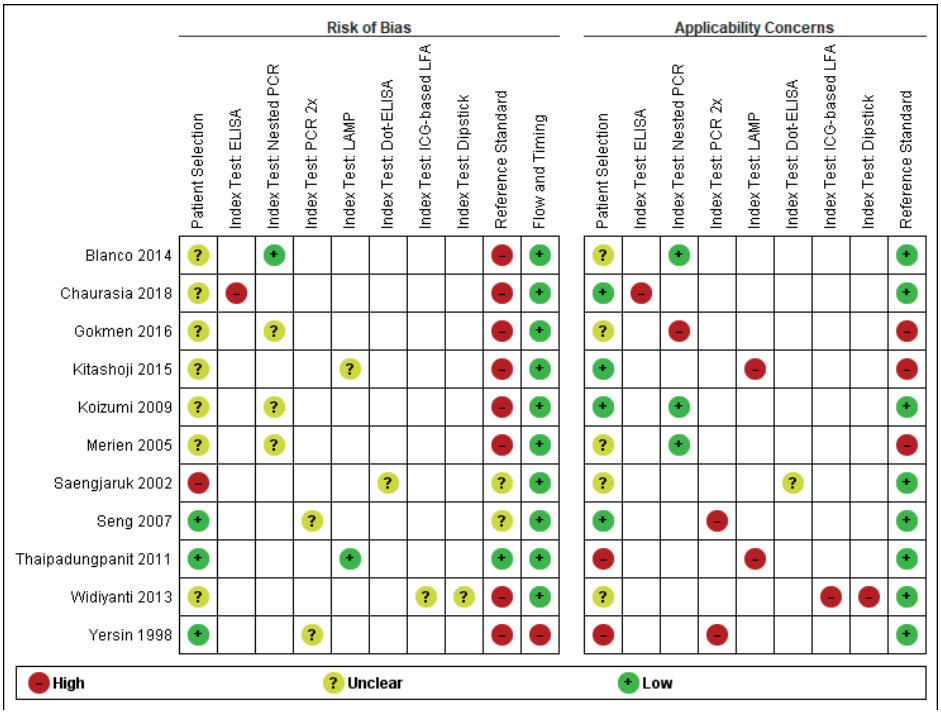

Figure 8. Studies of nested polymerase chain reaction (PCR), PCR performed twice (PCR 2x), loop-mediated isothermal amplification (LAMP), enzyme-linked immunosorbent assay (ELISA), dot-ELISA, immunochromatography-based lateral flow assay (ICG-based LFA), and dipstick assay: risk of bias and applicability concerns.

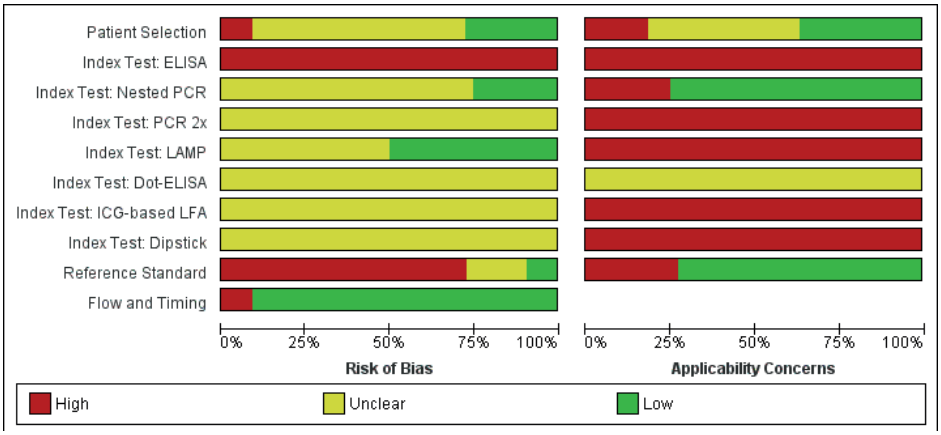

Figure 9. Studies of nested polymerase chain reaction (PCR), PCR performed twice (PCR 2x), loop-mediated isothermal amplification (LAMP), enzyme-linked immunosorbent assay (ELISA), dot-ELISA, immunochromatography-based lateral flow assay (ICG-based LFA), and dipstick assay: risk of bias and applicability concerns.

Risk of bias

Patient selection

Eight studies had a single-gate design (six cross-sectional studies, two single-gate case-control studies) with consecutive or random enrolment, and therefore, they were rated at low risk (Yersin 1998;[68] Seng 2007;[54] Ahmed 2009;[31] Thaipadungpanit 2011;[56,58] Agampodi 2012;[29] Agampodi 2016;[30] Biscornet 2017;[34] Riediger 2017[51]). However, in most studies, participant selection process was not or only very briefly described, leading to frequent 'unclear risk' judgements. Four studies employed a two-gate design and the risk of bias was therefore considered high (Samsonova 1997;[53] Ananyina 2000;[32] Saengjaruk 2002;[52] de Abreu Fonseca 2006).[40]

Index test

Studies seldom reported blinding of index test interpreters for reference standard results. Eight studies ensured adequate blinding (Samsonova 1997;[53] Ananyina 2000;[32] de Abreu Fonseca 2006;[40] Ahmed 2009;[31] Thaipadungpanit 2011;[56,58] Blanco 2014;[35] Vanasco 2016;[59] Woods 2018[66]), and two studies did the index test before the reference standard (Zhang 1992[69]; Riediger 2017[51]). No study reported that interpreters were unblinded. We considered the positivity threshold to be prespecified if readout methods for the index test led to a binary outcome (i.e. yes or no). This was the case for all index tests except real-time PCR and ELISA. Eight of 18 studies prespecified Cts for the real-time PCR (Ahmed 2009;[31] Waggoner 2014;[61,62] Waggoner 2015;[63] Denipitiya 2016;[28] Vanasco 2016;[59] Biscornet 2017;[34] Riediger 2017;[51] Woods 2018[66]).

Reference standard

We considered the risk of bias of the reference standard to be high in 22 studies. Only seven of 41 studies were at low risk for this domain (Zhang 1992;[69] Ananyina 2000;[32] de Abreu Fonseca 2006;[40] Ahmed 2009;[31] Thaipadungpanit 2011;[56,58] Vanasco 2016;[59] Riediger 2017[51]). Following QUADAS-2, we judged this domain based on two aspects: choice of reference standard and blinding of interpreters to index test results.

MAT was the sole reference standard in 22 studies with a single-gate design (17 cross-sectional studies, five single-gate case-controls studies) (Yersin 1998[68]; Fan 1999;[41] Merien 2005;[47] Ooteman 2006;[48] Riediger 2007;[50] Cardona 2008;[36] Koizumi 2009;[46] Chandrasiri 2010;[38] Agampodi 2012;[30] Villumsen 2012 BC;[60] Villumsen 2012 U;[60] Gonzalez 2013;[43] Widiyanti 2013;[65] Blanco 2014;[35] Waggoner 2014;[61,62] Kitashoji 2015;[45] Waggoner 2015;[63] Agampodi 2016;[29] Denipitiya 2016;[28] Gokmen 2016;[42] Chaurasia 2018;[39] Pakoa 2018[49]), which we regarded as high risk due to its imperfect sensitivity. Ten single-gate studies used a composite reference standard: two studies used MAT and IgM ELISA (Vanasco 2016;[59] Biscornet 2017[34]), 10 studies used MAT and culturing (Zhang 1992;[69] Wu 1996;[67] Wangroongsarb 2005;[64] Seng 2007;[54] Thaipadungpanit 2011;[56,58] Sonthayanon 2013;[55] Backstedt 2015;[33]

Riediger 2017;[51] Sukmark 2018;[57] Woods 2018[66]), and two studies used all three (Céspedes 2007;[37] Ahmed 2009[31]).

As an additional criterion, we required MAT to include paired samples for the judgement 'low risk'. All but one study (Wu 1996[67]) with a composite reference standard fulfilled this criterion. When MAT alone or culture alone was used as the reference standard in two-gate designs, risk of bias was considered low.

Two studies reported blinding of the reference standard interpreters, in which the blinding was adequate (Ahmed 2009;[31] Riediger 2017[51]). We also considered blinding to be adequate in nine studies in which the reference standard was done before the index test (Samsonova 1997;[53] Ananyina 2000;[32] de Abreu Fonseca 2006;[40] Thaipadungpanit 2011;[56,58] Villumsen 2012 BC;[60] Villumsen 2012 U;[60] Waggoner 2014;[61,62] Waggoner 2015;[63] Vanasco 2016[59]).

Flow and timing

Risk of bias for flow and timing was low for 32 studies and unclear for one study (Riediger 2007). Eight studies were considered high risk, as they did not include all patients in the analysis, with reasons varying from decisions by clinicians not to request MAT to exclusion based on inadequate urine samples (Yersin 1998;[68] de Abreu Fonseca 2006;[40] Villumsen 2012 BC;[60] Villumsen 2012 U;[60] Waggoner 2014;[61,62] Pakoa 2018;[49] Sukmark 2018;[57] Woods 2018[66]). All two-gate studies did not apply the same reference standards for cases and controls (Samsonova 1997;[53] Ananyina 2000;[32] Saengjaruk 2002;[52] de Abreu Fonseca 2006[40]). However, we did not consider this as differential verification bias, as differential verification bias implies that the choice of reference standard depended on the result of the index test, which was not the case in these studies.

Concerns regarding applicability of results to clinical practice

Concerns regarding the representativeness of the patient population

As studies were largely heterogeneous in their population, our standard for a representative patient population was low. We considered the patient population to be representative if patients with both single and paired samples were included, and if the patient characteristics did not differ significantly from the expected recipients of the test in practice (e.g. not all patients were female, or not all had severe renal failure). However, 17 studies did not provide sufficient description regarding patient selection methods or characteristics, leading to frequent 'unclear concern' judgements (Zhang 1992;[69] Gravekamp 1993;[44] Wu 1996;[67] Samsonova 1997;[53] Fan 1999;[41] Ananyina 2000;[32] Saengjaruk 2002;[52] Merien 2005;[47] Wangroongsarb 2005;[64] de Abreu Fonseca 2006;[40] Riediger 2007;[50] Chandrasiri 2010;[38] Widiyanti 2013;[65] Blanco 2014;[35] Waggoner 2015;[63] Gokmen 2016;[42] Vanasco 2016[59]). We had high concerns for four studies that excluded patients with only a single blood sample instead of paired samples (Yersin 1998;[68] Thaipadungpanit 2011;[56,58] Agampodi 2012;[30] Gonzalez 2013[43]).

Although verification by MAT is more accurate with paired samples, excluding patients with single samples may not reflect a representative clinical population, as they may have been patients with a severe disease course who did not survive until the second blood sampling. We also had high concerns for one study that included only patients with a strong suspicion for advanced severe leptospirosis (Backstedt 2015[33]), and two studies that excluded patients that had used antibiotics (Villumsen 2012 BC;[60] Villumsen 2012 U[60]).

Concerns regarding the representativeness and reproducibility of the index test

Defining representativeness was difficult for the index test, since all of the included tests were inhouse tests. Since we assumed that only fresh patient samples would be used for testing in clinical practice, we defined the concern as being high when the studies used frozen samples. This was the case for 22 studies (Gravekamp 1993;[44] Samsonova 1997;[53] Yersin 1998;[68] Ooteman 2006;[48] Seng 2007;[54] Thaipadungpanit 2011;[56,58] Agampodi 2012;[30] Villumsen 2012 BC;[60] Villumsen 2012 U;[60] Gonzalez 2013;[43] Widiyanti 2013;[65] Waggoner 2014;[61,62] Backstedt 2015;[33] Kitashoji 2015;[45] Waggoner 2015;[63] Agampodi 2016;[29] Denipitiya 2016;[28] Gokmen 2016;[42] Vanasco 2016;[59] Biscornet 2017;[34] Riediger 2017;[51] Sukmark 2018[57]). We also had concerns regarding applicability in one study, which added salt buffer to patient samples (Wu 1996[67]). Five studies failed to provide detailed descriptions of the execution of the index test, leading us to have high concern whether repetition would be possible (Zhang 1992;[69] Ananyina 2000;[32] Chandrasiri 2010;[38] Chaurasia 2018;[39] Pakoa 2018[49]).

Concerns regarding the reproducibility of the reference standard

We also applied 'high concern' judgements for the reference standard when studies failed to provide detailed description of the execution of the reference standard (Zhang 1992;[69] Wu 1996;[67] Fan 1999;[41] Ananyina 2000;[32] Merien 2005;[47] Chandrasiri 2010;[38] Waggoner 2014;[61,62] Kitashoji 2015;[45] Gokmen 2016[42]).

FINDINGS

Conventional polymerase chain reaction

Seventeen studies reported test accuracy data for the PCR. Fifteen studied PCR on blood products (serum: nine studies: whole blood (ethylenediaminetetraacetic acid (EDTA)): four studies; blood or serum: one study; unspecified blood product: one study) and of them, three also studied PCR on urine. Two studies included exclusively urine samples. The sensitivity of PCR on blood products ranged from 13% to 100%, and the specificity from 0% to 100% (see Figure 10). The 12 studies analysing PCR on blood products did not report the timing of sample collection for the index test (further referred to as DPO). Three studies did report the

DPO: one reported a mean of five days (Vanasco 2016[59]), one reported a range of one to five days (Zhang 1992[69]), and one a range of one to seven days (Céspedes 2007[37]).

The sensitivity of PCR on urine ranged from 22% to 57%, and the specificity from 56% to 100% (see Figure 11).

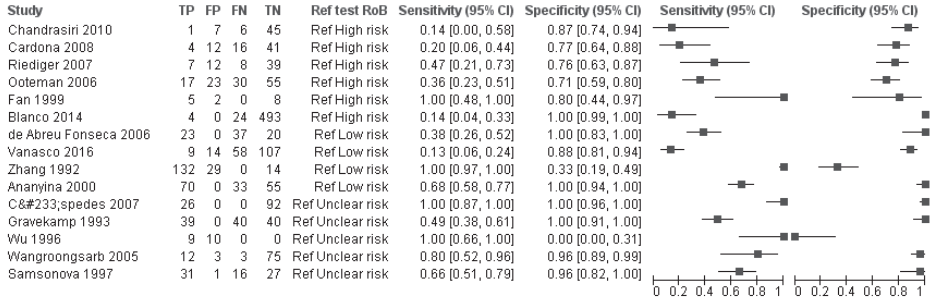

Figure 10. Forest plot of conventional polymerase chain reaction (PCR) on blood products. Ref test RoB: risk of bias for the 'reference standard' domain.

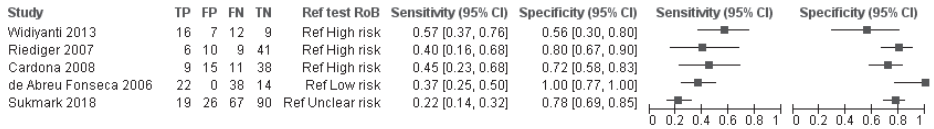

Figure 11. Forest plot of conventional polymerase chain reaction (PCR) on urine. Ref test RoB: risk of bias for the 'reference standard' domain.

Overall meta-analysis

We conducted a meta-analysis of PCR on blood products (see Figure 12). Using the bivariate model based on 15 studies (1884 participants, 660 with and 1224 without leptospirosis), the pooled sensitivity of PCR on blood products was 70% (95% CI 37% to 90%) and the pooled specificity was 95% (95% CI 75% to 99%). Based on a median prevalence of leptospirosis of 32.5%, the positive post-test probability (PPP) was 87% (95% CI 53% to 97%) and the negative post-test probability (NPP) was 87% (95% CI 71% to 95%). The positive likelihood ratio was 13.56 (95% CI 2.61 to 70.29) and the negative likelihood ratio was 0.32 (95% CI 0.12 to 0.82). There were too few studies for the PCR on urine to conduct a meaningful meta-analysis.

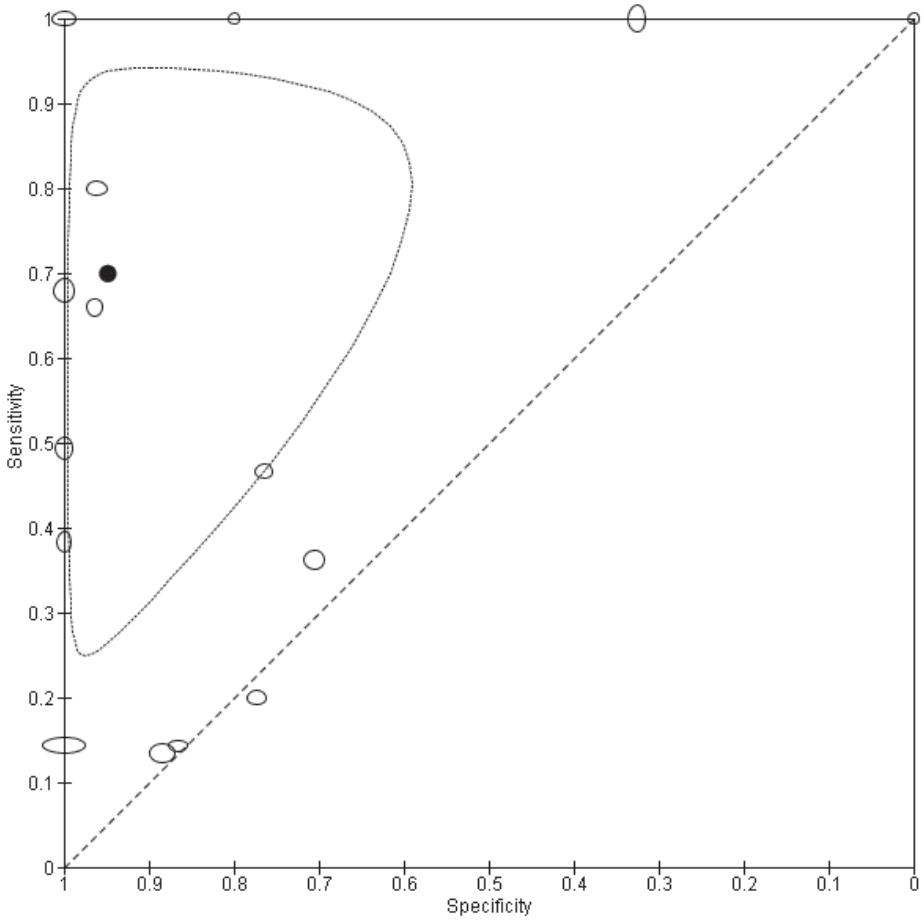

Figure 12. Summary ROC plot for conventional polymerase chain reaction (PCR) on blood products. *Transparent dots indicate the test accuracy of the individual studies included in the analysis; the black dot indicates the pooled test accuracy. The ellipse around the pooled test accuracy is the 95% confidence region. The size of the transparent dots represents the sample size, with the vertical diameter representing the number of cases and horizontal diameter representing the number of non-cases.*

Investigations of heterogeneity

We assessed heterogeneity only for PCR on blood products. We planned to investigate the following sources of heterogeneity: timing of sample collection, prevalence, blood sample type, target gene/primer, and brand of test.

- Timing of sample collection: timing of sample collection was usually unreported (12 studies) and the subgroups were too small to investigate heterogeneity.
- Prevalence: we investigated whether prevalence of leptospirosis was associated with test accuracy. Studies with a higher prevalence of leptospirosis had a significantly lower specificity (P = 0.0004). Prevalence was not associated with sensitivity (P = 0.2).

- Blood sample type for the index test: we included four studies using whole blood and nine studies using serum (see Figure 13). The pooled sensitivity of whole blood was 78% (95% CI 22% to 98%) and pooled specificity was 99% (95% CI 61% to 100%), and the pooled sensitivity of serum was 78% (95% CI 37% to 96%) and pooled specificity was 93% (95% CI 50% to 100%), meaning that these subgroups did not differ significantly from each other.
- Target gene/primers: a large variety of target genes and primers were used for the PCR (see Table 4), but the subgroups were too small to investigate heterogeneity.
- Brand of the index test: all tests were inhouse tests.

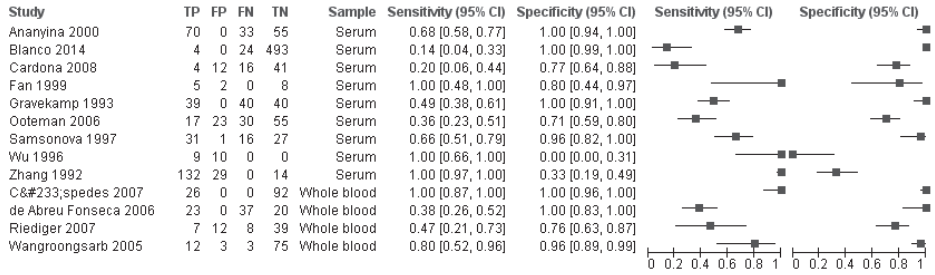

Figure 13. Forest plot of conventional polymerase chain reaction (PCR) on whole blood versus conventional PCR on serum.

Sensitivity analyses

See Table 5 for an overview of the analyses.

- Risk of bias: we excluded three studies that were at high risk of bias in the 'patient selection' domain of QUADAS-2 (Samsonova 1997;[53] Ananyina 2000;[32] de Abreu Fonseca 2006[40]). This resulted in a pooled sensitivity of 76% (95% CI 31% to 96%) and pooled specificity of 91% (95% CI 60% to 98%), showing no important difference from the overall meta-analysis. Likewise, we excluded six studies with high risk of bias in the 'reference standard' domain (Fan 1999;[41] Ooteman 2006;[48] Riediger 2007;[50] Cardona 2008;[36] Chandrasiri 2010;[38] Blanco 2014[35]). The resulting pooled sensitivity was 87% (95% CI 44% to 98%) and pooled specificity was 97% (95% CI 60% to 100%). While the sensitivity of the PCR increased, the CIs were very wide, with substantial overlap with the results of the overall meta-analysis.
- Antibiotic use: we excluded one study that reported the use of antibiotics in the patient population (Ananyina 2000[32]). Sensitivity analysis with the 14 remaining studies (13 did not report on antibiotic use, and one study reported that antibiotics were not used (Chandrasiri 2010[38])) resulted in a pooled sensitivity of 71% (95% CI 34% to 92%) and pooled specificity of 93% (95% CI 71% to 99%). These results did not differ from the overall meta-analysis.

Table 5. Pooled sensitivity and specificity of the conventional polymerase chain reaction (PCR) overall meta-analysis and the sensitivity analyses
CI: 95% confidence intervals

Analysis	Sensitivity (95% CI)	Specificity (95% CI)
Overall meta-analysis PCR	70% (37% to 90%)	95% (75% to 99%)
Exclusion of studies at 'high risk of bias' for patient selection	76% (31% to 96%)	91% (60% to 98%)
Exclusion of studies at 'high risk of bias' for reference standard	87% (44% to 98%)	97% (60% to 100%)
Exclusion of studies that reported the use of antibiotics	71% (34% to 92%)	93% (71% to 99%)
Meta-analysis with the lower MAT threshold dataset	70% (36% to 90%)	95% (75% to 99%)
Exclusion of studies that were only reported as abstracts	74% (40% to 93%)	96% (74% to 99%)

- Lower MAT threshold: two studies reported each two threshold values for the MAT (Ooteman 2006;[48] Cardona 2008[36]). For the overall analyses, we selected the higher threshold dataset. Sensitivity analysis with the lower threshold dataset in these two studies made no difference to the findings (pooled sensitivity: 70%, 95% CI 36% to 90%; pooled specificity: 95%, 95% CI 75% to 99%).
- Abstract-only study: we repeated the analysis excluding one study that was only reported as an abstract (Chandrasiri 2010[38]). The pooled sensitivity was 74% (95% CI 40% to 93%) and the pooled specificity was 96% (95% CI 74% to 99%), demonstrating no important change from the overall meta-analysis.

Comparison of different conventional polymerase chain reaction methods

Four studies reported direct comparisons (i.e. comparisons between different conventional PCR methods studied in the same study population): different timing of sample collection (one study) and different sample types for the PCR (three studies).

- Timing of sample collection: Céspedes 2007[37] compared the results of PCR on whole blood when samples from three different time frames were tested: 1 DPO to 7 DPO, 8 DPO to 9 DPO and 1 DPO to 9 DPO.
  - For 1 DPO to 7 DPO, sensitivity of PCR was 100% (95% CI 87% to 100%) and specificity was 100% (95% CI 96% to 100%).
  - For 8 DPO to 9 DPO, sensitivity was 30% (95% CI 18% to 45%) and specificity was 100% (95% CI 74% to 100%).
  - For 1 DPO to 9 DPO, sensitivity was 55% (95% CI 43% to 67%) and specificity was 100% (95% CI 97% to 100%).
- Sample types for PCR: the reported direct comparisons were serum versus urine (Cardona 2008[36]) and whole blood versus urine (de Abreu Fonseca 2006;[40] Riediger 2007[50]).

- In Cardona 2008,[36] the sensitivity of serum PCR was 20% (95% CI 6% to 44%) and specificity was 77% (95% CI 64% to 88%), and sensitivity of urine PCR was 45% (95% CI 23% to 68%) and specificity was 72% (95% CI 58% to 83%).
- In de Abreu Fonseca 2006,[40] the sensitivity of whole blood PCR was 38% (95% CI 26% to 52%) and specificity was 100% (95% CI 83% to 100%), and sensitivity of urine PCR was 37% (95% CI 25% to 40%) and specificity was 100% (95% CI 77% to 100%).
- In Riediger 2007,[50] the sensitivity of whole blood PCR was 47% (95% CI 21% to 73%) and specificity was 76% (95% CI 63% to 87%), and sensitivity of urine PCR was 40% (95% CI 16% to 68%) and specificity was 80% (95% CI 67% to 90%).

Real-time polymerase chain reaction

Eighteen studies assessed the accuracy of the real-time PCR. Sixteen studies used blood products as sample type: serum or plasma (nine studies), whole blood (six studies), serum or whole blood (three studies), blood culture samples (one study), and buffy coat samples (one study). Two studies used urine samples. Six studies each reported two sets of data: Agampodi 2012[30] and Riediger 2017[51] reported data for whole blood and serum, while Backstedt 2015,[33] Thaipadungpanit 2011,[56,58] and Woods 2018[66] reported data for two real-time PCRs, each using a different target gene (*rrs*, *lipL32*, or *rrs/lipL32*). Waggoner 2014[61,62] reported data for a monoplex and multiplex (detecting also dengue and malaria) real-time PCR. Ahmed 2009[31] reported three sets of data, each evaluating the test at different DPOs (1 DPO to 4 DPO, 5 DPO to 10 DPO, and 1 DPO to 10 DPO). Because we considered 1 DPO to 10 DPO to be the most representative time of sample collection, we included the dataset of 1 DPO to 10 DPO in the meta-analysis. Seven studies of real-time PCR on blood products did not report the DPO (Gonzalez 2013;[43] Sonthayanon 2013;[55] Backstedt 2015;[33] Waggoner 2015;[63] Biscornet 2017;[34] Riediger 2017;[51] Pakoa 2018[49]). The other studies all reported DPOs of the index test under 10 days.

The sensitivity of real-time PCR on blood products ranged from 0% to 100%, and the specificity ranged from 0% to 100% (Figure 14).

Two studies assessed real-time PCR on urine (Villumsen 2012 U;[60] Woods 2018[66]). One study assessed real-time PCR on blood culture samples (Villumsen 2012 BC[60]), and one study assessed real-time PCR on buffy coat samples (Woods 2018[66]). Due to the lack of enough studies for urine, blood culture, and buffy coat real-time PCR, we did not perform a meta-analysis. The sensitivities and specificities of these PCRs are displayed in Table 6.

Table 6. Sensitivity and specificity of real-time polymerase chain reaction (PCR) on urine, blood culture, and buffy coat samples
CI: 95% confidence intervals

Study ID	Sample type	Target gene	Sensitivity	Lower CI	Upper CI	Specificity	Lower CI	Upper CI
Woods 2018	Urine	*rrs* (Slack 2007)	17%	6%	36%	90%	87%	92%
Woods 2018	Urine	*rrs/lipL32* (Woods 2018)	14%	4%	32%	99%	98%	100%
Villumsen 2012 U	Urine	*rrs* (Smythe 2002)	100%	29%	100%	98%	90%	100%
Villumsen 2012 U	Urine	*lipL32* (Villumsen 2012)	100%	29%	100%	98%	90%	100%
Villumsen 2012 BC	Blood culture	*rrs* (Smythe 2002)	100%	59%	100%	95%	77%	100%
Villumsen 2012 BC	Blood culture	*lipL32* (Villumsen 2012)	86%	42%	100%	100%	85%	100%
Woods 2018	Buffy coat	*rrs* (Slack 2007)	3%	0%	16%	99%	98%	100%
Woods 2018	Buffy coat	*rrs/lipL32* (Woods 2018)	12%	3%	28%	99%	98%	100%

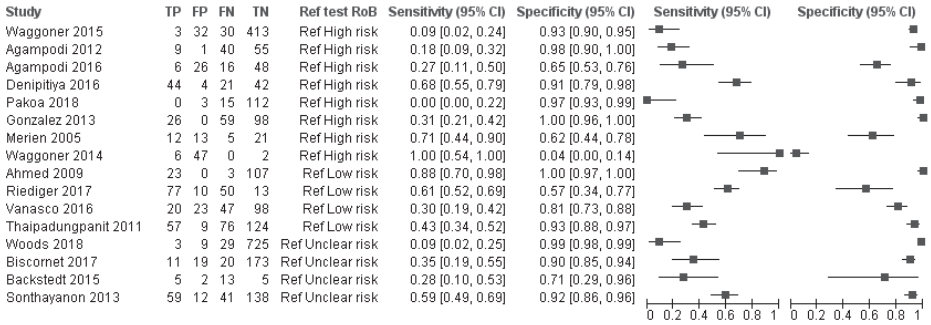

Figure 14. Forest plot of real-time polymerase chain reaction (PCR) on blood products. Ref test RoB: risk of bias for the 'reference standard' domain.

Overall meta-analysis

We conducted a meta-analysis only for real-time PCR on blood products using the HSROC model. As described previously, seven studies reported multiple data sets. However, each study may only contribute a single data set to the meta-analysis to prevent the study from being over-represented. Therefore, we randomly excluded the serum dataset of Agampodi 2012,[30] the *rrs* dataset of Backstedt 2015,[33] the *rrs* dataset of Thaipadungpanit 2011,[56,58] the serum dataset of Riediger 2017,[51] the *rrs* dataset of Woods 2018,[66] and the multiplex PCR dataset of Waggoner 2014.[61,62] For reasons mentioned earlier, we included only one of the datasets of Ahmed 2009[31] (1 DPO to 10 DPO) in the analysis.

The analysis included 16 studies with 3210 participants (826 with and 2384 without leptospirosis) (Figure 14; Figure 15). Because we anticipated that the thresholds of the real-time PCRs in the included studies would differ, we refrained from estimating a summary point. Instead, we constructed a summary curve. The summary curve is a graph of the values

of sensitivity and specificity that are obtained by varying the threshold across all possible values. To illustrate, we estimated the accuracy for three fixed specificity values of 85%, 90% and 95%. At 85% specificity, pooled sensitivity was 49% (95% CI 30% to 68%); at 90% specificity, pooled sensitivity was 40% (95% CI 24% to 59%); and at 95% specificity, pooled sensitivity was 29% (95% CI 15% to 49%). The median specificity of real-time PCR on blood products was 92%. The CIs were wide due to the heterogeneity of included studies. We did not estimate post-test probabilities or likelihood ratios for the real-time PCR as it would be unclear to which threshold values these estimates would correspond.

Investigations of heterogeneity

We restricted assessment of heterogeneity to real-time PCR on blood products. In summary, none of the analyses yielded a statistically significant difference.

- Timing of sample collection: we could not investigate timing of sample collection as a source of heterogeneity; although it was reported in nine studies, the reporting was too heterogeneous to form adequate subgroups (Table 4; Merien 2005;[47] Ahmed 2009;[31] Thaipadungpanit 2011;[56,58] Agampodi 2012;[30] Waggoner 2014;[61,62] Agampodi 2016;[29] Denipitiya 2016;[28] Vanasco 2016;[59] Woods 2018[66]).
- Prevalence: prevalence was not associated with test accuracy (P = 0.96).
- Blood sample type for the index test: nine studies used 'serum or plasma' (Merien 2005;[47] Agampodi 2012;[30] Gonzalez 2013;[43] Waggoner 2014;[61,62] Waggoner 2015;[63] Biscornet 2017;[34] Riediger 2017;[51] Pakoa 2018;[49] Woods 2018[66]), and six studies used whole blood (Thaipadungpanit 2011;[56,58] Agampodi 2012;[30] Sonthayanon 2013;[55] Backstedt 2015;[33] Denipitiya 2016;[28] Riediger 2017[51]). There was no statistically significant association between sample type and accuracy (P = 0.42).
- Target gene/primer: Table 4 shows an overview of the target genes for the real-time PCR. Due to the small number of studies in each subgroup, we refrained from analysing the effect of different primers.
- Threshold: reported thresholds for the real-time PCR were 35 Ct, 40 Ct, and 45 Ct. Due to the small number of studies in each subgroup, we refrained from analysing their effect. However, we used the HSROC model to take the threshold effect into account.
- Real-time PCR visualisation method: there was no statistically significant difference (P = 0.058) in the accuracy between studies of real-time PCR using SYBR green (five studies: Merien 2005;[47] Ahmed 2009;[31] Gonzalez 2013;[43] Backstedt 2015;[33] Denipitiya 2016[28]), and studies of real-time PCR using TaqMan probes (12 studies: Thaipadungpanit 2011;[56,58] Agampodi 2012;[30] Sonthayanon 2013;[55] Waggoner 2014;[61,62] Backstedt 2015;[33] Waggoner 2015;[63] Agampodi 2016;[29] Vanasco 2016;[59] Biscornet 2017;[34] Riediger 2017;[51] Pakoa 2018;[49] Woods 2018[66]). The pooled diagnostic odds ratio (DOR) for the SYBR green real-time PCR was 46.2 (95% CI 0.89 to 2383.68), while the pooled DOR for the TaqMan real-time PCR was 3.09 (95% CI 1.25 to 7.63).

- Brand of the index test: there were no variations among studies regarding the brand of the test, as all were inhouse tests.

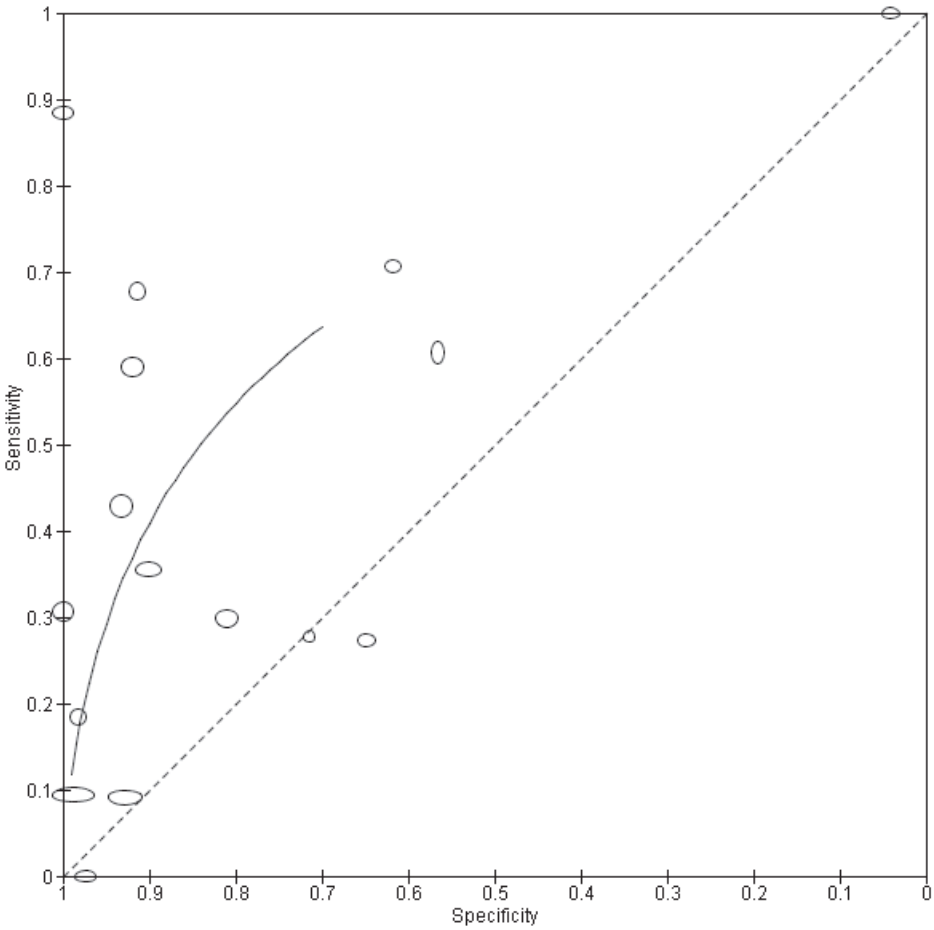

Figure 15. Summary ROC plot for real-time polymerase chain reaction (PCR) on blood products. *Transparent dots indicate the test accuracy of the individual studies included in the analysis. The solid black line (summary ROC curve) is a graph of the values of sensitivity and specificity that are obtained by varying the threshold across all possible values. The size of the transparent dots represents the sample size, with the vertical diameter representing the number of cases and horizontal diameter representing the number of non-cases.*

Sensitivity analyses

- Risk of bias: according to our QUADAS-2 judgements, one study had high risk of bias for the 'patient selection' domain (Waggoner 2015[63]). Exclusion of this study from the meta-analysis yielded a pooled sensitivity of 33% (95% CI 18% to 52%) at a fixed specificity of 95% (Table 7). When we excluded eight studies that had high risk of bias for the 'reference standard' domain (Merien 2005;[47] Agampodi 2012;[30] Gonzalez 2013;

[43] Waggoner 2014;[61,62] Waggoner 2015;[63] Agampodi 2016;[29] Denipitiya 2016;[28] Pakoa 2018[49]), the pooled sensitivity was 37% (95% CI 15% to 66%) at a fixed specificity of 95%. Both analyses did not introduce important changes to the overall result.

- Alternative datasets for the overall meta-analysis: we repeated the analyses with datasets which were previously randomly excluded from the overall meta-analysis. These are the serum dataset of Agampodi 2012[30] and Riediger 2017;[51] the *rrs* dataset of Backstedt 2015,[33] Thaipadungpanit 2011,[56,58] and Woods 2018;[66] and the multiplex PCR dataset of Waggoner 2014.[61,62] The repeated analyses with these datasets made no difference to the findings (pooled sensitivity 32%, 95% CI 17% to 52% at a fixed specificity of 95%).
- Antibiotic use: we excluded three studies in which participants used antibiotics before the index test (Thaipadungpanit 2011;[56,58] Sonthayanon 2013;[55] Woods 2018[66]). The pooled sensitivity was 28% (95% CI 12% to 53%) at a fixed specificity of 95%, demonstrating no important change from the overall meta-analysis.
- Lower MAT threshold: sensitivity analysis with the lower MAT threshold dataset in two studies did not lead to different results (Waggoner 2015;[63] Denipitiya 2016[28]). The pooled sensitivity was 29% (95% CI 16% to 47%) at a fixed specificity of 95%.

Table 7. Pooled sensitivity and specificity of the real-time polymerase chain reaction (PCR) overall meta-analysis and the sensitivity analysis

Analysis	Sensitivity (95% CI)	Specificity (fixed at 95%)
Overall meta-analysis real-time PCR	29% (15% to 49%)	95%
Exclusion of studies at 'high risk of bias' for patient selection	33% (18% to 52%)	95%
Exclusion of studies at 'high risk of bias' for reference standard	37% (15% to 66%)	95%
Alternative datasets for the overall meta-analysis	32% (17% to 52%)	95%
Exclusion of studies in which participants used antibiotics	28% (12% to 53%)	95%
Meta-analysis with the lower MAT threshold dataset	29% (16% to 47%)	95%

Comparison of different real-time polymerase chain reaction methods

We identified several direct comparisons pertaining to sample type, timing of sample collection, and target genes.

- Timing of sample collection: Ahmed 2009[31] compared samples collected at 1 DPO to 4 DPO, 5 DPO to 10 DPO, and 1 DPO to 10 DPO.
  - At 1 DPO to 4 DPO, sensitivity was 100% (95% CI 74% to 100%) and specificity was 100% (95% CI 94% to 100%).
  - At 5 DPO to 10 DPO, sensitivity was 69% (95% CI 41% to 89%) and specificity was 100% (95% CI 92% to 100%).
  - At 1 DPO to 10 DPO, sensitivity was 88% (95% CI 70% to 98%) and specificity was 100% (95% CI 97% to 100%).

- Sample type: Agampodi 2012[30] and Riediger 2017[51] compared whole blood samples with serum samples. In Agampodi 2012,[30] sensitivity of whole blood real-time PCR was 18% (95% CI 9% to 32%) and specificity was 98% (95% CI 90% to 100%), and sensitivity of serum was 51% (95% CI 36% to 66%) and specificity was 98% (95% CI 90% to 100%). In Riediger 2017,[51] sensitivity of whole blood was 61% (95% CI 52% to 69%) and specificity was 57% (95% CI 34% to 77%), and sensitivity of serum was 29% (95% CI 21% to 38%) and specificity was 87% (95% CI 66% to 97%). Woods 2018[66] reported direct comparisons of serum, buffy coat, and urine samples in two different real-time PCRs (one targeting *rrs* (Slack 2007) and one targeting *rrs/lipL32* (Woods 2018[66])). For clarity, the six pairs of sensitivity and specificity of Woods 2018[66] are shown in Table 8.
- Target gene/primer: four studies compared *rrs* and *lipL32* target genes in the same population (Thaipadungpanit 2011;[56,58] Villumsen 2012 BC;[60] Villumsen 2012 U;[60] Backstedt 2015[33]). The results of the four studies are displayed in Table 9. Woods 2018[66] also reported comparisons of *rrs* (Slack 2007) and *rrs/lipl32* (Woods 2018[66]) real-time PCRs on serum, buffy coat, and urine samples, which are shown in Table 8.
- Waggoner 2014[61,62] compared two types of real-time PCR in the same population, namely a multiplex real-time PCR for leptospirosis, dengue, and malaria, and a monoplex real-time PCR which used the same primer from the multiplex assay combined with a new probe for pathogenic leptospires. The sensitivity of the multiplex real-time PCR was 100% (95% CI 54% to 100%) and specificity was 0% (95% CI 0% to 7%) and the sensitivity of the monoplex real-time PCR was 100% (95% CI 54% to 100%) and specificity was 4% (95% CI 0% to 14%).

Table 8. Woods 2018:[66] direct comparison of serum, buffy coat, and urine real-time polymerase chain reaction (PCR)

Sample type	Target gene	Sensitivity	Lower CI	Upper CI	Specificity	Lower CI	Upper CI
Serum	*rrs/lipl32* (Woods 2018)	9%	2%	25%	99%	98%	99%
Buffy coat	*rrs/lipl32* (Woods 2018)	12%	3%	28%	99%	98%	100%
Urine	*rrs/lipl32* (Woods 2018)	14%	4%	32%	99%	98%	100%
Serum	*rrs* (Slack 2007)	9%	2%	25%	99%	97%	99%
Buffy coat	*rrs* (Slack 2007)	3%	0%	16%	99%	98%	100%
Urine	*rrs* (Slack 2007)	17%	6%	36%	90%	87%	92%

Nested polymerase chain reaction

Four studies reported accuracy data for the nested PCR (Merien 2005;[47] Koizumi 2009;[46] Blanco 2014;[35] Gokmen 2016[42]). All were cross-sectional studies using serum as the sample type, and all studies used MAT as the reference standard. The reported mean timing of

Table 9. Studies that report direct comparisons of *rrs* and *lipL32* real-time polymerase chain reaction (PCR)

Study ID	Target gene	Sample type	Sensitivity	Lower CI	Upper CI	Specificity	Lower CI	Upper CI
Thaipadungpanit 2011	*rrs* (Slack 2007)	Whole blood	56%	47%	64%	89%	83%	94%
	lipL32 (Stoddard 2009)	Whole blood	43%	34%	52%	93%	88%	97%
Villumsen 2012 BC	*rrs* (Smythe 2002)	Blood culture	100%	59%	100%	95%	77%	100%
	lipL32 (Villumsen 2012)	Blood culture	86%	42%	100%	100%	85%	100%
Villumsen 2012 U	*rrs* (Smythe 2002)	Urine	100%	29%	100%	98%	90%	100%
	lipL32 (Villumsen 2012)	Urine	100%	29%	100%	98%	90%	100%
Backstedt 2015	*rrs* (Backstedt 2015)	Whole blood	56%	31%	78%	14%	0%	58%
	lipL32 (Stoddard 2009)	Whole blood	28%	10%	53%	71%	29%	96%

sample collection was 5 DPO (Merien 2005[47]) and 7 DPO (Koizumi 2009[46]), but was not reported for Blanco 2014[35] and Gokmen 2016.[42] The sensitivity of nested PCR ranged from 0% (95% CI 0% to 13%) to 95% (95% CI 76% to 100%) and the specificity ranged from 42% (95% CI 23% to 63%) to 100% (95% CI 99% to 100%) (see Figure 16). Since only four studies were available, we did not conduct a meta-analysis or formal assessments of heterogeneity.

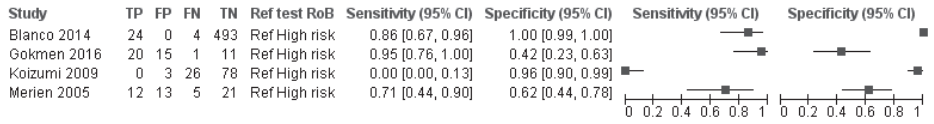

Figure 16. Forest plot of nested polymerase chain reaction (PCR) on serum. *Ref test RoB: risk of bias for the 'reference standard' domain.*

Comparison of different nested polymerase chain reaction methods

One study compared the *rrs* (Merien 1992) nested PCR to the *lipL32* (Bomfim 2008) nested PCR (Gokmen 2016[42]). The sensitivity of *rrs* nested PCR was 95% (95% CI 76% to 100%) and specificity was 42% (95% CI 23% to 63%). The sensitivity of the *lipL32* nested PCR was 90% (95% CI 70% to 99%) and specificity was 42% (95% CI 23% to 63%).

Conventional polymerase chain reaction performed twice

Two studies reported data for PCR done on serum samples taken at two different times (Yersin 1998;[68] Seng 2007[54]). The PCR was considered positive if one of the two samples was positive. The first sample was taken at admission and the second sample approximately 14 days later. Both studies were cross-sectional. One study used a composite reference standard (MAT and culturing; Seng 2007[54]), while the other used only MAT (Yersin 1998[68]). Seng

2007[54] reported sensitivity of 75% (95% CI 19% to 99%) and specificity of 94% (95% CI 88% to 98%), while Yersin 1998[68] reported sensitivity of 47% (95% CI 34% to 60%) and specificity of 96% (95% CI 87% to 100%).

Loop-mediated isothermal amplification

Two studies using single-gate designs evaluated the test accuracy of the LAMP (Thaipadungpanit 2011;[56,58] Kitashoji 2015;[45] Figure 17). LAMP was done on whole blood, plasma, or urine samples. The median timing of sample collection was 6.5 DPO for Kitashoji 2015[45] (plasma samples only, unreported for urine), but for Thaipadungpanit 2011[56,58] the timing was separately reported for cases (median 4 DPO) and non-cases (median 6 DPO). Kitashoji 201[45] reported results for LAMP on plasma (sensitivity 14%, 95% CI 9% to 22%; specificity 83%, 95% CI 76% to 89%) and LAMP on urine samples (sensitivity 14%, 95% CI 7% to 24%; specificity 91%, 95% CI 83% to 95%). Thaipadungpanit 2011[56,58] reported results for LAMP targeting *rrs* (Sonthayanon 2011[55]) (sensitivity 44%, 95% CI 35% to 52%; specificity 83%, 95% CI 76% to 89%) and LAMP targeting *lipL41* (Lin 2009) (sensitivity 38%, 95% CI 29% to 46%; specificity 90%, 95% CI 84% to 95%).

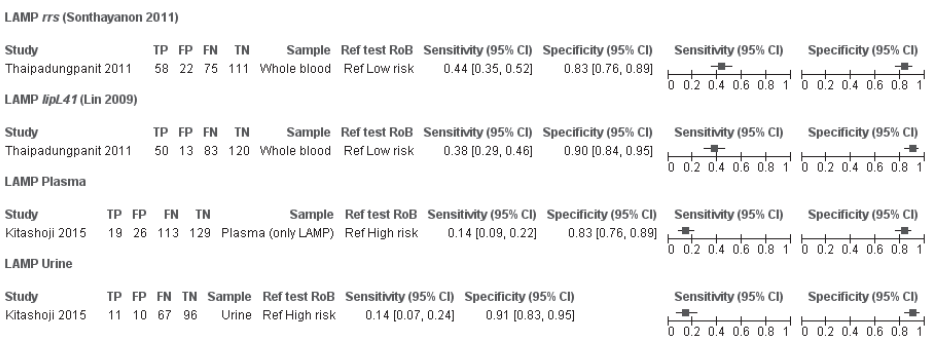

Figure 17. Forest plot of loop-mediated isothermal amplification (LAMP), on whole blood, plasma or urine. *Ref test RoB: risk of bias for the 'reference standard' domain.*

Comparison of different loop-mediated isothermal amplification methods

Thaipadungpanit 2011[56,58] compared *rrs* and *lipL41* as target genes and Kitashoji 2015[45] compared plasma and urine samples for the LAMP (Figure 17).

Enzyme-linked immunosorbent assay

One cross-sectional study conducted in India assessed the test accuracy of ELISA on urine samples (Chaurasia 2018[39]), The timing of sample collection and the threshold were not reported. The study used single-sample MAT as reference standard to classify 23 participants as cases and six as non-cases. The ELISA was done using seven different target antigens

(*LipL32*, *Fla1*, *LipL41*, *HbpA*, *SphCD210*, *Sph2*, and *Sph4*). For clarity, the sensitivities and specificities of these tests are shown in Table 10.

Table 10. Chaurasia 2018:[39] direct comparison of target antigens for enzyme-linked immunosorbent assay (ELISA)

Target antigen	Sensitivity	Lower CI	Upper CI	Specificity	Lower CI	Upper CI
LipL32	100%	85%	100%	67%	22%	96%
Fla1	91%	72%	99%	50%	12%	88%
LipL41	78%	56%	93%	83%	36%	100%
HbpA	91%	72%	99%	67%	22%	96%
SphCD210	100%	85%	100%	67%	22%	96%
Sph2	91%	72%	99%	67%	22%	96%
Sph4	39%	20%	61%	83%	36%	100%

Dot-enzyme-linked immunosorbent assay

One two-gate case-control study evaluated the test accuracy of a monoclonal antibody-based dot-ELISA for the detection of leptospiral antigens in urine samples (Saengjaruk 2002[52]). The study was conducted in Thailand with 42 participants, of which 25 were leptospirosis cases confirmed by culture and 17 were people with other illnesses. Timing of sample collection was not reported. The sensitivity of dot-ELISA was 64% (95% CI 43% to 82%) and specificity was 100% (95% CI 81% to 100%).

Immunochromatography-based lateral flow assay

One cross-sectional study assessed the accuracy of an ICG-based LFA using monoclonal antibodies specific to the *Leptospira* lipopolysaccharide (Widiyanti 2013[65]). The study tested the urine samples of 44 participants with suspected leptospirosis, and classified 28 as cases based on MAT alone. The mean timing of sample collection was 5.5 DPO. The sensitivity of LFA was 96% (95% CI 82% to 100%) and specificity was 56% (95% CI 30% to 80%).

Dipstick assay

Widiyanti 2013[65] evaluated a monoclonal antibody-based dipstick assay on urine, specific to the Leptospira lipopolysaccharide. The same urine samples were tested, with a mean timing of sample collection of 5.5 DPO. The sensitivity of the dipstick assay was 89% (95% CI 72% to 98%) and specificity was 63% (95% CI 35% to 85%).

Comparison of index tests

Several studies performed a comparison of index tests in the same patient population. Since each comparison contained only one study, we could not perform a meta-analysis.

- PCR versus real-time PCR: Vanasco 2016[59] reported a comparison of PCR and real-time PCR, both performed on whole blood or serum samples. PCR had a sensitivity of 13% (95% CI 6% to 24%) and a specificity of 88% (95% CI 81% to 94%). Real-time PCR had a sensitivity of 30% (95% CI 19% to 42%) and a specificity of 81% (95% CI 73% to 88%).
- PCR versus nested PCR: Blanco 2014[35] reported a comparison of PCR and nested PCR. PCR had a sensitivity of 14% (95% CI 4% to 33%) and a specificity of 100% (95% CI 99% to 100%). Nested PCR had a sensitivity of 86% (95% CI 67% to 96%) and a specificity of 100% (95% CI 99% to 100%).
- Real-time PCR versus nested PCR: Merien 2005[47] compared real-time PCR with nested PCR. The study reported identical results for the two tests: sensitivity was 71% (95% CI 44% to 90%) and specificity was 62% (95% CI 44% to 78%).
- PCR versus ICG-based IFA versus dipstick assay: Widiyanti 2013[65] reported a comparison between PCR, ICG-based IFA, and dipstick assay, with all three tests performed on urine samples.
    - For PCR on urine, sensitivity was 57% (95% CI 37% to 76%) and specificity was 56% (95% CI 30% to 80%).
    - For ICG-based IFA, sensitivity was 96% (95% CI 82% to 100%) and specificity was 56% (95% CI 30% to 80%).
    - For dipstick assay, sensitivity was 89% (95% CI 72% to 98%) and specificity was 63% (95% CI 35% to 85%).
- Real-time PCR versus LAMP: Thaipadungpanit 2011[56,58] reported a comparison of *rrs* (Slack 2007) and *lipL32* (Stoddard 2009) real-time PCR and *rrs* (Sonthayanon 2011[55]) and *lipL41* (Lin 2009) LAMP.
    - For real-time PCR targeting *rrs*, sensitivity was 56% (95% CI 47% to 64%) and specificity was 89% (95% CI 83% to 94%).
    - For real-time PCR targeting *lipL32*, sensitivity was 43% (95% CI 34% to 52%) and specificity was 93% (95% CI 88% to 97%).
    - For LAMP targeting *rrs*, sensitivity was 44% (95% CI 35% to 52%) and specificity was 83% (95% CI 76% to 89%).
    - For LAMP targeting *lipL41*, sensitivity was 38% (95% CI 29% to 46%) and specificity was 90% (95% CI 84% to 95%).

DISCUSSION

Summary of main results

In this systematic review, we summarised the diagnostic test accuracy of nucleic acid and antigen detection tests for human symptomatic leptospirosis, verified by (a combination of)

currently established tests – MAT, culture, and IgM ELISA. We identified 41 studies in the literature evaluating nine index tests, of which conventional PCR and real-time PCR were the most frequently evaluated tests. While we have performed a meta-analysis for PCR and real-time PCR on blood products, individual study results suggested very high between-study heterogeneity (PCR sensitivity ranging from 13% to 100% and PCR specificity from 0% to 100%). Therefore readers should interpret the meta-analytic result as a weighted mean of all the heterogeneous settings in which the index tests were evaluated, rather than an estimate that is applicable across settings. Summary of findings table 1, Summary of findings table 2, Summary of findings table 3, and Summary of findings table 4 give an overview of the most important findings.

Interpretation of the conventional polymerase chain reaction meta-analysis

From a meta-analysis of 15 studies evaluating PCR on blood products collected during various stages of disease, the pooled sensitivity was 70% (95% CI 37% to 90%) and the pooled specificity was 95% (95% CI 75% to 99%). This means that in a hypothetical cohort of 1000 people, with a prevalence of 32.5% (325 diseased), 98 (95% CI 32 to 205) cases would be missed by the PCR and 35 (95% CI 6 to 168) non-diseased people would be incorrectly diagnosed with leptospirosis. If the prior probability of an individual to have leptospirosis is 32.5%, the PPP was 87% (95% CI 53% to 97%) and the NPP was 87% (95% CI 71% to 95%). If the PCR would be used in a setting such as in the Netherlands where the prevalence is lower (9.7% in 2016; Collaborating Centre for Reference and Research on Leptospirosis in Amsterdam, unpublished data), 97/1000 people would have leptospirosis. In such a cohort, 29 (95% CI 9 to 61) diseased people would be missed and 47 (95% CI 8 to 225) non-diseased people would be incorrectly diagnosed with leptospirosis. If the prior probability of leptospirosis in an individual is 9.7%, the PPP is 59% (95% CI 20% to 89%) and the NPP is 97% (95% CI 92% to 99%). See Summary of findings table 1.

When the PCR meta-analysis was repeated without the studies at high risk of bias for the 'reference standard' domain (i.e. only including studies with composite reference standards or two-gate studies with MAT as reference standard, based on nine studies), the pooled sensitivity was 87% (95% CI 44% to 98%), and the pooled specificity was 97% (95% CI 60% to 100%). See Summary of findings table 2. This means that in a cohort of 1000 people with 325 leptospirosis patients, 42 (95% CI 5 to 183) cases would be missed and 17 (95% CI 1 to 272) non-diseased people would be incorrectly diagnosed with leptospirosis. In an individual with 32.5% prior probability of leptospirosis, the PPP is 94% (95% CI 41% to 100%) and the NPP is 94% (95% CI 70% to 99%). If the prevalence is 9.7%, 13 (95% CI 2 to 55) diseased people would be missed and 23 (95% CI 1 to 363) non-diseased people would be incorrectly diagnosed with leptospirosis. If an individual has 9.7% prior probability

of leptospirosis, the PPP is 78% (95% CI 13% to 99%), and the NPP is 99% (95% CI 91% to 100%).

A repeated meta-analysis of PCR without studies at high risk of bias for the 'patients' domain did not lead to important changes in our results.

Interpretation of the real-time polymerase chain reaction meta-analysis

For the real-time PCR, we estimated an SROC curve instead of a summary sensitivity and specificity, since we expected the positivity threshold (which was often not reported) to vary between studies. The median specificity of real-time PCR on blood products was 92%. For illustrative purposes, if we were to select a point on the curve with 95% specificity, the pooled sensitivity would be 29% (95% CI 15% to 49%) at an unknown threshold (Summary of findings table 3). Translating these numbers to a cohort of 1000 people of whom 325 are diseased, would mean that 230 (95% CI 167 to 276) diseased people would be missed and 34 non-diseased people would be incorrectly diagnosed with leptospirosis. Again, in a setting with a prevalence of 9.7% this would imply that 69 (95% CI 50 to 82) diseased people would be missed and 45 non-diseased people would be incorrectly diagnosed with leptospirosis. We did not provide PPP and NPP for real-time PCR, as threshold values needed to produce these estimates were unknown.

When we excluded real-time PCR studies at high risk of bias for the 'patients' domain or the 'reference standard' domain, there were no important changes in the pooled estimate.

The position of conventional polymerase chain reaction and real-time polymerase chain reaction in the clinical pathway

Based on the properties of PCR and real-time PCR, we examined the possible role of these tests in the diagnostic pathway for leptospirosis. Leptospirosis is a potentially life-threatening disease, meaning that efforts should be undertaken to minimise false-negative results. In the clinic, while most patients with suspicion of a severe infection are likely to receive broad-spectrum antibiotics, a false-negative result impedes the optimisation of the antibiotic therapy and assessment of the prognosis. In an outbreak setting, a missed case of leptospirosis will delay outbreak response and facilitate further dissemination of disease.

For patients presenting in the early disease stage, PCR and real-time PCR on blood products are preferable as first-line tests in the clinical pathway based on their ability for early detection. However, whether additional testing is needed to verify a positive or negative test result depends on test accuracy and the prevalence of leptospirosis. While PCR-based methods have been described as sensitive tests in the literature,[16,70,71] our results show that the sensitivity of PCR and real-time PCR vary greatly between studies, with the CI for PCR sensitivity ranging from 37% to 90%. This can partly be explained by differences in methodological quality, but it is also likely that there is true heterogeneity, such as differences in timing of sample collection. Furthermore, the reliability of a positive or negative test

result depends on the prevalence. In our review, prevalence of leptospirosis varied greatly between studies (range 3% to 85%). Consequently, whether PCR-based methods can be used alone, or together with other follow-up tests depends on regional considerations such as prevalence, factors that are likely to influence accuracy (e.g. timing of sampling), and downstream consequences of a positive or negative result. For example, in settings with a high prevalence, PCR and real-time PCR may not have a high enough negative post-test probability to confidently rule out leptospirosis. In this case, additional testing to verify negative results should be considered.

Comparison between index tests

This review did not find enough evidence to formally compare the diagnostic accuracy of included tests. Direct comparison studies (where two or more index tests are evaluated in the same patient population) are needed to draw valid conclusions about the differences in diagnostic test accuracy between tests, but such studies were lacking. Although the results of the meta-analyses seem to imply that real-time PCR has a lower sensitivity than PCR, this is not a valid comparison. The meta-analysis results are composed of mostly single-test studies and any differences between real-time PCR and PCR could arise from other reasons than the differences in the tests themselves, such as differences in study design or spectrum of disease. Therefore, any comparison between the meta-analytic results of PCR and real-time PCR must be interpreted with caution.

Heterogeneity of included studies

Substantial heterogeneity, as demonstrated by the wide CIs, complicated the interpretation of our findings.

An important covariate, timing of sample collection, could not be explored in heterogeneity analysis. Three of 15 studies assessing PCR on blood products reported the timing of sample collection (range 1 DPO to 7 DPO of symptoms), and in 9/16 studies assessing real-time PCR on blood products (range 1 to 19 DPO). However, the subgroups were either too small or the reporting of the timing variable was too heterogeneous for analysis. Two studies comparing test accuracy in patients who presented early with test accuracy in patients who presented later appear to support the hypothesis that the sensitivity of PCR and real-time PCR is greater in the first few days of illness (Céspedes 2007;[37] Ahmed 2009[31]). This is consistent with the current pathophysiological understanding of leptospirosis, that leptospiraemia declines rapidly and becomes undetectable after 10 DPO.[12] However, this could also be caused by patients with a higher bacterial load presenting earlier to the clinic due to a more severe clinical presentation than those with a lower bacterial load. Moreover, one study reported that the sensitivity of real-time PCR was not associated with timing of sample collection in patients presenting with fewer than 10 DPO (P = 0.33) (Agampodi

2012[30]). Ultimately, more studies are needed to confirm the association between timing and test accuracy.

In the case of PCR, statistical heterogeneity may be partly explained by the prevalence of leptospirosis in the study population. Specificity was inversely correlated with prevalence (P = 0.0004). A number of explanations for this association are possible. The prevalence of alternative diagnoses may be higher in places where leptospirosis prevalence is high, causing false-positive results on the PCR. The PCR may be detecting lower levels of infections that occur more frequently in high-prevalence settings, that are missed by MAT, and, therefore, recorded as false-positive results. The laboratories in high-prevalence settings may be less well-equipped and more often contaminated, and, therefore, allow more false-positive results. We did not examine the inverse correlation between prevalence and sensitivity or specificity for real-time PCR studies, since the HSROC model by default examines the association between prevalence and accuracy (alpha parameter) instead of sensitivity and specificity.

Other covariates that could possibly influence test accuracy, such as real-time PCR threshold and specific target genes or primers used in PCR-based methods, have not been ruled out as possible explanations for the significant heterogeneity. It is theoretically possible that the heterogeneity in sensitivity of PCR-based methods could be explained by differences in Leptospira species, as a primer may not able to detect a particular species. However, this is not very probable as it is usual practice to account for all existing species when developing PCR or its variants (unless a new species emerges). Since all index tests were inhouse tests, there may be other potential sources of heterogeneity (e.g. use of different laboratory equipment or protocols) that cannot be measured reliably or be reported in sufficient detail. For this reason, readers should be cautious when applying summary estimates of test accuracy in their own clinical settings.

Risk of bias

A major point of attention in our review was the use of MAT as reference standard, which is considered to have an imperfect sensitivity. If the reference standard is not sensitive, the specificity of the index test is likely to be underestimated. It is furthermore not inconceivable that some index tests, for example, PCR-based methods, may be more sensitive than MAT alone. We aimed to address this problem by including composite reference standards and rating the risk of bias as high when MAT was used as the sole reference standard, and when single samples were used (instead of paired). However, only a minority of studies used another reference standard alongside the MAT, leading to a 'high risk of bias' judgement in the majority of studies for this domain. In the case of the PCR, sensitivity increased when studies at 'high risk of bias' for the reference standard were excluded, but specificity was unchanged (Table 5). Other covariates that may be of importance, such as the cut-off value for MAT, the use of adequate regional panels for MAT, and the differences between composite reference standards, were not taken into account in our review to avoid excessive complexity.

Another issue is the inclusion of four two-gate case-control studies (Samsonova 1997;[53] Ananyina 2000;[32] Saengjaruk 2002;[52] de Abreu Fonseca 2006[40]). Cases and controls in these studies are selected separately and do not reflect the spectrum of disease in the clinical population.[26] Another concern in these studies is the possibility of coinfections of leptospirosis and another infectious disease. In two-gate designs, since controls are not MAT negatives but people with a condition resembling leptospirosis, coinfections with leptospirosis may be present. Treating these people as controls may underestimate the specificity of the index test. These studies have been excluded in the abovementioned sensitivity analyses as they were considered 'high risk' for patient selection (Table 5; Table 7).

Blinding of the index test result to the reference standard result interpreters (or vice versa) was largely unreported. Interpreters were blinded because either blinding methods were used, or by the virtue of their study design (e.g. blinding of the index test interpreter was not needed when the index test was done first). In the index test, eight studies reported to have used a form of blinding in their methods. However, we noted that only one study reported explicit methods for blinding (Samsonova 1997[53]). It was unclear if the remaining studies used proper blinding methods. Considering other possible biases in our review (Figure 4; Figure 6; Figure 8), readers are advised to weigh the results against the quality of evidence.

Other index tests

For other tests included in our review (nested PCR, PCR performed twice, LAMP, ELISA, dot-ELISA, ICG-based LFA, and dipstick assay), we could not conduct meta-analyses or investigations of heterogeneity due to the small number of studies.

Strengths and weaknesses of the review

The strength of our review lies in the fact that we used an extensive search strategy including 16 national and regional databases, without any limitations on languages and without using search filters or keywords containing terms related to diagnostic accuracy. We also contacted authors for full-text articles in case the studies did not report complete data for the construction of two-by-two contingency tables. Furthermore, we aimed to include all nucleic acid tests and antigen detection tests that we could find in the literature. And lastly, we included studies that used MAT with convalescent samples as a reference standard, as well as studies that used MAT with a single, acute sample. This is a strength, because it provides a good reflection of the day-to-day reality in clinics and laboratories, but at the same time, it is one of the major limitations of our review. It is known that antibodies appear in the blood only after several days to weeks.[72] Thus a serological test, such as the MAT, is not applicable for diagnosis of leptospirosis in the early stages. Inclusion of studies using MAT only on acute samples could have led to false-negative results by the reference standard. This lack of a perfect reference standard implies that the test accuracy of nucleic acid and antigen tests

presented here simply reflects the extent of agreement between the index test and MAT, and not necessarily the true test accuracy of the index tests.

Other limitations of our review are as follows. First, studies that potentially satisfy our inclusion criteria could not be included due to lack of clarity or inconsistencies in the full-text article. Second, due to poor reporting in primary studies, many aspects of the index test and methodological quality remain unclear and limit the potential to generalise our findings. Third, as we have discussed previously, we could not explain the substantial heterogeneity in study results due to the lack of statistical power. Although we have conducted meta-analyses for PCR and real-time PCR, it is debatable to what extent the pooled results are applicable to clinical practice, since numerous unexplored covariates are likely to have contributed to the pooled sensitivities and specificities.

Applicability of findings to the review question

We identified some concerns regarding the applicability of the results to our review question when the used research methods differed significantly from clinical practice (Figure 4; Figure 6; Figure 8).

Regarding the selection of participants, four studies excluded participants due to unavailability of convalescent samples for MAT (Yersin 1998;[68] Thaipadungpanit 2011;[56,58] Agampodi 2012;[30] Gonzalez 2013[43]). Although this is a reasonable decision, since verification by MAT is more accurate with paired acute and convalescent samples, it does not necessarily reflect the patient population in clinical practice, as participants with a fatal course of disease were likely to be excluded from the study as a result. In 19 studies, the patient selection method, inclusion criteria, and characteristics (including the timing of sample collection) were not well reported. These studies stated that the participants were 'clinically suspected for leptospirosis' without stating which signs, symptoms, and risk factors the participants had that made them clinically suspected. Furthermore, with baseline characteristics not reported, it was not possible to determine whether a particular age group or sex was over-represented in the study.

For the applicability of the index test and the reference standard, we did not only consider whether the method of testing differed from clinical practice, but also whether the execution of the test was reported in such detail that the test could be reproduced in full elsewhere. In four studies, we found that this was not the case for the index test (Ananyina 2000;[32] Chandrasiri 2010;[38] Chaurasia 2018;[39] Pakoa 2018[49]), and in five studies, the procedure (including the cut-off value) for the MAT was not reported, or there was a reference to an irretrievable study (Samsonova 1997;[53] Ananyina 2000;[32] Merien 2005;[47] Chandrasiri 2010;[38] Waggoner 2014[61,62]). At least 11 studies used frozen samples for the index test rather than fresh samples. We were uncertain if this could have influenced the test accuracy, so we considered the concern regarding applicability to be high.

AUTHORS' CONCLUSIONS

Implications for practice

The validity of review findings are limited by the poor reporting of methodological quality items and the use of suboptimal reference standards. We conclude that there is substantial between-study variability in the accuracy of conventional polymerase chain reaction (PCR) and real-time PCR, as well as substantial variability in the prevalence of leptospirosis. Consequently, the position of conventional PCR and real-time PCR in the clinical pathway depends on regional considerations such as prevalence, factors that are likely to influence accuracy (such as timing of sampling), and downstream consequences of test results. There is insufficient evidence to conclude which of the nucleic acid and antigen detection tests are the most accurate in the early stage of leptospirosis. There is preliminary evidence that conventional PCR and real-time PCR are more sensitive on blood samples collected early in the disease stage, but this needs to be confirmed in future studies. Evidence regarding other index tests was very limited.

Implications for research

Our review demonstrates that while there is a wealth of publications on new nucleic acid and antigen detection tests, there is a marked scarcity on well-designed, well-performed, and well-reported validations of such tests. More high-quality studies are needed with larger samples sizes, especially a larger group of cases to estimate sensitivity more precisely. Future investigators should follow the reporting guidelines of STARD (Standards for the Reporting of Diagnostic Accuracy Studies), to allow the assessment of potential biases in the study, as well as the assessment of the clinical value of the estimated test accuracy. Single-gate designs, such as the cross-sectional study, with consecutive enrolment have our recommendation above two-gate designs because of their lower risk of spectrum bias. The choice of reference standard should not only include MAT, but also culture, and if possible immunoglobulin M enzyme-linked immunosorbent assay (IgM ELISA), to minimise false-negative results from occurring. The emphasis should be on paired sampling, to show a possible rise in antibody titres. In order to compare and select the best performing tests, multiple index tests should be evaluated on the same participants so that direct comparison of their accuracy is possible. Last, we encourage future investigators to explore the effects of varying times of sample collection on test accuracy as a potential source of heterogeneity.

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SUPPLEMENTARY MATERIALS

Supplementary materials can be accessed at
https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD011871.pub2/full

The supplementary materials not printed here include:

Characteristics of included studies (p41-133)
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PART 2

Rickettsioses

Chapter 6

Under-diagnosis of rickettsial disease in clinical practice: a systematic review

Sophia G de Vries*, Louise E van Eekeren*, Jiri F P Wagenaar, René Spijker, Martin P Grobusch, Abraham Goorhuis

**authors contributed equally*

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ABSTRACT

Background

Rickettsial diseases present as acute febrile illnesses, sometimes with inoculation eschars.

Methods

We performed a systematic review of studies published between 1997 and 2017 to assess the underestimation of non-eschar rickettsial disease (NERD) relative to eschar rickettsial disease (ERD), as a cause of acute fever in patients with rickettsial diseases that commonly present with eschar(s): scrub typhus (ST), Mediterranean spotted fever (MSF), and African tick-bite fever. We compared ERD/NERD ratios according to study design: 'complete approach' studies, with testing performed in all patients with 'unspecified febrile illness'; versus 'clinical judgement' studies, with testing performed if patients presented with specific symptoms.

Results

In 'complete approach' studies, ERD/NERD ratios were significantly lower, suggesting a considerable under-diagnosis of NERD in 'clinical judgement' studies. Based on these results, we estimate that the diagnosis of rickettsial disease was missed in 66.5% of patients with ST, and in 57.9% of patients with MSF.

Conclusions

Study design influences the reported eschar rates in ST and MSF significantly. NERD is likely to be a vastly underdiagnosed entity, and clinicians should consider and test for the disease more often.

PROSPERO registration number

CRD 42016053348

INTRODUCTION

Rickettsial diseases (RD) present as acute febrile illness. They are caused by intra-cellular, gram-negative bacteria of the Rickettsiales order,[1] and are typically transmitted by various arthropods (e.g. ticks, mites, fleas and lice).[2] Clinical manifestations of rickettsial diseases range from mild, self-limiting to severe or fatal illness[3] and cause substantial mortality and morbidity worldwide.[4-11] Early diagnosis, facilitating early antibiotic treatment, reduces morbidity and mortality.[12] However, this may be cumbersome, as it may be clinically difficult to differentiate rickettsial diseases from other acute febrile illnesses. Moreover, rickettsial diseases are largely endemic in low-resource areas that lack diagnostic capacity. Additionally, available standard tests have a low sensitivity, especially in the early stages of disease.[13,14] Conversely, diagnosis can be straightforward, when a patient presents with one or more inoculation eschars ('tache noire'), which are typical painless necrotic skin lesions that may develop at the site of the vector bite in some, but not all cases of several rickettsial diseases.[10]

Traditionally, pathogens causing disease in humans are divided in three groups: the 'Spotted Fever Group' (SFG rickettsial diseases), 'Typhus Group' and the 'Scrub Typhus Group'. However, increasing numbers of pathogenic *Rickettsia* spp. are being identified,[1] and advances in molecular taxonomic methods have led to an ongoing reorganization of the classification,[10,15,16] which is difficult to be meaningfully disentangled by the practising clinician. From a clinical perspective, rickettsial diseases could be divided in eschar-rickettsial disease ('ERD') and non-eschar-rickettsial disease ('NERD'). Commonly diagnosed rickettsial diseases that can clinically present as either ERD or NERD are: Scrub typhus (ST, caused by *Orientia tsutsugamushi*), Mediterranean spotted fever (MSF, caused by *Rickettsia conorii*), and African tick-bite fever (ATBF, caused by *Rickettsia africae*).

Scrub typhus is transmitted by larval trombiculid mites, and mainly occurs in Asia and the Pacific islands, although recent reports suggest a wider geographical distribution.[8,11,17] In Southeastern Asia, where the disease is most common in rural areas, there are an estimated 1 million cases per year,[6] contributing to a substantial part of hospital admissions for acute undifferentiated fever.[18] Average mortality is around 12.7%, varying across regions.[8]

Mediterranean Spotted Fever is transmitted by *Rhipicephalus sanguineus* (sensu lato) ticks, and is endemic in the Mediterranean areas of Europe and North Africa, but has also been reported in sub-Saharan Africa.[10] Most cases occur in the warmer months, and in recent years, more fatal cases of MSF ('malignant MSF') have been reported.[19]

African Tick Bite Fever, transmitted by various ticks, is endemic in sub-Saharan Africa and the eastern Caribbean.[20] Reports of illness in the indigenous population are scarce; it is thought that infection usually occurs at a young age, taking a mild course.[20] The disease is common in travellers, often occurring after a safari trip in southern Africa, presenting as a relatively mild, acute febrile illness.[21]

Among returned travellers, rickettsial diseases are considered as amongst the most common causes of fever apart from malaria, typhoid fever, leptospirosis and arboviral infections.[5,22]

Among patients with unspecified febrile illness, we suspect a vast under-diagnosis of rickettsial diseases, especially in the absence of an inoculation eschar ('NERD'), because other symptoms, such as a skin rash, are unspecific. The frequency in which eschars are reported in these diseases varies.[23] Theories to explain this variation, such as geography and associated strains,[24-26] have been postulated, but have not been proven so far.[6]

We hypothesize that a large portion of the reported variation in eschar occurrence is caused by study design. For example, in studies evaluating rickettsial diseases (among others) in all patients with febrile illness, higher proportions of 'NERD' would be found compared to studies that only evaluate rickettsial diseases on the basis of clinical presentation, in which the proportion of 'ERD' would be overestimated, due to missed diagnoses of NERD. This would result in different ERD/NERD ratios depending on the diagnostic approach of rickettsial disease.

We hypothesize that this ratio will be closest to reality in studies in which diagnostic testing was performed independent of clinical presentation, thus in all patients presenting with unspecified febrile illness. However, the ERD/NERD ratio will be higher in studies in which diagnostic testing was performed according to clinical judgment, in patients presenting with symptoms compatible with rickettsial disease. A difference in ERD/NERD ratios depending on study design would enable us to quantify the extent of under-diagnosis of NERD.

This hypothesis is not applicable to rickettsial diseases that never or rarely present with an eschar, such as murine typhus (caused by *Rickettsia typhi*), Rocky Mountain spotted fever (caused by *Rickettsia rickettsii*), or to diseases caused by Rickettsia-like organisms such as *Coxiella spp.* and *Bartonella* spp. Neither does the hypothesis apply to other diseases that can present with eschar-like skin lesions (e.g., leishmaniasis, tularaemia, melioidosis, etc.).

Objectives of this study were (i) to assess whether the ERD/NERD ratio reported in studies varies depending on the diagnostic approach in rickettsial diseases that can present as either ERD or NERD, using Scrub Typhus, Mediterranean Spotted Fever, and African Tick Bite Fever as examples; and (ii), using the variation in ERD/NERD ratios, to determine to what extent NERD is an underestimated cause of acute febrile illness in endemic areas.

METHODS

A review protocol was developed and registered in the PROSPERO database, an international database of prospectively registered systematic reviews in health and social care (registration number: CRD 42016053348). Recommendations made by the Preferred Reporting Items for

Systematic Reviews and Meta-Analyses (PRISMA) group were followed in the conduct and reporting of this review.27

Search strategy and selection criteria

Medline, Embase, and Global Health Library were searched, applying the search strategies provided in Appendix 1. Records published in the past 20 years, from the 1st of January 1997 onwards, were included in the search. The last search was performed on 24 March 2017. Additionally, included studies and systematic reviews covering related topics were checked for relevant references.

Inclusion criteria were: (i) studies evaluating ATBF, MSF and/or ST in symptomatic patients; ii) evaluation of the presence of an eschar in patients with ATBF, MSF, or ST; iii) diagnosis of ATBF, MSF or ST is made according to laboratory diagnostics in the majority of patients.

Exclusion criteria were: (i) inappropriate study design (reviews, conference abstracts, and case series reporting on less than ten cases); (ii) studies in which patients were included based only on eschar presence; and (iii) studies in which the ERD/NERD ratio could not be calculated; for example, in cases of doubtful eschar presence (co-infections with other tick-borne diseases, unspecified SFG rickettsial diseases). A diagnosis of 'SFG rickettsial disease' was a reason for exclusion, as differentiation between *R. conorii conorii*, *R. africae* and other species of the SFG rickettsial diseases was impossible. In case the same study population was described in multiple reports, or evidence towards this existed, only the article evaluating the largest population sample was included.

Identified citations were imported to a Covidence database (VERITAS Health Innovation Ltd, www.covidence.org), and duplicate records were removed. Titles and abstracts were screened by two authors (LvE and SdV) independently. Disagreement was resolved by discussion. Full texts of included citations were retrieved; irretrievable articles – those not available online, from the university library, or through contacting authors – were excluded. The full texts of selected articles were screened by two independent authors (LvE and SdV), using an inclusion checklist. Disagreement was resolved by discussion.

Data extraction and data analysis

We extracted data using data extraction forms in Microsoft Word (Microsoft Corporation 2010). The form was piloted independently by two authors for two studies (SdV and LvE), after which the forms were refined accordingly. Data were extracted by one reviewer (LvE or SdV), and checked by a second reviewer (SdV or LvE); disagreements were resolved by discussion. For each study, the following data items were extracted: place and period in which the study was conducted; study objective(s); selection methods, including in- and exclusion criteria; laboratory diagnostic methods; number of patients assessed for eschar presence; percentage of patients presenting with eschar(s); mean or median age; and sex ratio. Within the population of patients diagnosed with RD in each study, we assessed the proportion of

patients that presented with one or more eschars. To define the sub-region wherein patients acquired the rickettsial disease, the United Nations geo-scheme was applied.[28] For the patient selection method, the studies were classified according to two scenarios: 'clinical judgement' or 'complete approach'. In studies with a selection approach according to the 'complete approach' scenario, diagnostics were performed independent of clinical presentation, in all patients presenting with 'unspecified febrile illness'. Studies were labelled as having a 'clinical judgement' selection approach, if diagnostics were performed according to clinical judgement, or if multiple pre-specified symptoms were applied as inclusion criteria, apart from fever. If the selection method was not clearly described, it was marked as 'unclear', 'clinical judgement/unclear', or 'complete approach/unclear', if the study was leaning towards either of the scenarios. A second reviewer reviewed all studies and classified the patient selection method independently. A final decision was made by consensus and, if necessary, the last author (AG) was consulted. If a study compared multiple groups, data were merged into one imaginary group to receive a larger sample. If this was not possible, data from the group with the largest sample of patients were extracted.

Assessment of risk of bias in individual studies

Studies were assessed for quality and risk of bias using a quality assessment tool composed of items of the case series quality assessment tools by NICE[29] and the Joanna Briggs Institute[30] (Appendix 2). Two authors (LvE and SdV) assessed quality independently for all studies. Disagreements were resolved by discussion. Based on the quality assessment, we assigned each study a rating: high quality (++), medium quality (+), or low quality (–).

Statistical analysis

Statistical analyses were performed applying the Statistical Package for the Social Sciences (SPSS, version 24.0, IBM, Chicago, IL, USA). Continuous variables were compared between the 'clinical judgement' and 'complete approach' groups using the unpaired *t*-test. Simple linear regression analyses were performed to assess whether continuous data were significantly associated. A chi-square test was performed to assess whether categorical data were significantly associated. The continuous data are expressed as mean ± standard deviation and all calculated values are weighted for the number of patients unless otherwise specified. P-values less than 0.05 were considered to be statistically significant.

For 'unclear', a worst/best case scenario analysis was performed, in which studies were subjected to the 'clinical judgement' group in the first analysis, and to the 'complete approach' group in a second analysis.

The estimation of the under diagnosis of NERD (missed eschar percentages) was calculated according to formulas depicted in Appendix 3.

RESULTS

Figure 1 depicts the study selection process. Database searches identified 2,293 unique records. 460 citations were selected for full-text assessment; 14 were irretrievable. Citation searching identified three additional records.[31-33] Forty-six articles were excluded because of evidence of overlap of datasets with included studies (Appendix 4). In total, 121 studies were included in this review; 98 reported on scrub typhus;[24,31-127] 21 on MSF;[7,26,128-146] one on both MSF and ATBF in travellers;[147] and one on ATBF in travellers.[148] Detailed characteristics of the included studies can be found in Appendix 5 and 6. Results of the quality assessment are depicted in Appendix 7.

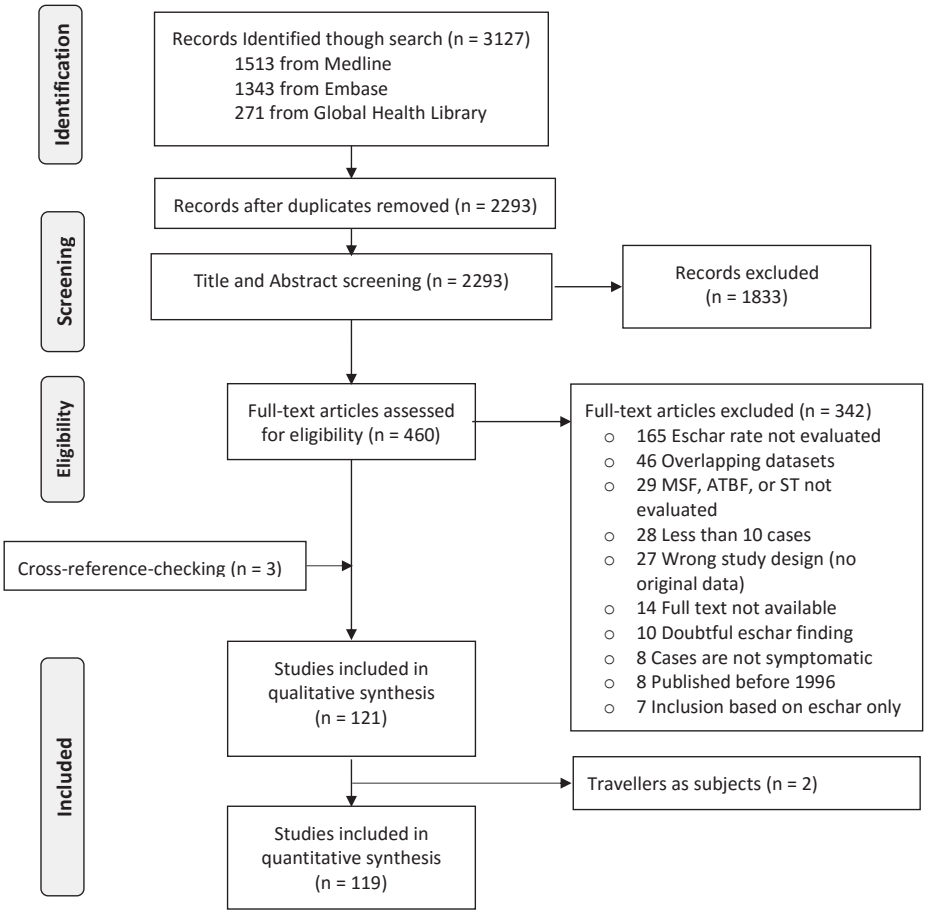

Figure 1. Study selection process

Scrub typhus

Figure 2 provides a graphic overview of eschar rates found in the studies.

Eschar presence was evaluated in a total number of 20,142 scrub typhus patients.[24,31-127] The mean overall percentage of eschar presence was 64.3% (standard deviation (SD) 25.4). Studies were conducted in India (n=39),[31,32,44-80] South Korea (n=19),[83-101] Thailand (n=13),[33,115-126] China (n=10),[24,35-43] Taiwan (n=9),[106-114] Japan (n=2),[81,82] Laos (n=2),[102,103] Bangladesh (n=1),[34] Malaysia (n=1),[104] Sri Lanka (n=1),[105] and Vietnam (n=1).[127] Among these 98 studies, 30 were assessed as having been performed according the 'complete approach' scenario,[31-33,44-50,52-59,76,102,106,107,115-122] and four were classified as complete approach/unclear'.[35,60,61,104] Of these 34 'complete approach' or 'complete approach/unclear' studies, eight were of high quality;[44,45,52,55,76,102,119,121] 22 of medium quality;[32,33,35,46-50,56-61,106,107,115-118,120,122] and four of low quality.[31,53,54,104] Forty studies were classified as 'clinical judgement',[24,37-42,51,70-75,77-80,82,87-101,105,114,124-127] and 24 as 'clinical judgement/unclear'.[34,36,43,62-69,81,83-86,103,108-113,123] Of these 64 'clinical judgement' or 'clinical judgement/unclear' studies, none was of high quality, 35 were of medium quality,[24,34,37,38,40-42,51,62,71,72,75,77,78,80,81,87,89-94,98,99,103,105,108-110,112,114,124,126,127] and 29 were of low quality.[36,39,43,63-70,73,74,79,82-86,88,95-97,100,101,111,113,123,125] 'Complete approach' and 'complete approach/unclear' studies had a significantly higher quality than 'clinical judgement' and 'clinical judgement/unclear studies' ($p < 0.001$).

Baseline characteristics on age and sex are presented in Table 1. In the 'complete approach' group, the mean or median age was lower compared to 'clinical judgement' studies (38.1 ± 7.6 years vs. 47.2 ± 18.2 years, $p < 0.001$), and there were fewer males (47.4% ± 13.1% vs 48.7% ± 10.3%, $p < 0.001$).

Mean percentages of eschar presence (Table 1) were significantly different between the 'complete approach' and 'clinical judgement' studies (23.6% ± 20.1% vs. 70.4% ± 19.8%, $p < 0.001$). As stated in our hypothesis, we assumed that the ERD/NERD ratio in the 'complete approach'-group is closest to the actual ratio. Using the calculations detailed in Appendix 3, we estimated that a diagnosis of ST was missed in 34,744 patients in the 'clinical judgement' group, which represents 87.0% (34,744/39,930) of the patients with NERD and 66.5% (34,744/52,263) of the total group of patients with ST, as shown in Figure 3.

When all unclear studies (both 'clinical judgement/unclear' and 'complete approach/unclear') were assigned to the 'clinical judgement' studies ('best case scenario'), the eschar rates remained significantly lower in complete approach studies compared to 'clinical judgement' studies ('clinical judgement' + 'clinical judgement/unclear' + 'complete approach/unclear': mean = 70.0 ± 20.3 (17,655 patients) vs. 'complete approach': mean = 23.8 ± 20.3 (2,487 patients); $p < 0.001$). The same applied to the 'worst case scenario', when all unclear studies were assigned to the 'complete approach' group ('clinical judgement': mean = 66.8 ± 22.5 (8,035 patients) vs. 'complete approach' + 'complete approach/unclear' + 'clinical judgement/unclear': mean = 62.6 ± 27.0 (12,107 patients); $p < 0.001$).

Figure 4 provides an overview of reported mean eschar rates per region.

Table 1. Baseline characteristics and eschar rates of included studies

	CJ	CJ/unclear	All CJ	CA	C/unclear	All CA	All CJ vs. all CA
Scrub Typhus							
Patients, n (studies)	8,035 (40)	9,484 (24)	17,519 (64)	2,487 (30)	136 (4)	2,623 (34)	
Age (mean or median)	50.8 ± 16.3 (6,311 values)	32.7 ± 18.0 (1,582 values)	47.2 ± 18.2 (7,893 values)	38.0 ± 7.9 (1,520 values)	39.0 ± 2.2 (124 values)	38.1 ± 7.6 (1,644 values)	**P < 0.001**
Sex (males, %)	46.4 ± 8.9 (6,888 values)	57.1 ± 10.9 (1,918 values)	48.7 ± 10.3 (8,806 values)	47.4 ± 13.3 (1,626 values)	47.1 ± 10.4 (136 values)	47.4 ± 13.1 (1,762 values)	**P < 0.001**
Eschar, % (mean, SD)	66.8 ± 22.5	73.4 ± 16.6	70.4 ± 19.8	23.8 ± 20.2	19.8 ± 16.6	23.6 ± 20.1	**P < 0.001**
Mediterranean spotted fever							
Patients, n (studies)	3,613 (12)	1,421 (8)	5,034 (20)	55 (1)	-	55 (1)	
Age (mean or median)	43.1 ± 6.6 (2,209 values)	47.7 ± 9.0 (780 values)	44.3 ± 7.6 (2,989 values)	5.6 ± 0.0 (55 values)	-	5.6 ± 0.0 (55 values)	**P < 0.001**
Sex (males, %)	53.6 ± 6.3 (1,786 values)	60.2 ± 8.6 (1,253 values)	56.4 ± 8.0 (3,039 values)	52.7 ± 0.0 (55 values)	-	52.7 ± 0.0	**P < 0.001**
Eschar, % (mean, SD)	71.4 ± 5.9	63.3 ± 9.5	69.1 ± 8.0	29.1 ± 0.0	-	29.1 ± 0.0	**P < 0.001**

* CA = 'complete approach'; CJ = 'clinical judgement'; SD = standard deviation

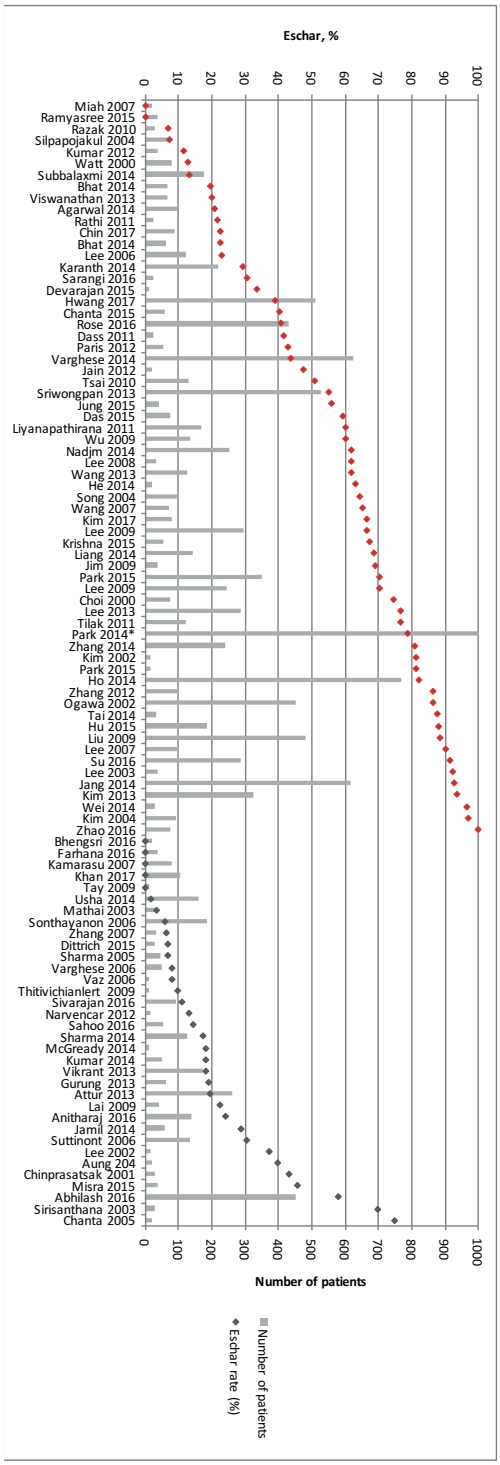

Figure 2. Eschar rates in scrub typhus reported per study
Number of patients included per study are depicted in the grey bars; red dots indicate the eschar rate in 'clinical judgement' studies, grey dots indicate eschar rates in 'complete approach' studies.

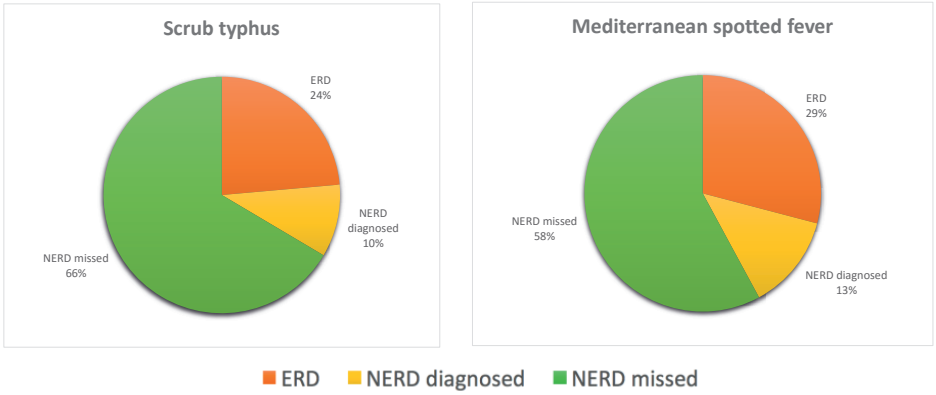

Figure 3. The underestimation of NERD
The circle diagrams visualize the extent of the underestimation of non-eschar scrub typhus and non-eschar Mediterranean spotted fever

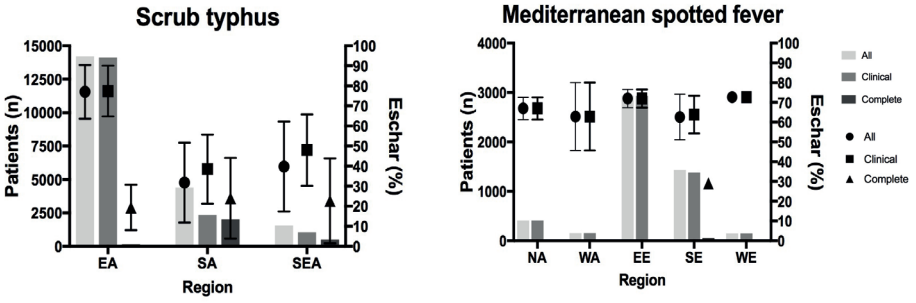

Figure 4. Eschar rates per region
The grey bars indicate the number of patients overall, and per study type (clinical judgement and complete approach). The dots indicate the overall eschar rates, with their 95% confidence intervals.
EA = Eastern Asia; SA = Southern Asia; SEA = South-Eastern Asia; NA = Northern Africa; WA = Western Asia; EE = Eastern Europe; SE = Southern Europe; WE= Western Europe

Mediterranean Spotted Fever

Figure 5 provides a graphic overview of eschar rates found in the studies.

Among the 21 studies evaluating Mediterranean Spotted Fever in residents, eschar presence was evaluated in a total of 5089 patients.[7,26,128-146] The overall percentage of eschar presence was 68.7% (SD 8.9).

Studies were conducted in Italy (n=3),[133-135] Portugal (n=3),[26,136,137] Spain (n=3),[139-141] Turkey (n=3),[144-146] Bulgaria (n=2),[129,130] Tunisia (n=2),[142,143] Algeria (n=1),[128] Croatia (n=1),[131] France (n=1),[7] Greece (n=1),[132] and Romania (n=1).[138] One study was classified as 'complete approach', and was of medium quality.[135] Twenty studies were classified 'clinical judgement' or 'clinical judgement/unclear'. Of those, one was of high quality,[139] 14 were of medium quality,[7,26,128,130-134,136,138,140,142,143,145] and five of low quality.[129,137,141,144,146] Quality did not differ

significantly between the two groups. Both studies that included travellers as subjects were of medium quality.[147,148]

The mean percentages of eschar presence were significantly different between 'clinical judgement' and 'complete approach' studies (69.1 ± 8.0 vs. 29.1 ± 0.0, $p < 0.001$) (Table 1).

We calculated that the diagnosis of MSF was missed in 6,922 patients in the clinical judgement group, which represents 81.6% (6,922/8,478) of the patients with NERD and 57.9% (6,922/11,956) of the total group of patients with MSF, as presented in Figure 3. For both age and percentage of males the means were significantly different ($p < 0.001$ and $p < 0.001$) between the 'clinical judgement' and 'complete approach' studies (Table 1).

When all 'unclear' studies (both 'clinical judgement/unclear' and 'complete approach/unclear') were assigned to the 'clinical judgement' group (best case scenario), the eschar rates remained significantly lower in complete approach studies compared to clinical judgement studies (clinical judgement + clinical judgement/unclear + complete approach/unclear: mean = 69.1 ± 8.0 vs. complete approach: mean = 29.1 ± 0.0, $p < 0.001$). The same applied when all unclear studies were assigned to the complete approach-group ('worst case scenario') (clinical judgement: mean = 71.4 ± 5.9 vs. complete approach + complete approach/unclear + clinical judgement/unclear: mean = 62.1 ± 11.3, $p < 0.001$).

African Tick-Bite Fever

We retrieved two studies that evaluated the eschar presence in travellers with African Tick-Bite fever. One study evaluated the presence of eschars in Norwegian travellers with ATBF who had returned from sub-Equatorial Africa, 52.6% of whom presented with an eschar.[148] In the second study, in which most patients had recently returned from southern Africa, an eschar percentage of 87.2% was found. However, in both studies, diagnostic laboratory tests did not allow for differentiation between ATBF and MSF.[147]

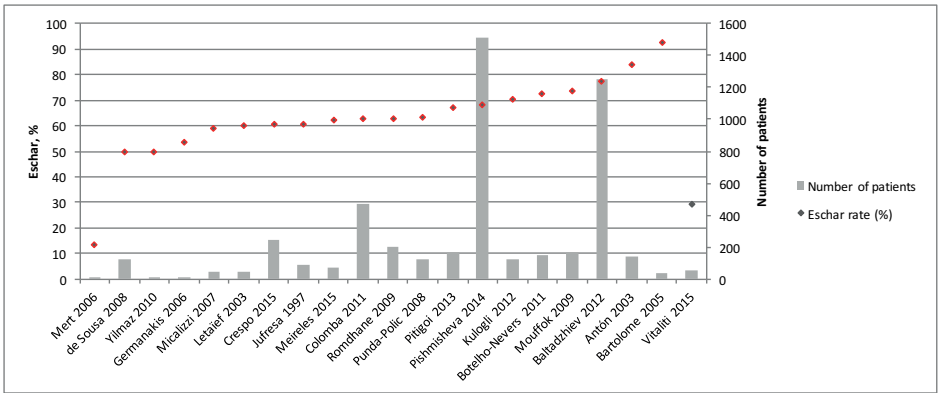

Figure 5. Eschar rates in Mediterranean spotted fever reported per study
Number of patients included per study are depicted in the grey bars; red dots indicate the eschar rate in 'clinical judgement' studies, grey dots indicate eschar rates in 'complete approach' studies.

DISCUSSION

Rickettsial diseases, and especially non-eschar forms, are an underestimated cause of febrile illness in endemic areas. In this study, we hypothesised that the ERD/NERD ratio in 'complete approach' studies was closest to reality. Ratios in 'clinical judgement' and 'complete approach' studies differed significantly. For scrub typhus, we estimated that among the studies included here, the diagnosis was missed in 66.5% of all patients. In MSF, this was up to 57.9%.

An expected underestimation of these rickettsial diseases has been mentioned before,[128,145,149] but no systematic evaluation and informed estimation of the scale of this problem had been performed to date.

For scrub typhus, overall percentages of eschar prevalence in patients (64.3%) are in line with a previously published review.[150] This is, however, likely to constitute an over-estimation, because the largest population included in this review came from 'clinical judgement' studies, which we have shown to be have significantly higher ERD/NERD ratios than 'complete approach' studies. Theories on eschar occurrence relating to geography and associated strains[6,24-26] are neither supported, nor can they be refuted by this review. Although strains of *O. tsutsugamushi* were not taken into account (neither were they frequently investigated) within countries, eschar prevalence rates showed considerable variance, often depending on the study design ('complete approach' vs. 'clinical judgement'). Countries with more 'complete approach' studies, such as India and Thailand, showed lower average eschar rates, as expected. High average eschar rates were found in countries with no, few, or small 'complete approach' studies, such as South Korea, China, and Taiwan. For example, within India (39 studies, of which 20 with a 'complete approach' study design), reported eschar rates ranged from 0%[47,49,50,65] to 76.7%,[79] independent of geography (in this case provinces). In Taiwan, average eschar rates were high (62.5%), with a range of 22.5%[106] up to 91.3%,[109] but few patients came from studies with a 'complete approach' design. Overall, eschar rates reported in Eastern Asia were higher than in South-Eastern and Southern Asia. We suspect this is due to the lower number of studies with an 'complete approach' study design in these regions; among 'complete approach' studies, reported eschar rates were similar across all regions. That notwithstanding, this hypothesis should be confirmed by studies including all patients presenting with febrile illness, comprising *O. tsutsugamushi* strain genotyping as well. It is also possible that pre-existing immunity among inhabitants of endemic areas, would lead to a lower prevalence of eschars,[151,152] but no longitudinal studies on this subject have been conducted to date.

For Mediterranean Spotted Fever, an overall eschar percentage of 68.7% was found, similar to other reports.[10] Only one study with a 'complete approach' study design was identified (with an eschar prevalence of 29.1% among 55 patients),[135] making our findings for this disease less robust.

In MSF, the *R. conorii conorii,* Malish strain causes eschars in similar percentages, whereas for the Israeli Spotted Fever (ISF) strain (*R. conorii* subsp. *israelensis*) much lower eschar rates are reported, around 38%.[10,26] The ISF strain is also believed to be more virulent, with higher fatality rates.[10] It is possible that the patients in the 'complete approach' study included in this review[135] suffered from ISF instead of MSF, explaining the low eschar rates. But given the facts that most clinicians do not have access to sophisticated diagnostic facilities to be able to differentiate between strains, and that the diseases are endemic in the same geographic areas,[10] this is of less importance for the clinical practice. The extent of *R. conorii* and its subspecies as a cause of NERD in endemic areas should be studied further.

For both diseases, significant differences were observed in age and sex between 'complete approach' and 'clinical judgement' studies. Patients included in complete approach studies were younger and there were fewer males. This is most likely due to heterogeneity, but is remarkable either way. It is reported that in areas where the disease is hyper-endemic, the percentage of patients presenting with an eschar is lower due to partial immunity acquired by prior exposure [153]. Thus, since most of the studies included here were performed in highly endemic areas, we expected to find lower eschar rates coming with higher age. Yet, we found the opposite, supporting our hypothesis that study design was the major factor influencing reported eschar rates.

Few studies on African tick bite fever were identified; of which only two were conducted in travellers.[147,148]. Both had a 'complete approach' study design. Therefore, we could conclude that schars are highly prevalent in travellers infected with *R. africae*; however, with a total population of 116 patients, evidence is moderate at best.

It has been postulated that, especially in international travellers, rickettsial disease is underreported in the published literature.[154] This was stressed by our finding that only a minority of the studies that we included reported on this disease in travellers. However, with an ever-growing number of tourists to areas highly endemic for rickettsial diseases,[155] and mosquitos being identified as new potential transmitting vectors,[156] this disease group is of high importance in travellers. Rickettsial disease, and especially NERD should be considered in every returning traveller with acute febrile illness. Travellers may be more likely than residents to present with an eschar due to a lack of acquired immunity, as was supported by the findings of the included studies reporting on travellers.[147,148] However, the proportion of patients with a missed diagnosis of NERD could potentially be even greater in returned travellers compared to residents, due to lack of awareness among physicians in non-endemic areas. The burden of rickettsial diseases as a cause of febrile illness in travellers should be studied more extensively in prospective, 'complete approach' designs.

Strengths of this review are that the recommendations made by PRISMA[27] were followed. A comprehensive literature search was performed by an experienced librarian, and all steps in data extraction, assessment, and analysis were performed by two reviewers. Studies were included irrespective of language, in an attempt to reduce publication bias.

Limitations of the study are that some Korean and Chinese articles were not retrievable, possibly introducing a bias towards studies in English from different countries. Heterogeneity between the included studies was high. Studies with different designs were included. Different microbiological methods were used, varying in reliability and quality, and thus in diagnostic accuracy, which may have influenced our results. However, the net effect is possibly mitigated by the fact these differences were distributed over both 'complete approach' and 'clinical judgement' studies, reducing the influence on the ERD/NERD ratios. In order to account for components of heterogeneity for which we could not control, we performed quality assessments for all studies instead. Also, bacterial strains as a cause of variance in reported eschar rates were not taken into account: firstly, most included studies used serological methods, thus we could not differentiate between strains; but most importantly, as clinicians, we tried to approach the problem from a clinical perspective. In daily clinical practice, genotyping is not readily available and as a result has little consequences for further medical management.

We did not analyse skin rashes in this review, because this is a prevalent finding in both ERD and NERD, and many other causes of febrile illness. Presence or absence of a skin rash does therefore not contribute to the estimation of under-diagnosis of rickettsial disease. On the contrary, ERD/NERD ratios between the two study designs would have been more similar, or even equal, should rickettsial disease have been considered more often in febrile patients presenting with a skin rash, but without eschar.

We analysed studies performed in all sub-regions together. Across different geographic locations, variation in circulating strains and immunity in local populations is to be expected, both influencing eschar occurrence. In addition, differences in skin colour of the included patients could partially explain a lower frequency of observed eschars in some regions, as eschars may be more difficult to diagnose in patients with a darker skin.[68,69] In warm and moist areas, eschars can be missed due to detachment of the crust, resulting in an ulcerous lesion. Differences in overall eschar rates between sub-regions were substantial, but if only studies applying 'complete approach' selection criteria were included, eschar rates were similar. This underpins our hypothesis that study design could be the most important explanation for the variation in reported eschar rates.

The strongest evidence for the under-diagnosis of NERD was found among patients with ST. Current surveillance guidelines for scrub typhus[149] emphasize the presence of eschars for diagnosis of the disease, as this is an important diagnostic clue. The absence of an eschar does not rule out scrub typhus. We argue that the importance of NERD recognition should be stressed in guidelines, also because the absence of an eschar in ST is associated with increased severity and mortality of the disease.[96,157]

For MSF, more studies in patients with febrile illness should be conducted to investigate our hypothesis, but here we also suspect a vast underestimation of the NERD and recommend clinicians in endemic areas to consider the disease with a low threshold.

The underestimation of rickettsial disease is caused by the fact that out of the total spectrum of clinical presentation (ERD/NERD combined), NERD is the presentation that is most often missed. In scrub typhus for example, the total underestimation (NERD/ERD combined) was 66.5%, but the underestimation of NERD alone was 87.0%. This latter higher percentage could also apply for rickettsial diseases in which an eschar is rarely or never seen, such as murine typhus, caused by *Rickettsia typhi* and Rocky Mountain Spotted Fever, caused by *Rickettsia rickettsii*. This was supported by a recent study from Croatia,[154] in which a strong discrepancy was observed between the prevalence of anti-rickettsial antibodies and the substantially lower number of reported cases of clinical disease. This discrepancy was most evident for murine typhus (in comparison to Mediterranean spotted fever).

We recommend that clinicians working in endemic areas, or treating travellers returning from endemic areas, should consider rickettsial disease, and especially NERD, with a low threshold of suspicion. The most-commonly available serologic diagnostic methods, have a poor sensitivity in the early phase of the disease, and to establish the diagnosis, a consecutive sample is often required. Pending diagnosis, and to prevent treatment delay, empirical antibiotic therapy should therefore be considered, when rickettsial disease is suspected clinically.

In conclusion, this review indicates that rickettsial disease, a potentially fatal but treatable disease, should be considered in all patients presenting with acute febrile illness in, and returning from, endemic areas, irrespective of eschar presence (NERD). For future research, more 'complete approach' studies investigating causes of febrile illness should be performed in all geographic areas, because local clinicians need to have a good knowledge of the local epidemiology. The under-diagnosis of rickettsial disease in returned travellers with fever should also be studied. And last but not least, current surveillance standards should be revised, in order to increase awareness of NERD among clinicians. A clinical suspicion of the possibility of NERD and the initiation of appropriate laboratory diagnostic tests and early (empirical) antibiotic treatment should be facilitated in a broader population, to prevent unnecessary morbidity and mortality.

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SUPPLEMENTARY MATERIALS

The supplementary materials provide details on the search strategy, quality assessment tool, calculation logarithms, excluded studies, and extensive tables with study characteristics and quality assessment forms. This data can be found, in the online version, at https://www.sciencedirect.com/science/article/pii/S1477893918300176?via%3Dihub#appsec1

Appendix 1 Search strategy
Appendix 2 Quality Assessment Tool
Appendix 3 Underestimation Calculation Logarithms
Appendix 4 Excluded studies due to overlapping datasets
Appendix 5 Characteristics of included studies
Appendix 6 Data Extraction Forms
Appendix 7 Quality Assessment Forms

Chapter 7

Searching and finding the hidden treasure: Rickettsial disease among Dutch international travelers – a retrospective analysis

Sophia G de Vries, Louise E van Eekeren, Hans van der Linden, Benjamin J. VIsser, Martin P Grobusch, Jiri F P Wagenaar, Marga G.A. Goris , Abraham Goorhuis

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ABSTRACT

Background

Rickettsial disease (RD) is a prevalent and underestimated cause of febrile illness worldwide, especially in the absence of an inoculation eschar. We attempted to quantify this under estimation at our clinic, by investigating past cases of febrile illness in travelers who had tested negative for leptospirosis, a disease that can initially present similar to non-eschar RD, and which we routinely consider when other important causes of unspecified febrile illness have tested negative.

Methods

We performed a retrospective analysis in febrile returned travelers from Asia, Africa or the Americas between 2010-2017, who had tested negative for leptospirosis. Serologic immunofluorescence assays were performed for *Orientia tsutsugamushi* (scrub typhus), Typhus Group (TG) and Spotted Fever Group (SFG) RD. We performed a chart review of all patients who tested positive. In case of a fitting medical history, cases were deemed either confirmed (based on convalescent serology) or suspected (based on single serology).

Results

Among 97 patients, convalescent serology was available in 16 (16.5%) patients, and a single serology in 81 (83.5%) patients. RD was the likely diagnosis in 8/16 (50.0%) patients with convalescent serology, and in 8/81 (9.9%) with single serology. Of the 16 confirmed/suspected cases, 11 (69%) had been missed and 7 (44%) had not received adequate empiric antibiotic therapy.

Conclusions

This study shows that non-eschar RD is an important and poorly recognized cause of illness in travelers, even in a specialized travel clinic. A lower threshold to test and treat for RD is warranted in returning travelers with febrile illness.

INTRODUCTION

Rickettsial diseases (RD) are zoonotic infections, transmitted to humans by predominantly arthropod vectors,[1] although leeches and mosquitoes have also been described as vectors.[2,3] The disease may be mild to life-threatening,[4] especially when treatment is delayed.[5,6] Substantial morbidity is reported worldwide in autochthonous populations, as well as in travelers.[7-15] RD generally presents as an indifferent acute febrile illness, with non-specific accompanying symptoms such as nausea, vomiting, lymphadenopathy, headache, skin rash, and sometimes an inoculation eschar. The prevalence of the latter varies widely per specific RD:[16] from 0% in patients with murine typhus (cause by *R. typhi*), to 30-90% in patients with African tick bite fever (caused by *R. africa*e).[11] Clinically, the symptomatology of RD is often similar to other acute febrile illnesses such as malaria, dengue fever, and leptospirosis,[17] especially if an eschar is absent at presentation.

The disease is caused by intracellular bacteria of the *Rickettsiaceae* family, ordered into two genera: *Orientia* (consisting of *Orientia tsutsugamushi*, causing scrub typhus) and *Rickettsia.*[18] The *Rickettsia* genus is divided in four biogroups: (1) the spotted fever group (SFG), which, among others, includes *R. conorii* (causing Mediterranean spotted fever), *R. africae* (causing African Tick Bite Fever) and, *R. rickettsii* (causing Rocky Mountain spotted fever); (2) the typhus group (TG), which comprises *R. typhi* and *R. prowazekii*, causing endemic and epidemic typhus, respectively; (3) a translational group, including *R. felis, R. australis* and *R. akari;* and (4), a non-pathogenic group.[18,19] Rickettsial organisms have been identified on all continents, except Antarctica.[20] *R. typhi* and *R. felis* and are distributed globally; SFG RD has been reported on all continents; Scrub typhus (cause by *O. tsutsugamushi*) is traditionally prevalent in the tropical Pacific triangle, but there are recent reports from South America and sub-Saharan Africa.[20]

Currently, the cornerstone of diagnosis is still the indirect detection of *Rickettsia*-specific antibodies in patient sera by serologic methods, such as immunofluorescence or western blotting. Because antibodies are detected at a later stage after infection, typically 15 days or more,[21-23] these methods have limited clinical impact in the acute stage of disease, when most initial diagnostic testing is done [5]. Additionally, there is cross-reactivity between species.[24] For a specific diagnosis in the acute phase of illness, molecular detection methods are preferred,[25-28] but these are not widely available. Also, reported diagnostic accuracy of the different tests varies considerably, also based on the specimen type (e.g., whole blood, serum), and reference tests are suboptimal, with differences in applied techniques, and targets.[29]

Because of the unspecific clinical presentation of RD, and difficulties in laboratory diagnostics in the early phase of disease, patients may be un- or misdiagnosed. In a previous study based on reported literature, we estimated that the diagnosis of RD was missed in 66.5% of patients with scrub typhus (ST), and in 57.9% of patients with Mediterranean

spotted fever (MSF) in autochthonous populations.[16] However, these percentages applied to patients who presented with or without an inoculation eschar. Among patients in whom an inoculation eschar was absent, RD was missed in 87.0% of patients with ST and 81.6% of patients with MSF.

In travelers, this proportion could even be higher due a low index of suspicion by physicians in areas that are not endemic for the disease. This underestimation is of growing concern, given the expansion of international travel to endemic regions such as Asia and Africa, resulting in increased numbers of imported infections such as RD.[30]

We hypothesize that in the absence of an inoculation eschar, the diagnosis of RD is missed in a substantial proportion of returned travelers presenting with acute febrile illness. Our hospital houses the Dutch Leptospirosis Reference Center (NRL), which means that testing for leptospirosis can be easily performed upon clinical suspicion. The disease is usually considered when diagnostic routine testing for other important causes of unspecified febrile illness turns out negative (i.e. malaria, typhoid fever, dengue, chikungunya and zika virus infection), even in the absence of evident exposure to fresh water, as this is often difficult to ascertain in retrospect. Therefore, and because leptospirosis and RD can have clinical similarities at initial presentation, we hypothesized that missed diagnoses of RD would likely be found among patients who had presented with unspecified febrile illness and who had tested negative for leptospirosis. Finding these missed diagnoses would provide us a rough indication of the under diagnosis of non-eschar RD at our travel clinic. In this study, we retrospectively assessed sera of a group of leptospirosis-negative returned travelers for the presence of antibodies to SFG and TG rickettsioses, and *O. tsutsugamushi*.

METHODS

This retrospective cohort study was performed as a collaboration of the NRL and the Center for Tropical and Travel Medicine, both part of the Amsterdam UMC.

We selected samples from adult (≥18 years) travelers, in whom leptospirosis had been clinically suspected, but had tested negative. All had presented at the Center of Tropical Medicine and Travel Medicine of the Amsterdam UMC between January 2010 and July 2017, and had recently returned from Africa, the Americas, or Asia, and had an available stored serum sample.

Laboratory diagnostics

Diagnostic tests were performed in December 2015 and June 2017 at the NRL. Serum samples had been stored at -20°C. If available, convalescent samples were tested. All samples were tested with several immunofluorescence assays (IFA). Two different kits were used:

1) The "*Rickettsia* Screen IFA Antibody Kit", IgG and IgM (*Fuller Laboratories, Fullerton, California, USA*), using *R. conorii and R. typhi* substrate antigens. A positive result was defined as a titer ≥1:128 (IgG) and ≥1:64 (IgM), a ≥4-fold titer-rise between acute and convalescent samples, or seroconversion.
2) The "*Orientia tsutsugamushi* IFA Antibody Kit", IgG and IgM, (*Fuller Laboratories, Fullerton, California, USA)*, using the Boryong, Gilliam, Karp and Kato antigen strains of *O. tsutsugamushi.* A positive result was defined as a titer ≥1:128 (IgG) and ≥1:64 (IgM), a ≥4-fold titer-rise between acute and convalescent samples, or seroconversion.

Cut-off titers were determined based on the low prevalence of RD in the research population, as the occurrence of autochthonous infections in the Netherlands is rare.[31] The IFAs were performed by two trained individuals (SdV and HvdL). In case of positivity or doubt, both interpreted all sample results independently. For a subset of samples, further dilutions were prepared once the sample was positive.

Chart review

The medical records of all patients who tested positive for RD were reviewed. Epidemiological and clinical data were extracted, including travel history, reason for travel, tick exposure during travel, whether or not the differential diagnosis had included RD, whether or not the patient had initially been tested for RD, the final clinical diagnosis, whether or not the patient had received treatment with anti-rickettsial drugs, and the follow-up. Countries of exposure were grouped. Tetracyclines, macrolides and fluoroquinolones were considered as effective treatments for RD. Finally, all clinical data of patients with positive laboratory tests were reviewed by two clinicians (SDV and AG), to assess whether RD was indeed the most likely diagnosis.

Case definitions

A "*laboratory-confirmed case"* was defined as a ≥4-fold titer-increase, or seroconversion in convalescent samples.

A "*laboratory-suspected case*" was defined as an IFA positive single serum sample, with the earlier mentioned cut-off titers.

A "*definitive-confirmed case*" was defined as a "laboratory confirmed case" in combination with a compatible clinical course and no other likely or confirmed diagnosis.

A "*definitive-suspected case*" was defined as a "laboratory suspected case" in combination with a compatible clinical course and no other likely or confirmed diagnosis.

Laboratory- and definitive-confirmed and suspected cases were categorized in four groups: Spotted Fever Group (SFG) rickettsiosis, Typhus Group (TG) RD, indeterminate RD (either SFG or TG, but IFA could not differentiate between the two), and scrub typhus.

Analysis

Data were anonymized, organized and analyzed using Microsoft Excel (*Microsoft Corporation, 2010*). Data were de-identified and not attributable to individual patients. For numerical variables with a normal distribution, including age and laboratory values, mean and standard deviation was calculated. For numerical variables with a non-normal distribution, including variables about the disease course, median and interquartile range was calculated.

RESULTS

Figure 1 provides the study flow and main results. In short, 97 patients met the inclusion criteria, of whom 16 (16.5%) had a convalescent sample available and 81 (83.5%) only a single sample. In total, 32 (33%) patients tested IFA-positive: 10/16 (62.5%) of patients with a convalescent sample (laboratory-confirmed cases), and 22/81 (27.2%) of patients with a single sample (laboratory-suspected cases).

Chart consolidation

Of the 32 patients who were IFA-positive (10 laboratory-confirmed and 22 laboratory-suspected), medical data were extracted. After chart review, 2/10 laboratory-confirmed cases were excluded, resulting in 8/16 (50%) definitive-confirmed cases among patients with a convalescent sample, which is 8/97 (8.2%) definitive-confirmed cases in the whole cohort. The two excluded cases comprised immunocompetent patients; one with a PCR-proven shigellosis, and one with PCR-proven Epstein Barr virus infection.

Of the 22 laboratory-suspected cases, 14 were excluded, resulting in 8/81 (9.9%) definitive-suspected cases among patients with a single sample, which is 8/97 (8.2%) definitive-suspected cases in the whole cohort. The 14 excluded patients comprised four with a dengue infection (two PCR-confirmed, two with positive IgM and dubious IgG); one with acute hepatitis A infection (anti-HAV IgM-positive); one with-PCR proven influenza B infection; one with a blood smear-positive *Plasmodium falciparum* malaria; one with a streptococcal infection complicated by glomerulonephritis; one with a recent (IgM positive) EBV infection; one with a lobar pneumonia; one with a bacterial cellulitis of the leg; one with an auto-immune mediated encephalitis, one with a cerebral and retinal vasculitis (although the latter could have been due to RD), and one with relapsing fevers.

In total, we thus identified 16/97 (16.5%) patients with either definitive-confirmed RD (8 patients) or definitive-suspected RD (8 patients).

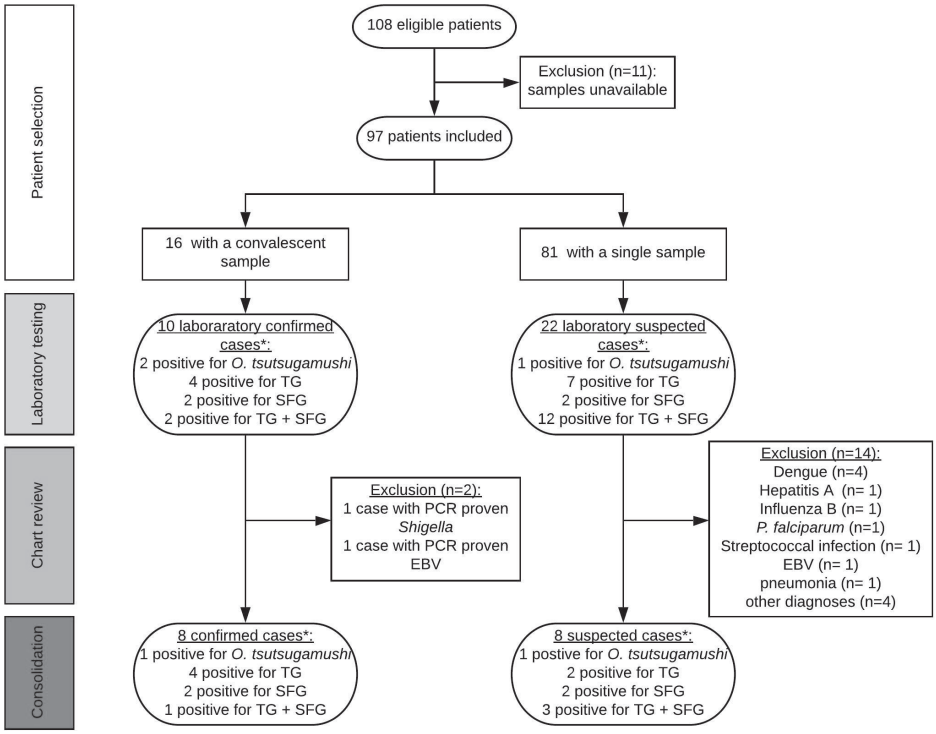

Figure 1 Flow diagram and main results

Demographics and Laboratory findings

Demographic characteristics are depicted in **Table 1**. Of the 16 definitive-confirmed/suspected cases, two were IFA-positive for *O. tsutsugamushi*, six for TG RD, four for SFG RD and in four cases, reactivity was indeterminate TG/SFG (**Figure 1**). Details of the laboratory findings can be found in **Table 2.**

Clinical findings

Table 3 summarizes general clinical characteristics and laboratory findings of the 16 definitive-confirmed/suspected patients. **Table 2** provides a detailed overview of clinical and diagnostic information of all definitive-confirmed/suspected cases. A total of five patients (31.3%) had initially been diagnosed with RD by the treating clinician, four of them based on diagnostics performed at the reference laboratory. Of the 16 definitive-confirmed/suspected patients, nine (56.3%) had received adequate antibiotic treatment. The course of illness of the eight who had not received treatment was not well documented.

Table 1. Demographics

Demographic data	All (n=97)	Definitive confirmed & suspected cases (n=16)
Male, n (%)	52 (53.6)	11 (68.8)
Mean age ± SD (range)	37.5 ± 14.5 (8.5 - 70.6)	44.8 ± 14.0 (24.0 – 68.2)
Region of travel, n (%)		
Southeast Asia	58 (59.8) (Asia all regions)	9 (56.3)
Sub-Saharan Africa	23 (23.7) (Africa all regions)	3 (18.8)
Latin America & Caribbean	16 (16.5) (Americas)	3 (18.8)
Northern Africa		1 (6.3)
Rickettsial disease included in differential diagnosis, n (%)	NA	9 (56.0%)
Initially diagnosed with rickettsiosis	NA	4 (25.0%)
Day post onset of disease at collection positive rickettsiosis sample, mean ± SD (range)	NA	17.3 ± 7.6 (1 - 36)
Hospital admission, n (%)	NA	5 (33.3%)
Deaths, n (%)	NA	0 (0)

DISCUSSION

In this study, we provided a rough estimate of the extent of missed diagnoses of RD among ill returning travelers, by investigating a patient cohort who had tested negative for leptospirosis, a disease that can initially present similar to RD, and which we routinely consider when other important causes of unspecified febrile illness have tested negative. Among 97 patients, we identified 16 (16.5%) patients with definitive confirmed or suspected RD, based on both laboratory and clinical criteria. Of these 16 patients, five (31.3%) had actually been correctly diagnosed by the treating physician, whereas 11 (68.7%) had been missed. Only 9 (56.3%) patients had received adequate empirical antibiotic treatment.

Interestingly, the highest proportion (9/16; 50%) of RD was found in the group of patients who twice tested negative for leptospirosis in convalescent samples, as opposed to 10% (8/81) in the group who was only tested once. Obviously, this was driven by the desire of the clinician to establish a diagnosis in a patient in whom pathology was highly suspected. To turn this around: if a patient had tested negative for leptospirosis in single sample testing, there was a 10% chance that RD was the missed underlying cause, which increased to 50% in case of a negative convalescent test, ordered by the treating physician for clinical reasons.

There are no other clinical studies that have tried to estimate the under-diagnosis of RD in travelers, only the recently published finding from our group among autochthonous populations, that in the absence of an inoculation eschar, 82-87% of RD were missed.[16] In our setting of a specialized academic travel clinic, where clinicians are familiar with RD, we also missed almost 70% of non-eschar RD. Therefore, one can assume that the under-diagnosis in general clinics is much higher.

Table 2. Clinical and laboratory details of definitive confirmed and suspected cases

Nr	Sex, age	Destination	Main symptoms	Initial diagnosis	Antibiotics administered?	Convalescent sample?	Duration illness (days)	IFA positive for	Laboratory findings
		Definitive confirmed cases							
81	Male, 33	Malaysia + Borneo	Fever, headache, arthralgia, myalgia, rash	Arbovirus or nematode infection	Yes (doxycyclin)	Yes	6 + 27	*O. tsutsugamushi*	Day 6: IgM-, IgG- Day 27: IgM 1:512, IgG 1:128
79	Male, 35	Thailand	Fever, chills, headache, arthralgia, myalgia, rash, nausea, vomiting, diarrhoea, abdominal pain, elevated CRP	Leptospirosis	Yes (ceftriaxone)	Yes	4 + 18	TG	Day 4: IgM-, IgG- Day 18: IgM +, IgG-
27	Female, 61	Indonesia	Fever, chills, arthralgia, myalgia, cough, dyspnoea, nausea, diarrhoea, rectal blood loss, anorexia, elevated CRP	TG Rickettsial disease	Yes (amoxicillin, ceftriaxone and doxycyclin)	Yes	10 + 20	TG	Day 10: IgM 1:64, IgG - Day 20: IgM 1:64, IgG 1:128
32	Female, 58	Congo	Fever, chills, headache, myalgia, cough, throat pain, conjunctival suffusion, improvement after treatment with doxycycline for 2 days	Rickettsial disease or flu-like illness	Yes (doxycyclin)	Yes	9 + 20	TG	Day 9: IgM -, IgG- Day 20: IgM 1:64, IgG 1:128
44	Female, 29	Uganda	Presentation after hospital admission Uganda for malaria. Headache, arthralgia, myalgia, abdominal pain, cough, dyspnoea, icterus, splenomegaly HB 4.7, elevated liver enzymes + bilirubin	Haemolytic anaemia after malaria	Yes (ciprofloxacin)	Yes	18 + 85	TG	Day 18: IgM 1:64, IgG- Day 85: IgM 1:64, IgG 1:128
92	Male, 62	South Africa	Fever, chills headache, arthralgia, myalgia	Rickettsial disease	Yes (doxycyclin)	Yes	6 + 72	SFG	Day 6: SFG and TG IgM 1:64 Day 72: SFG IgG 1:128

Table 2. Clinical and laboratory details of definitive confirmed and suspected cases (continued)

Nr	Sex, age	Destination	Main symptoms	Initial diagnosis	Antibiotics administered?	Convalescent sample?	Duration illness (days)	IFA positive for	Laboratory findings
63	Male, 68	Morocco	Fever, chills, nausea, petechias	SFG Rickettsial disease	No	Yes	19 + 39	SFG	Day 19: IgM 1:64, IgG- Day 39: IgM 1:64, IgG 1:128
23	Male, 29	Indonesia	Fever, myalgia, headache, itchy rash,	Viral infection	Yes (doxycyclin)	Yes	4 + 18	Mixed TG/SFG	Day 4: IgM- Day 18: IgM 1:512 TG/SFG
		Definitive suspected cases							
25	Female, 47	Thailand	Fever, nausea, vomiting, diarrhoea	Leptospirosis	Yes (ceftriaxone, gentamicin)	No	1	SFG	IgM 1:512
66	Male, 40	Suriname	Fever, chills, arthralgia, myalgia, rash, red eyes, lymphadenopathy, elevated CRP	Self-limiting arboviral infection	No	No	5	SFG	IgM 1:64
51	Female, 60	Thailand	Fever, cough	Viral infection (not specified)	No	No	16	Mixed TG/SFG	IgM 1:64 TG/SFG
69	Male, 24	French Guyana	Headache, myalgia, chills, anorexia, rash	Dermatomycosis	No	No	36	Mixed TG/SFG	IgM 1:64 IgG 1:128 TG/ SFG
88	Male, 30	Puerto Rico	Fever, headache, arthralgia, dyspnoea, nausea, rash	Viral infection (not specified)	No	No	14	Mixed TG/SFG	IgM 1:64 TG/SFG
11	Male, 42	Thailand	Headache, myalgia, rash, lymphadenopathy, transaminase elevation	CMV	No	No	22	*O. tsutsugamushi*	IgM 1:256
4	Male, 48	Indonesia	Headache, myalgia, sore throat	TG Rickettsial disease	Yes (doxycyclin)	No	24	TG	IgM 1:256
26	Male, 51	Thailand + Cambodia	Fever, chills, headache, arthralgia, abdominal pain, elevated CRP	Viral infection (not specified)	No	No	2 (but 19 days after return)	TG	IgM 1:256

Table 3. Symptoms and clinical laboratory findings of definitive confirmed and suspected cases*

Symptoms and signs, n (%)	All (n = 16)±
Fever	13 (81.3)
Headache	12 (75.0)
Myalgia	10 (62.5)
Arthralgia	9 (56.3)
Chills	9 (56.3)
Gastrointestinal symptoms (≥ 1)	8 (50.0)
Respiratory symptoms (≥ 1)	8 (50.0)
Skin rash	5 (31.3)
Lymphadenopathy	2 (12.5)
Symptoms of bleeding (≥ 1)	1 (6.3)
Urogenital symptoms (≥ 1)	1 (6.3)
Eschar	0 (0.0)
Laboratory abnormalities, n (%)	
Elevated CRP (> 5 mg/L)	5/12 (41.7)
Elevated ALAT (SGPT) (> 45 U/L)	5/16 (31.3)
Elevated ASAT (SGOT) (> 40 U/L)	4/13 (30.8)
Leukocytosis (> $10.5*10^9$/L)	4/16 (25.0)
Elevated bilirubin (> 17 µmol/L)	2/11 (18.2)
Low platelets (< $150*10^9$/L)	2/14 (14.3)
Elevated creatinine (> 110 µmol/L)	2/16 (12.5)
Low hemoglobin (M < 8,5/ F < 7,5 mmol/L)	1/16 (6.3)
Leukocytopenia (< $4.5*10^9$/L)	1/16 (6.3)
Hypokalemia (< 3.5 mmol/L)	0/7 (0.0)

** all symptoms and laboratory findings were recorded at the day of presentation to the clinic*
± denominator varies as not all clinical symptoms were available for all patients
Gastrointestinal symptoms include: nausea, vomiting, diarrhea and abdominal pain
Respiratory symptoms include: cough, sore throat, hemoptysis, dyspnea
Symptoms of bleeding include: hematemesis, melena, rectal bleeding
Urogenital symptoms include: dysuria, hematuria, oliguria

The currently existing body of evidence on RD in travelers mainly comprises a multitude of case reports and case series, of which an overview can be found in a review by Delord and colleagues.[14] Additionally, a few cohort studies have been published.[9,10,32-38] However, in these studies, patients were retrospectively identified based on the diagnosis made by the treating physician, which makes underestimation very likely, precluding the possibility to estimate under-diagnosis.[9,10,32,33] Five studies used prospective methods,[34-38] but investigated diagnosed infections, or only RD presenting with an inoculation eschar, precluding the possibility to assess under-diagnosis of non eschar RD.

The results presented here should be interpreted with caution, as there are several limitations.

Firstly, all patients had presented to a specialized travel clinic in an academic medical center, with a lower than average threshold of suspicion for RD.

Secondly, the group of patients in our study is not representative for the overall group of travelers with fever. Because we were interested in under diagnosis of RD, and studied a specific subset of patients who had tested negative for leptospirosis, we "missed" the typical presentations of RD who had presented with an eschar. These patients are readily diagnosed at our clinic based on the clinical presentation, precluding the need for further diagnostic testing for leptospirosis or other diseases. The fact that the diagnostic process for leptospirosis had been initiated, typically implies that more common causes of fever had already been excluded (e.g., malaria, dengue, chikungunya, Zika virus infection, common bacterial infections). Thus, we studied a selected group of patients with a higher a-priori likelihood of less common illnesses, such as non-eschar-RD. For this study however, this was intentional, because we expected to find missed cases of non-eschar RD in this population. Obviously, an important criterion to test for leptospirosis is exposure to fresh water, which means that we missed additional cases of non-eschar RD among patients who were never tested for leptospirosis because they were not exposed to fresh water. It is possible that this population was tested for RD more frequently.

Thirdly, important limitations apply to the laboratory methods. The diagnostic process for RD is changing rapidly.[29] Whereas many reference laboratories are still working with IFA or the micro-immunofluorescence assay (MIF) as reference standards,[20] molecular detection methods are gaining popularity,[29] as they can diagnose the illness in its early stage. Because of restrictions in the type and quality of samples available for this study, we only used serology-based methods. It is known that there are many limitations to IFA in general: (i) poor sensitivity in the acute phase of illness (and thus limited diagnostic value of single samples); (ii) high variation and lack of consensus in cut-off limits; (iii) inter-reader heterogeneity; (iv) cross-reactivity of IgM with other species and antibody persistence beyond the acute phase of illness.[20,23,29,39] All these limitations apply to this study. For the majority of patients, only a single sample was available. Therefore, dynamics in antibody titers could not be assessed; resulting in unconfirmed or even missed diagnoses of RD. Also, due to material constraints, not all samples underwent further diluting; presented dilutions could have been higher for some samples. Almost certainly, some positive IgM titers were based on cross-reactivity, or on previous infections. Although the latter is less likely in the Dutch population, co-infections with tick-borne *Rickettsiae* have been described in the Netherlands.[40] Remarkably, we observed cross-reactivity between SFG and TG groups in a considerable number of samples. It is possible that this has been caused by *R. felis* infections, a rickettsial illness that has been on the rise globally in the past years.[41]

Finally, the retrospective nature of this study introduced limitations by itself.

For example, the clinical information was extracted from patient files, and was often incomplete. Also, though not expected,[42] long-term freezing could have affected the quality of the serum samples.

The most important message from this study is that even in a specialized travel clinic, where clinicians are familiar with the diagnosis of RD, this diagnosis is still missed in a substantial proportion of patients, especially when an inoculation eschar is absent. In retrospect, in our study, 68.7% of the confirmed/suspected RD cases had been missed and 43.7% did not receive adequate (empiric) antibiotic therapy. Although no deaths occurred in this small group of patients, the hospitalization rate was high (33.3%), which emphasizes the importance of timely recognition and treatment of this disease. In a non-specialized clinical setting, the proportion of missed diagnoses of RD will probably be higher, as we also estimated earlier [16].

There is a dire need for properly conducted prospective studies among febrile travelers, in order to reach a credible estimation of the burden of this disease as an imported cause of febrile illness. A lower threshold to test for RD by clinicians is justified, and RD should be included in the testing algorithm of febrile illnesses.

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PART 3

Epilogue

Chapter 8

Summary and general discussion

Over the past years, the extent and burden of leptospirosis and rickettsial disease has increasingly been elucidated,[1-4] in both autochthonous as well as travelling populations. This has been due to an increased research interest, improved diagnostic techniques, and also new epidemiological methods to calculate disease burden. Also, climate change, with its accompanying increasing temperatures and wetter weather, contributes to an increase in caseload. That notwithstanding, the exact epidemiology and extent of both illnesses seems to be vastly underestimated worldwide.

SUMMARY

In **Part 1**, we focused on leptospirosis. **Chapter 2** focused on summarising all studies on leptospirosis known for the sub-Saharan African region, as the epidemiological situation on the continent was unclear. We found that most data was known on the occurrence of the illness in animals on the continent; probably due to the larger economic impact of the illness when livestock gets infected. Many different animals can carry *Leptospira* in their renal tubules, and shed them with urine. They can be asymptomatic ('maintenance hosts'), or develop symptoms. In cattle and pigs, infection can cause considerable reproductive problems, such as abortions and reproductive failure.[5] In animals, the disease seemed to be widespread on the continent; suggesting plenty of potential infection risk for humans. Data in humans is scarce, however, especially for the Central African region. Seroprevalence studies in healthy subjects showed exposure to the pathogen in many populations throughout the continent; in some regions, it was also found to be a frequent cause of febrile illness. Having said that, there were only a few well-performed studies in febrile patients, and the amount of studies that was done was very small for such a large continent. Therefore, the data presented in this chapter probably only represent the tip of the iceberg (or the ears of the hippopotamus, in more tropical terms), and the true burden of the illness on the continent is likely much higher. As the leptospirosis adage goes:

> "*Wherever leptospires and leptospirosis is searched for, they are invariably discovered.*"[6]

In **Chapter 3** and **4**, we studied cases of 'imported', or 'travel-related' leptospirosis. In our study of leptospirosis in the Netherlands (**Chapter 3**) from 2009 to 2016, we observed an increase of cases from 2014 onwards, both autochthonous and imported. The increase in autochthonous cases was thought to be mainly due to warm weather,[7] increasing the chances of survival of leptospires. The reason for the increase of imported cases, however, was multifactorial. An increased number of samples had been submitted to the National Collaborating Centre for Reference and Research on Leptospirosis (NRL); this could have been an indication of travellers engaging in more high-risk activities (e.g. jungle trekking, rafting),

or that clinicians considered the diagnosis more often. The positivity rate also increased from 2014 onwards; this could have been influenced by the introduction of polymerase chain reaction (PCR) as a standard test from September 2012 onwards at the NRL. PCR can detect leptospirosis in the early stages of the illness, unlike the widely-used serology-based methods, that require convalescent samples, or samples taken in a later stage of the illness. Of the 224 imported cases, the majority had travelled to Southeast Asia, the traditional 'leptospirosis-hotspot'; only 7 (3.1%) had travelled to sub-Saharan Africa. Most patients were male and relatively young; all characteristics that are also described in other studies on the subject. We studied extensive clinical characteristics of 41 patients with imported leptospirosis that had presented to the AMC. More than half of them (53.7%) had to be hospital-admitted, indicating the severity of the illness; and it took on average four days from symptom onset until they received antibiotics. We highlighted three cases that demonstrated the broad spectrum of clinical presentations, and the importance of a swift diagnosis.

In **Chapter 4**, we analysed confirmed leptospirosis cases that were reported to the GeoSentinel Surveillance Network, a network monitoring travel-related morbidity through travel and tropical medicine clinics. We analysed 180 confirmed cases of travel-related leptospirosis that were reported between 1999 and 2016. They showed similar characteristics as the Dutch cases; the majority was young, male, and had travelled to Southeast Asia. We also conducted a survey about leptospirosis diagnostic and therapeutic practices among GeoSentinel clinicians, most of which working in academic institutions. Remarkably, clinicians did often not consider the illness in the absence of the classic exposure history or clinical symptoms, even though it is known that these might often be absent. Also, the applied diagnostic methods were sub-optimal. These findings imply that even in highly specialised travel clinics, there could be a considerable under-estimation of cases.

The diagnostic methods for leptospirosis are complicated. For long, diagnosis depended on the Microscopic Agglutination Test (MAT) and culture, two laborious and highly specialized techniques, not suited to establish a diagnosis swiftly. Over the past decades, molecular detection tests, such as PCR, have gained terrain in the diagnosis of many infectious diseases. PCR can demonstrate the illness before the antibodies are formed, thus in the early stages. In **Chapter 5**, we performed a systematic review and meta-analysis on the diagnostic accuracy of nucleic acid and antigen detection tests for the diagnosis of acute leptospirosis. We identified 41 studies, and were able to do a meta-analysis for PCR and real-time PCR. There was, however, a very high heterogeneity between the studies; reports were often of poor methodological quality and study settings and disease prevalence varied widely. The interpretation of the review and analyses are therefore not straightforward. Also, we used the MAT as a reference standard, which has an imperfect sensitivity, and therefore likely underestimates the specificity of the index tests. We tried to address this problem by using "composite reference standards", MAT combined with IgM ELISA and/or culture. MAT alone as a reference standard was considered at high risk of bias. For PCR, we included 15 studies.

The pooled sensitivity was 70% (95% CI 37% to 90%) and the pooled specificity was 95% (95% CI 75% to 99%). This improved to a pooled sensitivity of 87% (95% CI 44% to 98%), and a pooled specificity of 97% (95% CI 60% to 100%) when studies using only MAT as a reference test were removed. For real-time PCR, an SROC curve was calculated, as the positivity threshold varied between studies. The median specificity was 92%. When 95% specificity was selected on the curve, the pooled sensitivity was 29% (95% CI 15% to 49%). Because of the high variability between studies, it is hard to draw firm conclusions about the accuracy of these tests, and their position in the testing pathway of leptospirosis.

In **Part 2**, we focused on the underestimation of rickettsial disease. In **Chapter 6** we made an estimation of the under-estimation of rickettsial disease in native populations. We performed a systematic review, including studies reporting on Mediterranean Spotted Fever, scrub typhus and African Tick Bite Fever, as these are rickettsial disease that an present with an eschar, but certainly also without. The reported rates of presentation with an eschar varies between studies.[8] We hypothesised that this variation in reported eschar rates is caused by the design of the studies. Many studies seem to include patients based on 'clinical suspicion', which will automatically lead to higher reported rates of eschar prevalence, as clinicians often associate these illnesses with eschars. We expected to find higher rates of non-eschar rickettsial disease (NERD) in studies including all patients with febrile illness. In this way, we could make an estimation of the under-reporting of NERD. We included 121 studies, of which the majority (98) reported on scrub typhus, 21 reported on Mediterranean Spotted Fever and two on African Tick Bite Fever. We were able to confirm our hypothesis: in scrub typhus, NERD was missed in 66% of patients. In Mediterranean Spotted Fever, this applied to 58% of patients. For African Tick Bite Fever, an estimate could not be provided, due to the limited number of studies found. All studies were performed in autochthonous populations, with consequently local clinicians that are aware of the local epidemiology. We therefore think this is an important finding; and earlier suspicion of the disease, and with that earlier initiation of treatment, could reduce morbidity and even mortality.

In **Chapter 7**, we aimed to test the hypothesis outlined above in returned travellers with febrile illness. We retrospectively performed serological tests for Spotted Fever Group rickettsioses, typhus group rickettsioses and scrub typhus in returned travellers who had presented to the AMC with an unspecified febrile illness, and were suspected of leptospirosis, but had tested negative. We hypothesised that rickettsial diseases were missed frequently in travellers returning with fever, but without an eschar. We included 97 patients who had returned from Asia, Africa or the Americas, of which16 were confirmed and suspected cases, 11 (69%) had been missed, and 7 (44%) had not received empiric antibiotics. This study certainly had limitations, however, the important message is, that even in a highly specialised academic travel clinic the diagnosis of non-eschar rickettsial disease is missed frequently, and it should be included in standard testing algorithms.

Future perspectives

Leptospirosis and rickettsioses are under-estimated causes of febrile illness with substantial morbidity and mortality worldwide. This thesis gives an overview of the current epidemiological concerns of both diseases.

For leptospirosis, we conclude that it is an entity prevalent in sub-Saharan Africa (**Chapter 2**), although the true scale of the problem is largely unknown due to a lack of regional epidemiological studies. For long, all febrile illness was attributed to malaria, but in the past decade, the over-diagnosis problem has emerged.[9,10] It is of vital importance that more surveys on the causes of febrile illness will be performed, as has increasingly been done the past years.[11-14] Only a better understanding of causes of illness can improve local clinical practice and subsequent patients outcomes, and enhance local governments to take adequate preventive measures. For rickettsial diseases, despite being a more widely recognised disease entity worldwide, the problem of lack of awareness at a local level is also a problem, as we demonstrated in **Chapter 6**. As febrile illnesses remain a highly prevalent challenge for clinicians worldwide, it is important that in the research on the causes of it, an open view is always retained. Traditional ideas of presentations of diseases may turn out wrong; with "non-eschar rickettsial disease" being a telling example. The downside is, that cause-of-fever research is expensive and resource-consuming, and funding not always available in a world directed by pharmaceutical giants. Smart algorithms for prospective studies to bypass this problem should be designed.

Not only clinicians in tropical parts of the world struggle with the cause-of-fever conundrum, as we demonstrated in **Chapters 3, 4 and 7**. Travel medicine is a specialised part of medicine, in which physicians deal with non-local, imported pathogens on a daily basis. In the Netherlands, the first step for a patient is usually the general practitioner, who often has very little knowledge on foreign illnesses and epidemiology. This can cause considerable delay in treatment, and worse outcomes for the individual patient (**Chapter 3**). However, even highly-specialised travel-medicine physicians can struggle to recognise the cause of febrile illness in their patients (**Chapter 4** and **Chapter 7**), especially when the "big causes", such as malaria and dengue fever, have been excluded. Broad prospective studies on causes of fever in travellers should be conducted.

Part of the problem of the under-estimation of leptospirosis and rickettsioses, are the complicated, laborious and poorly available diagnostic tests. Molecular detection test, such as (real-time) PCR, hold a promise in the swift and timely diagnosis of both illnesses, as the traditional antibody detection tests fail diagnosis in the early stages of illness. In **Chapter 5**, we demonstrated that the basis of these tests for leptospirosis is not yet very solid, as too little well-performed research exists. Robust methodological studies on the diagnostic accuracy of these tests should be performed.

In conclusion, this thesis showed that leptospirosis and rickettsioses are underestimated causes of febrile illness worldwide, of which the true extend and epidemiological patterns

still have to be elucidated. To reach this goal, improved knowledge on the value of the available diagnostic tests is key.

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Chapter 9

Nederlandse samenvatting

In de afgelopen jaren is steeds meer duidelijkheid ontstaan over de omvang en de ziektelast van leptospirose en rickettsiosen,[1-4] bij zowel autochtone als reizende bevolkingsgroepen. Dit dankzij toegenomen belangstelling voor onderzoek, verbeterde diagnostische technieken en nieuwe epidemiologische methoden om de ziektelast te berekenen. Ook draagt klimaatverandering, met de daarbij behorende stijgende temperaturen en natter weer, bij aan een toename van de ziektebelasting. Desalniettemin lijkt de exacte epidemiologie en omvang van beide ziekten wereldwijd enorm onderschat.

SAMENVATTING

In **Deel 1** hebben we ons gericht op leptospirose. In **Hoofdstuk 2** zijn alle onderzoeken naar leptospirose die bekend zijn voor Afrika ten zuiden van de Sahara samengevat, aangezien er veel onduidelijkheid van over de epidemiologische situatie op het continent bestaat. We ontdekten dat de meeste gegevens bekend waren over het voorkomen van de ziekte bij dieren op het continent; waarschijnlijk vanwege de grote economische impact wanneer vee wordt besmet. Veel verschillende dieren kunnen *Leptospira* in hun niertubuli dragen en ze met urine uitscheiden. Ze kunnen asymptomatisch zijn ("onderhoudsgastheren") of symptomen ontwikkelen. Bij runderen en varkens kan infectie aanzienlijke reproductieve problemen veroorzaken, zoals abortus en infertiliteit.[5] Bij dieren bleek de ziekte wijdverbreid op het continent; dit suggereert veel potentieel infectierisico voor mensen. Gegevens bij mensen waren echter schaars, vooral voor de Centraal-Afrikaanse regio. Seroprevalentie onderzoeken bij gezonde proefpersonen toonden blootstelling aan de ziekteverwekker bij veel populaties over het hele continent; in sommige regio's bleek het ook een veelvoorkomende oorzaak van koortsende ziekte te zijn. Er waren echter maar een paar goed uitgevoerde onderzoeken bij patiënten met koorts, en het aantal uitgevoerde onderzoeken was erg klein voor zo'n groot continent. Daarom vertegenwoordigen de gegevens in dit hoofdstuk waarschijnlijk slechts het topje van de ijsberg (of de oren van het nijlpaard, in meer tropische termen), en is de werkelijke ziektelast op het continent waarschijnlijk veel hoger. Zoals het leptospirose gezegde luidt:

> "*Waar naar leptospieren en leptospirose wordt gezocht, worden ze ontdekt.*"[6]

In **Hoofdstuk 3** en **4** hebben we gevallen van "geïmporteerde" of "reis-gerelateerde" leptospirose bestudeerd. In ons onderzoek naar leptospirose in Nederland van 2009 tot 2016 (**Hoofdstuk 3**) constateerden we een toename van het aantal gevallen vanaf 2014, zowel autochtoon als geïmporteerd. Aangenomen wordt dat de toename van autochtone gevallen voornamelijk te wijten was aan warm weer,[7] waardoor de overlevingskansen van leptospieren toenamen. De reden voor de toename van geïmporteerde gevallen was echter

multifactorieel. Een groter aantal monsters was ingediend bij het Nationaal Referentielaboratorium voor Leptospirosen (NRL); dit zou een aanwijzing kunnen zijn dat reizigers meer risicovolle activiteiten ondernamen (bv. trektochten door de jungle en raften), of dat clinici de diagnose vaker overwogen. Vanaf 2014 is ook het percentage positieve monsters gestegen; dit kan zijn beïnvloed door de introductie van polymerasekettingreactie (PCR) als standaardtest vanaf september 2012 bij het NRL. PCR kan leptospirose in de vroege stadia van de ziekte detecteren, in tegenstelling tot de veelgebruikte serologische methoden, waarvoor monsters nodig zijn die in een later stadium van de ziekte worden afgenomen. Van de 224 geïmporteerde gevallen was de meerderheid naar Zuidoost-Azië gereisd, de traditionele "leptospirose-hotspot"; slechts 7 (3,1%) waren in Sub-Sahara Afrika geweest. De meeste patiënten waren mannelijk en relatief jong; allemaal kenmerken die ook worden beschreven in andere studies over het onderwerp. We bestudeerden de klinische kenmerken van 41 patiënten met geïmporteerde leptospirose die in het AMC waren behandeld. Meer dan de helft van hen (53,7%) moest in het ziekenhuis worden opgenomen, wat de ernst van de ziekte aangeeft; en het duurde gemiddeld vier dagen vanaf het begin van de symptomen totdat ze antibiotica kregen. We belichtten drie gevallen die het brede klinische spectrum van de ziekte en het belang van een snelle diagnose aantoonden.

In **Hoofdstuk 4** analyseerden we bevestigde gevallen van leptospirose die werden gemeld aan het GeoSentinel Surveillance Network, een netwerk dat reis-gerelateerde morbiditeit bewaakt via reis- en tropische geneeskunde-klinieken. We analyseerden 180 bevestigde gevallen van reis-gerelateerde leptospirose die werden gemeld tussen 1999 en 2016. Ze vertoonden vergelijkbare kenmerken met de Nederlandse gevallen; de meerderheid van de patiënten was jong, man, en naar Zuidoost-Azië gereisd. Ook hielden we een enquête onder de clinici van GeoSentinel, waarvan de meeste in academische instellingen werken, over de diagnostische en therapeutische praktijken van leptospirose. Opmerkelijk genoeg overwogen de clinici de ziekte vaak niet zonder de klassieke blootstellingsgeschiedenis of klinische symptomen, hoewel bekend is dat deze vaak afwezig zijn. Ook waren de toegepaste diagnostische methoden niet optimaal. Deze bevindingen impliceren dat zelfs in zeer gespecialiseerde reisklinieken, de ziekte aanzienlijk zou kunnen worden onderschat.

De diagnostische methoden voor leptospirose zijn ingewikkeld. De diagnostiek was lange tijd gebaseerd op de microscopische agglutinatietest (MAT) en kweek, twee bewerkelijke en zeer gespecialiseerde technieken, die niet geschikt zijn om snel een diagnose te stellen. In de afgelopen decennia hebben moleculaire detectietests, zoals PCR, terrein gewonnen bij de diagnostiek van veel infectieziekten. PCR kan de ziekte aantonen voordat de antilichamen worden gevormd, dus in een vroege stadium van de ziekte. In **Hoofdstuk 5** hebben we een systematische review en meta-analyse uitgevoerd over de diagnostische nauwkeurigheid van nucleïnezuur- en antigeendetectietests voor de diagnose van leptospirose. We identificeerden 41 onderzoeken en konden een meta-analyse uitvoeren voor PCR en real-time PCR. Er was echter een zeer hoge heterogeniteit tussen de onderzoeken; de onderzoeksrapporten waren

vaak van slechte methodologische kwaliteit en de studieomgevingen en de prevalentie van ziekte liepen sterk uiteen. De interpretatie van de beoordeling en analyses is daarom niet eenvoudig. Daarnaast hebben we de MAT gebruikt als referentiestandaard, die een onvolmaakte sensitiviteit heeft en daarom waarschijnlijk de specificiteit van de indextests onderschat. We hebben geprobeerd dit probleem aan te pakken door gebruik te maken van "samengestelde referentiestandaarden", MAT gecombineerd met IgM ELISA en / of kweek. Als alleen MAT als referentiestandaard werd gebruikt, beschouwden we dit als een hoog risico op vertekening van resultaten ("bias"). Voor PCR hebben we 15 onderzoeken geïncludeerd in onze studie. De gepoolde sensitiviteit was 70% (95% betrouwbaarheidsinterval (BI) 37% tot 90%) en de gepoolde specificiteit was 95% (95% BI 75% tot 99%). Dit verbeterde tot een gepoolde sensitiviteit van 87% (95% BI 44% tot 98%) en een gepoolde specificiteit van 97% (95% BI 60% tot 100%) wanneer studies die alleen MAT als referentietest gebruikten werden verwijderd. Voor real-time PCR werd een SROC-curve berekend, aangezien de positiviteits-drempel tussen studies varieerde. De mediane specificiteit was 92%. Wanneer 95% specificiteit op de curve werd geselecteerd, was de gepoolde sensitiviteit 29% (95% BI 15% tot 49%). Vanwege de grote variabiliteit tussen studies, is het moeilijk om harde conclusies te trekken over de nauwkeurigheid van deze tests en hun positie in het testtraject van leptospirose.

In **Deel 2** hebben we ons gericht op de onderschatting van rickettsiosen. In **Hoofdstuk 6** hebben we de onderschatting van rickettsiosen bij autochtone populaties berekend, door een systematische review te doen over studies die rapporteren over "Mediterranean Spotted Fever", scrubtyfus en "African Tick Bite Fever" (Afrikaanse tekenbeetkoorts), omdat deze rickettsiosen zich zowel met, als zonder eschar presenteren. De gerapporteerde percentages van presentatie met een eschar variëert tussen studies.[8] We veronderstelden dat deze variatie in gerapporteerde eschar-percentages wordt veroorzaakt door de opzet van de studies. Veel studies includeren patiënten op basis van 'klinische verdenking', wat automatisch zal leiden tot hogere gerapporteerde percentages van eschar-prevalentie, aangezien clinici deze ziekten vaak associëren met eschars. We verwachtten hogere percentages van niet-eschar-rickettsiosen (NERD) te vinden in onderzoeken die alle patiënten met koorts includeerden. Op deze manier konden we een inschatting maken van de onderrapportage van NERD. We hebben 121 onderzoeken geïncludeerd, waarvan de meerderheid (98) rapporteerde over scrubtyfus, 21 over Mediterranean Spotted Fever en twee over African Tick Bite Fever. Onze hypothese werd bevestigd: bij scrubtyfus werd NERD bij 66% van de patiënten gemist. Bij Mediterranean Spotted Fever gold dit voor 58% van de patiënten. Voor African Tick Bite Fever kon vanwege het beperkte aantal gevonden onderzoeken geen schatting worden gemaakt. Alle onderzoeken zijn uitgevoerd in autochtone populaties, met lokale clinici die op de hoogte zijn van de lokale epidemiologie. We denken daarom dat dit een belangrijke bevinding is; eerder vermoeden van de ziekte, en daarmee een eerdere start van de behandeling, zou de morbiditeit en zelfs de mortaliteit kunnen verminderen.

In Hoofdst**uk 7** hebben we de hierboven geschetste hypothese getest bij terugkerende reizigers met koorts. We voerden retrospectief serologische tests uit voor Spotted Fever Group-rickettsiosen, tyfusgroep-rickettsiosen en scrubtyfus bij teruggekeerde reizigers die zich bij het AMC hadden gepresenteerd met aspecifieke koorts en verdacht werden van leptospirose, maar hierop negatief waren getest. We veronderstelden dat rickettsiosen vaak werden gemist bij reizigers die terugkeerden met koorts, maar zonder eschar. We namen 97 patiënten op die waren teruggekeerd uit Azië, Afrika of Amerika. We ontdekten 16 bevestigde en vermoedelijke gevallen, waarvan 11 (69%) waren gemist en 7 (44%) geen empirische antibiotica hadden gekregen. Deze studie had beperkingen, maar de belangrijke boodschap is dat zelfs in een zeer gespecialiseerde academische reiskliniek de diagnose van niet-eschar rickettsiosen (NERD) vaak wordt gemist, en een plaats zou moeten krijgen in de standaard testalgoritmen.

Toekomstperspectieven en conclusie

Leptospirose en rickettsiosen zijn onderschatte oorzaken van met koorts gepaard gaande ziekten, met aanzienlijke morbiditeit en mortaliteit wereldwijd. Dit proefschrift geeft een overzicht van de huidige epidemiologische stand van zaken rondom beide ziekten.

Voor leptospirose concluderen we dat het een entiteit is die voorkomt in Afrika ten zuiden van de Sahara (**Hoofdstuk 2**), hoewel de ware omvang van het probleem grotendeels onbekend blijft vanwege een gebrek aan goede regionale epidemiologische studies. Lange tijd werd alle koortsende ziekte toegeschreven aan malaria, maar in het afgelopen decennium is het probleem van de over-diagnostiek naar voren gekomen.[9,10] Het is van cruciaal belang dat er meer onderzoeken worden uitgevoerd naar de oorzaken van met koorts gepaard gaande ziekten, zoals de afgelopen jaren steeds vaker is gedaan.[11-14] Alleen een beter begrip van de oorzaken van koorts kan de lokale klinische praktijk en de klinische uitkomst van patiënten verbeteren, en de lokale overheden ertoe aanzetten om adequate preventieve maatregelen te nemen. Voor rickettsiosen, een over het algemeen wereldwijd beter erkende ziekte-entiteit, is het gebrek aan bewustzijn op lokaal niveau ook een probleem, zoals we in **Hoofdstuk 6** hebben aangetoond. Aangezien koortsende ziekten een veel voorkomende uitdaging blijven voor clinici over de hele wereld, is het belangrijk dat in het onderzoek naar de oorzaken ervan, men dit met open vizier blijft doen. Traditionele ideeën over ziektepresentaties kunnen verkeerd uitpakken; met "niet-eschar rickettsiose" (NERD) als sprekend voorbeeld. Het nadeel is dat onderzoek naar de oorzaken van koorts kostbaar en intensief is, en de financiering niet altijd beschikbaar is in een wereld geleid door de farmaceutische industrie. Er zijn slimme algoritmen nodig voor prospectieve studies om dit probleem te omzeilen.

Niet alleen clinici in tropische delen van de wereld worstelen met de oorzaak van koorts, zoals we lieten zien in de **Hoofdstukken 3**, **4** en **7**. Reisgeneeskunde is een gespecialiseerd onderdeel van de geneeskunde, waarbij artsen dagelijks te maken hebben met niet-lokale,

geïmporteerde pathogenen. In Nederland is de eerste stap voor een patiënt is meestal de huisarts, die vaak weinig kennis over buitenlandse ziekten en epidemiologie heeft. Dit kan een aanzienlijke vertraging in de behandeling veroorzaken en slechtere uitkomsten voor de individuele patiënt (**Hoofdstuk 3**). Maar zelfs zeer gespecialiseerde artsen in de reisgeneeskunde kunnen moeite hebben om de oorzaak van met koorts gepaard gaande ziekten bij hun patiënten te herkennen (**Hoofdstuk 4** en **Hoofdstuk 7**), vooral wanneer de "grote oorzaken", zoals malaria en knokkelkoorts, zijn uitgesloten. Er moeten brede prospectieve onderzoeken naar de oorzaken van koorts bij reizigers worden uitgevoerd.

Een deel van het probleem van de onderschatting van leptospirose en rickettsiosen zijn de gecompliceerde, bewerkelijke en slecht beschikbare diagnostische tests. Moleculaire detectietests, zoals (real-time) PCR, kunnen beide ziekten snel en tijdig diagnosticeren, waar de traditionele antilichaamdetectietests de diagnose in de vroege stadia van de ziekte niet kunnen stellen. In **Hoofdstuk 5** hebben we aangetoond dat de basis van deze tests voor leptospirose nog niet erg solide is, omdat er te weinig goed uitgevoerd onderzoek bestaat. Er moeten robuuste methodologische onderzoeken naar de diagnostische nauwkeurigheid van deze tests worden uitgevoerd.

Concluderend toonde dit proefschrift aan dat leptospirose en rickettsiosen wereldwijd onderschatte oorzaken zijn van koortsende ziekte, waarvan de ware omvang en epidemiologische patronen nog moeten worden opgehelderd. Om dit doel te bereiken is verbeterde kennis over de waarde van de beschikbare diagnostische tests essentieel.

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ADDENDUM

List of publications
Portfolio
Curriculum Vitae
Word of thanks

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PHD PORTFOLIO

Sophia G de Vries
PhD supervisors: Prof. Dr. M.P. Grobusch & Dr. A. Goorhuis

Courses at AMC Graduate School	Year
Writing systematic reviews (Cochrane)	2013
Basic Course Legislation and Organisation for Clinical Researchers (BROK)	2013
Endnote	2013
Basic Laboratory Safety	2013
Clinical epidemiology	2013
Advanced qPCR	2016
Courses at other institutions	
Diploma in Tropical Medicine and Hygiene (Liverpool School of Tropical Medicine)	2014
Clinical Epidemiology Course, Schiermonnikoog (LUMC)	2017
(Inter)national conferences and symposia	
Ad-hoc Scientific Panel for Developing Guidance on Interventions for Tuberculosis Prevention and Control in Hard-to-Reach and Vulnerable Populations", ECDC, Stockholm, Sweden	2015
2nd ELS Meeting on leptospirosis and other rodent-borne haemorrhagic fevers, Amsterdam	
Speaker at workshop "Helminths and Atopy" - LUMC / RELACS, Lambaréné, Gabon	2015
Speaker at HookVac annual meetings (Gent, Belgium; Amsterdam)	2015, 2016
Teaching and Supervision	
Research Project of Bada Yang (Master thesis, UvA/AMC): *"Molecular and antigen detection tests for leptospirosis"*	2014-2015
Research Project of Yael Ashruf (Master thesis, UvA/AMC): "*Start-up of a hospital-based, clinical study in Lambaréné, Gabon: the Lambaréné Leptospirosis Study (LALES)"*	2016
Research Project of Suzan Bouwman (Master thesis, UvA/AMC): *"Establishment of the Recombinase Polymerase Amplification Assay (RPA) for the diagnosis of acute leptospirosis in febrile patients in Lambaréné, Gabon"*	2016
Research Project of Maud Bekedam (Master thesis, UvA/AMC): "*Retrospective study on travel related leptospirosis in the Netherlands 2009-2016 and a case series on leptospirosis in returned travelers diagnosed at the Academic Medical Centre"*	2016-2017
Research Project of Klaas-Henk Binnendijk (Master thesis Maastricht University): *"Recombinase Polymerase Amplification Assay (RPA) for the diagnosis of human leptospirosis"*	2017
Research Project of Rona Brokkelkamp (Master thesis, VUMC): *"Causes of Non-Malarial Fever in Lambaréné, Gabon"*	2017
Grants	
European Center for Disease Prevention and Control – Grant for the conduct of a systematic review *"Systematic literature review on interventions for tuberculosis prevention and control in hard to reach and vulnerable populations"* Co-Applicant, 47.000 €	2014
Miscellaneous	
Study Coordinator, HV001 study – a Phase 1 RCT on a novel co-administered Hookworm Vaccine in Gabonese adults, Lambaréné, Gabon	2014-2016
Lead Writer, development of clinical study protocol and SOPs HV002 study - a Phase 1 RCT on a novel Hookworm Vaccine in Gabonese children, Lambaréné, Gabon	2016-2017
Development of clinical study protocol HV003 study - a Phase 1 RCT on a novel Hookworm Vaccine in Gabonese adults, Lambaréné, Gabon	2017

CURRICULUM VITAE

Sophia de Vries was born in Amsterdam on the 5th of November, 1985. She attended the "Sint Antonius" primary school in the Nieuwmarkt area, and obtained her pre-university education at the Vossius Gymnasium in 2004.

She had wanted to become a doctor for long, but had always had a great interest in the African continent. In 2005 she went to Ghana to work in an orphanage and for the Guinea Worm Eradication Program. She also travelled through Burkina Faso and Mali and visited Timbuctoo. In the meantime, she cooperated in the founding, coordination and fundraising of an NGO for young women empowerment (providing vocational training and means to start up own businesses), in Tamale, Ghana. She went back to Ghana several times in the years after.

In September 2005, she started the study Medicine at the Academic Medical Center (AMC), University of Amsterdam (UvA) and graduated in May 2013. During the clinical rotations she developed and interest in internal medicine and infectious diseases, and did the elective "developing countries" at the St. Lukes Hospital in Malosa, Malawi. After graduating, she went back to Malawi to volunteer for a project supporting and monitoring health needs of disabled children in rural and urban communities in Northern Malawi.

In October 2013, she started the PhD trajectory under supervision of Prof. Martin Grobusch. To prepare for the work in Lambaréné, Gabon, she obtained the Diploma of Tropical Medicine and Hygiene (DTM&H) at the Liverpool School of Tropical Medicine in 2014. She went to Gabon later in 2014, where she coordinated a Randomized, controlled phase 1 study on a novel Hookworm vaccine. In the mean time she worked on several other clinical research projects, and systematic reviews.

During her work in Gabon, she realised that she has a broad clinical interest and enjoyed working in a generalist way. Since September 2017, she is being trained as a general practitioner at the AMC.

DANKWOORD / WORD OF THANKS

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