Leptospirosis:
Epidemiology, clinical aspects and diagnosis

Marga Goris

502468-L-bw-Goris

Colofon

Leptospirosis: Epidemiology, clinical aspects and diagnosis

ISBN: 978-94-028-0103-3

This thesis was prepared at KIT Biomedical Research
(KIT, Koninklijk Instituut voor de Tropen / Royal Tropical Institute).

The publication of this thesis was financially supported by Cirion foundation.

Lay-out and design by Joska Sesink
Printed by Ipskamp Printing BV, Enschede

Leptospirosis:
Epidemiology, clinical aspects and diagnosis

Leptospirose:
Epidemiologie, klinische aspecten en diagnose

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof.dr. H.A.P. Pols
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 6 april 2016 om 13:30 uur
door

Maria Gerarda Aleida Goris
geboren te Loo

Erasmus University Rotterdam

502468-L-bw-Goris

Promotiecommissie:

Promotoren:	Prof.dr. E.C.M. van Gorp
	Prof.dr. P.R. Klatser
Overige leden:	Prof.dr. M.P.G. Koopmans
	Prof.dr. J.H. Richardus
	Prof.dr. M.P. Grobusch
Copromotoren:	Dr. R.A. Hartskeerl
	Dr. K.R. Boer

CONTENTS

CHAPTER 1

Introduction

INTRODUCTION

Leptospirosis is one of the most wide spread zoonotic diseases in the world. It occurs at every continent except the polar regions where the disease has not been reported. The epidemiology of leptospirosis is complex and dynamic and there is a wide variety of (a)specific clinical manifestations with a broad differential diagnosis that makes diagnosis difficult. This explains that the disease is vastly underdiagnosed, underreported and hence ignored[1]. Leptospirosis is among the most neglected infectious diseases and even is lacking on many lists of neglected infectious diseases[2].

Causative agent

Leptospires, the bacteria causing leptospirosis, are long (6-20 µm) and thin (<0.2 µm) coiled bacteria that belong to the genus *Leptospira*. In the past, *Leptospira* were divided into non-infectious bacteria that were capable of living in the environment and infectious leptospires requiring a host for multiplication, denoted as the species *Leptospira biflexa* and *L.interrogans*, respectively. Leptospires are classified on the basis of serological features. The serovar is the basic taxon and serologically related serovars are placed into serogroups. These serogroups have no taxonomic status but have a practical value in serovar typing. To date, over 300 serovars have been established, including about 250 pathogenic serovars[3].

In the late 1980's, *Leptospira* speciation based on DNA homology was introduced. Initially DNA homologies were deduced from DNA hybridization experiments, later on supplemented and ultimately substituted by other molecular techniques, including DNA sequencing. Currently, the molecular speciation system recognizes 22 species grouped into three clades: group I and group II infectious species and saprophytic species. Group I species contains pathogenic, potentially life threatening species. Group II contains phylogenetic intermediate species with an unclear pathogenic status, mostly found in asymptomatic carriers or in patients with very mild disease. There is little correlation between the serological and the molecular classification systems.

Epidemiology

Most mammalian species can be carriers of infectious *Leptospira*. Infectious serovars have an affinity for distinct hosts but differ in the extent of adaptation to a certain host. One host might be carrier of several serovars, whereas one serovar might be found in several distinct hosts. Adaptation to new hosts as well as migration of host populations, either or not caused by climate and ecological changes, are the main cause of high dynamics in the distribution of *Leptospira* serovars and substantiate the need for continuous surveillance. Infectious *Leptospira* live in the urogenital tract of their host and are excreted with the urine in the environment. In the environment, infectious leptospires can survive for a long time, depending on favorable i.e. warm and wet, conditions. Infection of the accidental hosts occurs by direct contact with the reservoir host or indirectly by the contaminated environment.

Table 1 | Global burden of disease study 2010 of listed neglected tropical diseases (NTDs) (adapted from Hotez *et al.*[12])

Disease	Number of cases (expected in 2010)	Deaths	DALYs (millions)
Intestinal nematode infections	1,723 million	2,700	5.19
Leishmaniasis	10 million	51,600	3.32
Schistosomiasis	252 million	11,700	3.31
Leptospirosis*	1 million	**58,900**	2.90[13]
Lymphatic filariasis	36 million	-	2.78
Food-borne trematodiasis	16 million	-	1.88
Rabies	1,100	26,400	1.46
Dengue	197,000# (~200 million)	14,700	0.83

*Not a listed NTD (severe cases only)
Incident (acute) symptomatic cases only

Indirect transmission is the main route of infection; leptospirosis is particularly endemic in (sub)tropical regions were environmental conditions are favorable for survival.

Recent studies lead by the WHO[1,4] on the global burden of human leptospirosis have estimated more than 1 million severe cases of leptospirosis with over 60,000 deaths annually[5,6]. Hence, leptospirosis would rank high in the list of formal neglected tropical diseases (NTDs), see Table 1.

There is little known about the occurrence of post-leptospirosis complaints in humans. The number of mild cases is unknown but probably is a multitude of severe cases. The burden of veterinary leptospirosis has not been assessed but is likely to be considerable. Animals are exposed to leptospires in the environment and might have subclinical infections (Figure 1).

Disease

Leptospires survive in a wide variety of situations; they need to adapt to reservoir hosts, to survive in the environment and subsequently evade, at least for a period, the host defense systems. This adaptation is reflected in the many distinct serovars and a variety of genomically distinguishable strains. While reservoir hosts usually are asymptomatic carriers, all serovars together cause a wide variety of clinical signs and symptoms in the accidental host. There is no strict correlation between clinical manifestations and a certain serovar but part of the serovars are inclined to cause severe, potentially fatal disease, while others usually are associated with asymptomatic infection or mild disease.

Leptospirosis is a protean disease. In addition to the spectrum of disease severity, clinical signs and symptoms show a wide variation, mimicking several other diseases that often are

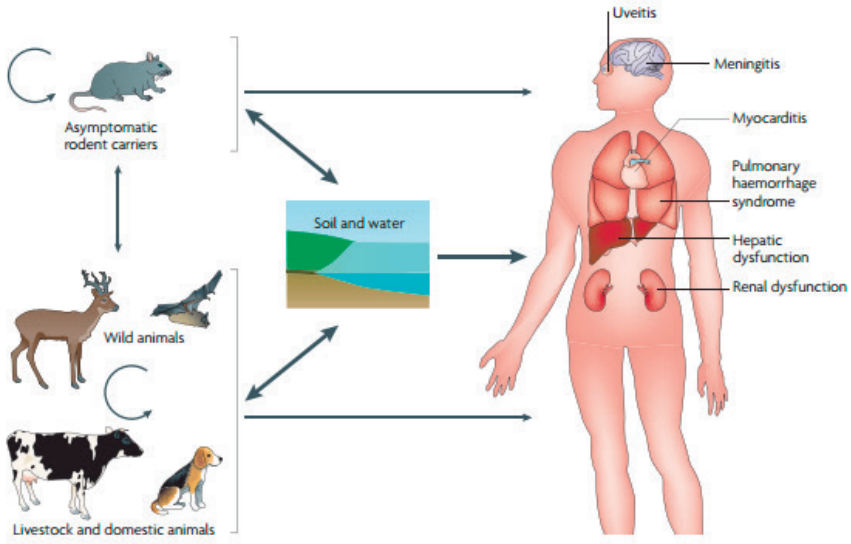


Figure 1 | The cycle of leptospiral infection (reproduced from Ko *et al.*[14])

endemic and epidemic in the same geographical and climatologic conditions as leptospirosis. Notably at the early acute phase of leptospirosis, complaints of fever, head- and muscle pain are largely non characteristic and are easily attributed to other infectious diseases. Fever might be bi-phasic, non-characteristic. Late acute complications comprise (again) a variety of symptoms. The WHO guidelines[7] mention 24 signs and symptoms, each of which alone and/or in different combinations may be due to leptospirosis (Table 2) and 20 other diseases that should be considered in the differential diagnosis (Table 3).

Hepato-renal disorders associated with bleeding tendency is referred to as Weil's disease when caused by serovars Icterohaemorrhagiae and Copenhageni with the rat as main reservoir or as Weil's syndrome, when caused by another serovar and other reservoir host. Depending on availability of adequate supportive and antibiotic treatment the case fatality rate (CFR) of Weil's disease ranges from 2% to 40%. Leptospirosis pulmonary haemorrhage syndrome (LPHS) is an emerging severe complication of leptospirosis with a CFR up to 80%. Apart from Weil's disease, leptospirosis has many synonyms, often reflecting symptoms, place and season of infection, occupation or reservoir host. To date, synonyms and attempts to group leptospirosis according to similar symptoms is increasingly evaded. Leptospirosis is separated into severe and mild leptospirosis. The CFR of mild leptospirosis is low. Little is known about mild leptospirosis, which probably in most cases is under- or misdiagnosed as influenza. Mild leptospirosis is supposed to be self-limiting with complete recovery in many cases. However, the disease might be associated with sequels. Uveitis is a well-known late complication. The occurrence of late or persistent complaints is recognized but not well

Table 2 | Signs and symptoms of leptospirosis in humans (adapted from WHO[7])

1	fever
2	severe headache
3	myalgias
4	conjunctival suffusion
5	jaundice
6	general malaise
7	stiff neck
8	chills
9	abdominal pain
10	joint pain
11	anorexia
12	nausea
13	vomiting
14	diarrhoea
15	oliguria/anuria
16	haemorrhages
17	skin rash
18	photophobia
19	cough
20	cardiac arrhythmia
21	hypotension
22	mental confusion
23	psychosis
24	delirium

Table 3 | Diseases to be considered in the differential diagnosis of leptospirosis (adapted from WHO[7])

1	influenza
2	dengue and dengue haemorrhagic fever
3	hantavirus infection, including hantavirus pulmonary syndrome or other respiratory distress syndromes
4	yellow fever and other viral haemorrhagic fevers
5	rickettsiosis
6	borreliosis
7	brucellosis
8	malaria
9	pyelonephritis
10	aseptic meningitis
11	chemical poisoning
12	food poisoning
13	typhoid fever and other enteric fevers
14	viral hepatitis
15	pyrexia of unknown origin (PUO)
16	primary HIV seroconversion
17	legionnaire's disease
18	toxoplasmosis
19	infectious mononucleosis
20	pharyngitis

documented and investigated. Leptospirosis in animals shows similar patterns as the disease in humans, although sub-clinical and persistent infections might be more common. Dominant clinical manifestations might vary between animal species. For example; dogs might develop fulminant, fatal 'Morbus Weil', while horses usually merely suffer from equine recurrent uveitis (ERU) and pigs mostly suffer from abortion.

Pathogenesis

Leptospires penetrate through wounded skin and mucous membranes. Penetration through water weakened skin is often indicated but not well supported by data. After penetration, leptospires multiply, cross tissue barriers and disseminate to all organs in the body, probably

via the blood. After two to 30 days, usually one to two weeks, the first symptoms of sudden high fever and/or muscle pain and headache occur. Pathogenic *Leptospira* are able to survive in the non-immune host by evading complement-mediated killing by binding factor H, a strong inhibitor of the complement system, on their surface. Furthermore, they withstand ingestion and killing by neutrophils, monocytes and macrophages. During the immune phase antibodies appear and *Leptospira* disappear from the blood. They do however persist in several organs, including liver, lung, kidney, heart and brain; post mortem findings show multi-organ involvement in (severe) disease[8].

In the kidneys both acute tubular damage and interstitial nephritis can occur. Acute tubular lesions evolve in time to interstitial oedema and acute tubular necrosis (ATN).

Patients who survive long enough can develop interstitial nephritis with renal failure. In general no widespread hepatocellular necrosis is found in the liver but histopathology may show focal necrosis, foci of inflammation and plugging of bile canaliculi. Bleedings and petechiae are seen in the lungs, kidneys, liver, gastrointestinal tract, heart, pancreas, muscles, prostate, testis and the brain. In several studies an association between haemorrhages and thrombocytopenia is shown[9,10]. Although the underlying mechanisms of thrombocytopenia are not elucidated, platelet consumption probably plays an important role. Disseminated intravascular coagulation (DIC) has been demonstrated in several studies[9-11]. Pathogenic *Leptospira* can activate endothelial cells *in vitro* and can disrupt the endothelial cell barrier function, which promotes dissemination. Platelets can aggregate to activated endothelium in the human lung and although histology shows swelling of activated endothelial cells, there is no evident vasculitis or necrosis. Humoral response is mainly directed at the lipopolysaccharides (LPS) of leptospires and hence is to an extent serovar-specific. Cross reactions to other serovars are notably present in the acute phase. It is unclear whether other antigens might play a significant role in protective humoral immunity. The dogma of the absence of a cell mediated immunity (CMI) is challenged by the finding of a correlation of the presence of CMI and the efficacy of vaccines in cattle. The role of the innate immunity is increasingly studied, as well as the usefulness of measuring biomarkers to get insight in the disease severity.

Diagnosis

Leptospirosis can be treated with antibiotics but efficacy depends on the initiation of the treatment at an as early possible stage of the disease. Therefore early diagnosis is important. Because of the variety of clinical manifestations, diagnosis of leptospirosis on clinical grounds only is challenging and laboratory confirmation is required. However, the available diagnostic tests have several drawbacks such as being complicated and requiring expertise and experience, laborious, unreliable, slow and/or expensive. Diagnostic tests can be divided into conventional tests, that all provide confirmation at a relatively late stage of the disease, and novel molecular tests. Conventional tests are culturing leptospires from clinical samples,

the microscopic agglutination test (MAT), which is the reference test and the enzyme-linked immunosorbent assay (ELISA). Both MAT and ELISA detect antibodies against leptospires. Molecular tests, mainly the polymerase chain reaction (PCR) provide a tool for diagnosis at the early acute stage. Recently, rapid diagnostic tests have been developed based on detecting antibodies and giving a result within 15 minutes. It is unknown how well these conventional diagnostic tests perform in suspected patient populations.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to investigate (i) epidemiological and clinical parameters of leptospirosis in humans and animals and (ii) the value of conventional tests in the laboratory diagnosis of leptospirosis.

The section 'Epidemiology and clinical aspects' starts with **Chapter 2** in which the trends in human leptospirosis in the Netherlands in the period 1925-2008 are analysed followed by **Chapter 3** in which the investigation into the sudden increase of leptospirosis in 2014 in humans and dogs in the Netherlands is described. In **Chapter 4** the burden of human leptospirosis is assessed; the occurrence of persistent complaints and duration of hospital stay is estimated and risk factors potentially impacting on the occurrence of post-leptospirosis complaints are investigated. **Chapter 5** describes the typing of two new leptospiral serovars from human patients in Costa Rica and the implications of these findings for public health. In **Chapter 6** healthy dogs and horses are checked for agglutinating antibodies against pathogenic *Leptospira* by MAT to explore exposure and existence of subclinical infections.

The section 'Diagnosis' starts with **Chapter 7** in which the process of establishing a valid laboratory case definition for human leptospirosis is formulated. The reference test is based on positive culture and the optimal cut-off titers of both MAT and ELISA are determined. This case definition is applied in **Chapter 8** in which three rapid serological diagnostic tests for leptospirosis are prospectively evaluated. **Chapter 9** presents a systematic review and meta-analysis of the diagnostic accuracy of serological laboratory tests for diagnosis of human leptospirosis in patients presenting with clinical symptoms.

Finally, in **Chapter 10** the findings of the studies in this thesis are reviewed and summarized with a view on future research in the field of leptospirosis.

REFERENCES

1. World Health Organization. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group, 2011.
2. Moran M, Guzman J, Chapman N, *et al.* G Finder 2014 Neglected Disease Research and Development: Emerging Trends 2014 (accessed 29 September 2015).
3. Adler B, de la Pena MA. *Leptospira* and leptospirosis. *Vet Microbiol* 2010; **140**(3-4): 287-96.
4. World Health Organization. Report of the First meeting of the Leptospirosis Burden Epidemiology Reference Group, 2010.
5. Costa F, Hagan JE, Calcagno J, *et al.* Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Negl Trop Dis* 2015; **9**(9): e0003898.
6. Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLoS Negl Trop Dis* 2015; **9**(9): e0003843.
7. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control; 2003.
8. Arean VM. The pathologic anatomy and pathogenesis of fatal human leptospirosis (Weil's disease). *Am J Pathol* 1962; **40**: 393-423.
9. Wagenaar JFP, Goris MGA, Partiningrum DL, *et al.* Coagulation disorders in patients with severe leptospirosis are associated with severe bleeding and mortality. *Trop Med Int Health* 2010; **15**(2): 152-9.
10. Chierakul W, Tientadakul P, Suputtamongkol Y, *et al.* Activation of the coagulation cascade in patients with leptospirosis. *Clin Infect Dis* 2008; **46**(2): 254-60.
11. Wagenaar JFP, Goris MGA, Sakundarno MS, *et al.* What role do coagulation disorders play in the pathogenesis of leptospirosis? *Trop Med Int Health* 2007; **12**(1): 111-22.
12. Hotez PJ, Alvarado M, Basanez MG, *et al.* The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. *PLoS Negl Trop Dis* 2014; **8**(7): e2865.
13. Torgerson PR, Hagan JE, Costa F, *et al.* Global Burden of Leptospirosis: Estimated in Terms of Disability Adjusted Life Years. *PLoS Negl Trop Dis* 2015; **9**(10): e0004122.
14. Ko AI, Goarant C, Picardeau M. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat Rev Microbiol* 2009; **7**(10): 736-47.

PART 1

Epidemiology and clinical aspects

CHAPTER 2

Human leptospirosis trends, the Netherlands, 1925-2008

Marga G.A. Goris
Kimberly R. Boer
Tamara A.T.E. Duarte
Suzanne J. Kliffen
Rudy A. Hartskeerl

Royal Tropical Institute, Department of Biomedical Research, Amsterdam, the Netherlands

Emerging Infectious Diseases 2013; 19(3): 371-8

ABSTRACT

To increase knowledge of leptospirosis in the Netherlands and identify changing trends of this disease over time, we analyzed historical passive surveillance reports for an 84-year period (1925–2008). We found that 2,553 mainly severe leptospirosis cases were diagnosed (average annual incidence rate 0.25 cases/100,000 population). The overall case-fatality rate for patients with reported leptospirosis was 6.5% but decreased over the period, probably because of improved treatment. Ninety percent of reported leptospirosis cases were in male patients. Most autochthonous leptospirosis infections were associated with recreational exposures, but 15.5% of the cases were attributed to accidents that resulted in injury and to concomitant water contact. Since the end of the 1950s, the proportion of imported infections gradually increased, reaching 53.1% of the total during 2005–2008. Most (80.1%) imported infections were associated with sporting and adventurous vacation activities.

INTRODUCTION

Leptospirosis is a zoonotic disease caused by infection with *Leptospira* spp. bacteria[1]. Pathogenic leptospires live in the kidneys of many mammalian hosts, including rodents, insectivores, and livestock. Leptospires are shed into the environment, where they can survive for several months in favorable (warm and wet) conditions. Thus, leptospirosis is particularly endemic to warm and humid tropical and subtropical regions[2]. Humans are infected by direct contact with infected animals or indirectly by contact with a contaminated environment.

Leptospirosis is an emerging public health problem globally[3–6]. However, this disease is often overlooked because it is difficult to clinically diagnose and because laboratory-based diagnosis is cumbersome. Because mild leptospirosis frequently goes unrecognized and notification systems are mostly absent, the global incidence of leptospirosis is underestimated. An international survey conducted by the International Leptospirosis Society reported ≥350,000 cases of severe leptospirosis annually[7]. This estimate is supported by data from an assessment of the global incidence of leptospirosis[8], which indicated a mean global incidence rate for leptospirosis of 5 cases/100,000 population.

In Europe, leptospirosis has been studied and diagnosed since the 1920s. Historical reviews from Germany[9] and France[10] have contributed to a better understanding of the epidemiology of leptospirosis. In the Netherlands, passive surveillance of human leptospirosis began in 1924. Reporting of cases of this disease is mandatory, and laboratory diagnosis has been centralized in 1 institution. To increase knowledge of leptospirosis, we analyzed historical passive surveillance reports in the Netherlands for 84 years (1925–2008) to determine changing trends of this disease over time.

Passive surveillance

The Royal Tropical Institute (KIT) in Amsterdam is associated with the World Health Organization/Food and Agricultural Organization/World Organisation for Animal Health and the National Collaborating Centre for Reference and Research on Leptospirosis (NRL), which confirms ≈99% of the suspected cases of leptospirosis in the Netherlands. Detailed records on serologic, clinical, and epidemiologic features are archived at the NRL. Since 1928, leptospirosis has been a mandatory reportable disease in the Netherlands[11]. A case of leptospirosis is defined as laboratory confirmation of infection as described in this report and by Hartskeerl[12] and fever or 2 of the following signs and symptoms: shivering, headache, muscle pain, conjunctival injection, bleeding in skin and mucosa, rash, jaundice, myocarditis, meningitis, renal failure, pulmonary hemorrhage with respiratory failure.

Study population

Reportable disease data for leptospirosis are compiled from passive surveillance reports received for the entire population of the Netherlands. General practitioners and consulting

clinicians suspecting leptospirosis send clinical specimens to the NRL for laboratory evaluation. During the period covered by this review, the total number of samples submitted for testing was estimated to be ≈50,000.

Changes in laboratory diagnosis

Laboratory tests have changed over time. However, all diagnoses in the Netherlands have been based on identification of leptospires by culture or antibodies against *Leptospira spp*. by agglutination tests[12]. During 1924–1963, culture of patient specimens was routinely performed by inoculation of blood or urine into guinea pigs or hamsters. In 1964, in vitro culturing was introduced and has been used exclusively since 1972.

Beginning in 1924, the agglutination test was used for diagnosis of leptospirosis; the test was performed as described by Martin and Pettit[13] using serovars Pyrogenes and Rachmat and unidentified isolates from patients. Serovar Copenhageni was included in 1927, serovars Icterohaemorrhagiae and Canicola in 1934, serovar Grippotyphosa in 1941, serovars Pomona and Bataviae in 1942, and serovar Ballum in 1945. During 1960–1990, the panel was increased by the addition of serovars Tarassovi (1961), Poi (1963), Bratislava (1964), Saxkoebing (1964), Hardjoprajitno (1980), Hebdomadis (1981), Hardjo-bovis (1983), Proechimys (1987), and Sejroe (1987). This panel was later supplemented with serovars Ballico, Celledoni, Cynopteri, Mini, Panama, and Shermani to include the representative serovars that that cause leptospirosis worldwide[1].

The agglutination test was modified in 1954 in accordance with recommendations of Wolff[14] and in 1978 in accordance with recommendations of Cole *et al*.[15]. An in-house IgM ELISA was introduced in 1984[16]. Laboratory diagnosis is currently based on a positive culture, a microscopic agglutination test titer ≥160 and IgM ELISA titer ≥80, or seroconversion[12]. Most presumptive infecting serogroups are deduced from the highest titers against ≥1 serovars in the microscopic agglutination test. Such titers are only indicative for serogroups[17]. Therefore, we report data on infecting serogroups. In cases in which a leptospiral isolate has been typed to serovar level, the corresponding serogroup has been used.

We also assessed differences between autochthonous and imported leptospirosis infections. Autochthonous infections are those most likely acquired in the Netherlands. Imported infections are those most likely acquired during a visit to another country <1 month before the day of symptom onset.

Data collection

During 1924–1964, when a case of leptospirosis was confirmed in the Netherlands, demographic, epidemiologic, and clinical data were collected by using a standardized questionnaire sent to the consulting physician by the NRL. When persons with confirmed leptospirosis were hospitalized, the physician was requested to send a copy of the patient's discharge letter to

the NRL. Because of changes in privacy laws, during 1964–1999 questionnaires were sent directly to the patients, but since 1999, questionnaires and informed consent forms have been sent to consulting physicians.

Data analysis

Archived information was entered into a database by using SPSS version 15.0 software (IBM, Armonk, NY, USA). Data were analyzed for trends over time, differences between autochthonous and imported infections, and differences related to sex of the patients.

Total annual incidence for male and female patients was calculated by using the population of the Netherlands as obtained from the Dutch Central Bureau for Statistics (The Hague, the Netherlands). Annual case-fatality rates (CFRs) were calculated by dividing the number of deaths by the total number of confirmed cases per year. Patient data on hospitalization, antimicrobial drug treatment (available since 1950), hemodialysis (available since 1961), and treatment in an intensive care unit (ICU) (available since 1955) were used to give an overview of clinical management of leptospirosis in the Netherlands. Missing data were recoded as "no" for the analysis.

To assess the severity of disease, data were analyzed for changes over time for 10-year intervals during 1925–2008 by using the $\chi 2$ test for trend. Severe leptospirosis was defined as disease for which hospitalization, admission to an ICU, or dialysis was indicated, or for which death occurred. Severity calculations are limited to the period when such supportive treatment was available. These characteristics were analyzed by using the $\chi 2$ test, Student *t* test, or Fisher exact test when appropriate.

Differences between autochthonous and imported infections regarding patient characteristics, likely source of exposure, treatment course, and infecting serogroups were tested by using the $\chi 2$ test and Fisher exact test for values <5. Likely source of exposure was determined by type of contact (recreational activities, accidental, job-related) and route of infection (water, animal or other source); source and route are mutually exclusive, in contrast to assessment of animal hosts.

We examined whether bacterial exposure and disease severity differed by patient sex. Patient data concerning mean age, imported disease, type of contact, and treatment course are presented by sex of the patient. Differences between male and female patients were analyzed by using the $\chi 2$ test. A p value ≤ 0.05 was considered significant.

Ethical issues

This study was exempted from ethical review of human subject research by the Medical Ethical Review Committee of the Academic Medical Centre, University of Amsterdam (protocol W12_075#12.17.0092). All data have been de-identified and were not attributable to individual patients.

Cases

During 1925–2008, the NRL reported 2,588 leptospirosis infections. Thirty-five case-patients were excluded: 22 living outside the Netherlands, 1 whose case was reported more than once, and 12 whose cases were retrospectively reclassified. Thus, the study sample comprised 2,553 confirmed case-patients. An overview of patient characteristics by autochthonous and imported infections is shown in Tables 1 and 2. Male patients accounted for 91.1% of all infections. Deaths were almost exclusively reported in men who had autochthonous infections with serogroup Icterohaemorrhagiae. Most (80.8%) imported leptospirosis cases and a substantial (44.4%) proportion of autochthonous cases were associated with recreational activities. A substantial number of infections (14.4%) were attributed to injury (i.e., traffic accidents and concomitant water exposure).

Table 1 | Characteristics for case-patients with leptospirosis, the Netherlands, 1925-2005*

Characteristic	Total cases, n=2,553	Autochthonous cases, n=2,231**	Imported cases, n=318**	p value
Male sex†	2,306 (91.1)	2,025 (91.6)	278 (87.4)	0.014
Mean (SD) age†	33.8 (17.1)	34.0 (17.5)	32.6 (14.4)	0.848
Type of contact				
Recreational activity	1,250 (49.0)	990 (44.4)	257 (80.8)	< 0.001
Job-related	685 (26.8)	664 (29.8)	21 (6.6)	< 0.001
Accident	367 (14.4)	345 (15.5)	22 (6.9)	< 0.001
Unknown	250 (9.8)	232 (10.4)	18 (5.7)	
Likely route of infection				
Water	1,457 (57.1)	1,219 (54.6)	236 (74.2)	< 0.001
Water and animals	500 (19.6)	446 (20.0)	53 (16.7)	0.162
Animals	351 (13.7)	346 (15.5)	4 (1.3)	< 0.001
Others#	16 (0.6)	14 (0.6)	2 (0.6)	1.000
Unknown	229 (9.0)	206 (9.2)	23 (7.2)	
Host exposure***	(n=851)	(n=792)	(n=57)	
Rats	443 (52.1)	411 (51.9)	32 (56.1)	< 0.535
Mice	123 (14.6)	120 (15.2)	3 (5.3)	0.004
Other rodents	31 (3.6)	28 (3.5)	3 (5.3)	0.789
Cows	231 (27.1)	224 (28.3)	6 (10.5)	< 0.001
Dogs	172 (20.2)	158 (19.9)	13 (22.8)	0.603
Other animals	185 (21.7)	168 (21.2)	16 (28.1)	0.088

Trends over time

The average annual incidence rate of leptospirosis in the Netherlands was 0.25 cases/100,000 population (Figure). For male patients, the average incidence was 0.46 cases/100,000 boys and men, which is >10-fold higher than the rate for female patients (0.04 cases/100,000 girls and women).

The mean (SD) age of patients was 33.8 (17.1) years. There was a gradual increase in the mean (SD) age over time: 29.1 (14.6) years during 1925–1934 to 38.0 (16.1) years during 2005–2008 (p<0.001).

Table 1 | Continued

Characteristic	Total cases, n=2,553	Autochthonous cases, n=2,231**	Imported cases, n=318**	p value
Serogroup				
Icterohaemorrhagiae	1,702 (66.7)	1,588 (71.2)	111 (34.9)	< 0.001
Grippotyphosa	196 (7.7)	174 (7.8)	22 (6.9)	0.595
Sejroe	128 (5.0)	116 (5.2)	12 (3.8)	0.771
Canicola	93 (3.6)	87 (3.9)	6 (1.9)	0.230
Pomona	54 (2.1)	45 (2.0)	9 (2.8)	0.107
Autumnalis	16 (0.6)	1 (0.1)	15 (4.7)	< 0.001
Bataviae	11 (0.4)	0	11 (3.5)	< 0.001
Others ##	54 (2.1)	12 (0.5)	42 (13.2)	< 0.001
Unknown	299 (11.7)	208 (9.3)	90 (28.3)	< 0.001
Cultures performed	1,335 (52.3)	1,151 (51.6)	182 (57.2)	0.060
Positive result	306 (22.9)	256 (22.2)	49 (26.9)	0.162

*Values are no. (%) unless otherwise indicated.

**Data for autochthonous and imported cases could not be obtained for 4 patients;

†Sex was recorded for 2,532 patients; 2,210 patients with autochthonous cases and 318 patients with imported cases; Age was recorded for 2,427 patients; 2,105 patients with autochthonous cases and 318 patients with imported cases.

#Laboratory accidents or contact with mud/soil.

***Animal specified is not mutually exclusive; 1 patient could have been in contact with >1 animal.

##For total infections, Australis (n=12); Celledoni (n=7); Sejroe/Hebdomadis/Mini complex (n=7); Javanica (n=6); Pyrogenes (n=6); Hebdomadis (n=4); Shermani (N=4); Ballum (n=2); Cynopteri (n=2); Tarassovi (n=2); Celledoni/Javanica complex (n=1); Mini (n=1).

For autochthonous infections, Australis (n=6); Ballum (n=2); Tarassovi (n=2); Javanica (n=1); Sejroe/Hebdomadis/Mini complex (n=1). For imported infections: Celledoni (n=7); Australis (n=6); Pyrogenes (n=6); Sejroe/Hebdomadis/Mini complex (n=6); Javanica (n=5); Hebdomadis (n=4); Shermani (n=4); Cynopteri (n=2); Celledoni/Javanica complex (n=1); Mini (n=1).

Table 2 | Treatment parameters for and deaths among case-patients with leptospirosis, the Netherlands, 1925-2008*

Characteristic	No. (%) total cases	No. (%) autochthonous cases	No. (%) imported cases	No. patients	p value
Deaths					
Total	166 (6.5)	162 (7.3)	4 (1.3)	2,553	< 0.001
Male patients	160 (97.0)	156 (96.9)	4 (100.0)	2,306	0.861
Treatment course**					
Hospitalization	1,851 (72.5)	1,612 (72.3)	235 (73.9)	2,553	0.539
Antimicrobial drugs	1,216 (68.5)	994 (68.2)	219 (69.5)	1,597†	0.652
Dialysis	119 (8.8)	103 (9.8)	15 (5.0)	796§	0.009
ICU	106 (6.5)	83 (6.3)	23 (7.3)	626¶	0.485

*ICU, intensive care unit

**Percentages were calculated from available data (all missing values were regarded as no). It was assumed that analyzing data that contained large amounts of missing values would result in findings that could not be generalised and lead to substantial overestimated values.

†Antimicrobial drug data were available for 1,597 of 1,776 infected patients, 1,306 of 1,457 patients with autochthonous cases and 288 of 315 patients with imported cases (January 1950 to December 2008). Before 1950, treatment with antimicrobial drugs was not expected to be used.

§Dialysis treatment data were available for 796 of 1,360 infected patients, 559 of 1,053 patients with autochthonous cases and 234 of 303 patients with imported cases (January 1961 to December 2008). Before 1961, dialysis treatment was not expected to be used.

¶ICU data were available for 626 patients of 1,641 infected patients, 441 of 1,324 patients with autochthonous cases and 183 of 313 patients with imported cases (April 1955 to December 2008). Before 1955, treatment in an ICU was not expected to be used

Although leptospirosis is endemic to most of the Netherlands, there have not been any large outbreaks. Peak periods of incidence rate increases were seen in 1932 (1.08 cases/100,000 population), 1941 (0.64 cases/100,000 population), and 1961 (0.64 cases/100,000 population), and to a lesser extent in 1967 (0.37 cases/100,000 population) and 1988 (0.42 cases/100,000 population) (Figure).

During 1925–2008, a total of 166 persons with leptospirosis died (CFR 6.5%) (Table 2). The annual number of deaths decreased over time, probably as a result of the introduction of dialysis treatment in 1961; there were 67 deaths (10.5%) in the first 20 years and 21 (3.9%) during 1975–1994 ($p<0.001$). Since 1995, the NRL has only recorded 5 (1.2%) deaths. During 1925–2008, the mean (SD) age of patients who died was 49.2 (15.6) years, and the mean (SD) age of patients who survived was 32.8 (16.9) years ($p<0.001$). The mean (SD) age of patients who died increased from 43.6 (16.0) years during 1925–1934 to 64.2 (12.7) years over the last 4 years of the study ($p<0.05$).

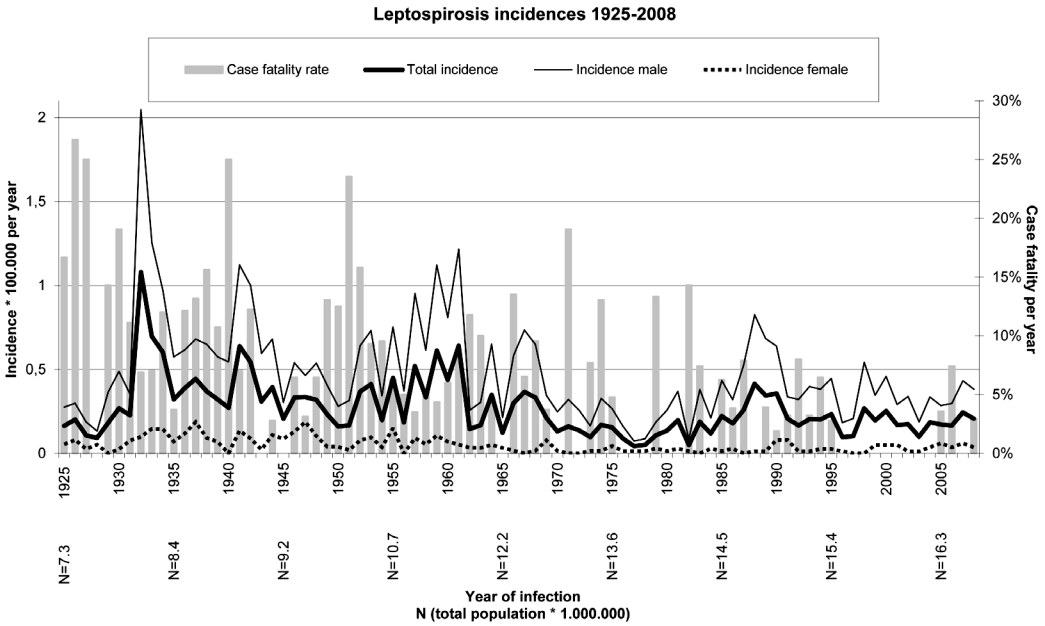


Figure | Incidence rates of leptospirosis, the Netherlands, 1925–2008. Grey bars indicate case-fatality rate (percentage of deaths/no. of confirmed cases), thick black line indicates total incidence rate (no. cases/100,000 population), thin black line indicates incidence rate among male patients (no. cases in male patients/100,000 male population), and dashed line indicates incidence rate among female patients (no. cases in female patients/100,000 female population). The total population of the Netherlands was 7.3 million in 1925, 8.4 million in 1935, 9.2 million in 1945, 10.7 million in 1955, 12.2 million in 1965, 13.6 million in 1975, 14.5 million in 1985, 15.4 million in 1995, and 16.3 million in 2005.

A total of 1,851 (72.5%) of the 2,553 patients were hospitalized. The proportion of patients hospitalized increased from 37.4% during 1925–1934 to 92.1% during 1955–1964 ($p<0.001$) and then decreased to 74.4% during 2005–2008 ($p<0.001$). Overall, 6.5% of patients were treated in an ICU and 8.8% received dialysis treatment.

The first ICU admissions for leptospirosis were reported in 1978. During 1975–1984, a total of 7.5% of patients were reported as being admitted to an ICU. The percentage of ICU admissions increased to 17.8% during 2005–2008. Dialysis treatment was introduced and recorded for the first time in 1961; its use increased markedly from 3 (2.0%) patients with leptospirosis during 1955–1964 to 16 (12.4%) patients during 2005–2008. Widespread use of penicillin to treat leptospirosis first began during World War II. Treatment with antimicrobial drugs increased from 53.3% of patients during 1945–1954 to 83.7% of patients during 2005–2008.

Table 3 | Treatment course and deaths, by infecting *Leptospira* spp. serogroups, for case-patients with leptospirosis, the Netherlands, 1925-2008*

Serogroup	No. patients	No. (%) hospitalized	No. (%) treated with dialysis	No. (%) treated in ICU	No. (%) died
Icterohaemorrhagiae	1,702	1,311 (77.0)	90 (11.9)	68 (7.0)	90 (5.3)
Grippotyphosa	196	124 (63.3)	3 (2.2)	2 (1.2)	0
Sejroe	128	48 (37.5)	3 (2.4)	2 (1.6)	1 (0.8)
Canicola	93	60 (64.5)	0	0	3 (3.2)
Pomona	54	40 (74.1)	3 (5.6)	7 (13.0)	0
Others	81	55 (67.9)	3 (3.8)	6 (7.6)	1 (1.9)
Unknown	299	213 (71.2)	17 (8.6)	21 (9.3)	71 (23.7)

*ICU, intensive care unit. Data were available for 2,553 patients who were hospitalized or died, 1,360 patients treated with dialysis, and, 1,641 patients treated in an ICU.

During 1925–2008, the main infecting serogroup in autochthonous cases was Icterohaemorrhagiae (1,588 identifications; 71.2%). Other common serogroups were Grippotyphosa (174 identifications; 7.8%), Sejroe (116 identifications; 5.2%), Canicola (87 identifications; 3.9%), and Pomona (45 identifications; 2.0%). All infections with serogroup Canicola occurred during the first 50 years of the study, and there was a peak during 1945–1954. Serogroup Icterohaemorrhagiae appeared to be the major cause of fatal leptospirosis, followed by serogroup Canicola (Table 3). Within the group of patients infected with serogroup Icterohaemorrhagiae, 90 patients died (CFR 5.3%). This number represents 94.7% of the patients who died for whom the infecting serogroup is known.

The CFR for patients infected with serogroup Canicola was 3.2%. None of the patients infected with serogroup Pomona died, although the percentages of hospitalization, ICU admission, and dialysis treatment were higher among these patients. Because all patients infected with serogroup Canicola were observed before 1967, dialysis and ICU treatment were not available for these patients, but absence of these treatments does not indicate milder clinical illness. Serogroups Grippotyphosa and Sejroe appeared to cause less severe disease, although 1 patient infected with serogroup Sejroe died (Table 3). Fatal leptospirosis can have a rapid, fulminate course, which often makes identification of infecting serogroups impossible. Information for the causative serogroup was available for only 95 of the 166 patients who died. The average CFR was 4.2% for patients in whom the infecting serogroup was determined and 23.7% for patients in whom the infecting serogroup was not identified.

Autochthonous versus imported leptospirosis

In the Netherlands, the total number of leptospirosis patients infected outside the country through 2008 was 318 (12.5% of all reported patients). The annual proportion of imported

leptospirosis cases has gradually increased over time; >50% of all infections during 2005–2008 were acquired outside the Netherlands (Table 4). In the early years of the study, a substantial proportion of imported infections occurred after exposure in other countries in Europe, mainly during vacations. Since the mid-1970s, the number of leptospirosis infections acquired outside Europe has increased markedly, mostly from exposures in Asia (134 cases; 42.1%), notably Thailand (Table 4). More than 80% of the imported leptospirosis infections were associated with water-related sport and adventure activities, such as whitewater rafting. In contrast, 44.4% of the autochthonous infections were attributed to recreational activities (Table 1), 29.8% were attributed to occupational exposures, and 15.5% were attributed to accidents. Except during 1985–1994, the ratio of infections related to recreational activities, occupations, and accidents has remained similar over time.

Sex differences

Of 2,532 patients, 2,306 (91.1%) were male patients and 226 (8.9%) were female patients (Table 5). On the basis of CFR data, male patients were more likely to have had a more severe leptospirosis infection; 160 male patients died (CFR 6.9%) and 6 female patients died (CFR 2.7%) (Table 2). In addition, a higher percentage of male patients were treated with dialysis (Table 5). The greater severity of leptospirosis among male patients does not appear to be attributable to infections caused by more virulent serovars: 67.2% of male patients were infected with serogroup Icterohaemorrhagiae compared with 61.1% of female patients ($p = 0.06$). However, male patients were older than female patients: mean (SD) was 34.5 (17.0) years for male patients and 26.4 (16.5) years for female patients ($p<0.001$).

Table 4 | Characteristics for case-patients with leptospirosis, the Netherlands and other regions, 1925–2008*

	Period								
Characteristic	1925-1934	1935-1944	1945-1954	1955-1964	1965-1974	1975-1984	1985-1994	1995-2004	2005-2008
No. case-patients	289	352	271	434	259	159	379	281	129
Hospitalized, %	37.4	68.8	78.6	92.1	83.8	84.3	62.5	72.6	74.4
Antimicrobial drug treatment, %	NA	NA	53.3	63.8	61.8	57.9	72.3	82.9	83.7
Dialysis, %	NA	NA	0	2.0	5.0	17.6	8.2	10.0	12.4
ICU, %	NA	NA	0	0.0	0.0	7.5	6.6	16.4	17.8
CFR %	11.1	9.9	9.6	6.0	8.1	3.8	4.0	0.4	3.1

Table 4 | Continued

	Period								
Characteristic	1925-1934	1935-1944	1945-1954	1955-1964	1965-1974	1975-1984	1985-1994	1995-2004	2005-2008
Imported infections	0	2 (0.6)	3 (1.1)	20 (4.6)	22 (8.5)	17 (0.7)	92 (24.3)	94 (33.5)	68 (53.1)
Europe	0	2	2	19	20	9	40	27	13
Asia	0	0	1	0	1	4	38	48	42
Sub-Saharan Africa	0	0	0	0	0	0	2	4	1
South America	0	0	0	0	1	4	6	7	7
Central and North America	0	0	0	0	0	0	2	6	5
Middle East	0	0	0	1	0	0	0	1	0
Australia	0	0	0	0	0	0	0	1	0
Likely route of infection									
Water									
autochthonous	222 (92.8)	251 (82.3)	194 (78.7)	333 (87.4)	189 (88.3)	111 (79.9)	157 (60.4)	162 (88.5)	46 (79.3)
Imported	NA	2 (100)	2 (66.7)	18 (94.7)	19 (95.0)	15 (93.8)	84 (98.8)	89 (100.0)	60 (98.4)
Animal									
autochthonous	15 (6.3)	52 (17.0)	48 (19.5)	44 (11.5)	25 (11.7)	27 (19.4)	103 (39.6)	21 (11.5)	11 (19.0)
Imported	NA	0	0	1 (5.3)	1 (5.0)	1 (6.2)	1 (1.2)	0	0
Other									
autochthonous	2 (0.8)	2 (0.7)	4 (1.6)	4 (1.0)	0	1 (0.7)	0	0	1 (1.7)
Imported	NA	0	1 (33.3)	0	0	0	0	0	1 (1.6)

*Values are no. (%) except as indicated. ICU, intensive care unit; CFR, case-fatality rate; NA, not available

Table 5. Characteristics for 2,532 case-patients with leptospirosis, by sex, the Netherlands, 1925–2008*

Characteristic	Male patients, n=2,306	Female patients, n=226	p value
Mean age, y (SD)	34.5 (17.0)	26.4 (16.5)	<0.001
Patients with imported cases	278 (12.1)	40 (17.8)	0.014
Type of contact			
Recreational activity	1,086 (47.1)	162 (71.7)	0.001
Job-related	666 (28.9)	18 (8.0)	< 0.001
Accident	339 (14.7)	27 (11.9)	0.235
Unknown	215 (9.3)	19 (8.4)	
Treatment course†			
Hospitalization	1,683 (73.0)	163 (72.1)	0.781
Dialysis§	118 (9.3)	1 (1.1)	0.007
ICU¶	102 (6.7)	4 (3.4)	0.063
Death†	160 (6.9)	6 (2.6)	0.013

*Values are no. (%) unless otherwise indicated. Data for age and sex were available for 2,422 patients, and data for autochthonous or imported infections and sex were available for 2,528 patients. ICU, intensive care unit.
†Percentages were calculated from available data (missing values were regarded as no). It was assumed that analysing data that contained large amounts of missing values would result in findings that could not be generalized and lead to substantial overestimated values.
§Dialysis data were available for 795 of 1,360 patients (732 of 1,263 male patients, 63 of 93 female patients) (January 1961 to December 2008). Before 1961, dialysis was not expected to be used.
¶ICU data were available for 625 of 1,641 patients (573 of 1,518 male patients, 52 of 119 female patients) (April 1955 to December 2008). Before 1955, ICU was not expected to be used.

CONCLUSIONS

Leptospirosis is endemic to the Netherlands. During the study period, the average incidence was 0.25 cases/100,000 population. The reported incidence probably reflects the more severe end of the clinical spectrum for leptospirosis because mild forms of this disease are more likely to go unrecognized[1,8,18,19]. In the 84 years covered by this study, there were 5 years with notably increased annual incidences. The peaks in 1932 and 1941 coincided with the global economic depression and compulsory reporting of leptospirosis (1932)[20] and with World War II (1941). The increase in 1988 was associated with a dairy fever outbreak among farmers who were infected while handling leptospirosis-infected cattle[18,21]. No specific situations or events are known to be associated with the peaks in 1961 and 1968.

The overall CFR of 6.5% was high and exceeded CFRs reported in some countries with a higher prevalence of leptospirosis[3,4,22,23]. The CFR decreased over the analysis period, probably

because of improved treatments. No deaths were recorded during 1995–2005. However, this finding was probably caused by underreporting because transient stringent privacy regulations hampered identification of deaths. Thus, the actual average CFR might be higher than calculated for years after 1999.

An explanation for the high CFR might be that most (71.2%) autochthonous leptospirosis cases identified in the Netherlands were caused by more virulent serovars of serogroup Icterohaemorrhagiae; infections with this serogroup are less common among imported cases. Another reason might be that clinicians in the Netherlands are more proactive in treating severe leptospirosis in travelers than in persons with locally acquired infections. Potentially fulminant leptospirosis leading to rapid death, in combination with limited diagnostic potential in countries to which this disease is endemic, often prevents confirmation of leptospirosis cases[24] and might lead to underestimation of the CFR[3,4]. Serogroups Grippotyphosa, Sejroe, Canicola, and Pomona frequently cause leptospirosis in the Netherlands. Infections with serovar Hardjobovis in serogroup Sejroe were found mainly in dairy fever cases among farmers from 1985 until early 1990. Infections with serogroup Pomona were more recent and mostly caused by the newly identified serovar Mozdok[25].

Infections with serogroup Canicola were not found after 1966. This finding was probably caused by introduction of bivalent Copenhageni/Canicola canine vaccines. Dogs are the reservoir of serovar Canicola, and vaccination interfered with the transmission cycle, resulting in elimination of serovar Canicola infections in the dog population in the Netherlands[26]. Lack of dialysis and ICU treatments in patients infected with serogroup Canicola was mainly caused by absence of these treatments and does not indicate less severe or underestimated symptoms.

Recreational activities accounted for most (44.4%) autochthonous leptospirosis cases in this analysis, and ≈80% of imported infections were acquired during recreational water contact. Our data indicate that infections acquired during holidays in tropical countries are increasing. An increase in the incidence of leptospirosis related to exposures in tropical countries has been reported[9]. Therefore, clinicians should consider leptospirosis in the differential diagnosis for patients with a febrile illness and a history of travel abroad.

A total of 15.5% of autochthonous and 6.9% of imported cases were reportedly caused by accidents with water exposure, indicating a need to further study this issue[22]. In Germany accidental exposure has been reported and represented 3% of all reported cases during 1997–2000[9].

The total incidence of leptospirosis in the Netherlands showed a small decrease over the 84-year study period. A decreasing trend in incidence has also been observed in France by Baranton and Postic[10], albeit, more pronounced. These authors attribute the decrease to

changes in lifestyle and the rural environment. In the Netherlands, overall incidence is not decreasing as rapidly because of the increase in imported cases. The decrease in autochthonous infections in the Netherlands may reflect the success of the surveillance system and associated dairy control measures and vaccination of dogs, as shown by elimination of infections with serogroup Canicola since 1974[12,18,21].

During the study period, most leptospirosis cases in the Netherlands were in male patients. However, surveillance systems based on passive reporting are biased toward including more severe cases, which are found more often in male patients[27]. Therefore, if milder symptoms develop in female patients, these cases might also be more likely to be underdiagnosed. Certain occupations are more likely to be performed by men, and work-related exposure differences might contribute to the disproportional number of male patients given a diagnosis of leptospirosis. However, similar exposure risks during travel do not necessarily indicate similar rates of disease for persons of both sexes[5,9]. In the Netherlands, risky vacation activities are found equally among men and women. However, these activities do not indicate an equal distribution of leptospirosis in men and women.

In addition, the disparity in the incidence of leptospirosis by sex of the patient was unlikely to be caused by differences in infecting serogroups because similar proportions of male and female patients were infected with serogroup Icterohaemorrhagiae. Therefore, we propose that sex of the patient might play a role in disease progression, which might influence the likelihood of diagnosis and reporting. Differences in health care–seeking behavior between male and female patients might also play a major role[28]. However, genetic and physiologic differences that may affect disease manifestations in men should also be considered[29–31]. Further research is needed to substantiate this hypothesis.

Our analysis has several major limitations. Use of passive surveillance data probably underestimates the total number of infections because mild cases of leptospirosis are less likely to be diagnosed. Furthermore, nationwide access to laboratory confirmation of leptospirosis has changed over time. In the early years of the study period, many features of the surveillance system were different, including diagnostic methods available, knowledge of existing serovars, collation of data, and clinician awareness of leptospirosis. However, the early data have contributed to our understanding of changes in leptospirosis over time because this dataset is comprehensive and includes an entire national cohort for >80 years.

We conclude that the effective surveillance system in the Netherlands, combined with adequate control measures, has reduced the incidence of leptospirosis in this country. Efforts to prevent imported infections should include providing better information on risks to travelers and greater awareness by clinicians about development of leptospirosis in persons with a history of travel abroad.

ACKNOWLEDGMENTS

We thank Stella van Beers, Birgit van Benthem, Mirjam Bakker, Pauline Scheelbeek, Merel Lammers van Toorenburg, Andre Smorenburg, Esther van der Meer, Louise van Mourik, and Vanessa Kikken for help with data entry and analysis and for statistical advice.

REFERENCES

1. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control; 2003.
2. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001;**14**(2):296–326.
3. Agampodi S, Peacock SJ, Thevanesam V. The potential emergence of leptospirosis in Sri Lanka. *Lancet Infect Dis* 2009; **9**(9):524–6.
4. McCurry J. Philippines struggles to recover from typhoons. *Lancet* 2009; **374**(9700):1489.
5. Meites E, Jay MT, Deresinski S, Shieh WJ, Zaki SR, Tompkins L, *et al*. Reemerging leptospirosis, California. *Emerg Infect Dis* 2004; **10**(3):406–12.
6. Vijayachari P, Sugunan AP, Shriram AN. Leptospirosis: an emerging global public health problem. *J Biosci*. 2008; **33**(4):557–69.
7. Hartskeerl RA. Leptospirosis: current status and future trends. India*n J Med Microbiol* 2006; **24**:309.
8. World Health Organization. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group, 2011.
9. Jansen A, Schoneberg I, Frank C, Alpers K, Schneider T, Stark K. Leptospirosis in Germany, 1962–2003. *Emerg Infect Dis* 2005; **11**(7):1048–54.
10. Baranton G, Postic D. Trends in leptospirosis epidemiology in France. Sixty-six years of passive serological surveillance from 1920 to 2003. *Int J Infect Dis* 2006; **10**(2):162–70.
11. van Vliet J. History of notification [in Dutch]). *Tijdschrift voor Infectieziekten* 2009; **4**(2):51–60.
12. Hartskeerl RA. Leptospiroses [in Dutch]. In: van Steenbergen J, Timen A, Beaujan DJ, eds. LCI guidelines infectious disease control. Bilthoven (the Netherlands):LCI, Coordinator Infectious Disease Netherlands; 2008.
13. Martin L, Pettit A. Sero-diagnosis of the icterohaemorrhagic spirochetosis [in French]. *Bull Mem Soc Med Hop Paris* 1918; **42**:672–5.
14. Wolff J. The laboratory diagnosis of leptospirosis. In: Dalldorf G, editor. American lectures in tests and techniques. Springfield (IL): Charles C. Thomas; 1954.
15. Cole JR Jr, Sulzer CR, Pursell AR. Improved microtechnique for the leptospiral microscopic agglutination test. *Appl Microbiol* 1973; **25**(6):976–80.
16. Terpstra WJ, Ligthart GS, Schoone GJ. ELISA for the detection of specific IgM and IgG in human leptospirosis. *J Gen Microbiol* 1985; **131**(2):377–85.
17. Levett PN. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clin Infect Dis* 2003; **36**(4):447–52.
18. Hartskeerl RA, Goris MG. Leptospirosis in 2006: almost half of the cases were contracted in the Netherlands [in Dutch]. *Infectieziekten Bulletin* 2007; **18**:402–3.
19. Hartskeerl RA, Collares-Pereira M, Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clin Microbiol Infect*2011; **17**(4):494–501.

20. Schüffner W. Recent work on leptospirosis. *Trans R Soc Trop Med Hyg* 1934; **28**(1):7–31.

21. Goris MG, Hartskeerl RA. Leptospirosis: rat disease or not? [in Dutch]. *Analyse* 2008; **62**(10):295–8.

22. Leptonet [cited 2011 Aug 16]. http://www.leptonet.net/

23. Zaki SR, Shieh WJ. Leptospirosis associated with outbreak of acute febrile illness and pulmonary haemorrhage, Nicaragua, 1995. The Epidemic Working Group at Ministry of Health in Nicaragua. *Lancet* 1996; **347**(9000):535–6.

24. Speelman P, Hartskeerl RA. Leptospirosis. In: Fauci AS, Braunwald E, Kasper DL, Hauser SL, Longo DL, Jameson JL, *et al*., eds. *Harrison's internal medicine*; 2008. p. 1048–51.

25. te Gussinklo JT, Venker R, Bode AD, Schuurman JL, van der Werf D, van Kuijk SJ, *et al*. Leptospirosis caused by serovar Pomona [in Dutch]. *Infectieziekten Bulletin* 1998; 9(7):165–8.

26. Houwers DJ, Goris MG, Abdoel T, Kas JA, Knobbe SS, van Dongen AM, *et al*. Agglutinating antibodies against pathogenic *Leptospira* in healthy dogs and horses indicate common exposure and regular occurrence of subclinical infections. *Vet Microbiol* 2011; **148**(2-4):449–51.

27. Notifiable disease surveillance and notifiable disease statistics— United States, June 1946 and June 1996. *MMWR Morb Mortal Wkly Rep* 1996; 45:530–6.

28. Tolhurst R, de Koning K, Price J, Kemp J, Theobald S, Squire SB. The challenge of infectious disease: time to take gender into account. *Journal of Health Management* 2002; **4**(2):135–51.

29. Hubacek JA, Stuber F, Frohlich D, Book M, Wetegrove S, Ritter M, *et al*. Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide binding protein in sepsis patients: gender-specific genetic predisposition to sepsis. *Crit Care Med* 2001; **29**(3):557–61.

30. Schlagenhauf P, Chen LH, Wilson ME, Freedman DO, Tcheng D, Schwartz E, *et al*. Sex and gender differences in travel-associated disease. *Clin Infect Dis* 2010; **50**(6):826–32.

31. Jansen A, Stark K, Schneider T, Schoneberg I. Sex differences in clinical leptospirosis in Germany: 1997–2005. *Clin Infect Dis* 2007; **44**(9):e69–72.

CHAPTER 3

Marked increase in leptospirosis infections in humans and dogs in the Netherlands, 2014

Roan Pijnacker[1]
Marga G.A. Goris[2]
Margreet J.M. te Wierik[1]
Els M. Broens[3]
Joke W.B. van der Giessen[1,4]
Mauro de Rosa[1,5]
Jaap A. Wagenaar[3,4]
Rudy A. Hartskeerl[2]
Daan W. Notermans[1]
Kitty Maassen[1]
Barbara Schimmer[1]

1. Centre for Infectious Disease Control (CIb), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
2. Leptospirosis Reference Centre, Royal Tropical Institute (KIT), KIT Biomedical Research, Amsterdam, the Netherlands
3. Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands
4. Central Veterinary Institute of Wageningen University and Research Center (WUR), the Netherlands
5. Veterinarian Incident and Crisiscentre, Food and Consumer Product Safety Authority (NVWA), the Netherlands

Accepted Eurosurveillance

ABSTRACT

In the Netherlands, 60 autochthonous cases of leptospirosis were notified in 2014, mostly during June to November. This represents a 4.6-fold increase in autochthonous cases compared to 2010-2013. This marked increase in humans coincided with an increase of leptospirosis in dogs. The majority of the autochthonous cases were linked to recreational exposure, such as during swimming and fishing. A possible explanation for this increase is the preceding mild winter of 2013-2014 followed by the warmest year in three centuries, possibly enabling rodents and *Leptospira* to better survive. Also a slight increase of imported leptospirosis was observed in Dutch tourists that mostly acquired the infection in Thailand. More awareness and early recognition of this mainly rodent-borne zoonosis by medical and veterinary specialists is warranted.

BACKGROUND

Leptospirosis is a zoonosis caused by pathogenic *Leptospira* species and may result in a broad clinical spectrum of disease, ranging from asymptomatic infections to severe disease manifestations known as Weil's syndrome, characterized by the triad of jaundice, acute renal failure and bleeding manifestations, and severe pulmonary haemorrhage syndrome (SPHS) with a high case fatality rate[1-3]. Transmission to humans usually occurs via direct or indirect contact with urine of infected animals. A wide variety of animal species, primarily mammals such as rodents, cattle and dogs, may serve as a reservoir of leptospires[1]. The usual port of entry is the skin via abrasions or cuts but infection may also occur via the conjunctiva[2]. In dogs, leptospirosis can cause severe, life threatening infections with vascular damage, liver and renal failure and more recently pulmonary symptoms were reported as well[4]. There are nearly 300 pathogenic *Leptospira* serovars, often specific for particular host reservoirs, belonging to 29 serogroups, and therefore an indication for the most likely source of human infections[2].

In the Netherlands, leptospirosis is a mandatory notifiable disease in humans since 1928[5]. It mainly occurs as a sporadic disease and is primarily caused by two serogroups of *Leptospira* spp.; Icterohaemorrhagiae (serovars Icterohaemorrhagiae and Copenhageni) with rats as reservoir and Grippotyphosa (serovar Grippotyphosa type Duyster) from mice. In animals, only leptospirosis caused by *Leptospira borgpetersenii* serovar Hardjo is a notifiable disease. In the late 1980s and early 1990s, dairy cattle was a major source of serovar Hardjo[6]. Due to an effective control and monitoring programme in the 1990s, serovar Hardjo became rare in Dutch cattle[7], resulting in a marked decrease in autochthonous dairy farm fever (Hardjo) cases[8]. Since 2000, approximately 30 human leptospirosis cases were diagnosed annually in the Netherlands, mostly associated with recreational exposures[6,9]. Leptospirosis has an annual peak incidence occurring in late summer and fall in temperate regions like the Netherlands[2]. Due to increasing globalization, the proportion of imported human cases

gradually increased over time. Most of them acquired leptospirosis outside Europe, mainly in countries in Southeast Asia[6].

In September 2014, an increase in notified leptospirosis cases was observed by the National Leptospirosis Reference Centre (NRL), which alerted the National Institute of Public Health and the Environment (RIVM) as part of their national reference tasks. The NRL, which is also WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, shared this alert with the WHO Collaborating Centre on Leptospirosis in France, which, in turn, confirmed a coinciding increase in leptospirosis in mainland France. They posted their joint findings in an urgent inquiry in the Epidemic Intelligence Information System for Food and Waterborne Diseases (UI-272, EPIS) on 31 October 2014. In parallel, an increase in confirmed leptospirosis in dogs and inquiries by veterinarians about suspected cases was noted by the Dutch Veterinary Microbiological Diagnostic Center in October 2014. In this report we combined all available data to describe this marked increase in leptospirosis infections in humans and dogs, and provide case characteristics such as symptoms, travel history, possible sources of exposure and serogroup information.

METHODS

We used surveillance reports stored in the national surveillance database at the National Institute for Public Health and the Environment (RIVM). Clinicians and general practitioners sent clinical specimens of patients suspected for leptospirosis to the National Leptospirosis Reference Centre (NRL) for laboratory evaluation using microscopic agglutination test (MAT) and an in-house developed IgM-ELISA for diagnostic confirmation based on detection of antibodies. When patient serum is collected before the 11th day after date of symptom onset, tests to detect leptospiral antigen (culture and Polymerase Chain Reaction (PCR)) are performed as well; on urine PCR is always performed because leptospiral DNA can be detected in urine at all stages of the disease. The presumptive serogroup was deduced from the highest MAT titer with a pathogenic serovar in a follow-up sample. A case of leptospirosis is considered confirmed positive for *Leptospira* by culture and/or PCR and/or serology (MAT or IgM ELISA) and has fever or at least 2 of the following symptoms: rigors, headache, myalgia, running eyes, bleeding in skin and mucosa, rash, jaundice, myocarditis, meningitis, renal failure or pulmonary hemorrhagic symptoms.

Patients with confirmed leptospirosis are reported by the NRL to the Municipal Health Service that collects case characteristics, performs source tracing and, if needed, instigates control measures[3]. Detailed travel history in the month before date of symptom onset and the most likely source of infection determine whether a case is classified as autochthonous or imported. The Municipal Health Service notifies each laboratory confirmed case that adheres to the clinical case definition to the national surveillance database at the RIVM[3]. The

Municipal Health Service also notifies autochthonous cases to the Dutch Food and Consumer Product Safety Authority (NVWA) if site investigation is necessary, for instance if a petting farm is suspected as source of human infection[10].

For this study, we compared all notified leptospirosis cases in 2014 with diagnosed patients in the NRL patient database based on birth year, sex and 4-digit postal code, for completeness and confirmation of serogroup details and laboratory method. Case characteristics such as date of symptom onset, symptoms, travel history, relevant exposures and serogroup information were analyzed. Diagnostic delay is defined as the median time period between day of symptom onset and laboratory confirmation by NRL. The Veterinary Microbiological Diagnostic Center (VMDC) receives sera of dogs in the Netherlands showing clinical signs of leptospirosis which are confirmed by a combination of IgM and IgG-ELISA[11]. No information is available about the infecting serogroups in dogs. The VMDC also acts as an information desk for Dutch veterinary practitioners about dogs suspected for leptospirosis, for which received phone calls are registered. A voluntary serovar Hardjo-free programme of dairy cattle was started by GD Animal Health in 1995. Farmers could obtain a leptospirosis free status by testing of bulk milk from dairy herds or (slaughterhouse) blood samples from non-dairy herds using ELISA. In 2003, they implemented a nationwide system for animal health surveillance, including leptospirosis.

RESULTS

Humans

In 2014, a total of 97 human cases (incidence 0.57/100,000 inhabitants) were notified in the Netherlands (Figure 1, Table 1). Twenty-five cases tested positive based on serology and culture or PCR. Thirty-three cases tested positive for culture or PCR and 39 cases only had positive serology. The majority of these cases (60/97; 62%) were autochthonous as they most likely contracted the infection in the Netherlands, representing a 4.6-fold increase compared to 2010-2013. Most of them became symptomatic from June to November, with a peak in August. The rise was one month earlier compared to 2010-2013. Also a 1.6 fold increase (33/97; 34%) in imported cases was observed. Country of infection was unknown for 4 cases. The median age was 48 years (range: 10-75 years) and 42 years (range: 13-64 years) for autochthonous cases and imported cases, respectively. The majority of autochthonous (49/60; 82%) and imported cases (26/33; 79%) were male. Autochthonous cases occurred sporadically based on the 4-digit postal code of their residential address and were mainly resident in the Western (28/60; 47%) and Eastern (20/60; 33%) regions of the Netherlands. A small proportion was resident in the Northern (9/60; 15%) and Southern (2/60; 3%) region (Figure 2). Imported cases were mainly resident in the agglomerated Western region (24/33; 73%) of the Netherlands.

Table 1 | Characteristics of autochthonous (n=60) and imported (n=33) leptospirosis cases in 2014 (column percentages), the Netherlands

Characteristics	Autochthonous (%)	Import (%)
Male sex	49 (82)	26 (79)
Median age in years (range)	48 (10-75)	42 (13-64)
Region		
North	9 (15)	1 (3)
West	28 (47)	24 (73)
East	20 (33)	5 (15)
South	2 (3)	3 (9)
Other[a]	1 (2)	-
Type of exposure		
Recreational	20 (33)	29 (89)
Swimming	10	12
Fishing	5	0
Water sports	2	8
Water contact[b]	3	9
Occupational	15 (25)	0
Farmer	6	-
Dredging	2	-
Rat catcher	1	-
Gardener	1	-
Handyman	1	-
Kite surf instructor	1	-
Water management	1	-
Sheet piling	1	-
Police trainee	1	-
Residential	11 (18)	-
Gardening	3	-
Rat/mice presence around home	3	-
Cleaning pond	2	-
Pet mice	1	-
Water/mud	1	-
Not specified	1	-
Accidental	7 (12)	-
Fell in water	4	-
Bite	3	-
Not specified	7 (12)	4 (11)

Table 1 | Continued

Characteristics	Autochthonous (%)	Import (%)
Most likely route of infection		
Surface water	37 (62)	29 (88)
Ditch	9	0
Lake	9	4
Canal/river	7	9
Pond	2	0
Indoors	2	0
Unknown	8	16
Animal	13 (22)	0
Rat	8	-
Mouse	2	-
Cow	1	-
Not specified	2	0
Soil	4 (7)	4 (12)
Unknown	6 (10)	
Rat presence reported		
Yes	21 (35)	na
No	18 (30)	na
Not reported	21 (35)	na
Serogroup	n=26	n=8
Icterohaemorrhagiae	9	1
Grippotyphosa	8	0
Javanica	3	0
Sejroe/Hebdomadis/Mini	2	0
Sejroe	2	1
Mini	1	1
Pomona	1	0
Australis	0	2
Cynopteri	0	1
Celledoni	0	2

[a]Not a Dutch resident

[b]Multiple types of water contact or type of water contact not further specified

Symptoms and hospitalization

Among cases for whom symptoms were reported, fever was the most frequently reported symptom 79/86 (92%). Other symptoms reported were, in order of prevalence, myalgia, headache, rigors, renal failure, jaundice (Table 2). Autochthonous cases more often presented with renal failure, jaundice and hemorrhagic symptoms compared to imported cases. Meningitis was reported in one autochthonous case and myocarditis in one imported case. Ninety percent (54/60) of the autochthonous and 70% (23/33) of the imported cases were hospitalized. No deaths were reported. The diagnostic delay was 15 days (range: 3-50 days)

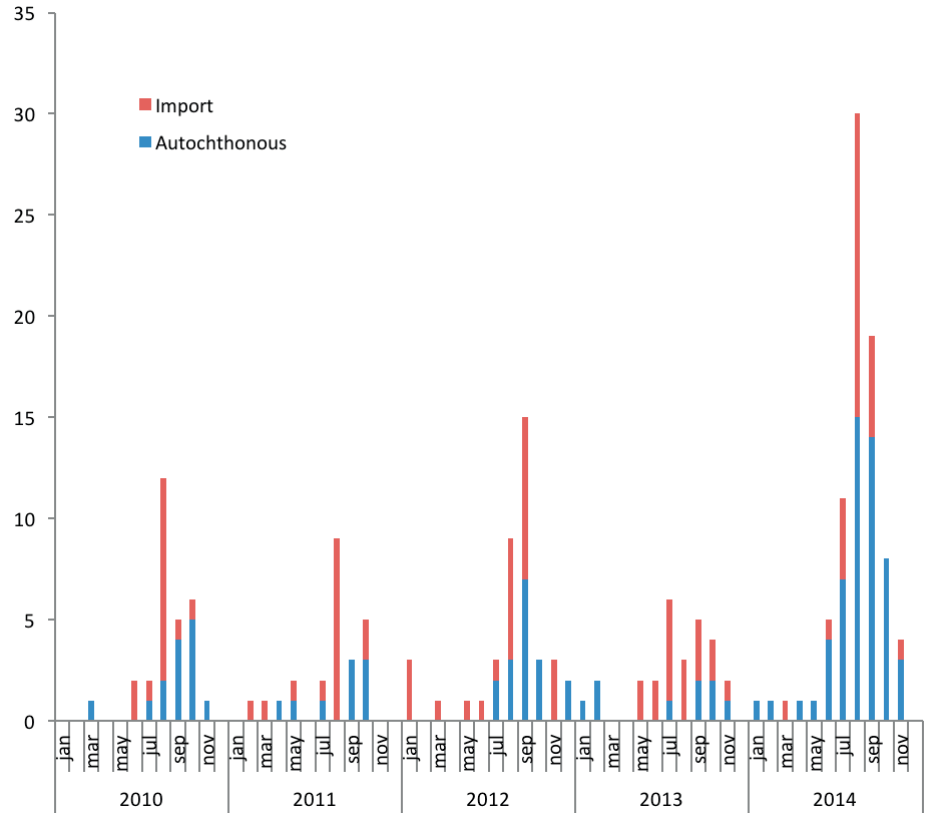


Figure 1 | Autochthonous and imported cases of leptospirosis based on month of illness onset, 2010-2014, the Netherlands

Table 2. Main symptoms, hospitalization and median diagnostic delays of autochthonous (n=60) and imported (n=33) leptospirosis cases in 2014, the Netherlands

	Autochthonous (%)	**Import (%)**
Symptoms	n=56	n=30
Fever	51 (91)	28 (93)
Myalgia	35 (63)	21 (70)
Rigors	31 (55)	17 (57)
Headache	26 (46)	25 (83)
Renal failure	21 (38)	6 (20)
Jaundice	17 (30)	4 (13)
Skin rash	8 (14)	5 (17)
Hospitalization		
Yes	54 (90)	23 (70)
No	4 (7)	7 (21)
Unknown	2 (3)	3 (9)
Median diagnostic delay in days (range)	15 (3-50)	12 (1-49)

Figure 2 | Geographical distribution of autochthonous and imported cases based on postal code of residence, 2014, the Netherlands

for autochthonous cases and 12 days (range: 3-49 days) for imported cases. From 2010 to 2013, the diagnostic delay was 14 days (range: 5-64 days) for autochthonous cases and 21 days (range: 3-84 days) for imported cases.

Serogroups

Among the autochthonous cases, 26/60 cases allowed the presumptive deduction of the infecting serogroup based on MAT titers; i.e. Icterohaemorrhagiae (9/26), Grippotyphosa (8/26), Javanica (3/26), Sejroe/Hebdomadis/Mini complex (2/26), Sejroe (2/26), Mini (1/26) and Pomona (1/26). Among imported cases, the presumptive serogroup could be deduced for 8/33 cases: Australis (2/8), Sejroe (1/8), Mini (1/8), Icterohaemorrhagiae (1/8), Cynopteri (1/8) and Celledoni (2/8). For the remaining 59 cases (63%), the serogroup could not be determined, mostly because no follow-up serum sample was received.

Country of infection

Imported cases mainly acquired leptospirosis in countries in Southeast-Asia, of which 55% (18/33) in Thailand. Other countries were Cuba (3 cases), Cambodia and Sri Lanka (each 2 cases), Indonesia, Laos, Malaysia, Nepal, Costa Rica, Guatemala, Suriname and France (each 1 case).

Transmission route and presence of rodents

Autochthonous cases mainly acquired leptospirosis during recreational activities (20/60) such as swimming (10/40) and fishing (5/40), followed by occupational activities (15/60), mostly observed among farmers (6/15). Cases also contracted leptospirosis during activities at their place of residence (11/60) such as gardening (3/11), and due to accidents (7/60), which included patients who fell in water (4/7) or were bitten by a mouse (3/7). About sixty percent (37/60) of the autochthonous cases were most likely attributable to surface water contact, including contact with water in ditches (9/29), lakes (9/29), canal/rivers (7/29), ponds (2/29) and indoor surface water (e.g. water in basement) (2/29). Direct animal contact (13/60), including rats (8/13), mice (2/13) and cows (1/13), and soil contact (4/60) were also reported. Thirty-five percent (21/60) reported rats or mice presence at the most probable site of infection. Imported cases were almost all attributable to contact with surface water (29/33) and contracted the disease during recreational activities (29/33) such as swimming (12/29) or other water sports (8/29).

Source investigations based on notified human cases

The NVWA received 26 notifications of autochthonous cases in 2014, mostly from a Municipal Health Service, accompanied with a request for animal source investigation. In 9 notifications, site investigations were performed, and if necessary animal or environmental samples were collected. In two site investigations, animal samples were found positive for *Leptospira* antibodies.

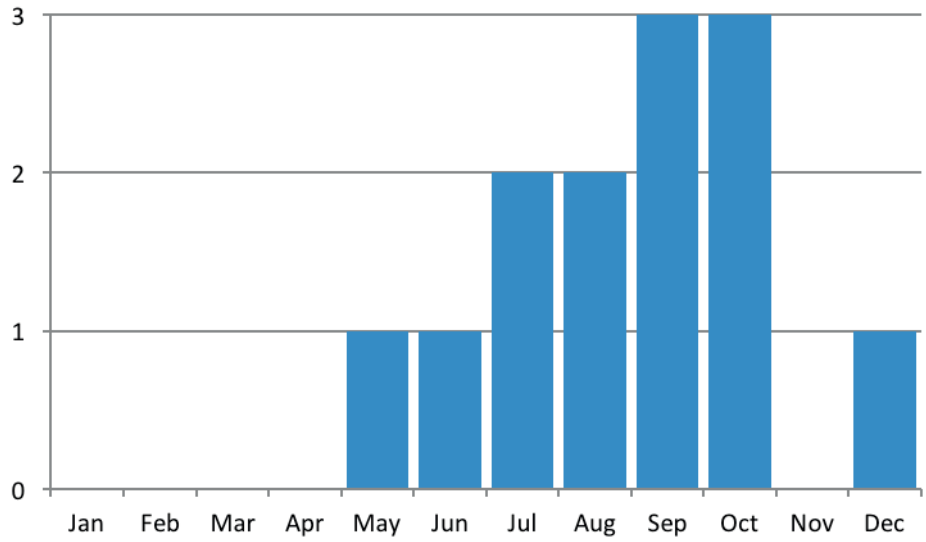


Figure 3 | The number of dogs diagnosed with leptospirosis by the Veterinary Microbiological Diagnostic Center (VMDC) based on month of diagnosis, 2014, the Netherlands

In August 2014, serovar Hardjo was identified in a Dutch farmer. Bulk milk of his cattle was tested positive for the presence of *Leptospira* antibodies using ELISA in March 2014. In April 2014, antibiotics were administered to cattle of which blood samples were tested positive for leptospirosis using ELISA. However, before antibiotic treatment cleared the infection, leptospirosis was further transmitted among dairy cattle, which most likely infected the farmer during milking in June 2014. Investigation by the NVWA revealed that his initially infected cattle most likely acquired the infection via German cattle, since they accidently grazed on the same pasture at the same time.

The second source investigation included a carp farmer positive for leptospirosis in November, who reported a rat infestation at his farm. A captured rat was tested PCR positive by the NRL. Culture and further characterization did not succeed, but the PCR melting curve results of the farmer and rat samples were similar and matched with *L. interrogans*.

Dogs

The VMDC reported 13 dogs with leptospirosis in 2014, mostly diagnosed between June and October (Figure 3). From 2010 to 2013, a median of 4.5 (range 2 to 6) dogs were diagnosed annually according to VMDC. The number of inquiries on suspected leptospirosis in dogs doubled in 2014 (n=54) compared to 2013 (n=24).

DISCUSSION

A marked increase in autochthonous cases of leptospirosis was observed in the Netherlands in 2014, particularly during the second half of the year, from June until November, resulting in one of the highest incidence rates in Europe[12].

Cases mainly acquired leptospirosis during recreational activities such as swimming and fishing, in contrast with other western-European countries, where autochthonous leptospirosis infections are predominantly associated with occupational activities[13-15]. A possible explanation for the increase of autochthonous cases is the preceding mild winter of 2013-2014 followed by the warmest year in three centuries in Europe[16,17], possibly enabling rodents and also excreted *Leptospira* to better survive[2,18,19]. Warm weather might also be related to increased outdoor recreational activities due to the early high temperatures in spring 2014, leading to more exposure, and an earlier seasonal rise in cases than the normal seasonal trend[20]. The increase in autochthonous cases supports a recent French study[13] hypothesizing an increase in leptospirosis burden in European countries due to global warming, increasing populations of urban rodents or other animal reservoirs[21], human population growth, urbanization and increasing international travels. Germany also noted a similar increase in autochthonous cases in 2014, which they likewise attributed to a humid and warm climate[22]. In the Netherlands, the number of imported cases was also elevated but to a lesser extent. This might be due to increased awareness for leptospirosis in Dutch travelers among medical specialists, indicated by the decreased diagnostic delay compared to 2010-2013.

In 2014, serogroup Sejroe/Hebdomadis/Mini complex was identified in two autochthonous cases in the Netherlands, which is remarkable as this serogroup has only been identified in one autochthonous case before in 1998[6]. One of them acquired leptospirosis after being bitten by a mouse intended feeding a snake and the other case had multiple possible sources of infection. For the first time in 16 years, serovar Hardjo was identified in a dairy cattle farmer in the Netherlands. This was surprising, because 99% of the dairy and beef cattle farms in the Netherlands had a Hardjo-free status in 2014[7]. However, source investigations revealed that he most likely acquired the infection via German cattle, in which serovar Hardjo is common[14].

Remarkably, although based on small numbers, is the concomitant increase in canine cases in the second half of 2014, strengthening the hypothesis of increased environmental exposure. A recently started monitoring programme in rodents in 2014 revealed that *Leptospira* are present and widespread in the rat population in the Netherlands (data not shown, personal information Joke van der Giessen, December 2014).

A major limitation of this study was the use of passive human surveillance data, likely reflecting the more severe hospitalized cases which leaves milder cases often unrecognized[1,23,24]. This

should be taken into account when interpreting the clinical presentation of cases described in this article. Also the number of canine leptospirosis cases is likely to be underestimated, as it depends on the veterinary clinicians' ability to identify leptospirosis in dogs. Unfortunately, the infecting serogroup based on MAT titers could only be presumed in less than half of the cases, because often follow-up samples were not received.

The results suggests that prevention efforts should be aimed at advising the general public and high risk occupational groups having direct or indirect contact with urine of rats or mice, about possible precautions to reduce exposure to *Leptospira*. In the future, monitoring programmes in rodents should focus at predicting risk of zoonotic transmission and developing preventive strategies[9]. Furthermore, vaccination of dogs should be promoted in the Netherlands, of which currently approximately 55% is vaccinated[9]. Preventive measures are generally advisable when a dog is suspected for leptospirosis. More awareness and early recognition of this mainly rodent-borne zoonosis by medical specialists is warranted.

ACKNOWLEDGEMENTS

We thank L. Wuyckhuise-Sjouke (GD Animal Health) for providing data on *L. borgpetersenii* serovar Hardjo in Dutch cattle farms and W. van Pelt (RIVM) for critical reading of the manuscript.

REFERENCES

1. World Health Organization. Human leptospirosis: guidance for diagnoses, surveillance and control. Geneva: WHO; 2003.
2. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001;**14**(2):296-326.
3. National Coordinating Body for Infectious Diseases (LCI). Guideline for Leptospirosis. Bilthoven: RIVM; 2011 [20-10-2015]. Available from: http://www.rivm.nl/Documenten_en_publicaties/Professioneel_Praktisch/Richtlijnen/Infectieziekten/LCI_richtlijnen/LCI_richtlijn_Leptospirose.
4. Sykes JE, Hartmann K, Lunn KF, Moore GE, Stoddard RA, Goldstein RE. 2010 ACVIM small animal consensus statement on leptospirosis: diagnosis, epidemiology, treatment, and prevention. *J Vet Intern Med 2011*;**25**(1):1-13.
5. Van Vliet JA. History of notification. *Tijdschrift voor Infectieziekten* 2009;4(2):51-60.
6. Goris MG, Boer KR, Duarte TA, Kliffen SJ, Hartskeerl RA. Human leptospirosis trends, the Netherlands, 1925-2008. *Emerg Infect Dis* 2013;**19**(3):371-8.
7. GD Animal Health. Monitoring animal health: headlines report third quarter 2014. Deventer: GD; 2014. Available from: http://www.gddiergezondheid.nl/~/media/Files/Monitoringsflyers/Rund/Monitoring%20diergezondheid%20rund%20Hoofdpunten%20Rapportage%20Derde%20kwartaal%202014.ash
8. Olszyna DP, Jaspars R, Speelman P, van Elzakker E, Korver H, Hartskeerl RA. Leptospirosis in the Netherlands, 1991-1995. *Ned Tijdschr Geneeskd* 1998;**142**(22):1270-3.
9. Houwers DJ, Wagenaar JA, Hartskeerl RA, *et al*. Leptospirosis in dogs: a risk for humans? *Tijdschr Diergeneeskd* 2009;**134**(9):3.
10. Veterinarian Incident and Crisiscentre of the Food and Consumer Product Safety Authority (NVWA). Script handling suspected leptospirosis. Wageningen: NVWA; 2014.
11. Hartman EG, van Houten M, van der Donk JA, Frik JF. Determination of specific anti-leptospiral immunoglobulins M and G in sera of experimentally infected dogs by solid-phase enzyme-linked immunosorbent assay. *Vet Immunol Immunopathol* 1984;**7**(1):43-51.
12. European Centre for Disease Prevention and Control. Annual epidemiological report on food-and waterborne diseases and zoonoses. Stockholm: ECDC; 2014. Available from: http://www.ecdc.europa.eu/en/publications/Publications/food-waterborne-diseases-annual-epidemiological-report-2014.pdf.
13. Dupouey J, Faucher B, Edouard S, *et al*. Human leptospirosis: an emerging risk in Europe? *Comp Immunol Microbiol Infect Dis* 2014;**37**(2):77-83.
14. Jansen A, Schoneberg I, Frank C, Alpers K, Schneider T, Stark K. Leptospirosis in Germany, 1962-2003. *Emerg Infect Dis* 2005;**11**(7):1048-54.
15. Van Alphen LB, Lemcke Kunoe A, Ceper T, *et al*. Trends in Leptospirosis in Denmark, 1980 to 2012. *Euro Surveill* 2015;**20**(4).
16. Van Oldenborgh GJ, Haarsma R, de Vries H, Allen MR. Cold extremes in North America vs. mild weather in Europe: the winter 2013/2014 in the context of a warming world. *Bull Amer Meteor Soc* 2015;**96**:707-714.

17. Photiadou C, Van der Schrier G, Oldenborgh GJ, Verver G, Tank AK, Plieger M, *et al*. 2014 warmest years on record in Europe: EURO4M; 2014 [20-10-2015]. Available from: http://cib.knmi.nl/mediawiki/index.php/2014_warmest_year_on_record_in_Europe.
18. Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *J Bacteriol* 1967;**94**(1):27-31.
19. Dufour B, Moutou F, Hattenberger AM, Rodhain F. Global change: impact, management, risk approach and health measures--the case of Europe. Rev Sci Tech 2008;**27**(2):529-50.
20. World Health Organization. Guidelines for the control of leptospirosis. Geneva: WHO; 1982.
21. Jansen A, Luge E, Guerra B, Wittschen P, Gruber AD, Loddenkemper C, *et al*. Leptospirosis in urban wild boars, Berlin, Germany. *Emerg Infectious Dis* 2007;**13**(5):739-42.
22. Bundes Institut für Risikobewertung, Robert Koch-Institut. Leptospirosis - a rare but increasingly prevalent disease. Berlin: BfR; 2014. Available from: http://www.bfr.bund.de/cm/343/leptospirose-eine-seltene-aber-immer-haeufiger-auftretende-erkrankung.pdf.
23. World Health Organization. Report of the second meeting of the Leptospirosis Burden Epidemiology Reference Group, 2011.
24. Hartskeerl RA, Collares-Pereira M, Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clin Microbiol Infect* 2011;**17**(4):494-501.

CHAPTER 4

Towards the burden of human leptospirosis: duration of acute illness and occurrence of post-leptospirosis symptoms of patients in the Netherlands

Marga G.A. Goris[1*]
Vanessa Kikken[2*]
Masja Straetemans[1]
Sandra Alba[1]
Marco Goeijenbier[3]
Eric C.M. van Gorp[3]
Kimberly R. Boer[1]
Jiri F.P. Wagenaar[1]
Rudy A. Hartskeerl[1]

1. WHO/FAO/OIE and National Leptospirosis Reference Center and section Epidemiology, Royal Tropical Institute, KIT Biomedical Research, Amsterdam, The Netherlands,
2. Athena Institute, Free University, Amsterdam, The Netherlands,
3. Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands

*these authors contributed equally to the work presented in this paper

PLoS One 2013; 8(10): e76549

ABSTRACT

Background

Leptospirosis is a global zoonotic disease. Although important for the assessment of the burden of leptospirosis, data on the duration of the illness and the occurrence of post-leptospirosis complaints are not well documented. Hence the main objective of this study was to estimate the occurrence of persistent complaints and duration of hospital stay in laboratory confirmed leptospirosis patients in the Netherlands during 1985 to 2010. Additionally, several risk factors potentially impacting on the occurrence of post-leptospirosis complaints were investigated.

Methods/Principal Findings

Methods/Principal Findings: The duration of the acute phase of leptospirosis was 16 days (IQR 12–23); 10 days (IQR 7–16) were spent hospitalized. Eighteen fatal cases were excluded from this analysis. Complaints of leptospirosis patients by passive case investigations (CPC) derived from files on ambulant consultations occurring one month after hospital discharge, revealed persistent complaints in 108 of 236 (45.8%) laboratory confirmed cases. Data on persistent complaints after acute leptospirosis (PCAC), assessed in 225 laboratory confirmed leptospirosis cases collected through questionnaires during 1985-1993, indicated 68 (30.2%) PCAC cases. Frequently reported complaints included (extreme) fatigue, myalgia, malaise, headache, and a weak physical condition. These complaints prolonged in 21.1% of the cases beyond 24 months after onset of disease. There was no association between post-leptospirosis complaints and hospitalization. However, individuals admitted at the intensive care unit (ICU) were twice as likely to have continuing complaints after discharge adjusting for age and dialysis (OR 2.0 95% CI 0.8-4.8). No significant association could be found between prolongation of complaints and infecting serogroup, although subgroup analysis suggest that infection with serogroups Sejroe (OR 4.8, 95%CI 0.9 – 27.0) and Icterohaemorrhagiae (OR 2.0, 95%CI 0.9 – 4.3 CI) are more likely to result in CPC than infections with serogroup Grippotyphosa.

Conclusion/Significance

In addition to the acute disease, persistent complaints have an impact on the burden of leptospirosis.

INTRODUCTION

Leptospirosis is a zoonotic disease with worldwide distribution[1]. Human leptospirosis is a neglected and underreported disease[2], due to a lack of awareness, misdiagnosis and technically demanding laboratory confirmation tests[2,3]. Recent international initiatives on the assessment of the global disease burden of leptospirosis have estimated the global annual incidence of endemic human leptospirosis exceeding 5 severe cases per 100.000 population, excluding cases from outbreaks[2]. However, this number probably presents a substantial underestimation; as this incidence is derived from underreported, laboratory confirmed cases. The true total incidence of mild and severe leptospirosis most likely presents only a small portion of the true number of mild to severe cases annually.

Leptospirosis is caused by pathogenic spirochaetes of the genus *Leptospira*[4]. A wide variety of mammalian hosts, both feral and (semi-) domestic, serve as infection reservoirs. In developing countries, urban leptospirosis is an increasing public health hazard due to hygiene and overcrowding issues, however, farmers still represent the major risk group for leptospirosis[5]. In developed, temperate countries leptospirosis is mainly a recreational disease, associated with water-contact activities[1,2].

Leptospirosis is a protean disease ranging from a sub-clinical illness to a potentially fatal disease with haemorrhage and multi-organ failure as its clinical hallmarks. Persistent or chronic symptoms are often variable in character and onset[6]. This complicates the association between acute disease and sequelae[6,7] and makes it difficult to truly measure the burden of disease. Apart from some anecdotal case reports, post-leptospirosis complaints are not well assessed[3]. Data on the impact and occurrence of persistent or chronic complaints, together with data on the period of hospitalization are badly needed for assessing the burden of leptospirosis in terms of disability-adjusted life years (DALYs)[2,3].

Therefore, in this paper we describe the estimate of the burden of hospitalized and outpatient human leptospirosis in the Netherlands with regard to the duration of the acute and chronic illness as well as the incidence of persistent symptoms by active and passive case finding. We estimate the effect of intensive care unit (ICU) stay adjusted for the potential confounding effect of age, gender, infecting serogroup and occurrence of the acute severe complications of lung (acute lung injury, (ALI)) and kidney (acute renal injury (ARI)), liver injury and haemorrhage. As well, several studies have shown that ICU admission is a risk factor for both physical and psychological symptoms up to 12 months after discharge from the hospital[8]. Psychological consequences include depressive symptoms, post-traumatic stress disorders (PTSD), anxiety and chronic fatigue syndrome (CFS). These sequelae were associated with traumatic memories during ICU or hospital stay[9-11]. In the light of these studies regarding persistent symptoms, in this study it was considered of particular importance, to determine whether there is an association between ICU stay and persistent symptoms after the acute phase of leptospirosis.

METHODS

Ethical statement

The Medical Ethical Review Committee of the Academic Medical Center, University of Amsterdam provided clearance for the execution of the active survey on persistent complaints (96.17.038). In addition, the Medical Ethical Review Committee waived the study for human subjects research ethical review for the data collected from passive case finding (W12_075#12.17.0092). All presented data have been de- identified and are not attributable to individual patients.

Study participants and data collection

The WHO/FAO/OIE and National Leptospirosis Reference Center (NRL) resides in the department of Biomedical Research of the Royal Tropical Institute in Amsterdam, the Netherlands. NRL forms the diagnostic center for leptospirosis in the Netherlands, confirming nearly 100% of the cases suspected of leptospirosis in the country. Confirmation was done in accordance with the national laboratory case definition[12], which is consistent with the definition of the WHO-Leptospirosis Epidemiology Reference Group (LERG)[2].

From 1985 until 2010 the laboratory received an estimated 20,000 samples for investigation. Detailed records on serological, clinical and epidemiological data, including letters of discharge and ambulant consultations after discharge from the hospitals are archived in the NRL. Abstracted data from this archive were used to assess post-leptospirosis complaints by active and passive case finding (see below), duration of illness, hospitalization and attendance of the ICU. Data on the health status of the general population in the Netherlands were retrieved from the Dutch Central Bureau for Statistics (CBS).

Persistent Complaints by Active Case finding

In 1997 an active survey on long-term persistent symptoms among leptospirosis confirmed patients was conducted. Questionnaires were sent to the general practitioners (GP) of all confirmed cases from the years 1985 to 1993. Complaints derived from the questionnaires are referred to as Persistent Complaints by Active Case finding (PCAC) and comprises both hospitalized patients and outpatients. The following questions were included in the questionnaire: (i) Did the patient have long-term complaints after the acute disease? (ii) If so, what were the complaints? (iii) What was the duration of these complaints?

Complaints determined by Passive Case finding

Complaints by passive case (CPC) finding were collected from all hospitalized leptospirosis patients from 1985 to 2010 that were confirmed by NRL. Archived data[13] included information on symptoms and signs, including those from ambulant consultations. These were usually collected one month after patient discharge from the hospital. For the purpose of determining

continued reported complaints following hospital discharge this data was compiled, entered and analysed.

Data processing and definitions

Fatality was used as an exclusion criterion in the analyses for several reasons; firstly, progression towards death is usually rapid and not a good indicator for the mean duration of a stay in the hospital and secondly, death itself is a major burden factor.

The total acute phase of illness is defined as the first day of illness till the day of discharge from the hospital, which includes the date of discharge. The total acute phase of illness was separated into number of days prior to hospital admission and number of days of hospital stay. ICU stay was defined as the first day of admittance to discharge from ICU. If either of these dates were missing, ICU stay was recorded as yes, but the length of time was not calculated and recorded as missing. Cases with incomplete data affecting a specific evaluation (for example, lacking data on the duration of hospital stay) were excluded only from the part of the analyses. Also, questionnaires that contained conflicting or incomplete information were excluded from the analyses.

Other factors that were included in the analyses were gender and age (<38 years; ≥38 years) in which 38 years of age was the median age in the study population during the acute illness. If information was recorded concerning difficulties at work it was included in the data analyses. Data on the infecting serogroup were deduced from the serovar giving the highest titers in the MAT[14]. Common complaints were classified as separate variables in the database. Data was collected on liver injury, haemorrhage, acute renal injury (ARI) and acute lung injury (ALI). WHO-LERG definitions were used for ARI and ALI[2]. In brief, ALI patients presents with respiratory distress indicated by dyspnoea and/or a respiratory frequency ≥28 per minute, bilateral crepitus, bilateral infiltrates in chest X-ray examination, a ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO2/FiO2) <300mm or report of the use of mechanical ventilation as a therapeutic intervention. ARI is defined by an acute onset of oliguria, uraemia, or abnormally elevated serum creatinin or blood urea nitrogen. Liver injury is defined by jaundice, hepatitis and/or abnormal laboratory findings with respect to blood albumin and/or bilirubin, alanine-aminotransferase (ALAT or SGPT), aspartate-aminotransferase (ASAT or SGOT) and/or lactate dehydrogenase (LDH), alkaline phosphatase and γ-glutamyl transpeptidase (γ-gt). The term 'haemorrhage' refers to a recorded bleeding such as petechiae and gastrointestinal bleeding.

Data analysis

Descriptive data is presented on the duration of acute illness, the presence of complaints after discharge from the hospital in the CPC group and presence, nature and duration of complaints in the PCAC group using median (IQR) and proportions. Mann Whitney tests were

conducted to determine differences in duration of acute illness by age and sex. Chi-square tests and fisher's exact tests were used to assess the association between reported complaints by age, gender, infecting serogroup and occurrence of the acute severe complications ALI, ARI, liver injury and haemorrhage and ICU stay.

Multivariate logistic regression analysis was performed to assess risk factors for ICU stay. Variables were considered for the multivariate model if it was associated with complaints and ICU stay with a $p<0.10$. Associations are presented by Odds Ratios (OR) with the corresponding 95% confidence intervals. Analyses were conducted using SPSS software version 17.0.

RESULTS

Incidence and duration of illness

During the period 1985 to 2010, 829 patients were confirmed with leptospirosis; 764 (92.3%) males and 64 (7.7%) females (Table 1). A total of 570 patients (78.9%) were known to be hospitalized, 106 patients (of whom 13 died) were reported to have been admitted to the ICU (Table 1). Reference to the ICU has been poorly documented, but from those with recorded data, ICU admission was usually within the first day of hospital admission.

18 males and 1 female died during the recorded period (Table 1). Based on available data of 369 (67%) of the 552 surviving hospitalized patients the median duration of total acute phase of illness was 16 days (IQR 12–23 days). The median duration of illness prior to hospital admission was 5 days and of hospital stay was 10 days. The median duration of ICU stay was 7 days (Table 2).

Current policies in the Netherlands aim at reducing the period of hospitalization. Consistently, in the years 2005 to 2010 the median duration of hospital attendance was lower (8 days IQR 6–11 days) compared to the entire period 1985-2010 (10 days; IQR 7–16 days) while the period 'prior hospitalization' remained virtually the same (median 4 days, IQR 3 – 7 days).

The median number of days prior to hospital admission did not differ by age (<38 and ≥38 years) or gender. However, males had a longer hospital stay compared to females (11 versus 7.5 day, $p=0.013$). Individuals aged ≥ 38 had longer hospital stay (12 versus 9 days, $p<0.001$).

Factors associated with Complaints by Passive Case finding (CPC)

Data was available on health status after discharge for 236 of the 552 hospitalized surviving patients, with a small difference in age and gender of those with data available and not available. The number of males in the included versus the excluded cases was 213 (90.6%) and 295 (93.4%), respectively and the median age was 37 years, (IQR 25-50) for those included and 39 years, (IQR 26-54) for those excluded.

Table 1 | Patient characteristics of leptospirosis patients separated according to available data*

Characteristic	Hospitalized and outpatients	Hospitalized patients	Hospitalized patients	Hospitalized and outpatients
	All laboratory confirmed patients	Days of acute illness known	Health status after discharge known; Complaints by Passive Case Finding Group	Eligible questionnaires; Persistent Complaints by Active Case finding Group
period	1985-2010	1985-2010	1985-2010	1985-1993
n	829	369	236	225
Male†	764 (92.3)	332 (90.5)	213 (90.6)	214 (95.5)
Age – median (25th and 75th percentile)	38 (26-52)	38 (25-51)	37 (25-50)	38 (27-51)
Antibiotic treatment **	653 (86.0)	327 (89.1)	205 (86.9)	171 (83.8)
Hospital admission#	570 (78.9)	368 (100)	236 (100)	129 (73.7)
Deceased no.(%)##	19 (2.3)	0	0	0
Dialysis on hospitalized patients††	79 (16.1)	46 (12.7)	24 (10.2)	20 (17.9)
ICU admission***	106 (21.3)	52 (14.5)	31 (13.4)	11 (10.3)
Serogroup known	676	298	192	205
Missing	153	71	44	20
Icterohaemorrhagiae	312 (46.4)	154 (51.7)	92 (47.9)	84 (41.0)
Grippotyphosa	114 (16.9)	57 (19.1)	39 (20.3)	33 (16.1)
Sejroe	110 (16.3)	18 (6.0)	8 (4.2)	67 (32.7)
Pomona	56 (8.3)	28 (9.4)	18 (9.4)	14 (6.8)
Other serogroups	84 (12.4)	41 (13.8)	35 (18.2)	7 (3.4)
Post-leptospirosis complaints				
Occurrence CPC	NA	NA	108 (45.8%)	NA
Occurrence PCAC00	NA	NA	NA	68 (30.2)
Extreme fatigue	NA	NA	NA	44 (67.7)
Myalgia	NA	NA	NA	13 (20.0)
Malaise	NA	NA	NA	11 (16.9)
Headaches	NA	NA	NA	10 (15.4)
'Hampered' physical condition	NA	NA	NA	10 (15.4)
Joint complaints	NA	NA	NA	6 (9.2)
Disability to work	NA	NA	NA	9 (13.8)
Others	NA	NA	NA	27 (41.5)

Table 1 | Continued

Characteristic		Hospitalized and outpatients	Hospitalized patients	Hospitalized patients	Hospitalized and outpatients
		All laboratory confirmed patients	Days of acute illness known	Health status after discharge known; Complaints by Passive Case Finding Group	Eligible questionnaires; Persistent Complaints by Active Case finding Group
Acute injuries					
Period		1985-2010	1985-2010	1985-2010	1985-1993
n		332	125	62	225
ARI	Yes	165 (61.8)	95 (78.5)	49 (83.1)	111 (59.7)
	No	102 (38.2)	26 (21.5)	10 (16.9)	75 (40.3)
	Missing	65	4	3	39
Haemorr-hage	Yes	62 (31.0)	36 (38.7)	19 (38.0)	43 (29.9)
	No	138 (69.0)	57 (61.3)	31 (62.0)	101 (70.1)
	Missing	132	32	12	81
Liver injury	Yes	184 (80.7)	91 (81.3)	46 (80.7)	128 (79.5)
	No	44 (19.3)	21 (18.8)	11 (19.3)	33 (20.5)
	Missing	104	13	5	64
ALI	Yes	27 (19.7)	15 (14.2)	7 (13.7)	15 (16.1)
	No	110 (80.3)	91 (85.8)	44 (86.3)	78 (83.9)
	Missing	195	19	11	132

*values are no. (%) unless otherwise indicated

NA is Not Applicable, † sex was registered for 828 patients

**data on antibiotic treatment available from respectively 759/829, 367/368, 236/236 and 204/225 patients

#data on hospital admission available from respectively 722/829, 368/368, 236/236 and 175/225 patients

##18 patients died in the hospital, 1 patient is unknown

††data on dialysis on hospitalized patients available from respectively 491/570, 361/368, 235/236 and 112/129 patients

***data on ICU admission on hospitalized patients available from respectively 497/570, 359/368, 231/236 and 107/122 patients

[00] symptoms for PCAC available for 65 cases

Table 2 | Days of acute illness of hospitalized, surviving patients in the period 1985 to 2010, stratified according to gender and age

Duration in Category	Number of patients	Median (25th and 75th percentile)	P-value*
Acute disease total	369	16 (12 – 23)	NA
Males	333	16 (12 – 23)	0.018
Females	35	13 (9 – 21)	
Age <38 years	185	14 (11 – 19)	<0.001
Age ≥38 years	187	19 (14 – 25)	
Prior hospitalization total	390	5 (3 – 7)	NA
Males	352	5 (3 – 7)	0.735
Females	37	5 (2 – 7)	
Age <38 years	191	4 (3 – 7)	0.066
Age ≥38 years	199	5 (4 – 7)	
Hospitalization total	366	10 (7 – 16)	NA
Males	331	11 (7 – 16)	0.013
Females	34	7.5 (6 – 12.5)	
Age <38 years	182	9 (6 – 13)	<0.001
Age ≥38 years	184	12 (8 – 18)	
ICU attendance total	20	7 (4 – 13.5)	NA
Males	19	7 (4 – 14)	0.223
Females	1	3	
Age <38 years	7	8 (3 – 12)	0.937
Age ≥38 years	13	13 (4 – 14.5)	

NA is Not applicable

* Mann Whitney test

The number of CPC was 108 (45.8%) (Table 1). Individuals of older age (≥ 38 years) were two times more likely to have CPC compared to individuals of younger age (OR 2.2, 95%CI 1.3 – 3.7) (Table 3). Complaints typically comprised non-specific aberrations noticed during physical examination during the follow-up visit by physician, such as (extreme) fatigue, headaches, hair loss at young age, mild jaundice and disability to work and/or supported by abnormal laboratory findings. No association was found between CPC and gender (males, OR 1.3, 95%CI 0.5 – 3.1) and the overall infecting serogroup (Table 3). However, subgroup analyses suggest that Sejroe group infections (OR 4.8, 95% CI 0.9 – 27.0) and Icterohaemorrhagiae group infections (OR 2.0, 95% CI 0.9 – 4.3) were more likely to result in CPC compared toserogroup Grippotyphosa. Remarkably, when taking ICU attendance as a marker for severity of acute illness, Sejroe group infections caused milder disease (none

Table 3 | Uni- and multivariate analysis of risk factors for post-leptospirosis complaints

Risk factors	Available data within the risk categories	CPC group	OR 95%CI	P value	PCAC group	OR 95%CI	P value
Univariate analysis		N=236			N=225		
Sex	Male	213	1.3 (0.5-3.1)	0.618°	214	4.1 (0.5-33.0)	0.289°°
	Female	22			10		
	Missing	1			1		
Age	≥38 years	117	2.2 (1.3-3.7)	0.003°	114	2.5 (1.4-4.5)	0.002°
	<38 years	119			111		
	Missing	0			0		
ICU	Yes	31	2.8 (1.3-6.3)	0.009°	11	2.2 (0.6-8.0)	0.203°
	No	200			96		
	Missing	5			118		
Dialysis	Yes	24	3.2 (1.3-8.0)	0.01°	20	1.2 (0.4-3.2)	0.763°
	No	211			92		
	Missing	1			17		
Serogroup	Grippo	39	Reference	0.047°	33	Reference	0.827°
	Ictero	92	2.0 (0.9-4.3)		84	0.6 (0.3-1.5)	
	Sejroe	8	4.8 (0.9-27.0)		67	0.7 (0.3-1.7)	
	Pomona	18	0.6 (0.2-2.1)		14	1.0 (0.3-3.6)	
	Other	35	1.1 (0.4-2.7)		7	0.7 (0.1-4.2)	
	Unknown*	44			20		
Acute injuries (1985-1993)		N=62			N=225		
ARI	Yes	49	1.0 (0.2-3.7)	0.612°°	111	1.6 (0.8-3.1)	0.171°
	No	10			75		
	Missing	3			39		
ALI	Yes	7	1.5 (0.3-7.3)	0.703°°	15	1.1 (0.3-3.7)	0.580°°
	No	44			78		
	Missing	11			132		
Liver injury	Yes	46	6.4 (1.2-33.0)	0.016°	128	1.2 (0.5-2.8)	0.658°
	No	11			33		
	Missing	5			64		
Haemorr-hage	Yes	19	0.7 (0.2-2.3)	0.608°	43	1.3 (0.6-2.7)	0.539°
	No	31			101		
	Missing	12			81		

Table 3 | Continued

Risk factors	Available data within the risk categories	CPC group	OR 95%CI	P value	PCAC group	OR 95%CI	P value
Multivariate analysis		N=236			N=225		
ICU stay	Yes	31	2.0 (0.8-4.8)	0.112		NA	
	No	200					
	Missing	5					
Age	≥38 years	117	2.0 (1.2-3.5)	0.010		NA	
	<38 years	119					
Dialysis	Yes	24	2.3 (0.9-6.2)	0.098		NA	
	No	211					
	Missing	1					

The table presents data from the period 1985 till 2010, unless otherwise indicated
NA Not Applicable
Ictero Icterohaemorrhagiae
Grippo Grippotyphosa
° Chi-square test
°°fisher's exact test
*excluded from the analysis

of 8 cases attended the ICU) compared to Icterohaemorrhagiae group infections of whom 19% (17/89) needed ICU care (data not shown). This might indicate a potential effect by distinct serogroups on the occurrence of CPC, apparently unrelated to case severity.

Factors associated with Persistent Complaints by Active Case finding (PCAC)

In total 321 questionnaires were distributed of which 256 (79.8%) were returned. Data from 31 questionnaires were excluded because the information provided was incomplete (n=28) or contained conflicting data (n=3). This resulted in 225 eligible participants' questionnaires. The median age was 38 years (IQR 20-46 years) of which 214 were male (95.5%). This is comparable to those participants excluded; i.e. 33 years (23-46 years) and 88 males (91.7%), respectively.

The number of PCAC was 68 (30.2%), and for 65 (95.6%) cases these persistent complaints were specified. Depression-compatible complaints were most frequently reported, such as extreme fatigue, headache and malaise, or complaints that seemed to present persistence, such as myalgia and joint pains (Table 1). Several participants reported back pain and vertigo (3 (4.6%) patients, each), depression and concentration problems (2 (3.1%) patients, each). One participant reported sleep disorders and one reported severe mental alterations. 27 of 65 participants (39.7%) reported 'other symptoms', this included typical thoracic pain, perspiration, painful shoulders, stomach pain, and tinnitus.

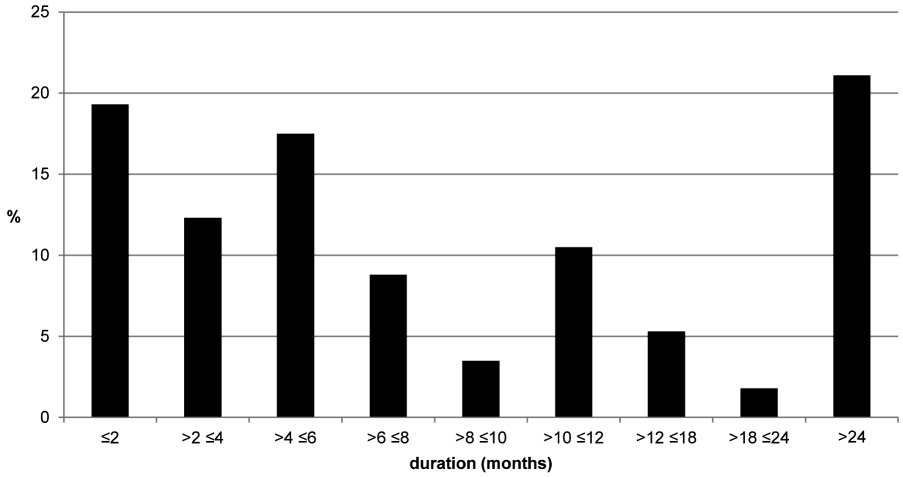


Figure 1 | Persistent complaints by active case finding (PCAC) stratified according to months of duration. The bars represent the percentage of the total of reported post-leptospirosis complaints separated according to duration, expressed in periods of months as indicated on the X-axis.

The duration of PCAC was reported for 57 cases, 11 (19.3%) cases reported ≤2 months, and 1 case reported 18-24 months (Figure 1). However, 12 (21.1%) cases had chronic complaints for more than 24 months. Difficulties in returning to work after the acute phase of leptospirosis were reported in 9 out of 68 (13.2%) questionnaires, comprising the inability to work in a dusty environment (1/9), disability to work for 2 months (2/9), bed rest for 2 months (1/9), the need of assistance at the farm for 6 months (1/9), increasing inability to do farmers work (1/9), resigning the job because of disability of the associated labour (1/9) and permanent disability to work (2/9).

Data in Table 3 show that higher age (≥38 years) was positively associated with the occurrence of PCAC (OR 2.5, 95% CI 1.4 – 4.5), and males trended towards having more complaints than females (OR 4.1, 95% CI 0.5 – 33.0), although not significant. There was no association between age groups and duration of PCAC or the type of symptoms, although higher age showed a trend towards a higher proportion of myalgia (OR 3.3, 95% CI 0.7 – 16.4) and physical condition problems (OR 5.3, 95% CI 0.6 – 44.9).

ICU stay and persistent complaints

Univariate analysis revealed an increasing trend of post-leptospirosis complaints associated with ICU attendance that was significant for CPC (OR 2.8, 95%CI 1.3-6.3), Table 3.

Furthermore, it seemed ICU attendance and renal injuries were not significantly associated with PCAC. Age was the only risk factor significantly associated with PCAC, therefore only

univariate analyses were performed. This suggests that ICU stay as well as the age of ≥ 38, dialysis and liver injury were independently associated with an increase in the odds of complaints (Table 3) in the CPC group but not in the PCAC group.

No significant association was found between ARI, ALI and haemorrhage and persistent complaints in both CPC and PCAC groups (Table 3). Dialysis and age were retained in the multivariate model to control for confounding (P<0.1). The association between ICU stay and age in the univariate model was 2.8 (95% CI 1.3-6.3); while in the multivariate model it was 2.0 (95% CI 0.8-4.8), Table 3. The odds ratio for dialysis decreased from 3.2 (95% CI 1.3-8.0) in the univariate analysis to 2.0 (95% CI 0.8-4.8) in multivariate analysis, suggesting some confounding due to ICU stay and age.

Agreement between PCAC and CPC groups

The occurrence of persistent symptoms was assessed through two methods, one using active case finding using questionnaires and one using passive finding using routine data from existing archive files. From 50 patients information was available both on CPC and PCAC. When comparing data from overlapping cases in both routes, results of CPC and PCAC corroborated for 32 cases (64%), which implies a slight agreement (Cohen's kappa = 0.298). Fourteen of the 27 cases with CPC (51.9%) reported prolonged complaints for 2 months or more. On the other hand, four PCAC cases had no report on CPC (S1).

DISCUSSION

The main objective of this study was to estimate the burden of human leptospirosis in the Netherlands with regard to the frequency and nature of post leptospirosis symptoms as well as the duration of both the acute and persistent phase. In addition, the effects of age, gender, severity of acute illness, including ICU attendance and infecting serogroups were investigated as potential risk factors on the occurrence of post-leptospirosis complaints.

Dutch hospitalized patients had a median stay in the hospital of 10 days and spend 5 days of illness before hospitalization. The total duration of the acute phase thereby consisted of a median 16 days. This concurs with previous data on the duration of acute leptospirosis in the Netherlands and in other parts of the world[3,15]. A limitation of this data is that it concentrates on acute illness from severely ill patients attending the hospital and therefore excludes less severe cases, for which no hospital data were available. However, hospitalization occurred in almost 80% of cases. Moreover, less severe leptospirosis was recognized as such, suggesting a relative high grade of severity for these cases (obviously, patients did seek medical care and the clinician considered leptospirosis), comparable or exceeding that of an influenza-like disease. Taking into account that recovery from a severe influenza-like disease usually

requires one to two weeks, ambulant cases are unlikely to considerably reduce the mean duration of acute leptospirosis. Additionally, it is estimated that at least 30% of severe patients in the Netherlands, are missed due to unawareness or mis-diagnosis[3,16]. Denial of effective treatment because of misdiagnosis probably results in a higher severity of complications and a longer duration of illness[17] and hence missed severe cases might well balance the decrease in hospital days caused by ambulant cases.

The duration of hospital stay showed a declining trend toward recent years. Although this does not exclude the increase of less severe forms of leptospirosis, this more likely reflects a general trend in the Netherlands aiming at the reduction of time of hospital attendance[18-20]. This can be partially explained by economic considerations in the Netherlands and can partially be attributed to developments in medical science and improved health awareness in the population[17]. Thus the decline in duration of hospital stay does not reflect a reduction of the burden of the acute phase of disease but rather indicates that its definition based on discharge of the hospital might become less appropriate.

Significant differences were found between the two age categories, with older patients having a longer hospital stay during the acute phase compared to younger patients. This is in accordance with various reports that indicate that increasing age is associated with increasing severity of leptospirosis[21]. This is not unique for leptospirosis but is commonly seen for a variety of infectious diseases in the Netherlands (CBS).

Significant differences were also found between males and females. Over 90% of the confirmed cases were male patients and they spent more time in the hospital than their females counterparts (11 versus 7.5 days). Similarly, the majority of ICU cases were males (89/93) with a median ICU stay of 7 days. This together indicates that males seem to acquire leptospirosis more frequently and develop more severe complications than females. This further substantiates earlier reports that suggested that males are more susceptible to severe leptospirosis than females[13,22]. The assessment of the economic impact of the disease burden is beyond the scope of this paper. However, it should be noted that the vast majority (86%) of severe cases are male patients between 20 and 65 years of age, which is the current working age in the Netherlands. Since females comprise less than 10% of those presenting with leptospirosis, it is likely they will have little effect on the national economic burden.

In 236 eligible participants from a total of 552 records information was collected on persistent complaints in the first month following the acute disease. This was most likely a representative sample, since the median age and sex ratio was comparable for those included and excluded cases. Nearly half of these patients (45.8%) reported continuing complaints one month after discharge from the hospital. This corroborates well with a previous prospective study in Brazil, where complaints were reported in 49% of former hospitalized patients at a follow up of about 20 days[7].

The assessment of PCAC comprised a sample of 225 eligible participants from a total group of 321 patients, with a comparable age and sex in the included and excluded groups. PCAC occurred for one third of the patients with a declining incidence towards a prolonged duration. However, one-fifth of the PCAC patients had complaints that continued more than two years. Complaints were generally depression-like symptoms such as extreme fatigue, malaise, and headache. Physical complaints included myalgia and joint pains affecting mobility. In this sample, only a few files included information on problems in executing ones job. However, it is well conceivable that these depression-like conditions also impacted on the ability to continue working and hence should be expected to constitute a burden to DALYs.

The PCAC study group was conducted from 1985 to 1993 and was contained within the CPC study group. This allowed for a comparison to investigate the association between the two data sets used for these two groups. The presence or absence of complaints was consistent for two-third of the patients who had both PCAC and CPC data. About half of the CPC group patients apparently had recovered from the post-leptospirosis complaints since PCAC was not indicated. However, for four PCAC group patients no CPC was documented. This can be explained by assuming that either the patients or the clinician considered CPC as normal at the ambulant consultation and did not include the data in the report. On the other hand, it is well conceivable that sequelae develop after apparent complete cure from the acute illness as has been reported for leptospirosis- related uveitis[23].

Evidence has consistently shown that serovars Icterohaemorrhagiae and Copenhageni of the Icterohaemorrhagiae group cause severe leptospirosis more often than serovar Hardjo-bovis (Sejroe group) and serovar Grippotyphosa (Grippotyphosa group)[12,14,24,25]. Assuming that sequelae notably occur after traumatic and stressful acute complications, one might expect to find persistent complaints largely corroborating with Icterohaemorrhagiae as the infecting serogroup. However, our data did not show any association between any infecting serogroup and PCAC, although we found an association compared to the Grippotyphosa serogroup, albeit not significant, between CPC and infections with the Icterohaemorrhagiae group. Surprisingly, however, this association was more pronounced with Sejroe as the infecting serogroup. These Sejroe infections are predominantly due to a dairy fever outbreak caused by serovar Hardjo-bovis in the period 1985-1995[14]. Since complications requiring ICU attendance apparently were absent, we argue that Sejroe group infections cause less severe forms of leptospirosis. Thus, while there seems to be a correlation between the causative infection serotype and severity of illness on one hand, this is not the case between disease severity and the occurrence of persistent complaints on the other hand. Consistently, delayed physical and psychological recovery after Hardjo infection, taking several months to more than a year have been reported by others[26]. Research among randomly selected dairy farms in 1986 and 1987 showed that 63% of persons seropositive for leptospirosis suffered from prolonged excessive fatigue, which led to a partial or complete disability to work[27]. Taking these arguments together, we conclude that less severely ill dairy fever patients do have a

relatively high risk for persistent complaints. The reason for this remains unclear, although it is tempting to speculate that the host-dependence of the serovar Hardjo[28] is associated with a greater invasive power of the central nervous system and/or induces other pathways in the host defence responses.

ICU stay is considered to be a traumatic cause of long lasting sequelae[8,9,11,29–31]. In concordance with this, ICU patients had about a twice-higher risk for persistent complaints that was significant for CPC. The risk even increased nearly a 12–fold when combined with dialysis as another stressful event[32].

Although data on the duration of acute leptospirosis and hospital and ICU stay have been presented in previous studies, these are scarce[3]. Hence, the data on incidence, duration of disease and the occurrence of persistent complaints assessed in this paper present a valuable contribution to the current initiatives to estimate the global burden of leptospirosis (WHO-LERG)[2].

This study has some caveats. Numbers, notably of ICU stay and of female cases are low and affect the statistical analyses robustness. Additionally, too few data on the length of ICU stay were available for adequately examining the association ICU stay and PCAC. Although the severity of illness was assessed by analysing several reliable indicators, such as ICU stay, dialysis, ALI and ARI, this study did not use a 'severity of illness scoring system' such as APACHE II score or SAPS score[31]. Finally, and probably most importantly, data on sequelae are based on persistent complaints after acute leptospirosis. This does not necessarily imply that these can be fully attributed to leptospirosis. Indeed, persistent complaints also have been reported for other infectious diseases and thus are not unique to leptospirosis. Uniqueness of persistent or chronic symptoms can only be made plausible when including a proper control group, which was not part of this study. Moreover, sequelae, generally consisting of depression-like symptoms, need to be corrected for similar complaints in the whole population in order to clarify that a proportion of the sequelae indeed can be exclusively attributed to the acute disease. We have addressed this point by consulting national databases on population-wide trends in health and lifestyle[33].

Compared to the persistent leptospirosis (CPC and PCAP of 45.8% and 30.2% respectively) the baseline data were much lower, i.e. 18.5% of the general Dutch population did not have a good health with several minor complaints summarized as malaise and likely only for part constitute complaints with a severity that has been reported for persistent leptospirosis. Additionally, there is little correlation between malaise complaints and age in the general population[33], while for leptospirosis such complaints increase with increasing age. Therefore, it is concluded that acute leptospirosis induces persistent and late sequelae that contribute to the disease burden. Reports on chronic leptospirosis usually concern anecdotic case

reports. This is one of the few, if not first, systematic reports on the burden of both acute and chronic leptospirosis and as such forms a highly valuable contribution to the assessment of its global burden.

ACKNOWLEDGMENTS

We are appreciative of the contributions of Drs. P. Speelman and W.J. Terpstra in the active case finding study. We also wish to thank the participants in the meetings of the World Health Organization, Leptospirosis Epidemiology Reference Group (LERG) and the Global Leptospirosis Environmental Action Network (GLEAN) for their fruitful discussions.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RAH MS KRB MGAG. Performed the experiments: VK MGAG MG. Analyzed the data: VK MGAG MS KRB SA. Contributed reagents/materials/analysis tools: RAH MS KRB SA. Wrote the manuscript: MGAG VK RAH ECMG JFPW KRB SA MS.

SUPPORTING INFORMATION

Table S1 | Agreement between CPC and PCAC study groups; period 1985 to 1993.

		PCAC group		
		Complaints	**No complaints**	**Total**
CPC group	Complaints	13	14	27
	No complaints	4	19	23
	Total	17	33	50

Cohen's kappa: 0.298

REFERENCES

1. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control, 2003.
2. World Health Organization. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group, 2011.
3. Hartskeerl RA, Collares-Pereira M, Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clin Microbiol Infect* 2011; **17**(4):494-501.
4. Farr RW. Leptospirosis. *Clin Infect Dis* 1995; **21**(1):1-6.
5. Leptonet. Available: http://www.leptonet.net/. Accessed: 25 July 2013.
6. Pond K. Water recreation and disease. Plausibility of associated infections: acute effects, sequelae and mortality. London, Seattle: IWA Publishing; 2005.
7. Spichler A, Athanazio D, Seguro AC, Vinetz JM. Outpatient follow-up of patients hospitalized for acute leptospirosis. *Int J Infect Dis* 2011; **15**(7): e486-e490.
8. Hatch R, McKechnie S, Griffiths J. Psychological intervention to prevent ICU-related PTSD: who, when and for how long? *Crit Care* 2011; **15**(2): 141.
9. Boer KR. Optimizing care for patients surgically treated for severe peritonitis. Dissertation 240pp 2007.
10. Dowdy DW, Bienvenu OJ, Dinglas VD, *et al*. Are intensive care factors associated with depressive symptoms 6 months after acute lung injury? *Crit Care Med* 2009; **37**(5):1702-7.
11. Griffiths JA, Gager M, Waldmann C. Follow-up after intensive care. *Contin Educ Anaesth Crit Care Pain* 2004; **4**(6): 202-5.
12. Hartskeerl RA. Leptospiroses [in Dutch]; Van Steenbergen J, Timen A, Beaujan DJMA, eds. LCI-Guidelines Infectious Disease Control. Bilthoven: LCI, National Coordination Centre for Infectious Disease Control, the Netherlands; 2008.
13. Goris MG, Boer KR, Duarte TA, Kliffen SJ, Hartskeerl RA. Human leptospirosis trends, the Netherlands, 1925-2008. *Emerg Infect Dis* 2013; **19**(3): 371-78.
14. Olszyna DP, Jaspars R, Speelman P, Van Elzakker E, Korver H, Hartskeer RA. [Leptospirosis in the Netherlands, 1991-1995]. *Ned Tijdschr Geneeskd* 1998; **142**(22): 1270-3.
15. Katz AR, Ansdell VE, Effler PV, Middleton CR, Sasaki DM. Assessment of the clinical presentation and treatment of 353 cases of laboratory-confirmed leptospirosis in Hawaii, 1974-1998. *Clin Infect Dis* 2001; **33**(11): 1834-41.
16. Centers for Disease Control and Prevention. Website about leptospirosis. Available: http://www.cdc.gov/leptospirosis/symptoms/ index.html. Accessed: 25 Jul 2013.
17. Gezondheid en zorg in cijfers (2008) [in Dutch]. Available: http:// www.cbs.nl/nl-NL/menu/themas/gezondheid-welzijn/publicaties/ publicaties/archief/2008/2008-c156-pub.htm. Accessed: 25 Jul 2013.
18. Van der Stegen RHM, Ploemacher J (1981-2005) Methodebeschrijving van tijdsreek diagnose statistieken op basis van de LMR. Dutch]. Available: http://www.cbs.nl/NR/rdonlyres/93DA2BC3-4376-407B-8E2C-C08ADE58B6DC/0/2009methodebeschrijvingtijdreeksdiagnosestatistiekenopbasisvanLM R19912005art.pdf. Accessed: 25 July 2013.

19. Ziekenhuisopnamen geslacht, leeftijd en diagnose-indeling ISHMT in Dutch]. Available: http://statline.cbs.nl/StatWeb/publication/? DM=SLNL&PA=71858ned&D1=0-8&D2=1. Accessed: 2013 July 25.
20. Doodsoorzaken korte lijst (belangrijke doodsoorzaken), leeftijd, geslacht [in Dutch]. Available: http://statline.cbs.nl/StatWeb/publication/? VW=T&DM=SLNL&PA=7052_95&D1=0-8. Accessed 2013 July 25
21. Ko AI, Galvao RM, Ribeiro Dourado CM, Johnson WD Jr., Riley LW. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. *Lancet* 1999; **354**(9181): 820-5.
22. Jansen A, Stark K, Schneider T, Schöneberg I. Sex differences in clinical leptospirosis in Germany: 1997-2005. *Clin Infect Dis* 2007; **44**(9): e69- e72.
23. Rathinam SR. Ocular leptospirosis. *Curr Opin Ophthalmol* 2002; **13**(6):381-6.
24. Goris MGA, Hartskeerl RA. Leptospirosis: rat disease or not? [in Dutch]. *Analyse* 2008; **62**(10): 295-8.
25. Hartskeerl RA. Leptospirose een onderschatte ziekte. *Ned Tijdschr Med Microbiol* 2003; **11**(4): 94-8.
26. Terpstra WJ, Bercovich Z. [Milkers' fever, the leptospirosis of cattlemen]. *Ned Tijdschr Geneeskd* 1984; **128**(22):1040-4.
27. Huitema SW, Pal TM, Groothoff JW [Milker's fever, an occupational disease on the increase]. *Ned Tijdschr Geneeskd* 1989; **133**(39):1939-41.
28. Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, *et al*. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci USA* 2006; **103**(39): 14560-5
29. Scragg P, Jones A, Fauvel N. Psychological problems following ICU treatment. *Anaesthesia* 2001; **56**(1):9-14.
30. Tagay S, Kribben A, Hohenstein A, Mewes R, Senf W. Posttraumatic stress disorder in hemodialysis patients. *Am J Kidney Dis* 2007; **50**(4):594-601.
31. Wunsch H, Linde-Zwirble WT, Angus DC. Methods to adjust for bias and confounding in critical care health services research involving observational data. *J Crit Care* 2006; **21**(1):1-7.
32. Hyre AD, Cohen AJ, Kutner N, Alper AB, Muntner P. Prevalence and predictors of posttraumatic stress disorder among hemodialysis patients following Hurricane Katrina. *Am J Kidney Dis* 2007 **50**(4): 585-93.
33. Botterweck A, Frenken F, Janssen S, Rozendaal L, De Vree M, Otten F. Plausibiliteit nieuwe metingen algemene gezondheid en leefstijlen 2001. Available: http://statline.cbs.nl/StatWeb/publication/? VW=T&DM=SLNL&PA=7052_95&D1=0-8. Accessed: 25 July 2013.

CHAPTER 5

New serovars of *Leptospira* isolated from patients in Costa Rica: implications for public health

Maria de los Angeles Valverde[1*]
Marga G.A. Goris[2*]
V González[3]
ME Anchia[4]
P Diaz[4]
Ahmed A. Ahmed[2]
Rudy A. Hartskeerl[2]

1. Costa Rican Institute for Research in Nutrition and Health (INCIENSA), National Reference Center for Leptospirosis, Tres Rios, Costa Rica
2. KIT Biomedical Research, WHO/FAO/OIE Leptospirosis Reference Centre, Amsterdam, the Netherlands
3. Area Rectora Salud Corredores (Ministerio de Salud), Corredores, Costa Rica
4. Hospital Ciudad Neilly (Caja Costarricense de Seguro Social), Corredores, Costa Rica

* These authors contributed equally to the paper

Journal of Medical Microbiology 2013; 62(9): 1263-71

ABSTRACT

Leptospira strains JICH 05 and INCIENSA 04 were isolated from hospitalized leptospirosis patients in the province of Puntarenas, Costa Rica. The isolates produced agglutination titres notably against members of serogroups Tarassovi and Pyrogenes, respectively, but appeared serologically unique in the cross agglutinin absorption test (CAAT). Therefore, JICH 05 and INCIENSA 04 were considered to represent two new serovars, designated Corredores and Costa Rica of the serogroups Tarassovi and Pyrogenes, respectively. Multilocus sequence genotyping revealed that both strain INCIENSA 04 and strain JICH 05 belong to *Leptospira santarosai*. These two new serovars are in addition to various other recently identified highly virulent serovars, including the new *L. santarosai*, serovar Arenal. Considering the fact that isolation and typing of leptospires from patients has only recently been introduced in Costa Rica, these findings suggest that various known and unknown virulent serovars of *Leptospira* are circulating in this country and probably beyond, thus posing a severe threat to public and probably veterinary health in the region.

INTRODUCTION

Leptospirosis is a worldwide zoonosis, which is particularly endemic in warm tropical and subtropical countries. It seems that in Costa Rica, located in Central America, the disease is highly endemic with a mean annual incidence of about 5 per 100,000 population during the period 1996–2005[1]. Leptospires are transmitted to humans via contaminated mud and water or by direct exposure to the urine of infected animals usually during occupational, recreational, or other vocational activities.

Development of severe clinical disease might depend on both host- and causative agent-related factors such as immunological competence, age, physical condition and virulence of the infecting leptospires. The causative agents of leptospirosis belong to the genus *Leptospira*, which contains both saprophytic and pathogenic species[2]. A multitude of mammalian species can be carriers of pathogenic leptospires. Animals with subclinical infections as well as those that recover from the clinical disease may become a potential source of infection for other susceptible hosts, because they continue to excrete leptospires for a prolonged period of time[3,4] .

The isolation and identification of an infecting *Leptospira* strain is cumbersome and time consuming. Leptospires are fastidious bacteria that are difficult to isolate by *in vitro* culturing[4,5]. The initial identification of *Leptospira* is morphological, by dark field microscopy. Definitive identification of the isolates requires the use of serological and molecular techniques[6,7,8]. Based on serological criteria, strains of *Leptospira* are differentiated into serovars, which represent the basic taxon[7,9]. Serovars that are antigenically related are placed into serogroups. Serogroups do not have an official taxonomic status, but are of clinical and epidemiological importance[8]. Currently more than 200 pathogenic serovars have been arranged in 26 serogroups. The genotypical classification system is based on DNA homology. In this scheme, leptospires are placed into 20 *Leptospira* species of a pathogenic, saprophytic and intermediate nature[6,10,11,12]. There is a poor correlation between the serological and genotypic classification systems[6,12].

It is notable that in countries such as Costa Rica where leptospirosis is highly endemic and where research on circulating pathogenic *Leptospira* types is still in its infancy, it is likely that a wide variety of serovars have remained unknown.

In this paper, we describe two new *Leptospiral* serovars isolated from the blood of severely ill leptospirosis patients in the canton Corredores, Costa Rica and discuss the implications for national public health.

METHODS

Description of patients

Case one: A 16-year-old man was hospitalized in the Ciudad Neilly Hospital, Corredores, Costa Rica with a 2 days history of fever, chills, back pain and myalgia. The patient was a school student who had been swimming in La Bonita Brook 15 days before onset of symptoms. At the day of admission, his temperature was 37.3° C and rapidly progressed to 39.9° C in the next few hours. He had tachycardia (107 beats per minute) and his blood pressure was 127/77 mmHg. Clinical examination showed no important clinical signs or an infectious focus. Laboratory test revealed normal hepatic enzymes, creatinine and ureic nitrogen values. The leukocyte count was 13, 4 x 10^3 /µL with 94% polymorph nuclear cells. Urinalysis was normal and malaria blood smears were negative. Abdominal ultrasound was normal. Leptospirosis was confirmed by seroconversion in the microscopic agglutination test (MAT, see below) with the highest titer of: 1:320 with serovar Tarassovi. Also the rapid screening test LeptoTek Dri Dot[13] gave a positive outcome (not shown). In addition, blood yielded a positive culture result.

The patient received treatment with penicillin for 7 days (2 x 10^6 units four times a day), which resulted in a resolution of symptoms. Oral treatment with doxycycline was continued for 12 more days.

Case two: A 39-year-old man was hospitalized in the Ciudad Neilly Hospital, canton Corredores, Costa Rica with a 1 day history of fever, headache and myalgia. The patient had been swimming in the river Corredores 7 days before the onset of symptoms. On the day of admission, his temperature was 39.2° C and his blood pressure was 113/82 mmHg. Clinical examination showed no important clinical signs or an infectious focus. Laboratory test revealed normal hepatic enzymes, creatinine and ureic nitrogen values. The leukocyte count was 10.3 x 10^3/µL with 87% polymorph nuclear cells. Urinalysis was normal and malaria blood smears were negative. Abdominal ultrasound was normal. Leptospirosis confirmation was based on a positive culture obtained from a blood sample collected at admission. The same blood sample was negative in the MAT. A follow-up sample for confirmation by seroconversion in the MAT was not obtained.

The patient received 3 days of treatment with penicillin (2 x 10^6 units four times a day), which resulted in a resolution of symptoms. Oral treatment with doxycycline was continued for 12 more days.

Strains: Apart from the isolates, all strains used in this study are from the *Leptospira* strain collection of the WHO/FAO/OIE (World Health Organization/Food and Agriculture Organization of the United Nations/World Organisation for Animal Health) and National Leptospirosis Reference Centre, KIT, Biomedical Research, Royal Tropical Institute, Amsterdam, the Netherlands.

Culture: Culturing was performed in liquid Ellinghausen and McCullough modified Johnson and Harris (EMJH) and semi-solid Fletcher culture medium[4,5,14]. For isolation, 0.1 ml aliquots of whole blood in heparin anticoagulant were inoculated into 6 ml EMJH culture medium. Incubation was at 30 °C and cultures were inspected by dark field microscopy for growth of leptospires at regular intervals. Isolates were sub-cultured and maintained in EMJH medium and in Fletcher medium supplemented with 5 fluorouracil (200µg/ml) as a selective inhibitor for contaminating micro-organisms[3,4,5,14,15].

MAT: The MAT was performed as per standard procedure[7,9,14] starting with a serum dilution of 1:20 up to 1:20480. The highest dilution of serum showing 50% reduction in free-moving leptospires under dark field microscope was considered to be the end-titre. The panel that was used in the MAT consisted of the following serovars: Australis, Autumnalis, Castellonis, Bataviae, Canicola, Cynopteri, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Copenhageni, Javanica, Panama, Pomona, Pyrogenes, Hardjo type Prajitno, Sejroe, Wolfii, Tarassovi and Patoc.

Serological typing

MAT with group sera and monoclonal antibodies: Rabbit anti-*Leptospira* sera were raised following standard procedures[9].

To identify the isolates up to serogroup level, MAT was performed following standard procedure using a panel of 43 anti-*Leptospira* rabbit reference sera[7,14]. Isolates were further typed at the serovar level by performing MAT with panels of monoclonal antibodies (mAbs) that characteristically agglutinate serovars from the serogroups Pyrogenes (F134C1, F134C2, F134C4, F134C5 and F134C6), Icterohaemorrhagiae, Sarmin and Javanica (F12C3, F20C3, F20C4, F52C1, F52C2, F70C4, F70C7, F70C13, F70C14, F70C20, F70C24, F70C26, F82C1, F82C2, F82C7, F82C8, F89C3, F89C12, F98C4, F98C5, F98C8, F98C12, F98C17, F98C19, and F98C20), Ballum (F74C1, F74C4, F74C7, and F74C12), and Tarassovi and Shermani (F151C1, F151C6, F151C7, F151C8, F151C9, F151C13, F151C17, F151C19 and F151C20) as previously described[14,16,17].

Cross agglutinin absorption test: The Cross Agglutinin Absorption Test (CAAT), which is the standard test for serovar determination was carried out by staff of the Costa Rican Institute for Research in Nutrition and Health (INCIENSA) as described elsewhere[7,14,18,19] and included all recognized serovars from relevant serogroups (*Leptospira* Library, Royal Tropical Institute; http://www.kit.nl/Leptospira-Library, accessed 3 December 2012). CAAT was performed in duplicate and independently by two people to assure reproducibility and results were confirmed by staff of the WHO/FAO/OIE and National Leptospirosis Reference Centre, Amsterdam, the Netherlands.

Genetic Characterization: *Leptospira* strains were propagated at 30 °C in EMJH culture medium. Genomic DNA was extracted, purified and eluted using the QIAamp DNA extraction kit according to the manufacturer's instructions (QIAamp, QIAGEN).

For subsequent multilocus sequence genotyping (MLST), the DNA was amplified and sequenced as described elsewhere[20,21,22]. DNA sequence alignments were generated with Vector NTI 10 software (Invitrogen). Phylogenetic analysis was conducted with molecular evolutionary genetics analysis (MEGA)5 software[23]. One thousand bootstrap replications were used to assess the degree of confidence to be placed in the nodes. The tree was constructed by the neighbor joining method in Jukes-Cantor mode[23]. *Leptospira* strains used in the MLST analysis are listed in Table 1.

Ethics: This study is an integral part of the project "Evaluation of the effect of decentralization of screening tests and culture media on the timeliness and quality of diagnosis and confirmation of cases of leptospirosis in Costa Rica", which has been reviewed and approved by the Ethical Committee of INCIENSA (IC 200308). All presented data have been de-identified and are not attributable to individual patients.

Table 1 | Strains used for multilocus sequence genotyping (MLST)

No.	Code	Genome species	Serovar	Strain
1	>1L.bor	*L.borgpetersenii*	Kenya	766*
2	>2L.bor	*L.borgpetersenii*	Kremastos/ Hebdomadis	873*
3	>3L.bor	*L.borgpetersenii*	Saxkoebing	1498*
4	>4L.bor	*L.borgpetersenii*	Ricardi /Saxkoebing	1522*
5	>5L.bor	*L.borgpetersenii*	Kenya	4880*
6	>6L.bor	*L.borgpetersenii*	Istrica	Bratislava M 84*
7	>7L.bor	*L.borgpetersenii*	Dehong	De 10*
8	>8L.bor	*L.borgpetersenii*	Zhenkang	L 82*
9	>9L.bor	*L.borgpetersenii*	Sejroe	M84*
10	>10L.bor	*L.borgpetersenii*	Poi	Poi*
11	>11L.bor	*L.borgpetersenii*	Kunming	RIM 139*
12	>12L.bor	*L.borgpetersenii*	Ricardi /Saxkoebing	RIM 156*
13	>13L.bor	*L.borgpetersenii*	Kunming	RIM 201*
14	>14L.bor	*L.borgpetersenii*	Mini	Sari*
15	>15L.bor	*L.borgpetersenii*	Javanica	Veltrat Batavia*
16	>16L.int	*L.interrogans*	Lora	2366*
17	>17L.int	*L.interrogans*	Kenya	4883*

No.	Code	Genome species	Serovar	Strain
18	>18L.int	*L.interrogans*	Proechimys	1161 U*
19	>19L.int	*L.interrogans*	Australis	Ballico*
20	>20L.int	*L.interrogans*	Australis	MG-392*
21	>21L.int	*L.interrogans*	Copenhageni	YASUODAMMA*
22	>22L.int	*L.interrogans*	Ramisi	MG-347*
23	>23L.int	*L.interrogans*	Ratnapura	TB-6*
24	>24L.int	*L.interrogans*	Saxkoebing	MG-73*
25	>25L.int	*L.interrogans*	Valbuzzi	MG-472*
26	>26L.int	*L.interrogans*	Hardjoprajitno	Hardjoprajitno*
27	>27L.int	*L.interrogans*	Copenhageni	Isolate 9*
28	>28L.int	*L.interrogans*	Lyme	K30B*
29	>29L.int	*L.interrogans*	Australis	K9H*
30	>30L.int	*L.interrogans*	Rushan	L01*
31	>31L.int	*L.interrogans*	Canicola	L14*
32	>32L.int	*L.interrogans*	Lai	Lai*
33	>33L.int	*L.interrogans*	Kenniwicki	LT1026*
34	>34L.int	*L.interrogans*	Copenhageni	M 20*
35	>35L.int	*L.interrogans*	Canicola	M12/90*
36	>36L.int	*L.interrogans*	Copenhageni	M9/99*
37	>37L.int	*L.interrogans*	Grippotyphosa	Moskva Y*
38	>38L.int	*L.interrogans*	Saxkoebing	Mus24*
39	>39L.int	*L.interrogans*	Portlandvere	MY1039*
40	>40L.int	*L.interrogans*	Pomona	Pomona*
41	>41L.int	*L.interrogans*	Icterohaemorrhagiae	RGA*
42	>42L.int	*L.interrogans*	Sumneri	Sumner*
43	>43L.int	*L.interrogans*	Valbuzzi	Valbuzzi*
44	>44L.kir	*L.kirschneri*	Sokoine	745*
45	>45L.kir	*L.kirschneri*	Sokoine	771*
46	>46L.kir	*L.kirschneri*	Mwogolo	845*
47	>47L.kir	*L.kirschneri*	Ricardi /Saxkoebing	1501*
48	>48L.kir	*L.kirschneri*	Qunjian	2980*
49	>49L.ki	*L.kirschneri*	Sokoine	4602*
50	>50L.kir	*L.kirschneri*	Mozdok	5621*
51	>51L.kir	*L.kirschneri*	Kuwait	136/2/2*
52	>52L.kir	*L.kirschneri*	Tsaratsovo	B 81/7*
53	>53L.kir	*L.kirschneri*	Vanderhoedeni	Kipod 179*
54	>54L.kir	*L.kirschneri*	Grippotyphosa	Moskva V*

Table 1 | Continued

No.	Code	Genome species	Serovar	Strain
55	>55L.kir	*L.kirschneri*	Kenya	Njenga*
56	>56L.kir	*L.kirschneri*	Schueffneri	Vleermuis 90C*
57	>57L.kir	*L.kirschneri*	Ratnapura	Wumalasena*
58	>58L.nog	*L.noguchii*	Panama	CZ214k*
59	>59L.nog	*L.noguchii*	Guaratuba	Isolate 4*
60	>60L.nog	*L.noguchii*	Myocastoris	LSU 1551*
61	>61L.nog	*L.noguchii*	Louisiana	LSU 1945*
62	>62L.san	*L.santarosai*	Recreo	380*
63	>63L.san	*L.santarosai*	Varela	1019*
64	>64L.san	*L.santarosai*	Guaratuba	An 7705*
65	>65L.san	*L.santarosai*	Bananal	2ACAP*
66	>66L.san	*L.santarosai*	Brasiliensis	An 776*
67	>67L.san	*L.santarosai*	Guaricura	Bov.G*
68	>68L.san	*L.santarosai*	Canalzonae	CZ188*
69	>69L.san	*L.santarosai*	Alexi/Guaratuba/ Princestown	Isolate 1*
70	>70L.san	*L.santarosai*	Weaveri/Rio	Isolate 2*
71	>71L.san	*L.santarosai*	Rama	Isolate 3*
72	>72L.san	*L.santarosai*	Rama	Isolate 5*
73	>73L.san	*L.santarosai*	Claytoni	Isolate 6*
74	>74L.san	*L.santarosai*	Georgia	LT117*
75	>75L.san	*L.santarosai*	Guaricura	M4/98*
76	>76INCIENSA04	*L.santarosai*	Costa Rica	INCIENSA 04#
77	>77JICH05	*L.santarosai*	Corredores	JICH 05#

*Ahmed *et. al*, 2006[20], # This paper.

RESULTS

Isolation

The cultures became positive after two weeks. The isolates were named strains JICH 05 (from case 1) and INCIENSA 04 (from case 2). Under dark field microscopy, strains INCIENSA 04 and JICH 05 showed typical *Leptospira* motility and morphology. The strains grew well in EMJH and Fletcher medium at 30 °C.

Serological characterization

Data generated by serological typing of strains INCIENSA 04 and JICH 05 are summarized in Table 2.

Strain INCIENSA 04: Testing INCIENSA 04 against a panel of 43 rabbit anti-*Leptospira* sera to determine the major serogroups, revealed a highest agglutination titer (reciprocal titer ≥1280) against serovar Pyrogenes of serogroup Pyrogenes. This was followed by reciprocal titers 640 and 20 against serovars Kenya and Ballum, respectively of the serogroup Ballum, a reciprocal titer of 320 against serovar Manhao 3 of the Manhao group and reciprocal titer 80 against serovar Weaveri of the Sarmin group.

Subsequently strain INCIENSA 04 was tested with three panels of mAbs against serogroups Ballum, Pyrogenes and Sarmin. Agglutinating mAbs against serovars of the Manhao group are not available, hence, mAb typing within this serogroup was not possible. None of the mAbs in the three panels agglutinated strain INCIENSA 04 (results not shown). This finding corroborates with the profiles of serovars Kenya and Peru of the Ballum group and Varela of the Pyrogenes group and thus is not conclusive. Cross agglutinations were performed to further investigate the identity of the serovar status of INCIENSA 04 using all reference strains and sera from the serogroups Ballum, Manhao and Pyrogenes.

Table 2 | Results of serogrouping and cross-agglutinin absorption test (CAAT) of strains INCIENSA 04 and JICH 05 with 43 reference sera.

INCIENSA 04				
Serogroup	**Reactive serovar (titer)**	**CrossAggl (serovar)†#**	**CAAT (%) ¶**	**Species**
Ballum	Ballum (20)	No	ND	*L. borgpetersenii*
	Kenya (640)	No	ND	*L. borgpetersenii*
Pyrogenes	Pyrogenes (≥1280)	Pyrogenes	50	*L. interrogans*
	*	Abramis	100	*L. interrogans*
	*	Alexi	100	*L. santarosai*
	*	Biggis	100	*L. interrogans*
	*	Guaratuba	100	*L. interrogans*
	*	Hamptoni	100	*L. borgpetersenii*
	*	Manilae	100	*L. interrogans*
	*	Menglian	50	*L. weilii*
	*	Myocastoris	50	*L. noguchii*
	*	Nigeria	100	*Unknown*
	*	Princetown	100	*L. santarosai*
	*	Robinsoni	25	*L. interrogans*
	*	Varela	100	*L. santarosai*
Manhao	Manhao 3 (320)	No	ND	*L.alexanderi*
	*	Lichuan	100	*L. inadai*
	*	Qingshui	100	*L. weilii*
Sarmin	Weaveri (80)	No	ND	*L. santarosai*

Table 1 | Continued

JICH 05				
Serogroup	**Reactive serovar (titer)**	**CrossAggl (serovar)†#**	**CAAT (%) ¶**	**Species**
Tarassovi	Tarassovi (640)	No	ND	*L. borgpetersenii*
	*	Atchafalaya	100	*L. santarosai*
	*	Atlantae	25	*L. santarosai*
	Bakeri (≥1280)	Bakeri	100	*L. santarosai*
	*	Darien	12.5	*L. santarosai*
	*	Gatuni	25	*L. santarosai*
	*	Gengma	100	*L. borgpetersenii*
	*	Guidae	100	*L. borgpetersenii*
	*	Kanana	25	*L. borgpetersenii*
	*	Kisuba	50	*L. borgpetersenii*
	Rama (320)	Rama	50	*L. santarosai*
	*	Kaup	100	*L. inadai*
	*	Langati	100	*L. weilii*
	Mogden (160)	Mogden	25	*L. weilii*
	*	Tunis	25	*L. borgpetersenii*
	*	Vughia	100	*L. weilii*
Shermani	Shermani (320)	No	ND	*L. santarosai*

†(Heterologous titer : homologous titer) x 100%; > 10% is significant

#Serovars that did not show agglutination are not listed in the table.

¶(Homologous titer after absorption: homologous titer before absorption) x 100%; ≤ 10% indicates similarity of the serovars.

*Indicates that the corresponding reference serum is not included in the serogrouping panel of 43 sera.

ND: Not done.

No: the percentage is not significant.

No significant cross-agglutinations (> 10% compared to the homologous agglutination) were observed with reference sera from the Ballum and Sarmin groups, virtually excluding the possibility that the strain belongs to any of these two serogroups. However, significant cross agglutinations were found with several serovars from the serogroups Manhao and Pyrogenes (Table 2). The serovar status is determined by serological features established by the CAAT. According to the definition of a serovar, two strains belong to different serovars if, after cross-absorption with adequate amounts of heterologous antigen, more than 10% of the homologous titer regularly remains in at least one of the two antisera in repeated tests[18,19]. In the forward CAAT, i.e. reference sera absorbed with strain INCIENSA 04, residual titers of 25%

Table 3 | Comparison of agglutination titers of strain JICH 05 and relevant reference serovars from serogroups Tarassovi and Shermani mAbs against these serogroups.

Monoclonal antibodies	JICH 05†	Atcha†	Atlan†	Bakeri†	Rama†	Langati†	Cari†	Luis†
F151C1	*	*	**160**	*	*	*	*	*
F151C6	≥20480	40960	81920	40960	40960	81920	20480	*
F151C7	80	**2560**	**5120**	**5120**	*	*	*	*
F151C8	640	1280	640	1280	*	*	*	640
F151C9	20	160	*	**320**	*	*	*	*
F151C13	*	*	*	*	*	*	*	*
F151C17	*	*	*	*	*	*	*	*
F151C19	*	**160**	*	*	*	*	*	*
F151C20	80	**1280**	**2560**	**2560**	*	*	*	*

†*Leptospira* strain,

*No agglutination. Inconsistent titers are in bold, i.e. with more than a 3-fold titer difference.

or higher were found (Table 2). This defines INCIENSA 04 as a new serovar without the need to execute the reversed agglutination reaction, i.e. anti-serum against INCIENSA 04 absorbed with candidate *Leptospira* reference strains.

Strain JICH 05: Applying the panel of 43 reference sera, JICH 05 showed the highest agglutination titers with serogroup Tarassovi, i.e. reciprocal titers of 160, 320, 640 and ≥1280 for serovars Mogden, Rama, Tarassovi and Bakeri, respectively, with one additional reciprocal agglutination titer of 320 against serovar Shermani of the Shermani group. Subsequent serotyping with panels of mAbs against serogroups Tarassovi and Shermani generated agglutination profiles that did not corresponds to any of the serovars in these serogroups (Table 3), suggesting that JICH 05 may also be an unknown serovar. Subsequent cross agglutination studies with all serovars of serogroups Tarassovi and Shermani revealed significant cross agglutinations with 17 serovars of the Tarassovi group only (Table 2). All these serovars were tested in the forward CAAT. None of the obtained residual titers revealed sufficient similarity of JICH 05 with any of these reference serovars to warrant identification (Table 2). This confirms its status as a new serovar.

Strains INCIENSA 04 and JICH 05 were named serovar Costa Rica and Corredores, respectively. Based on highest titers in the serogrouping as well as the number of reference sera cross agglutinating with the newly isolated strains, serovars Costa Rica and Corredores are placed into serogroup Pyrogenes and Tarassovi, respectively.

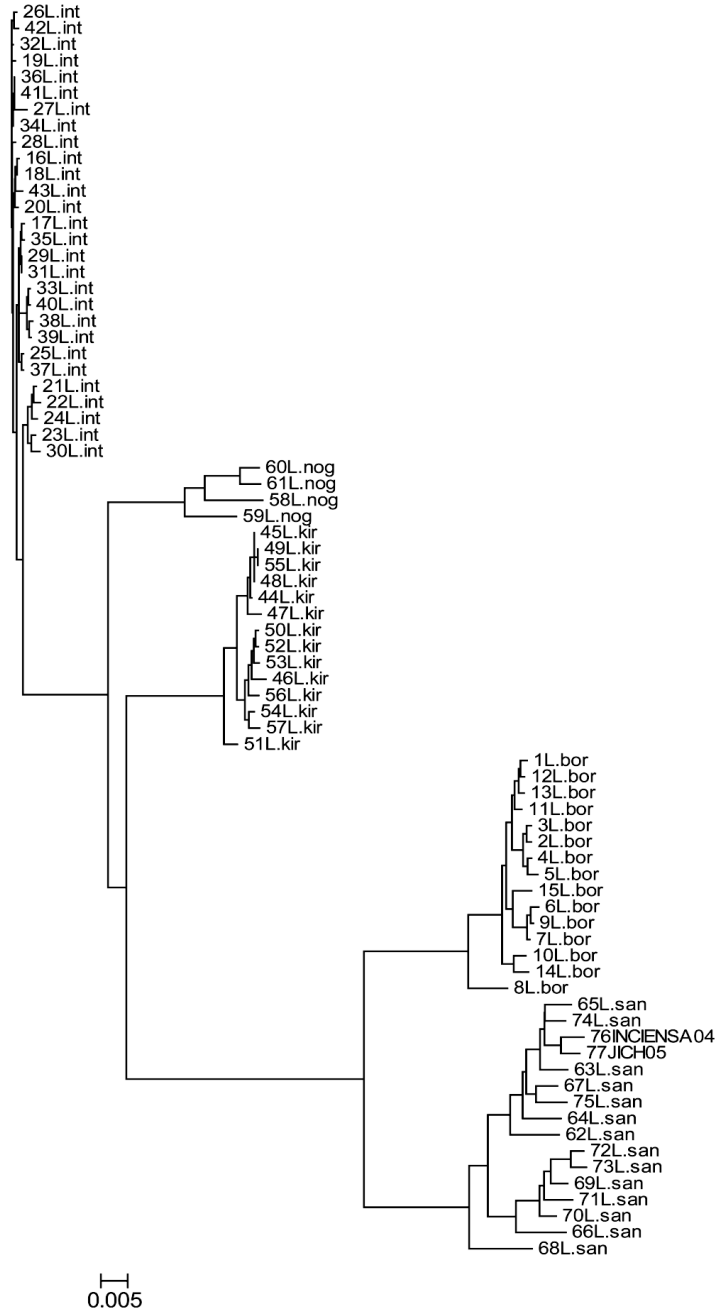


Figure 1 | Phylogenetic tree of *Leptospira* strains based on sequences of six loci according the MLST analysis. The evolutionary history was inferred using the Neighbor-Joining method[23].

Phylogenetic analyses were conducted in MEGA5[23]. Codes of strains are listed in Table 1.

Molecular characterization

Consistent with the pathogenic status, DNA from INCIENSA 04 and JICH 05 could be amplified in pathogenic-specific PCRs[22,24]. To determine the species of INCIENSA 04 and JICH 05, six loci of both strains were amplified and sequenced. Subsequently, sequences were used for MLST analysis including the known sequences of reference strains used in previous MLST studies[20,21]. The resulting phylogenetic tree is shown in Figure 1. Sequences of strain INCIENSA 04, serovar Costa Rica and strain JICH 05, serovar Corredores are highly similar but not identical and cluster within the species *L.santarosai*.

DISCUSSION

We describe the isolation and characterization of two novel *Leptospira* serovars isolated from Costa Rican patients.

The patients were admitted to the hospital with symptoms and signs compatible with severe leptospirosis. In one case, leptospirosis was serologically confirmed. The infection was probably acquired during swimming. Infection through water recreation activities is also highly probable for the second patient. However, the single sample collected from this patient was negative in the MAT and confirmation was merely based on the positive culture. Indeed, a positive culture provides proof of infection and is the actual gold standard in the laboratory case definition of leptospirosis[25]. Moreover, a negative MAT, notably on early acute single samples when anti-*Leptospira* antibodies have not yet produced in detectable amounts, is not exceptional. While MAT is often referred to as the gold standard or is used as the reference test, its diagnostic accuracy has shown to be questionable, mainly due to a relatively low sensitivity[25,26]. The failure to detect agglutination by MAT in this patient might present one of the examples of a 'false' negative MAT outcome and stresses the need for testing paired samples.

The morphology and motility of INCIENSA 04 and JICH 05 under dark field microscopy were consistent for the genus *Leptospira*. Serological determination with various methods, including the CAAT, revealed that INCIENSA 04 and JICH 05 represent new serovars. We have denoted these serovars as Costa Rica and Corredores, respectively, after the country and the canton Corredores near the residence and location of infection of the patients. Serovars Costa Rica and Corredores have been placed into the serogroups Pyrogenes and Tarassovi, respectively, based on highest agglutinating titers against multiple serovars within these groups[18]. Cross agglutination titers for serovar Costa Rica were also found in the serogroups Ballum, Manhao and Sarmin, and for Corredores with Shermani. This intra-serogroup cross agglutination is not unique and can be explained by a serologically similarity of several serogroups[14].

MLST sequence analysis indicated that both strain INCIENSA 04, serovar Costa Rica and strain JICH 05, serovar Corredores belong to species *L. santarosai*, which is almost exclusively distributed in the America's[6].

Serovars Costa Rica and Corredores are not the first new serovars recently identified in Costa Rica. Previously, we described another new serovar named Arenal[27]. Arenal has been isolated from various patients at various locations within Costa Rica, showing that this is a common serovar. To date, it is not clear to what extent serovars Costa Rica and Corredores are circulating in Costa Rica but it is likely that these also are not locally confined, exotic serovars. *Leptospira* isolation from patients and typing of such isolates has been introduced only during the last decade at INCIENSA and, hence has been operational only for a short period. Therefore, these findings support our suspicion that several, yet unknown serovars are circulating in Central America, as can be expected from a region that is highly endemic for leptospirosis [1,4,6,27]. Our findings might have a number of implications. Firstly, serovars Arenal, Costa Rica and Corredores were cultured from hospitalized and thus severely ill patients. Hence, these should be considered virulent serovars. Moreover, in the same period, a variety of other known virulent serovars, such as Copenhageni, Pomona, Canicola, Guaratuba, Rama and Claytoni have been isolated from Costa Rican patients[20]. This variety of virulent serovars identified in a short period of recent years may firstly imply that leptospirosis poses a serious threat to the public health in Costa Rica and probably neighboring countries, as reflected by high incidences of leptospirosis in the region[1]. Secondly, the relatively large proportion of unknown serovars might affect the sensitivity of the local MAT. This test performs optimally when using a panel of locally circulating serovars. The presence of unknown serovars implies the use of an incomplete panel of serovars in the MAT, holding the risk of missing patients by using this standard serological test, as occurred in case two. Indeed, while the panel of serovars used in the MAT included serovar Pyrogenes of serogroup Pyrogenes, the causative serovar Costa Rica of the same serogroup apparently differed too much to enable detection of antibodies generated against using this reference serovar. It is thus conceivable that the use of an apparently sub-optimal panel of serovars in the MAT implies an underestimation of the current (already high) incidence. These two consequences justify continuous research and surveillance on the distribution and risks of leptospirosis in the region and the improvement of the panel of strains for the MAT by adding locally relevant serovars.

Currently, infection reservoirs of serovars Costa Rica and Corredores have not been identified. Infection with both serovars was very likely to have been acquired by indirect transmission of leptospires excreted into surface waters. The environment of La Bonita Brook and the Corredores river makes it possible that it has been contaminated with urine of infected cattle and pigs. Therefore, it is tempting to speculate that these form the infection reservoirs of these new serovars. However, a variety of feral and (semi) domestic

hosts cannot be excluded as the infection source at this stage, and further research on potential infection sources in the region will be needed to confirm or refute domestic animals as the main source of infection.

In conclusion, *L.santarosai* serovars Costa Rica and Corredores, type strains INCIENSA 04 and JICH 05 have been deposited under this designation in the culture collections of the National Reference Center for Leptospirosis, Costa Rican Institute for Research in Nutrition and Health, Tres Ríos, Costa Rica and as KIT0251 and KIT0252, respectively at the WHO/FAO/OIE and National Collaborating Centre for Reference & Research on Leptospirosis, Royal Tropical Institute, Amsterdam, the Netherlands.

The identification of a wide variety of serovars in a short time period is indicative of a variety of sources of infection. Since infection only causes serovar-specific immunity[4], multiple repeated infections with other serovars leading to disease seems realistic. This represents a severe threat to both public and veterinary health in Costa Rica and probably the whole of Central America and substantiates the need for continuous surveillance in the region.

ACKNOWLEDGEMENTS

The authors wish to thank Mr J. I. Chaverri and Wendy van Zaanen for their technical assistance. The work was supported by EU-INCO grant ICA4-CT-2001-10086.

REFERENCES

1. Costa F, Martinez-Silveira MS, Hagan JE, Hartskeerl RA, Reis MG, Ko AI. Surveillance for leptospirosis in the Americas, 1996-2005: a review of data from ministries of health. *Rev Panam Salud Publica* 2012; **32**(3): 169-177.
2. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; **14**(2): 296-326.
3. Faine S (editor). Guidelines for the Leptospirosis control: Geneva: World Health Organization Offset Publication no. 67; 1982.
4. Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and Leptospirosis. Medi Sci; 1999.
5. Faine S. *Leptospira* and leptospirosis. Boca Raton, Florida USA CRC PRESS; 1994.
6. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS. Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int J Syst Bacteriol* 1999; **49**: 839-58.
7. Dikken H, Kmety E. Serological typing methods of leptospires. In: *Methods in Microbiology* 1978; pp. 260-295. New York: Academic Press.
8. Levett PN. *Leptospira* and *Leptonema.* In: *Manual of Clinical Microbiology*, pp. 929-936. Edited by Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH. Washington, DC: Press; 2003.
9. Anonymous. International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Leptospira*: Minutes of the Meeting, 6 and 10 August, 1982, Boston, MA, USA. *Int J Syst Bacteriol* 1984; **34:** 258-259.
10. Levett PN, Morey RE, Galloway RL, Steigerwalt AG. *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. *Int J Syst Evol Microbiol* 2006; **56**: 671-3.
11. Perolat P, Chappel RJ, Adler B, Baranton G, Bulach DM, Billinghurst ML, Letocart M, Merien F, Serrano MS. *Leptospira fainei* sp. nov., isolated from pigs in Australia. *Int J Syst Bacteriol* 1998; **48:** 851-8.
12. Yasuda PH, Steigerwalt AG, Sulzer KR, Kaufmann AF, Rogers F, Brenner DJ. Deoxyribonucleic Acid Relatedness between Serogroups and Serovars in the Family *Leptospiraceae* with Proposals for Seven New *Leptospira* Species. *Int J Syst Evol Microbiol* 1987; **37**(4), *407-15.*
13. Smits HL, van der Hoorn MA, Goris MG, Gussenhoven GC, Yersin C, Sasaki DM, Terpstra WJ, Hartskeerl RA. Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis. *J Clin Microbiol* 2000; **38**(3): 1272-5.
14. Hartskeerl RA, Smits HL, Korver H, Goris MGA, Terpstra WJ. International course on laboratory methods for the diagnosis of leptospirosis; 2006, Course Manual. Amsterdam, the Netherlands: Royal Tropical Institute.
15. Faine S, Stallman N. Amended Descriptions of the Genus *Leptospira* Noguchi 1917 and the Species *L. interrogans* (Stimson 1907) Wenyon 1926 and *L. biflexa* (Wolbach and Binger 1914) Noguchi 1918. *IJSEM* 1982; **32**: 461-3.
16. Alex JC, Korver H, Ratnam S. *Leptospira* isolates of Madras City. *Ind J Med Microbiol* 1993; 17: 269-73.

17. Korver H, Kolk AHJ, Vingerhoed J, van Leeuwen J, Terpstra WJ. Classification of the serovars of Icterohaemorrhagiae serogroup by monoclonal antibodies. *Isr J Vet Med Isr J Vet Med* 1988; **44**, 15-8.
18. Anonymous. International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Leptospira*: Minutes of the Meeting, 5 and 6 September 1986, Manchester, England. *Int J Syst Bacteriol* 1987; **37**: 472-3.
19. Kmety E, Dikken H. *Classification of the species Leptospira interrogans and history of its serovars*. Groningen, the Netherlands: University Press Groningen; 1993.
20. Ahmed A, Thaipadungpanit J, Boonsilp S, Wuthiekanun V, Nalam K, Spratt B, *et al*. Comparison of two multilocus sequence based genotyping schemes for *Leptospira* species. *PLoS Negl Trop Dis* 2011; 5(11): e1374.
21. Ahmed N, Devi SM, Valverde ML, Vijayachari P, Machang'u RS, Ellis WA, Hartskeerl RA. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Ann Clin Microbiol Antimicrob* 2006; **5**:28.
22. Victoria B, Ahmed A, Zuerner RL, Ahmed N, Bulach DM, Quinteiro J, Hartskeerl RA. Conservation of the S10-spc-alpha locus within otherwise highly plastic genomes provides phylogenetic insight into the genus *Leptospira*. *PLoS One* 2008; **3**(7): e2752.
23. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; **28**(10): 2731-9.
24. Gravekamp C, van de Kemp H, Franzen M, Carrington D, Schoone G J, Van Eys G J, Everard CO, Hartskeerl RA, Terpstra WJ. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *J Gen Microbiol* 1993; **139**(8): 1691-1700.
25. Goris MGA, Leeflang MMG, Boer KR, Goeijenbier M, van Gorp ECM, Wagenaar JFP, Hartskeerl RA. Establishment of Valid Laboratory Case Definition for Human Leptospirosis. *J Bacteriol Parasitol* 2012; **3**(2).
26. Limmathurotsakul D, Turner EL, Wuthiekanun V, *et al*. Fool's gold: Why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. *Clin Infect Dis* 2012; **55**(3): 322-31
27. Valverde ML, Ramirez JM, Montes de Oca LG, Goris MG, Ahmed N, Hartskeerl RA. Arenal, a new *Leptospira* serovar of serogroup Javanica, isolated from a patient in Costa Rica. *Infect Genet Evol* 2008; **8**(5): 529-33.

CHAPTER 6

Letter to the Editor: Agglutinating antibodies against pathogenic *Leptospira* in healthy dogs and horses indicate common exposure and regular occurrence of subclinical infections

Dirk J. Houwers[1]
Marga G.A. Goris[2]
Theresia H. Abdoel[2]
Jeroen A. Kas[1]
Sandra S. Knobbe[1]
Astrid M. van Dongen[3]
Fenna E. Westerduin[4]
Wim R. Klein[4]
Rudy A. Hartskeerl[2]

1. Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, the Netherlands
2. WHO/FAO/OIE and National Leptospirosis Reference Centre and Section Rapid Diagnostics, KIT Biomedical Research, Amsterdam, the Netherlands
3. Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, University of Utrecht, the Netherlands
4. Department of Equine Sciences, Faculty of Veterinary Medicine, University of Utrecht, the Netherlands

Veterinary Microbiology 2011; 148(2-4): 449-51

INTRODUCTION

Clinical leptospirosis in dogs is often associated with recent exposure, directly, or indirectly, to surface water contaminated by brown rat urine. Interestingly, Weil's disease, the most prominent clinical presentation of leptospirosis mostly occurs as an individual case, although more individuals have usually been exposed to the same presumed source. In horses, clinical leptospirosis has a low incidence and the main clinical presentation probably is abortion in a limited number of mares on a stud, typically without a likely or identified source of infection. Furthermore, Equine Recurrent Uveitis (ERU) apparently associates with intra-ocular antibodies against *Leptospira*[1]. Interestingly, serovar Grippotyphosa is clearly predominant among *Leptospira* isolates from ERU-eyes[2], which is supported by the results from testing for intra-ocular antibodies using the Microscopic Agglutination Test (Houwers *et al*., unpublished).

Serosurveys among domestic animals are limited, but recent studies available show that agglutinating antibodies may occur in healthy animals and that there is considerable variation among the predominant serovars[3-6]. It is unclear what influence the biotope in which the surveyed animals live bears on the survey results, e.g. is infection with a certain serovar associated with the way specific animal species live and where they live or is the exposure more or less ubiquitous. Since leptospires are considered to be able to survive in the environment for longer periods of time provided there is sufficient moisture and ambient temperatures are moderate, it is likely that exposure is related to the type of environment the animal lives in. Evidently, the occurrence of specific serovars is also depending on the presence and prevalence of specific host species that act as infection reservoir. Together, these factors are responsible for the considerable differences in the outcomes of serological surveys between continents and geographical areas within continents.

Pet dogs live in close contact with man and share a considerable part of his environment, but in their typical behaviour when they are being walked and particularly when they are let loose they also penetrate the biotope of a variety of other, mainly small, mammals.
Horses typically behave differently and live in a different environment which usually combines pasture and a stable, but these biotopes are also shared with a number of small mammals. In winter, most horses are fed roughage which is almost inevitably contaminated by mouse, and often rat, urine. So, dogs as well as horses are, mainly indirectly, exposed to animals that can be reservoirs of leptospires. Some leptospiral serovars are associated with particular reservoir species, e.g. Icterohaemorrhagiae and Copenhageni with brown rats (*Rattus norvegicus*), whereas other serovars have several, or as yet unknown, reservoir species.

In order to gain insight into the level of exposure to pathogenic leptospires present in the environment, we performed a serosurvey using the microscopic agglutination test (MAT) among healthy horses and dogs living in the Netherlands. Generally, dogs and horses

share only a part of their environments and they have totally different feeding patterns – carnivorous versus herbivorous – hence they are exposed to different environmental sources and routes of infection. Since man shares his environment with dogs and horses, the results of this survey may also shed light on the potential exposure of humans.

MATERIALS AND METHODS

During the spring of 2003, blood samples taken by local veterinary practitioners in the framework of routine clinical procedures from 112 healthy dogs and 112 horses were made available with their owners consent. Among the dog samples, 86 were obtained through 3 veterinary clinics with a mainly urban patient population situated in various parts of the country. Sera were collected at random, provided the dogs had no complaints suggestive of an infectious disease. In addition, 26 sera from healthy dogs in a non-vaccinated hunting pack were included.

During the autumn of 2009, a total of 100 dog samples were obtained by random collection of blood samples submitted to a clinical chemistry laboratory by veterinary practitioners across the Netherlands; samples were only enrolled if they had accompanying clinical information that did rule out the likelihood of the presence of an infectious disease.

The ages of the dogs ranged from 7 months to 16 years. Apart from those in the hunting pack, the vaccination statuses of the dogs were unknown.

The horse serum samples were obtained from two different geographical areas in the Netherlands, i.e. the west of country with clay-type soils with many permanently water-filled ditches surrounding the pastures (n = 58), and the eastern part with sandy soils and pastures mainly surrounded by fences (n = 54). The maximum number of samples per owner or establishment was four. Since a vaccine for horses is not available, no horse ever received leptospiral vaccination.

Sera were stored at -20° C until tested with MAT at the WHO/FAO/OIE and National leptospirosis reference laboratory at KIT Biomedical Research in Amsterdam (all sera were tested within 6 months after collection). The standard Dutch leptospires panel consisting of 16 strains (15 serovars) was employed. The panel included the non-pathogenic serovar Patoc, which is incorporated for its broad cross-reactivity. Since the absence of agglutination at serum dilution 1:20 was clearly detectable and a number of sera clearly showed no agglutination with any strain, titers of 1:20 or higher were considered specific and thus positive.

RESULTS

Dogs

Of the total of 86 healthy family dogs, 7 agglutinated serovar Canicola; they also agglutinated serogroup Icterohaemorrhagiae (serovars Copenhageni and Icterohaemorrhagiae). Since the available leptospiral vaccines only contain serogroups Canicola and Icterohaemorrhagiae and serogroup Canicola is considered virtually extinct in the Netherlands as a consequence of the relatively high degree of vaccination[7] these dogs were considered to have been vaccinated recently and their results were removed from the data. Of the remaining 79 dogs 57 (72%) had reciprocal titers ranging from 20 to 640 against at least one of the serovars in the panel; the most commonly agglutinated serovar was Copenhageni, closely followed by Patoc and to a much lesser degree Icterohaemorrhagiae.

Of the total of 100 dog samples obtained in 2009, 7 had MAT titers against serovars Canicola, Icterohaemorrhagiae and Copenhageni. These were also removed from the data for the above reason. Of the remaining total of 93 samples, 30 (32%) had MAT titers against one or more serovars with Copenhageni being predominant, also showing the highest titer (1:320).

A total of 22 of the 26 (87%) dogs in the hunting pack had titers: serovar Patoc was most commonly agglutinated, closely followed by Copenhageni and Bratislava, with Saxkoebing and Sejroe at some distance. Notably, these dogs have never been vaccinated.

Horses

A total of 89 of 112 horses (79%) had agglutinating antibodies against one or more serovars with Copenhageni and Patoc being predominant, followed by Bratislava and Grippotyphosa. Highest titers found were against Bratislava (1:320 and 1:640). The results for the two different biotopes – east/dry versus west/wet – did not clearly differ.

Note that titers against the saprophytic serovar Patoc do not signify homologous infection but indicate infection with a cross-reacting pathogenic serovar.

DISCUSSION

In view of the likelihood of a certain degree of crossreactivity between the strains in the panel, it is impossible to extract strain prevalences from these data, hence conclusions are restricted to the relative predominance of strains.

With respect to the horse samples, the results clearly show that antibodies against pathogenic serovars occur in horses that are healthy according to their owners. Even if it is argued that titers of 1:20 or 40 are less specific, this conclusion still stands because higher titers were

also recorded. Since horses in the Netherlands are not vaccinated against leptospirosis, the presence of these antibodies can only be explained by the occurrence of subclinical infections, consequently that the average equine biotope in the Netherlands associates with exposure to pathogenic leptospires. Notably, the dominant serovar is Copenhageni which, together with serovar Icterohaemorrhagiae, is associated with most cases of Weil's disease in humans and dogs. Thus, horses are exposed to Copenhageni and other pathogenic serovars, but infections apparently take a subclinical course in this host species.

The results of the three groups of dog samples also show predominance of Copenhageni, although the prevalence varied between the groups. Since most dogs in this study may have been vaccinated before sampling there is a chance of a vaccination bias. However, by removing the 14 dogs that agglutinated serovars Canicola and either Icterohaemorrhagiae or Copenhageni or both (closely related serovars that both belong to serogroup Icterohaemorrhagiae) the possibility of vaccination bias was strongly reduced. Moreover, the dogs in the non-vaccinated dog pack showed clear Copenhageni agglutinating titers. And, in addition, the results of the – unvaccinated – horses demonstrate that exposure to Copenhageni is common. Together, these findings suggest that the prevalence of antibodies and the predominance of Copenhageni are not caused by vaccination but are the result of subclinical infections. There is no straightforward explanation for the difference in frequency of MAT-positivity between the samples of 2003 and 2009, but a seasonal effect has been demonstrated[5,7] and differences in exposure over the years are likely. Nevertheless, the results of the dog samples show that dogs in their average biotope are also exposed to pathogenic *Leptospira* and that they more or less regularly undergo subclinical infections. In addition, the predominance of Copenhageni agglutination is similar to that in horses which indicates that the brown rat – the main reservoir of this serovar – contaminates both biotopes leading to similar levels of exposure. Our findings are fully in concordance with a recent paper, which reported a 7% excretion rate of pathogenic leptospires by healthy dogs from sanctuaries in Ireland, which suggests a considerable seroprevalence[8]. The logically arising question is why only very few dogs (and horses) apparently develop clinical disease while others might adapt to a commensal relationship. Basically, this could be determined by species-specific or individual host factors, or, alternatively, by differences in the infection doses or differences within the infecting serovar.

In conclusion, this study shows that dogs and horses in the Netherlands are commonly exposed to and regularly subclinically infected by pathogenic *Leptospira*. Since humans, dogs and horses in part share their biotopes, humans are also basically exposed. Note that most humans generally do not behave like dogs and horses; their hygiene will presumably result in fewer infections. These findings are in line with recent observations of others clearly demonstrating that also humans may experience subclinical infection with pathogenic leptospires and that only few of those exposed and infected fall ill[9].

REFERENCES

1. Wollanke B, Gerhards H, Brem S, Kopp H, Meyer P. [Intraocular and serum antibody titers to *Leptospira* in 150 horses with equine recurrent uveitis (ERU) subjected to vitrectomy]. *Berl Munch Tierarztl Wochenschr* 1998; **111**(4): 134-9.
2. Hartskeerl RA, Goris MG, Brem S, *et al*. Classification of *Leptospira* from the eyes of horses suffering from recurrent uveitis. *J Vet Med B Infect Dis Vet Public Health* 2004; **51**(3): 110-5.
3. Stokes JE, Kaneene JB, Schall WD, *et al*. Prevalence of serum antibodies against six *Leptospira* serovars in healthy dogs. *J Am Vet Med Assoc* 2007; **230**(11): 1657–64.
4. Iwamoto E, Wada Y, Fujisaki Y, *et al*. Nationwide survey of *Leptospira* antibodies in dogs in Japan: results from microscopic agglutination test and enzyme-linked immunosorbent assay. *J Vet Med Sci* 2009; **71**(9): 1191-9.
5. Gautam R, Guptill LF, Wu CC, Potter A, Moore GE. Spatial and spatio-temporal clustering of overall and serovar-specific *Leptospira* microscopic agglutination test (MAT) seropositivity among dogs in the United States from 2000 through 2007. *Prev Vet Med* 2010; **96**(1-2): 122-31.
6. Baverud V, Gunnarsson A, Engvall EO, Franzen P, Egenvall A. *Leptospira* seroprevalence and associations between seropositivity, clinical disease and host factors in horses. *Acta Vet Scand* 2009; **30**(51): 15.
7. Hartman EG. Epidemiological aspects of canine leptospirosis in the Netherlands. *Zentralbl Bakteriol Mikrobiol Hyg A* 1984; **258** ((2–3)): 350–9.
8. Rojas P, Monahan AM, Schuller S, Miller IS, Markey BK, Nally JE. Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. *Eur J Clin Microbiol Infect Dis* 2010; **29**(10): 1305-9.
9. Speelman P, Hartskeerl RA. Leptospirosis. Harrison's Internal Medicine; 2008.

PART 2

Diagnosis

CHAPTER 7

Establishment of valid laboratory case definition for human leptospirosis

Marga G.A. Goris[1]
Mariska M.G. Leeflang[2]
Kimberly R. Boer[1]
Marco Goeijenbier[4]
Eric C.M. van Gorp[4]
Jiri F.P. Wagenaar[1,3]
Rudy A. Hartskeerl[1]

1. Royal Tropical Institute (KIT), KIT Biomedical Research, Amsterdam, the Netherlands
2. Department of Clinical Epidemiology and Biostatistics, Academic Medical Center, Amsterdam, the Netherlands
3. Slotervaart Hospital, Department of Internal medicine, Amsterdam, the Netherlands
4. Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands

Journal of Bacteriology and Parasitology 2012; 3(2)

ABSTRACT

Laboratory case definition of leptospirosis is scarcely defined by a solid evaluation that determines cut-off values in the tests that are applied. This study describes the process of determining optimal cut-off titers of laboratory tests for leptospirosis for a valid case definition of leptospirosis. In this case the tests are the microscopic agglutination test (MAT) and an in-house IgM enzyme-linked immunosorbent assay (ELISA) both on single serum and paired samples using a positive culture as the reference test in the Dutch population. The specificity was assessed using panels of sera from healthy donors, cases with known other diseases and non-leptospirosis cases with symptoms compatible with leptospirosis. Cases were divided into three periods corroborating the acute phase (1-10 days post onset of illness (DPO)), the early convalescent (11-20 DPO) and the late convalescent phase (>20 DPO). Cut-off titers for MAT and IgM ELISA were determined as 1:160 and 1:80 respectively for all three periods. These cut-off titers combined 100% specificity with a sensitivity that changed according to the stage of disease for both tests. The low sensitivities in the early acute phase are consistent with the dynamics of the humoral immune response. IgM ELISA yielded higher sensitivities compared to MAT in the acute and early convalescent stages. Moreover, the optimal sensitivity of MAT, the gold standard was < 82%, implying that a significant part of global cases is missed by this recommended test. MAT and IgM ELISA manifested partly complementary, resulting in a higher sensitivity when combining the results of these two tests. The availability of paired samples and of adequate clinical and epidemiological data are other parameters that will significantly increase the sensitivity of laboratory confirmation. This study enables fine-tuning of the current laboratory definition towards an improved case finding and implies that solid validation of laboratory parameters for case definition will improve both the diagnosis for individual patient care and for estimating the disease burden at a worldwide scale.

INTRODUCTION

Leptospirosis, a zoonosis with a worldwide distribution, is an acute febrile illness caused by microorganisms of the genus *Leptospira*[1]. Leptospires enter the body through small cuts or abrasions, via mucous membranes such as the conjunctiva and possibly through wet skin and circulate in the blood stream, with a bacteremic phase lasting for up to 10 days post onset of the disease (DPO). The disease has a sudden onset with headache, fever, malaise, myalgia, conjunctival suffusion and sometimes a transient rash. Thereafter, the illness may rapidly develop into a severe, potentially fatal form with a high mortality rate.

Because of the wide diversity of clinical signs, diagnosis of leptospirosis is difficult and depends upon a variety of laboratory assays such as detection of specific antibodies in the blood by microscopic agglutination test (MAT) and enzyme-linked immunosorbent assays (ELISA). Leptospires or their components may be detected in blood, urine or tissues by culture, dark field microscopy, immuno-staining or PCR[2,3]. The diagnosis of leptospirosis is not usually done on bacterial detection, but is mainly based on serology. Culturing provides proof of infection but is tedious and slow. This results in a high percentage of false negative findings, whereas the sensitivity of culturing usually does not exceed 20%[4-6]. The MAT is the reference standard in serodiagnosis. It is the most widely used diagnostic test for leptospirosis in the world and presents the only serological test that can be applied on any infected mammalian species. The MAT has the advantage of being specific for serogroups, although cross reactions and paradoxical reactions between serogroups do occur[7]. However, there are limitations in sensitivity in the early phase of the disease since detectable titers of antibodies appear in the blood about 5–10 days after the onset of disease[2], and sometimes later, especially if antibiotic treatment is instituted[8]. In addition, cross-agglutination of antibodies against other diseases and repeated exposure to *Leptospira* in endemic regions cause background titers[8,9]. The performance of the MAT is further complicated because live cultures of different *Leptospira* serovars prevalent in a particular geographical area are required for optimal performance[8].

Thus, the leptospirosis case definition including laboratory parameters varies in different epidemiological backgrounds and ideally requires local assessments of the laboratory tests. However, valid determination of cut-off titers for case definition is only scarcely done[9] or are not publicly presented[10,11] and current case definitions are mainly based on general assumptions and experience of experts[8,12,13]. In addition, case definitions formulate clinical manifestations either too wide or specify only forms of severe disease with signs and symptoms that often develop in the immune phase of the disease, when antibiotic treatment is less effective[14,15]. The lack of well-defined laboratory criteria for regional case definitions is mainly due to a lacking knowledge on the assessment of optimal diagnostic accuracies of tests as well as on the easy availability of globally recommended criteria[8,13]. The global criterion for laboratory confirmation of a current *Leptospira* infection is usually defined as seroconversion or a four-fold rise in titer in paired serum samples or set at a single MAT titer ≥ 1:400 in the presence of clinical signs and appropriate history of animal contact[16].

This research describes the process of establishing optimal cut-off titers of tests for leptospirosis in order to obtain a valid laboratory case definition of leptospirosis, in this case the performance of MAT and ELISA in the population of the Netherlands. For this purpose, a positive culture is used as the reference test to define a case. For the clinical setting it is important to know how to interpret these diagnostic tests in relation to the time of sampling. Although cut-off titers will vary across distinct endemic situations, still this assessment will serve as a valuable example for others, irrespective the local situation. An improved, tailor-made case definition enables a better case finding and thus will contribute to an improved worldwide diagnosis of leptospirosis.

MATERIALS AND METHODS

Patients and sera

Culture positive patients: In the period of investigation, the population in the Netherlands increased from 14.1 to 16.4 million with a median of 15.6 million. The WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis (NRL) at KIT Biomedical Research in Amsterdam receives clinical material (whole blood, serum, EDTA blood, heparinized blood or urine) for testing from every patient suspected for leptospirosis in the Netherlands. Yearly, NRL receives about 600 specimens of suspected patients and confirms on average 30 cases[4,5]. Nowadays approximately 50% of the confirmed cases consist of imported infections, usually associated to adventurous activities such as white water rafting or jungle tracking during vacation in tropical countries[6,17]. Culture on blood is performed when sampled within 10 days after onset of symptoms. Culture on urine can be performed at any timepoint in the disease, although not often requested since the inoculation into the culture medium has to take place within two hours after voiding. From each blood sample routine diagnostics are performed by MAT and IgM ELISA. In the period 1980-2009 leptospirosis was confirmed by a positive culture in 109 patients, referred to as 'cases' in this paper. Samples were selected from these cases in order to have no more than one sample of each patient in each of the time periods 1 to 10 days post onset (DPO), 11 to 20 DPO and more than 20 DPO according to the most relevant periods for laboratory confirmation. The first period is the acute phase defined by low sensitivity because of the absence of detectable amounts of antibodies in most samples. Antibody levels reach a maximum after about 10 days[18] and are well detectable in the early convalescent phase and steadily decrease after 20 days[2]. When multiple samples of one patient were present in a period of time, the sample with the lowest DPO was chosen (Table 1).

Healthy controls: Sera (n=110) from healthy blood bank donors were obtained from the local blood bank and served as healthy controls to determine cross-reacting antibodies in the population.

Table 1 | Samples selected according to disease period expressed as days post onset (DPO)

Patients (n)	Samples (n) per patient	Sample taken from patients (n) at time period		
		DPO 1-10*	DPO 11-20	DPO >20
15	1	11**	0	4
58	2	26	26	
		29		29
			3	3
30	3	30	30	30

*Where 1-10 DPO is divided into 1-4 and 5-10 DPO the number of samples is 48 respectively 62.
** 5 of these patients have died therefore no follow up sample is available

Controls with known disease to determine specificity based on selectivity: The following controls with known disease were used to screen for cross-reactivity to determine specificity based on selectivity. Sera from patients with human immunodeficiency virus (HIV) infection (n=20), hepatitis A virus infection (n=10), hepatitis B virus infection (n=9), malaria (n=20), toxoplasmosis (n=11) and meningococcal meningitis (n=20) since these infectious agents could cause symptoms similar to leptospirosis. Moreover, a control group with proven syphilis (n=19), also a spirochete and related to *Leptospira* and a group with autoimmune disease (rheumatoid arthritis, n=10; systemic lupus erythematosus, n=20) were used since these diseases are notorious for generating cross-reactive antibodies. These samples are referred to as 'known controls'.

Controls with unknown disease: Sera were taken from patients suspected of leptospirosis but having a negative culture and for whom leptospirosis could not be confirmed. These samples are referred to as 'unknown controls' and were divided into an acute set, 1-10 DPO (n=62) and a convalescent set, >10 DPO (n=63).

Laboratory tests

Microscopic agglutination test: The microscopic agglutination test (MAT) was performed with a panel of live leptospires as described previously[19]. The panel consisted of 16 strains of the pathogenic serovars Bratislava, Ballum, Canicola, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Copenhageni, Poi, Pomona, Proechimys, Hardjo, Saxkoebing and Sejroe, and the non-pathogenic serovar Patoc. The sera from patients who were probably infected abroad were additionally examined by using a second panel of 12 strains representative of global leptospires, comprising the pathogenic serovars Australis, Rachmati, Bataviae, Celledoni, Cynopteri, Mini, Panama, Pyrogenes, Shermani and Tarassovi, and the non-pathogenic serovars Andamana and Semaranga. Non-pathogenic serovars were included

to detect cross-agglutinating antibodies against pathogenic serovars that are not represented in the panels. Titers with these saprophytic serovars have no diagnostic relevance and are excluded from this assessment. Agglutination was performed in microtiter plates with serial twofold dilutions of the sera starting at 1:20 (5 µl of serum, 45 µl of phosphate buffered saline, and 50 µl of antigen, incubated for 2-4 hours at 30 °C). Agglutination was examined by dark-field microscopy. The titer was defined as the highest dilution giving 50% agglutination in comparison with that of the negative control[8,20].

IgM ELISA: The in-house developed ELISA for the detection of *Leptospira*-specific IgM antibodies (IgM ELISA) was performed with antigen prepared from a well grown culture of the local strain Wijnberg (serovar Copenhageni, serogroup Icterohaemorrhagiae) in EMJH medium[21]. The culture was killed by formalin (final concentration 0.5% v/v), heated in a boiling water bath for 30 min and centrifuged for 30 min at 10,000 g. The supernatant was used for antigen. In each well of polystyrene micro titer plates (Greiner, Frickenhausen, Germany, Microlon 96 wells plates, medium binding) 100 µl of the supernatant was left to evaporate at room temperature. The coated plates were stored in a dry place at room temperature[21]. Before use, the plates were thoroughly washed with phosphate buffered saline (PBS) pH 7.2, containing 0.05% Tween 20 and serial twofold dilutions were made of the sera starting at 1:10 (10 µl serum and 90 µl of PBS containing 0.05% Tween 20 and 0.5% bovine serum albumin). After incubation with the serum dilutions for 1 h at 30 °C the micro titer plates were washed and subsequently incubated with peroxidase-conjugated anti-human IgM (Biorad, Veenendaal, the Netherlands). After 1 h incubation at 30 °C, the plates were washed again and finally incubated with substrate (5-aminosalicylic acid; Merck). After 2 h the reaction was read at wavelength 492 nm. The titer was the last dilution giving an absorbance of more than half the value of a positive control serum[20].

Culture: The culture medium used was of Ellinghausen-McCullough as modified by Johnson and Harris (EMJH) supplemented with 5-fluorouracil (200µg/ml) (EMJH-FU)[8,16], rabbit serum (EMJH-RS) and 1% (V/V) fetal calf serum (EMJH-FCS) or combinations. Fletcher medium was prepared according to standard protocols[8,16,20]. The culture was done on blood (approximately 150 µl) which was inoculated into the following culture media (6 ml): Fletcher medium, EMJH, EMJH-FU, EMJH-FCS-RS, and EMJH-FCS-RS-FU. Urine was diluted 1:10, 1:100 and 1:100 onto EMJH-FU and EMJH-FCS-RS-FU. Inoculated media are incubated for a maximum of 4 months at 30 °C and biweekly checked for leptospiral growth by darkfield microscopy.

Data collection

From each sample Patient ID, sample ID, highest titer in MAT with the pathogenic strains, IgM ELISA titer and days post onset of symptoms (DPO) were retrieved from the KIT archives and entered into EPI INFO version 6.

Analyses

Sensitivity and corresponding confidence intervals was calculated for each cut-off titer by dividing the number of cases having at least this titer by the total number of cases. This was done for three time periods (1-10 DPO, 11-20 DPO and >20 DPO). Specificity and corresponding confidence intervals was calculated for three subgroups: healthy controls, known controls and unknown controls. The unknown controls were divided into two time points, 1-10 DPO and >10 DPO. Receiver Operating Characteristic (ROC) curves were constructed in Microsoft Excel. These curves show the true positive rate (in the cases) against the false positive rate (for each of the control subgroups) for the different cut-off titers of the MAT and IgM ELISA. For both tests for each time period (1-10 DPO, 11-20 DPO and >20 DPO) the cut-off values yielding an optimal combination of sensitivity and specificity were chosen. During the acute phase, detectable levels of anti-*Leptospira* antibodies are produced, so it can be expected that the sensitivity of MAT and IgM ELISA is much lower in the early acute phase (DPO <5). Therefore the acute phase was divided into 1 to 4 days and 5 to 10 days. From the optimal values combinations of MAT AND/OR IgM ELISA were made, and sensitivity and specificity were calculated on the complete dataset. ROC curves were plotted from these combinations in order to find out whether the sensitivity and/or specificity can be increased by combining the results from both tests. In the diagnosis of leptospirosis the optimal cut-off values are defined as those titers having the highest specificity. Confidence intervals will aid in determining which sensitivity and specificity are most precise based on the data. Results are presented based on MAT and IgM ELISA values on single sera, combinations of MAT AND/OR IgM ELISA values on single sera and seroconversion in paired sera. Serology on paired sera is much more reliable compared to a single sample[12] since a follow-up sample will take into account the effect of background or cross-reacting titers. Thus an optimal specificity is reached at a lower titer. We therefore argued that a one-step lower cut-off titer can be used for MAT and IgM ELISA on paired samples, herewith increasing the test sensitivity. Hence, the sensitivity is calculated on follow up samples using one-step lower cut-off titers in the tests. Paired samples from 68 cases were used to check the integrity of this approach.

RESULTS

Patient samples

The total number of positive leptospirosis cases in the period 1980-2009 (based on culture and/or serology) is 919. One hundred and seven cases were found culture positive (11.6%). Leptospires were isolated from urine in 8 cases and from blood in 99 cases. Four cases were excluded from the analysis: first day of illness was not documented in three cases and ELISA results were missing in one case. The remaining group of 103 cases consisted of 95 men of whom 6 died (ranging from 6 to 30 DPO, median 7 DPO), and 8 women. Serovars belonging

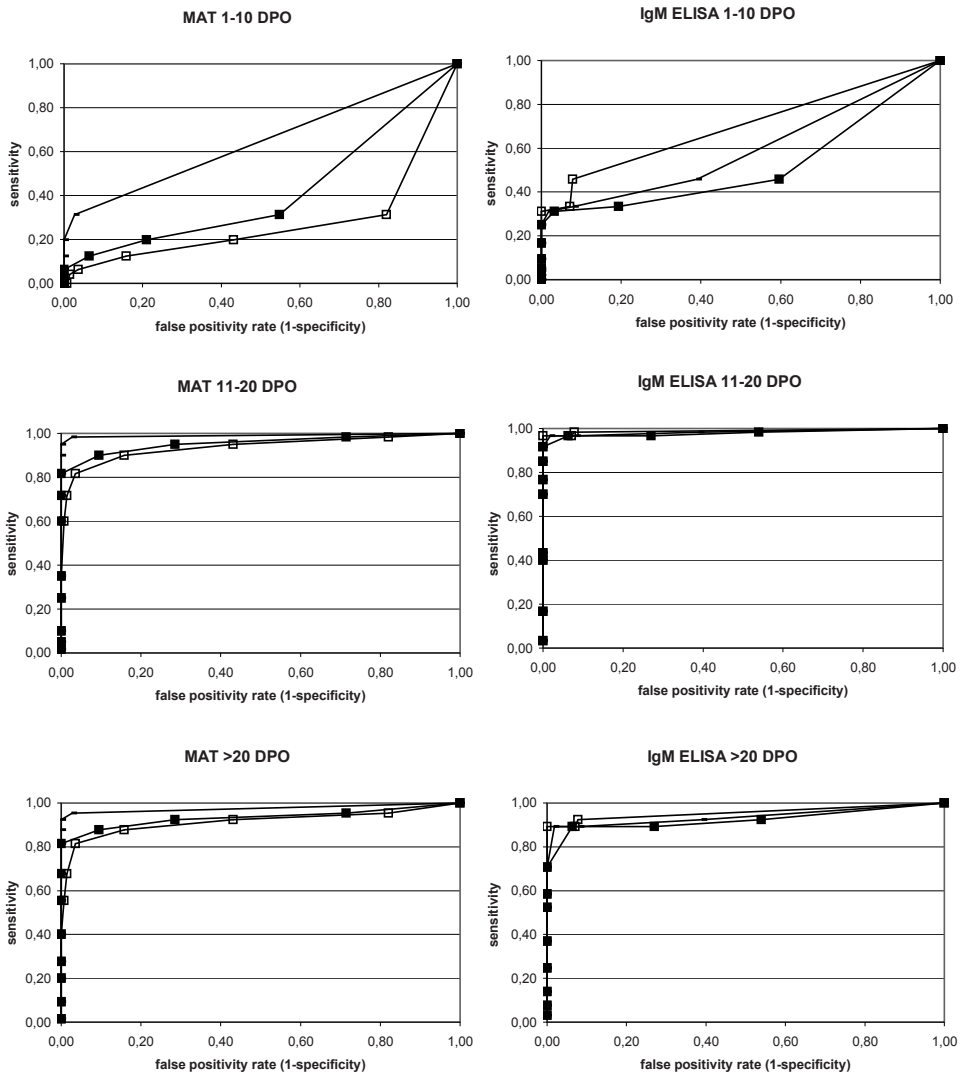


Figure 1 | Receiver Operating Characteristic (ROC) Curve of cut-off titers of MAT and IgM ELISA in different time periods.

Healthy controls: _
Known controls:▫
Unknown controls:▪

to the following serogroups were isolated: Icterohaemorrhagiae n=57, Grippotyphosa n=17. Autumnalis n=6, Celledoni n=3, Hebdomadis n=3, Pyrogenes n=3, Sejroe n=3, Bataviae n=2, Canicola n=2, Javanica n=2, Pomona n=2, Shermani n=2, Cynopteri n=1.

Determination of cut-off titers on single sera for MAT and IgM ELISA:
The ROC curves showing the relationship between sensitivity and specificity according to each the acute phase and the early and late convalescent phases are depicted in Figure 1. The ROC curves include the most optimal sensitivity-specificity distribution for the set of healthy blood donors and indicate, as expected, that the specificity of MAT and IgM ELISA is not limited by cross-reacting antibodies in the healthy population. ROC curves for the known and unknown controls largely coincided, albeit that the IgM ELISA seemed to perform more stable than MAT. Specificity based on known controls (selectivity) indicates that cross-reacting antibodies to other diseases may interfere with the diagnosis and as such have importance that cannot be ignored. However, this selectivity is biased by inclusion of diseases that are unlikely to be confused with leptospirosis and overrates their impact on the practical situation that is better presented by the specificity based on unknown controls. Therefore, specificity based on unknown controls was selected as the most important parameter for further assessment of optimal cut-off values. From the ROC plots, MAT and IgM ELISA titers were selected for which optimal sensitivity and specificity was achieved for each time period (Table 2). When several optimal values were close together on the ROC curve all those values were listed in the table, the combination with the highest specificity being marked. For IgM ELISA a cut-off titer of ≥ 1:80 signified 100% specificity in all periods and was selected as the optimal cut-off value. This was associated with sensitivities of 25.0%, 91.7% and 70.8% for the time periods 1- 10 DPO, 11-20 DPO and >20 DPO, respectively. Cut-off titers of ≥1:40 did give a higher sensitivity in all categories but were associated with a lower specificity (Table 2). For MAT, 100% specificity was reached at a cut-off titer of ≥1:160. This was associated with a lower selectivity of 96.4% (not shown) and corresponded to sensitivities of 6.3%, 81.7% and 81.5%, for periods 1-10 DPO, 11-20 DPO and >20 DPO, respectively.

To investigate the acute phase, the time period 1-10 DPO was divided into two periods of 1-4 DPO (early acute) and 5-10 DPO (late acute). As expected, at the cut-off titer of ≥1:80, the sensitivity of IgM ELISA was only 6.3% in the early acute phase but rapidly increased to 53.2% in the late acute phase. For MAT this was 2.1 and 24.2%, respectively (Figure 2). This supports the notion that serology has minor importance at the very early phase of disease and only becomes useful during the second half of the first week of illness. The sensitivities of MAT in the acute phase are markedly lower than those of IgM ELISA, showing that IgM ELISA tends to give an earlier positive outcome compared to MAT.

Determination of cut-off titers on single sera for MAT combined with IgM ELISA:
From the results in Table 2, sensitivities and specificities were calculated for all possible combinations of MAT and IgM ELISA in the samples and plotted in ROC curves (not shown).

Table 2 | Sensitivity and specificity and corresponding 95% confidence intervals (CI) of either MAT or IgM ELISA

DPO	Cut-off titer	MAT Sensitivity (%)	95% CI	MAT Specificity* (%)	95% CI	IgM ELISA Sensitivity (%)	95% CI	IgM ELISA Specificity* (%)	95% CI
1-10	≥1:40	19.8	13.1-28.9	79.0	67.4-87.3	31.3	22.9-41.1	96.8	89.0-99.1
	≥1:80	12.5	7.3-20.6	93.5	84.6-97.5	**25.0**	**17.4-34.5**	**100.0**	94.2-100
	≥1:160	**6.3**	**2.9-13.0**	**100.0**	94.2-100.0	16.7	10.5-25.4	100.0	94.2-100
11-20	≥1:40	95.0	86.3-98.3	71.4	59.3-81.1	96.7	88.6-99.1	93.7	84.8-97.5
	≥1:80	90.0	79.9-95.3	90.5	80.7-95.6	**91.7**	**81.9-96.4**	**100.0**	94.2-100.0
	≥1:160	**81.7**	**70.1-89.4**	**100.0**	94.2-100.0	85.0	73.9-91.9	100.0	94.2-100.0
>20	≥1:40	92.3	83.2-96.7	71.4	59.3-81.1	89.2	79.4-94.7	93.7	84.8-97.5
	≥1:80	87.7	77.5-93.6	90.5	80.7-95.6	**70.8**	**58.8-80.4**	**100.0**	94.2-100.0
	≥1:160	**81.5**	**70.4-89.1**	**100.0**	94.2-100.0	58.5	46.3-69.6	100.0	94.2-100.0

Bold text indicates the optimal cut-off for sensitivity and specificity based on the ROC plots with priority on a high specificity.

*specificity based on unknown controls

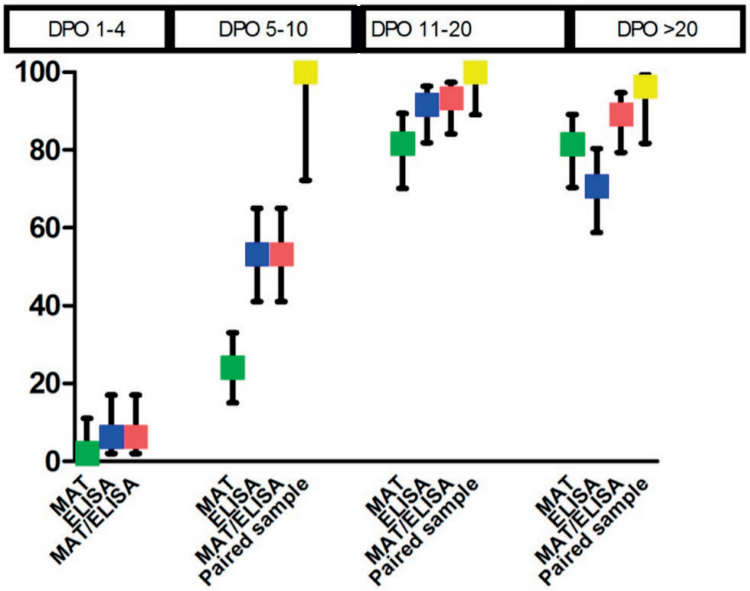


Figure 2 | Sensitivity and corresponding 95%CI in the periods 1-4 DPO, 5-10 DPO, 11-20 DPO and >20 DPO of MAT (cut-off titer ≥ 1:160), IgM ELISA (cut-off titer ≥1:80), combination "MAT ≥ 1:160) OR IgM ELISA ≥1:80", and "paired samples' of which follow up sample is taken in the designated time period.

The optimal values of these combinations are shown in Table 3. Considering the limitations inflicted on MAT by the selectivity, the optimal cut-off titer of the MAT was again set to ≥1:160. For the categories DPO 1-10, both the cut-off titer combinations 'MAT ≥1:160 OR IgM ELISA ≥1:80' and 'MAT ≥1:160 AND IgM ELISA ≥1:80' did give 100% specificity but the sensitivity was much higher (25%) in the combination 'OR'. As indicated by the results for the separate tests, serology on early acute sera samples is not very helpful, also when combining the titers of the two tests (Figure 2). However, for DPO 5-10, the cut- off titer combination 'MAT ≥1:160 OR IgM ELISA ≥1:80' represented a sensitivity of 53.2% with a specificity of 100% (Figure 2). In the period 1-10 DPO, all samples having a MAT titer ≥1:160 also had an IgM ELISA titer of ≥1:80, so the sensitivity of 'MAT ≥1:160 OR IgM ELISA ≥1:80' is determined by the IgM ELISA. The optimal combination for both the periods 11-20 DPO and >20 DPO was 'MAT ≥1:160 OR IgM ELISA ≥1:80'. Specificity was in both periods 100% and associated with a decrease of sensitivity from 93.3 to 89.2% in the later stage, reflecting the decrease of antibody levels in the late convalescent stage of disease[2].

Paired samples

Sensitivity for follow up samples or samples that are accompanied by clinical and epidemiological data compatible with leptospirosis was calculated for a cut-off titer 1:40 for IgM ELISA and 1:80 for the MAT, accounting both for seroconversion and a minimal 4-fold titer rise in both tests. For the time period 1-10 DPO, this implied a sensitivity of 12.5% and

Table 3 | Optimal sensitivity and specificity by combining MAT and IgM ELISA results

DPO	Cut-off titer MAT	AND/OR	Cut-off titer IgM ELISA	Sensitivity (%)	95% CI	Specificity (%)	95% CI
1-10	≥1:80	AND	≥1:40	10.4	5.8-18.1	100.0	94.2-100.0
	≥1:80	OR	≥1:40	33.3	24.7-43.2	90.3	80.4-95.5
	≥1:80	AND	≥1:80	10.4	5.8-18.1	100.0	94.2-100.0
	≥1:80	OR	≥1:80	27.1	19.2-36.7	93.5	84.6-97.5
	≥1:160	AND	≥1:40	6.3	2.9-13.0	100.0	94.2-100.0
	≥1:160	OR	≥1:40	31.3	22.9-41.1	96.8	89.0-99.1
	≥1:160	AND	≥1:80	6.3	2.9-13.0	100.0	94.2-100.0
	≥1:160	**OR**	**≥1:80**	**25.0**	**17.4-34.5**	**100.0**	**94.2-100.0**
11-20	≥1:80	AND	≥1:40	90.0	79.9-95.3	98.4	91.5-99.7
	≥1:80	OR	≥1:40	96.7	88.6-99.1	85.7	75.0-92.3
	≥1:80	AND	≥1:80	86.7	75.8-93.1	100.0	94.2-100.0
	≥1:80	OR	≥1:80	95.0	86.3-98.3	90.5	80.7-95.6
	≥1:160	AND	≥1:40	81.7	70.1-89.4	100.0	94.2-100.0
	≥1:160	OR	≥1:40	96.7	88.6-99.1	93.7	84.8-97.5
	≥1:160	AND	≥1:80	80.0	68.2-88.2	100.0	94.2-100.0
	≥1:160	**OR**	**≥1:80**	**93.3**	**84.1-97.4**	**100.0**	**94.2-100.0**
>20	≥1:80	AND	≥1:40	81.5	70.4-89.1	98.4	91.5-99.7
	≥1:80	OR	≥1:40	95.4	87.3-98.4	85.7	75.0-92.3
	≥1:80	AND	≥1:80	67.7	55.6-77.8	100.0	94.2-100.0
	≥1:80	OR	≥1:80	90.8	81.3-95.7	90.5	80.7-95.6
	≥1:160	AND	≥1:40	76.9	65.4-85.5	100.0	94.2-100.0
	≥1:160	OR	≥1:40	93.8	85.2-97.6	93.7	84.8-97.5
	≥1:160	AND	≥1:80	63.1	50.9-73.8	100.0	94.2-100.0
	≥1:160	**OR**	**≥1:80**	**89.2**	**79.4-94.7**	**100.0**	**94.2-100.0**

Bold text indicates the optimal cut-off for sensitivity and specificity combinations with priority on a high specificity.

31.3% for MAT and IgM ELISA, respectively (Table 2). For the category 11-20 DPO this was 90.0% and 96.7% respectively, declining to 87.7%, and 89.2% for >20 DPO. When combining the MAT and IgM ELISA results at these cut-off titers, the three categories had sensitivities of 33.3%, 96.7% and 95.4%, respectively (Table 3). In our study, 68 paired samples with initial titers not fulfilling the cut-off titer combination 'MAT ≥1:160 OR IgM ELISA ≥1:80', showed in 67 cases (98.5%) in the follow up sample seroconversion or at least a four-fold titer rise after 2 to 121 days (median 12 days). Sixty three cases did show seroconversion or titer rise in the

follow up sample exceeding the criteria set for a single sample, while another four did meet the criteria set for paired samples. The case not showing seroconversion was infected with serovar Grippotyphosa; samples were taken on 3 and 30 DPO.

DISCUSSION

Together with the difficult diagnosis of leptospirosis both in the clinic and in the laboratory, inadequate case definitions probably form a major cause for the global underestimation and unawareness of the disease. Good knowledge on how to assess optimal accuracy of laboratory tests is mostly lacking and general recommended criteria, rather than tailor-made ones, are adopted.

In this retrospective case control study, the optimal cut-off values for leptospirosis confirmation by MAT and IgM ELISA in the Netherlands are determined, using positive culturing results as the reference test. Taking into account the dynamics of the humoral response[2], single samples were analysed according to three time periods, i.e. the acute stage of illness (1-10 DPO), the early convalescent stage (11-20 DPO) and the late convalescent stage (>20 DPO). Cut-off titers of 1:80 and 1:160 for IgM ELISA and MAT were determined for all three periods. This was associated with a specificity of 100% for both tests. However, the sensitivities varied markedly from 6.3 to 81.7% for MAT and 25.0% to 91.7% for IgM ELISA in the acute and early convalescent phase. Thus, sensitivities of MAT and IgM ELISA are low at the acute stage of illness and require a follow-up sample for confirming seroconversion or a significant titer rise.

To assess the impact of the increasing immune response on the diagnostic outcome in more detail, the acute phase was split in an early (1-4 DPO) and late (5-10 DPO) stage. Rapid increases in sensitivity in both tests across the two stages were noticed; i.e. the sensitivity for MAT rose from 2.1% to 24.2% and the sensitivity of the IgM ELISA from 6.3% to 53.2%. Moreover, testing paired samples markedly increased the sensitivity of the diagnosis. This justifies two conclusions. (i) These findings support the notion that serology has minor importance at the very early phase of disease. (ii) These results underline the importance of using a follow up sample for confirmation by serology. However, unlike generally stated that paired samples should preferably be collected two weeks apart[12], much shorter intervals appear relevant. Seroconversion was observed in samples taken as few as two days apart. Therefore, we advocate the use of repeated samples, albeit at intervals of one week or shorter in case of urgent suspicion.

The MAT is the reference test in serodiagnosis of leptospirosis and is often referred to as the gold standard. This nomination is not supported by this study. MAT was at the best 81.7% sensitive in the convalescent stage, implying that 2 of 10 cases are missed when only MAT is used on a single sample. The IgM ELISA did perform much better (sensitivity 91.7%) and was

significantly more sensitive compared to MAT in the late acute stage of illness. It is therefore tempting to designate IgM ELISA as the new gold standard. There are, however, caveats. This study was performed on Dutch cases with low infection backgrounds and concerned an in-house ELISA. This ELISA is based on the determination of a titer from serial dilutions[19,21]. This approach is probably more robust than using single well platforms and, hence, our results cannot be translated to these other formats. Moreover, high prevalence countries might have higher background titers, requiring different cut-off titers of both MAT and ELISA that might favour the use of MAT. Additionally, unlike MAT, ELISA is a genus specific tests that does not enable the assessment of presumptive infecting serogroups, and as such lacks an important epidemiological application. In the Netherlands, MAT is always performed along with IgM ELISA. Therefore the impact of diagnostic value by combining the results of both tests was investigated. Combination of tests had no effect on the acute phase of the disease that was dominated by the sensitivity of the IgM ELISA but resulted in a markedly increased sensitivity in the early convalescent stage. Applying cut-off titers of 1:160 and 1:80 for MAT and IgM ELISA, a sensitivity of 93.3% was reached by combining MAT 'OR' IgM ELISA results, comparing to 81.7 and 91.7%, for each test, respectively. Apparently, the two tests are partly complementary at this stage of illness. This observation substantiates the importance of using two distinct tests.

This study unambiguously demonstrates that serology largely fails in the acute phase of the disease. This period is of most clinical importance because adequate antibiotic treatment has to be initiated as early in the illness as possible[14,15]. Thus, serology is not very helpful in an early stage of leptospirosis when confirmation is most required. Late confirmation by serological testing, presents a major obstacle in clinical decision making. Clinicians have to rely on their own judgment based on mostly uncharacteristic symptoms to initiate appropriate (antibiotic) treatment. On one hand, low titers during early illness are highly unreliable because of a low accompanying specificity (Table 2). On the other hand, such low titers might be a sign of a developing leptospirosis and as such, should not be ignored. We deal with this contradiction by informing the consulting clinician promptly upon finding low reactions and explain their significance. Following this strategy, the clinician is provided with early indications on a potential *Leptospira* infection that support decisions on the initiation or pursuit of a suitable treatment. Fortunately, nowadays validated real time PCRs are available[22,23]. These PCRs have a high diagnostic accuracy notably in this early acute phase, hence perfectly complementing serodiagnostic testing. We are aware that a lack of experience and financial potential might hamper the introduction of real time PCR notably in those regions where the disease is highly prevalent but, nevertheless, strongly recommend its implementation in the diagnostic services. In absence of real time PCR, laboratory testing will largely benefit from well-established criteria that define positive outcomes of serological testing. The study described in this paper serves as a template for other such studies in distinct epidemiological settings. Such local evaluation is of great importance because different cut-off titers in the MAT have been demonstrated in high compared to low endemic situations[9]. In our study, relatively

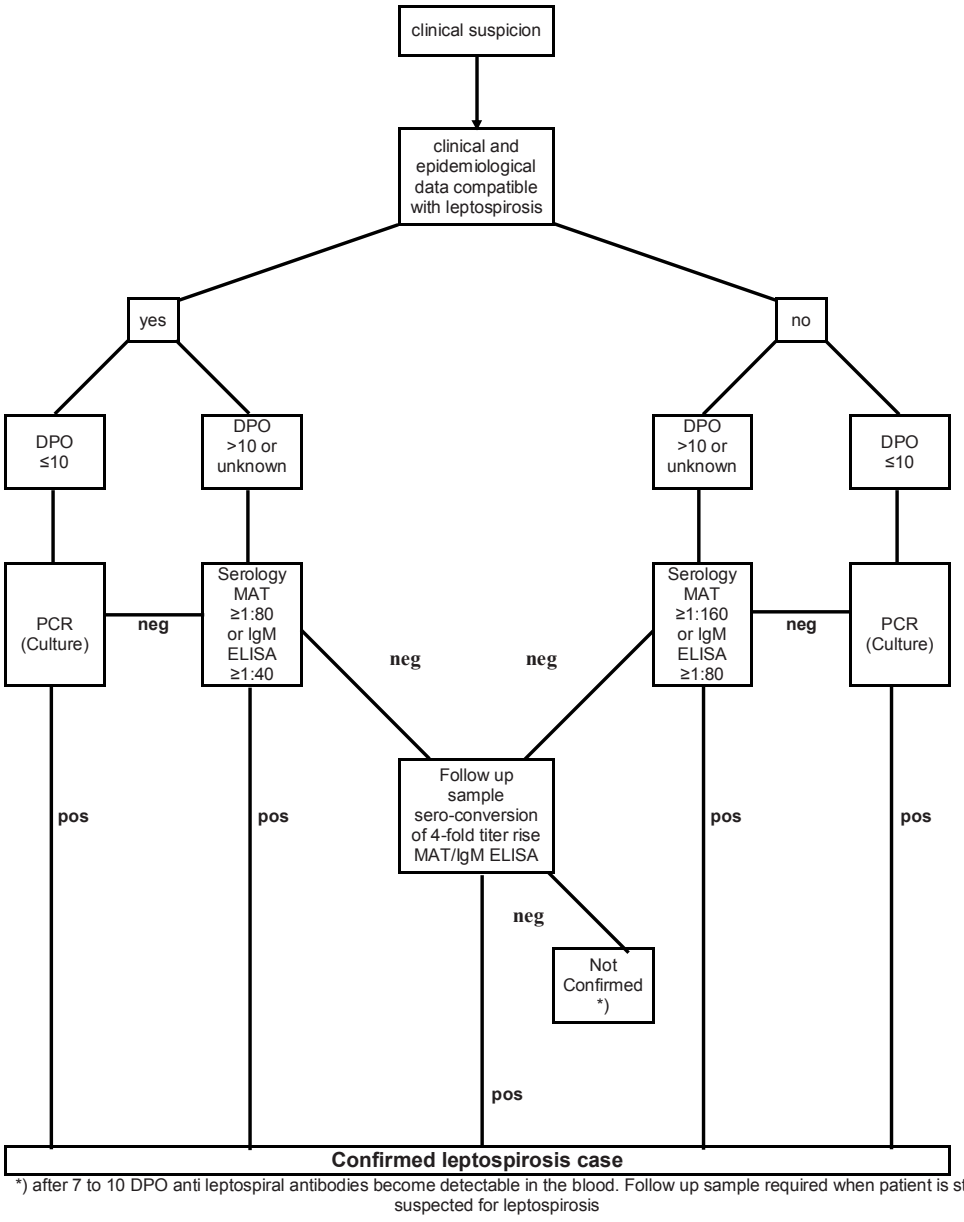


Figure 3 | Algorithm, assisting with interpretations and conclusions on the outcome of laboratory testing

low cut-off titers for MAT were defined, i.e., ≥1:160 for single samples and ≥1:80 for paired samples. There are two reasons for these low cut-off titers: (i) The infection background in the Netherlands is low and as such impacts to a lesser extent on the specificity. This also counts for the IgM ELISA. (ii) Historically, at NRL *Leptospira* strains are not diluted before performing the MAT as recommended[8] but full grown cultures are used. A higher density of *Leptospira* strains will reduce the titer by one dilution step at the minimum[16]. Consistently, in the MAT proficiency testing, we find our titers below the median titer[24]. Cut-off titers are based on single serum samples, without information on adequate clinical and epidemiological findings. It is common notice that knowledge of clinical and epidemiological data are highly valuable and should largely contribute to the confirmation of suspected leptospirosis[8,25]. For the same, testing of paired serum samples is strongly recommended and case definitions use lower cut-off titers on paired samples than on single ones[8,13]. In line with this argument, we applied cut-off titers that were one dilution step lower than those deduced for single samples. When testing this approach on a set of 68 paired samples, all but one fulfilled the criteria of these lower cut-off titers. The one exception was an infection with Grippotyphosa that did not give any detectable antibody response. Grippotyphosa infections are notorious for causing transient or delayed immune responses that might be missed[2,8,10]. Our evaluation allowed us to change our current and more stringent case definition based on preliminary data analysis on single serum samples[26] and consequently is expected to improve the case finding in the Netherlands. The use of different cut-off values in different epidemiological situations might lead to confusion. Therefore, we have designed an algorithm, assisting with interpretations and conclusions on the outcome of our laboratory testing (Figure 3). This algorithm includes application of a validated real time PCR[23]. We anticipate that the assessment of our case definition as well as the algorithm presented in this paper is not only useful for application in the Netherlands but can easily be adapted into a useful assessment tool in different epidemiological backgrounds.

As a concluding remark we mention that a lack of notification is not the only reason for the underestimation of leptospirosis[27]. As we have shown, inadequate case definition and a questionable sensitivity of the reference MAT both are also causes of missing cases.

REFERENCES

1. Farr RW. Leptospirosis. *Clin Infect Dis* 1995; **21**(1): 1-6.
2. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; **14**(2): 296-326.
3. Bharti AR, Nally JE, Ricaldi JN, *et al*. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis* 2003; **3**(12): 757-71.
4. Hartskeerl RA, Goris MGA. Leptospirose in Nederland in 2008 en 2009 (Leptospirosis in the Netherlands in 2008 and 2009). *Infectieziekten Bulletin* 2010; **21**(6): 185-7.
5. Hartskeerl RA, Goris MGA. Meer leptospirose in 2007 (more leptospirosis in 2007). *Infectieziekten Bulletin* 2008; **19**(10): 301-2.
6. Hartskeerl RA, Goris MGA. Leptospirose in 2005: veel infecties tijdens vakantie in de tropen (Leptospirosis in 2005: many infections during vacation in the tropics). *Infectieziekten Bulletin* 2006; **17**(8): 304-5.
7. Levett PN. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clin Infect Dis* 2003; **36**(4): 447-52.
8. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control; 2003.
9. Vijayachari P, Sugunan AP, Sehgal SC. Evaluation of microscopic agglutination test as a diagnostic tool during acute stage of leptospirosis in high & low endemic areas. *Indian J Med Res* 2001; **114**: 99-106.
10. Contribution to leptospirosis surveillance in France in 2005. 2011 2011. http://www.pasteur.fr/recherche/Leptospira/textcnre05.html.
11. Leptospirosis Laboratory Case Definition (LCD). 5/25/2007 2007. http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-phlncd-leptospirosis.htm.
12. Leptospirosis (Excerpt from "*WHO recommended standards and strategies for surveillance, prevention and control of communicable diseases"*). 2011. http://www.who.int/zoonoses/diseases/Leptospirosissurveillance.pdf.
13. World Health Organization. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group, 2011.
14. Watt G, Padre LP, Tuazon ML, *et al*. Placebo-controlled trial of intravenous penicillin for severe and late leptospirosis. *Lancet* 1988; **1**(8583): 433-5.
15. Guidugli F, Castro AA, Atallah AN. Antibiotics for treating leptospirosis. *Cochrane Database Syst Rev* 2000; (2): CD001306.
16. Faine S, Adler B, Bolin CA, Perolat P. *Leptospira* and Leptospirosis. MediSci; 1999.
17. Wagenaar JF, de Vries PJ, Hartskeerl RA. Leptospirosis with pulmonary hemorrhage, caused by a new strain of serovar Lai: Langkawi. *J Travel Med* 2004; **11**(6): 379-81.
18. McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. *Curr Opin Infect Dis* 2005; **18**(5): 376-86.
19. Terpstra WJ, Ligthart GS, Schoone GJ. Serodiagnosis of human leptospirosis by enzyme-linked-immunosorrbent-assay (ELISA). *Zentralbl Bakteriol A* 1980; **247**(3): 400-5.
20. Hartskeerl RA, Smits HL, Korver H, Goris MGA, Terpstra WJ. International course on laboratory methods for the diagnosis of leptospirosis; 2006.

21. Terpstra WJ, Ligthart GS, Schoone GJ. ELISA for the detection of specific IgM and IgG in human leptospirosis. *J Gen Microbiol* 1985; **131**(2): 377-85.
22. Slack A, Symonds M, Dohnt M, Harris C, Brookes D, Smythe L. Evaluation of a modified Taqman assay detecting pathogenic *Leptospira* spp. against culture and *Leptospira*-specific IgM enzyme-linked immunosorbent assay in a clinical environment. *Diagn Microbiol Infect Dis* 2007; **57**(4): 361-6.
23. Ahmed A, Engelberts MF, Boer KR, Ahmed N, Hartskeerl RA. Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. *PLoS One* 2009; **4**(9): e7093.
24. Chappel RJ, Goris M, Palmer MF, Hartskeerl RA. Impact of proficiency testing on results of the microscopic agglutination test for diagnosis of leptospirosis. *J Clin Microbiol* 2004; **42**(12): 5484-8.
25. Faine S. Guidelines for Leptospirosis Control: Geneva: WHO offset Publication 67; 1982.
26. Hartskeerl RA. Leptospiroses [in Dutch]. In: van Steenbergen J, Timen A, DJMA B, eds. LCI-Guidelines Infectious Disease Control. Bilthoven: LCI, Coordinator Infectious Disease Netherlands; 2008.
27. Abela-Ridder B, Sikkema R, Hartskeerl RA. Estimating the burden of human leptospirosis. *Int J Antimicrob Agents* 2010; **36 Suppl 1**: S5-S7.

CHAPTER 8

Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis

Marga G.A. Goris[1*]
Mariska M.G. Leeflang[2]
Martin Loden[1]
Jiri F.P. Wagenaar[1]
Paul R. Klatser[1,3]
Rudy A. Hartskeerl[1]
Kimberly R. Boer[1,3]

1. KIT Biomedical Research, Royal Tropical Institute (KIT), Amsterdam, the Netherlands
2. Department of Clinical Epidemiology and Biostatistics, Academic Medical Center, Amsterdam, the Netherlands
3. Department of Global Health, Academic Medical Center, Amsterdam, the Netherlands

PLoS Neglected Tropical Diseases 2013; 7(7): e2290

ABSTRACT

Background

Diagnosis of leptospirosis by the microscopic agglutination test (MAT) or by culture is confined to specialized laboratories. Although ELISA techniques are more common, they still require laboratory facilities. Rapid Diagnostic Tests (RDTs) can be used for easy point-of-care diagnosis. This study aims to evaluate the diagnostic performance of the RDTs, LeptoTek Dri Dot, LeptoTek Lateral Flow and Leptocheck-WB, prospectively.

Methodology

During 2001 to 2012, one or two of the RDTs at the same time have been applied prior to routine diagnostics (MAT, ELISA and culture) on serum specimens from participants send in for leptospirosis diagnosis. The case definition was based on MAT, ELISA and culture results. Participants not fulfilling the case definition were considered not to have leptospirosis. The diagnostic accuracy was determined based on the 1st submitted sample and paired samples, either in an overall analysis or stratified according to days post onset of illness.

Results

The overall sensitivity and specificity for the LeptoTek Dri Dot was 75% respectively 96%, for the LeptoTek Lateral Flow 78% respectively 95% and for the Leptocheck-WB 78% respectively 98%. Based on the 1st submitted sample the sensitivity was low (51% for LeptoTek Dri Dot, 69% for LeptoTek Lateral Flow, and 55% for Leptocheck-WB) but substantially increased when the results of paired samples were combined, although accompanied by a lower specificity (82% respectively 91% for LeptoTek Dri Dot, 86% respectively 84% for LeptoTek Lateral Flow and 80% respectively 93% for Leptocheck-WB).

Conclusions

All three tests present antibody tests contributing to the diagnosis of leptospirosis, thus supporting clinical suspicion and contributing to awareness. Since the overall sensitivity of the tested RDTs did not exceed 80%, one should be cautious to rely only on an RDT result and confirmation by reference tests is strongly recommended.

AUTHOR SUMMARY

Leptospirosis is one of the world's most spread zoonoses causing acute fever. The illness can rapidly develop into a severe, potentially fatal, form with a high mortality rate. Laboratory tests are needed to confirm the diagnosis. Culturing leptospires from patient material can take months to grow. Therefore, most used laboratory tests are based on detection of antibodies against leptospires. The microscopic agglutination test is considered the reference standard, but is only performed at specialized laboratories. In this study, we measured the diagnostic accuracy of three rapid diagnostic tests (RDTs) by doing a prospective evaluation during 11 years. These tests produce results within 15 minutes. The overall sensitivities (77%) and specificities (96%) were similar for the RDTs. Evaluating the first submitted specimen resulted in lower sensitivities (51% for LeptoTek Dri Dot, 69% for LeptoTek Lateral Flow, and 55% for Leptocheck-WB). When paired specimens were evaluated, the sensitivity increased although the specificity decreased (82% respectively 91% for LeptoTek Dri Dot, 86% respectively 84% for LeptoTek Lateral Flow and 80% respectively 93% for Leptocheck-WB). Based on these results confirmation by reference tests is still strongly recommended although the RDTs contribute to the diagnosis of leptospirosis, thus supporting clinical suspicion and contributing to awareness.

INTRODUCTION

Leptospirosis is caused by microorganisms of the genus *Leptospira*. It is one of the world's most wide-spread zoonoses, with a mean global incidence of endemic and epidemic leptospirosis of 5 per 100,000 and 14 per 100,000 population, respectively[1]. It causes an acute febrile illness[2] with a wide diversity of milder clinical signs such as headache, malaise, myalgia, conjunctival suffusion and sometimes a transient rash. However, the illness can rapidly develop into a severe, potentially fatal form with a high mortality rate[3]. Leptospirosis is often overlooked since it mimics many other diseases, including dengue, malaria, influenza and hantavirus infections[4], making differential diagnosis very difficult based on clinical grounds alone. Laboratory tests are therefore the basis of a confirmed case of leptospirosis.

The most commonly used laboratory tests are based on detection of antibodies against the leptospires. Pathogenic leptospires enter the body through small cuts or abrasions, or via mucous membranes and possibly through wet skin. After infection, leptospires circulate in the blood stream, with a bacteremic phase lasting for up to 10 days post onset of the disease (DPO). Detectable antibodies appear in the blood about 5-10 DPO[5], and sometimes later, especially if antibiotic treatment is instituted[4]. These antibodies can be detected by a variety of laboratory assays such as the microscopic agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT)[6]. Currently, the MAT is considered the reference standard in serodiagnosis and as such has a worldwide application. However, MAT and ELISA are technically demanding and relatively expensive tests and therefore not widely applicable in peripheral healthcare facilities, especially in tropical and subtropical developing regions where leptospirosis is most endemic. Culturing leptospires out of blood provides proof of infection but is insensitive[7] and has little clinical value for patient management as it can take weeks to months to confirm results. PCR on blood has proven to be useful in the first week of the disease[8], however many laboratories are not equipped to run PCR tests. Hence, for most clinical situations rapid diagnostic tests (RDTs) can play an important role in immediate case detection and clinical management. Most commonly used RDTs are based on the immunochromatographic lateral flow technology.

To date, a variety of RDTs have been described and evaluated in various papers[9–11], most of these being short term retrospective evaluations and often concern evaluations of a single RDT. Our aim was to evaluate the diagnostic accuracy of three RDTs, applied on serum specimens from suspected leptospirosis patients from the Netherlands in a prospective cohort of leptospirosis suspected patients. Additional aims were to assess whether there are differences between the three tests and whether using the tests at different times since patient's onset of symptoms leads to differences in diagnostic accuracy. The RDTs used in this research were available in the Netherlands, or could be easily imported.

MATERIALS AND METHODS

Standards for the Reporting of Diagnostic accuracy testing (STARD) checklist were adhered to throughout the text (Table S1)[12].

Study participants

The Royal Tropical Institute (KIT), Biomedical Research houses the WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis (NRL), which confirms about 99% of the suspected cases of leptospirosis in the Netherlands. The typical annual number of suspected cases is around 500, of which approximately 30 are confirmed leptospirosis cases. About 50% of the confirmed cases have contracted leptospirosis during travel abroad. In the period of evaluation, July 2001 to August 2012, the population in the Netherlands was stable at about 16 million. During this period, all human blood specimens sent by physicians practicing in the Netherlands to NRL for leptospirosis diagnosis were tested upon arrival by routine diagnostics. In most cases only one sample was received per participant, in other cases two or more samples. Further inclusion and exclusion criteria of samples and participants are depicted in a flow diagram (Figure 1). Laboratory tests routinely performed are MAT and in-house IgM-ELISA. Culture was done as described below. A single or a combination of two RDTs were prospectively performed for evaluation purposes.

Leptospirosis case definition

Patients were considered as having leptospirosis based on one or more of the following criteria: (i) single MAT titer with a pathogenic strain ≥1:160, (ii) single IgM-ELISA titer ≥1:160, (iii) positive culture or (iv) seroconversion/≥ four-fold titer rise MAT or IgM ELISA (titer ≤1:20 to ≥1:80) in paired samples taken at least 2 days apart[13]. The treating physician was encouraged to send multiple samples for laboratory testing for all participants.

Laboratory methods

RDTs were applied prior to and independent of routine diagnostic testing. All tests were performed by skilled staff of NRL (10 persons) who followed detailed protocols about interpretation of tests. NRL is accredited based on ISO 15189 since 2006. All serological tests were performed on serum specimens which were inactivated in a 56°C water bath for 30 minutes before testing.

Culture: Culture was initiated for blood, plasma or serum samples collected within the first 10 days of disease. Urine was cultured at all time points during the course of disease within 2 hours after voiding. Fletcher medium and Ellinghausen-McCullough as modified by Johnson and Harris (EMJH) culture medium was used[14]. EMJH was supplemented with 5-fluorouracil (200 mg/ml), 1% (V/V) rabbit serum and 1% (V/V) fetal calf serum or combinations[15]. Inoculated media were incubated for a maximum of 4 months at 30 °C and (bi)weekly checked for leptospiral growth by dark field microscopy.

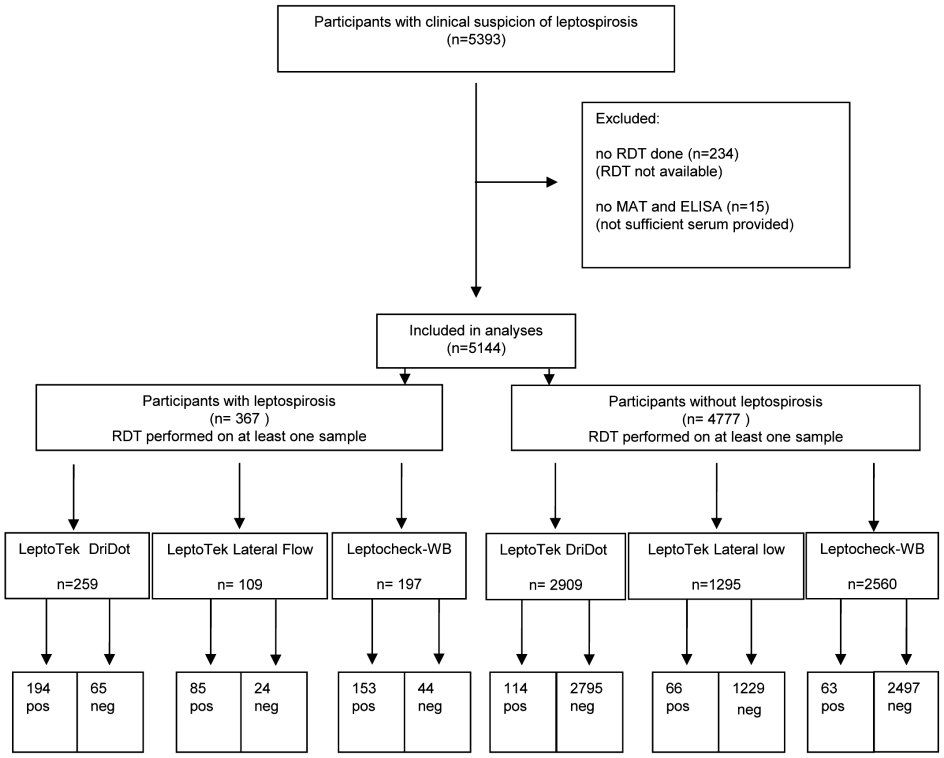


Figure 1 | Flow chart of participants and rapid diagnostic tests.

Microscopic agglutination test: The MAT was performed with a panel of live leptospires as described elsewhere[15]. The panel consisted of 16 strains of the pathogenic serovars Bratislava, Ballum, Canicola, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Copenhageni, Poi, Pomona, Proechimys, Hardjo, Saxkoebing and Sejroe, and the non-pathogenic serovar Patoc. Sera from patients who visited a country outside the Netherlands within one month prior to the day of onset of symptoms were also tested with an additional panel of 12 globally representative strains, i.e. the pathogenic serovars Australis, Rachmati, Bataviae, Celledoni, Cynopteri, Mini, Panama, Pyrogenes, Shermani and Tarassovi, and the non-pathogenic serovars Andamana and Semaranga.

IgM ELISA: In-house developed ELISA for the detection of *Leptospira*-specific IgM antibodies (IgM ELISA) was performed with antigen prepared from the local strain Wijnberg (serovar Copenhageni, serogroup Icterohaemorrhagiae)[16,17].

Rapid diagnostic tests: Three rapid diagnostic serological tests were used according to their availability: (i) 2001-2008 LeptoTek Dri Dot, Organon Teknika B.V., later bioMérieux B.V., Boxtel, the Netherlands. (ii) 2001-2004 LeptoTek Lateral Flow, Organon Teknika B.V. Boxtel, the

Netherlands. (iii) 2004-2012 Leptocheck-WB, Zephyr Biomedicals, Verna Goa, India. LeptoTek Lateral Flow and Leptocheck-WB are lateral flow immunochromatographic tests. These are both qualitative, sandwich immunoassays intended for the detection of *Leptospira*-specific IgM antibodies in humans. The test can be read after 10 to 15 minutes and can be used for serum/plasma or whole blood specimens. LeptoTek Dri Dot is a latex agglutination assay and detects *Leptospira*-specific antibodies (IgM and IgG) in human sera.

The rapid tests were performed according to the manufacturer's instructions. For the LeptoTek Lateral Flow 5 µl serum was spotted in the sample port of the device, running buffer was added and the test was read after 10 minutes. For the Leptocheck-WB 10 µl serum was spotted in the sample port of the device and 15 minutes after running buffer was added the test was read. Both tests were valid when the control band stained. Valid tests were scored positive when a test band was observed, negative when no band was observed and indeterminate when it was unclear whether a band was observed or not. Invalid tests were repeated. For the LeptoTek Dri Dot 10 µl of serum was mixed with the dried leptospiral-antigen-coated latex spot on the agglutination card. The test was read within 30 seconds and scored positive when agglutination was observed, negative when there was no agglutination and indeterminate when occurrence of agglutination was unclear.

Data analyses

Data were entered into a Laboratory Information System (LASSIST, Mechatronics Software Applications BV, the Netherlands) and exported and analyzed in SPSS (version 19, IBM, NY, USA). These included patient data obtained from the request form (i.e. gender, date of birth, date of onset, travel history). The results of each diagnostic test of every sample were entered into the database. Follow-up samples taken less than two days after the first sample were excluded. Indeterminate results were regarded as negative, unless otherwise stated.

Overall accuracy: In this analysis, the overall accuracy of RDTs for diagnosing leptospirosis for any submitted sample was estimated. Diagnostic accuracy was defined by sensitivity and specificity with 95% confidence intervals (CIs)[18]. For these analyses, participants were considered positive if they had a positive RDT result in at least one of the submitted samples (participant-level, not on individual samples received). Sensitivity was calculated in participants who fulfilled the case-definition, specificity on those who did not. The three RDTs were considered different from each other if the 95% confidence intervals did not overlap.

Overall Accuracy – first sample sent in and follow-up sample: To avoid potential overestimations of sensitivity and underestimations of specificity of the individual test in the above analyses, a subgroup analysis was completed on only the first sample that was sent in, and, if available, on the follow-up sample (paired samples), if taken within 1 month. This reflects clinical practice better than the previous analysis, as it represents the disease period when leptospirosis diagnostics are typically requested by the clinician. As well, this analysis does

not depend on a defined first day of illness. The three tests were considered different from each other if the 95% confidence intervals did not overlap.

Time trends: For those patients with data available on their first day of illness (50%), the diagnostic accuracy of the serologic tests was calculated at different time-periods, i.e., 0-4 days post onset of symptoms (DPO) (early acute), 5-10 DPO (late acute), 11-20 DPO (convalescent) and >20 DPO (late convalescent). If multiple samples of a participant were taken in the same time-period, the sample with the lowest DPO was included.

Sensitivity analyses: A substantial proportion of the samples were scored indeterminate in the RDTs. To assess the impact of the interpretation of indeterminate results as considered negative in the previous analyses, a sensitivity analysis of the diagnostic accuracy was conducted by allocating the indeterminate scores to either the negative test results or positive test results or by excluding these indeterminate scores for 1st and follow-up samples.

Furthermore the predictive value of an indeterminate versus a negative test result was assessed: from participants whose first test result was either indeterminate or negative, we looked at the RDT result in the follow-up sample to calculate the proportion of participants fulfilling the case definition. This denotes the proportion of patients changing from a negative or indeterminate RDT to a positive RDT.

An additional analysis was completed to determine the potential differences in diagnostic accuracy of the RDTs between infecting serogroups. This analysis considered infections with serogroup Icterohaemorrhagiae, Grippotyphosa, other serogroups and not classifiable serogroups for the 1st sample and paired samples when available.

To investigate the consistency of the diagnostic accuracy of these RDTs through the periods of use, sensitivity and specificity were compared for each diagnostic test for the 1st sample and paired samples by years the test was completed.

Ethical statement

This data collection was exempted from ethical review of human subjects research by the Medical Ethical Review Committee of the Academic Medical Centre, University of Amsterdam (W12_076#12.17.0092). All data presented have been de-identified and were not attributable to individual patients.

RESULTS

During the 11 years of data collection, blood specimens from 5393 participants suspected of leptospirosis were submitted to NRL for testing. The majority of participants (95.4%) were tested by MAT, IgM ELISA and one or more of the rapid tests (Figure 1); however there were short periods where no RDT could be performed due to their unavailability on the international market (Table S2). No RDT could be completed for 234 participants. Furthermore, 15 participants were excluded as there was no MAT or ELISA completed, as a prerequisite of the reference standard and case definition, leaving a total of 5144 patients. Follow-up specimens were received from 929/5144 participants and 53.1% of the participants had a documented DPO.

There were 367 (6.7%) leptospirosis cases fulfilling the case definition, with a male to female sex ratio of about 6:1. The sex ratio of non-leptospirosis cases was 2:1. The mean age of cases and non-cases was 39.7 and 42.1 years, respectively. Male leptospirosis cases were older (mean age 40.2, SD 15.7) than female cases (mean age 36.8, SD 17.3). Table 1 presents an overview of characteristics of the eligible study participants. Table 2 presents an overview of the participants fulfilling the case definition. There were no invalid test results reported for the RDTs, i.e. the control band in the LeptoTek Lateral Flow and Leptocheck-WB stained in all tests performed.

RDTs were performed on 1st and follow-up specimens from 861/929 participants (16.7 % of all participants); 80.7% of the leptospirosis cases, and 11.8% of the non-leptospirosis participants. The total median number of days between 1st and follow-up sample was 16 days (IQR 11 to 28). For the confirmed leptospirosis participants this was 14 days (IQR 8 to 22), versus 20 days (IQR 3 to 200) for the non-leptospirosis participants (P <0.05, Kruskal-Wallis test).

Overall accuracy

The overall sensitivity and specificity, calculated on all samples from early acute till the late convalescent phase showed a sensitivity of 75% (95% CI 69% to 79%) for LeptoTek Dri Dot, 78% (95% CI 69% to 85%) for LeptoTek Lateral Flow and 78% (95% CI 71% to 83%) for Leptocheck-WB. The specificity was 96% (95% CI 95% to 97%) for LeptoTek Dri Dot, 95% (95% CI 94% to 96%) for LeptoTek Lateral Flow and 98% (95% CI 97% to 98%) for Leptocheck-WB (Table 3). There were no marked differences between the three tests; the sensitivities and specificities were similar with overlapping confidence intervals.

Table 1 | Characteristics of participants

Characteristic	Total	Leptospirosis patients	Non leptospirosis patients
	(n=5144)	(n=367)	(n=4777)
Male† - no. (%)	3496 (67.9)	314 (85.6)	3181 (66.6)
Mean age* - years (SD)	41.9 (18.3)	39.7 (15.9)	42.1 (18.5)
Mean age males - years (SD)	42.9 (18.0)	40.2 (15.7)	43.2 (18.2)
Mean age females - years (SD)	39.6 (18.8)	36.8 (17.3)	39.7 (18.9)
Travel history no. (%)	1392 (27)	179 (48.8)	1213 (25.4)
Europe	268	36	232
Asia	531	108	423
Africa	198	5	193
South America	153	11	142
Central and North America	102	16	86
Middle East	58	2	56
Australia	12	0	12
Unknown	70	1	69
DPO known - no. (%)	2733 (53.1)	338 (92.1)	2395 (50.1)
Single serology sample - no. (%)	4215 (81.9)	48 (13.1)	4167 (87.2)
Multiple serology samples - no. (%)	929 (18.1)	319 (86.9)	610 (12.8)
DPO 1st serology sample# – median (IQR)	10 (5 - 22)	7 (5-11)	10 (5 – 24)
DPO follow up serology sample## – median (IQR)	24 (16 – 39)	21 (14 – 30)	28 (17 – 44)
Culture - no. (%)	1455 (28.3)	223 (60.8)	1232 (25.8)

† gender was registered for 5139 participants: 367 leptospirosis patients; 4772 non leptospirosis patients

*Age was registered for 5143 participants: 367 leptospirosis patients; 4776 non leptospirosis patients

#DPO of first sample was calculated from 2703 participants of whom first day of onset was known as well as date of sample collection: 330 leptospirosis patients; 2373 non leptospirosis patients

##DPO of follow up sample was calculated from 630 participants of whom first day of onset was known as well as date of sample collection: 276 leptospirosis patients; 354 not leptospirosis patients

Table 2 | Diagnostic test and serogroup of Leptospirosis positive patients (n=367)

Fullfillment of Case definition:	**Multiple positive features n=282**	**Single positive feature n=85**
Culture positive	31	6
MAT ≥1:160	253	20
IgM ≥1:160	234	45
Seroconversion MAT	140	4
Seroconversion IgM ELISA	108	10
Probable infecting serogroup*	**Autochthonous cases, n=188**	**Imported cases, n=179**
Grippothyphosa	27 (14.4%)	15 (8.4%)
Icterohaemorrhagiae	89 (47.3%)	33 (18.4%)
Other serogroups		
Australis	1	8
Autumnalis	-	10
Ballum	2	1
Bataviae	-	6
Canicola	-	2
Celledoni	-	5
Cynopteri	-	2
Hebdomadis/Sejroe/Mini complex	-	15
Javanica	2	2
Pomona	14	1
Pyrogenes	-	4
Shermani	-	1
not classifiable	53 (28.2%)	74 (41.3%)

* Probable infecting serogroup is based on titers in MAT and typing results of positive cultures (Autumnalis n=3, Bataviae n=2, Canicola n=2, Grippotyphosa n=5, Hebdomadis n=1, Icterohaemorrhagiae n=19, Javanica n=2, Pyrogenes n=2, Shermani n=1). Probable infecting serogroup could not be determined if patient was a case based only on IgM-ELISA or had several similar reacting serogroups in MAT.

Accuracy of first sample and follow-up sample

When considering only the first sample that was sent in for each patient, the sensitivity of each test dropped dramatically from 75% to 51% and from 78% to 55% for the LeptoTek Dri Dot and the Leptocheck-WB, respectively. The sensitivity of the LeptoTek Lateral Flow decreased from 78% to 69%, although not a statistically significant change. The specificity of all tests remained more or less the same. Test results from paired samples (either one of the samples positive) increased the sensitivity significantly from 51% to 82% for the LeptoTek Dri Dot and from 55% to 80% for the Leptocheck-WB. The increase from 69% to 86% for the LeptoTek Lateral Flow was not statistically significant. The corresponding decrease in specificity was significant, i.e. from 96% to 91% for the LeptoTek Dri Dot, from 96% to 84% for the LeptoTek Lateral Flow and from 98 to 93% for the Leptocheck-WB (Table 3).

Time trends

For 2733 participants (53.1% of study participants) the first day of onset of symptoms was known. All three tests show a lower sensitivity during the early acute phase of the disease (till DPO 4), which increased during DPO 5-10 and DPO 11-20, while the specificity of all tests remained relatively stable (Table 4). LeptoTek Lateral Flow was performing the best at DPO 0-4 (sensitivity of 62%, 95% CI 41% to 79% and specificity of 98%, 95 CI 93% to 99%).

Sensitivity analyses

The proportion of the indeterminate results for the 1st sample for LeptoTek Dri Dot were 10/256 (4%) in the participants fulfilling the case definition and 85/2903 (3%) in the participants not fulfilling the case definition. For the LeptoTek Lateral Flow, these proportions were 4/108 (4%), respectively 173/1292 (13%), and for the Leptocheck- WB 17/183 (9%), respectively 239/2551 (9%).

Table 3 | Overall case sensitivity and specificity of rapid diagnostic tests

Assay		Sensitivity	%	CI	Specificity	%	CI
LeptoTek Dri Dot	1st sample	131/256	51	45-57	2795/2903	96	96-97
	paired samples	137/167	82	76-87	261/286	91	87-94
	Any sample	194/259	75	69-79	2795/2909	96	95-97
LeptoTek Lateral	1st sample	74/108	69	59-77	1235/1292	96	94-97
Flow	paired samples	56/65	86	76-93	116/138	84	77-89
	Any sample	85/109	78	69-85	1229/1295	95	94-96
Leptocheck-WB	1st sample	100/183	55	47-62	2495/2551	98	97-98
	paired samples	103/129	80	72-86	162/174	93	88-96
	Any sample	153/197	78	71-83	2497/2560	98	97-98

Allocation of indeterminate results to positive scores did not substantially change sensitivity, but it did have an impact on specificity (Figure 2). For the LeptoTek Dri Dot, the specificity decreased from 96% to 93% for the 1st submitted sample and from 91% to 81% for the paired samples. The LeptoTek Lateral Flow showed a decrease of the specificity from 96% to 82% for the 1st sample and 84% to 62% for the paired samples, while the Leptocheck-WB showed a decrease from 98% to 88% and from 93% to 80% respectively.

About 28% of the participants with an initial indeterminate result for the LeptoTek Dri Dot and Leptocheck-WB were later confirmed with leptospirosis in follow-up testing, and had a positive RDT compared to only 10% of participants with an initial negative result. For the LeptoTek Lateral Flow, the numbers are somewhat different with 9% positive results after the first sample was indeterminate, and 4% positive results after the first sample was negative, but the same trend is present (Table S4).

Exclusion of indeterminate results showed an increasing sensitivity and decreasing specificity for all RDTs and for all time points, though not statistically significant. When stratifying the samples according to the defined time-periods of the disease, the same trend was observed (Table S3).

The sensitivity of RDTs appeared to depend on the infecting serogroup (Table S3). In general infecting serogroup Icterohaemorrhagiae yielded a higher sensitivity for all three RDTs compared to the other categories of serogroups. Differences were significant in the follow-

Table 4 | Sensitivity and specificity of rapid diagnostic tests at different days post onset (DPO)

Assay	DPO	Sensitivity	%	CI	Specificity	%	CI
LeptoTek Dri Dot	0-4		27	17-40		97	94-98
	5-10		55	47-63		96	94-98
	11-20		83	74-89		96	93-98
	>20		74	66-80		96	95-98
LeptoTek Lateral Flow	0-4		62	41-79		98	93-99
	5-10		75	62-84		94	89-96
	11-20		81	69-90		93	88-96
	>20		85	75-92		95	91-97
Leptocheck-WB	0-4		42	28-58		97	95-99
	5-10		65	55-74		96	94-97
	11-20		72	62-81		98	95-99
	>20		70	61-78		97	95-98

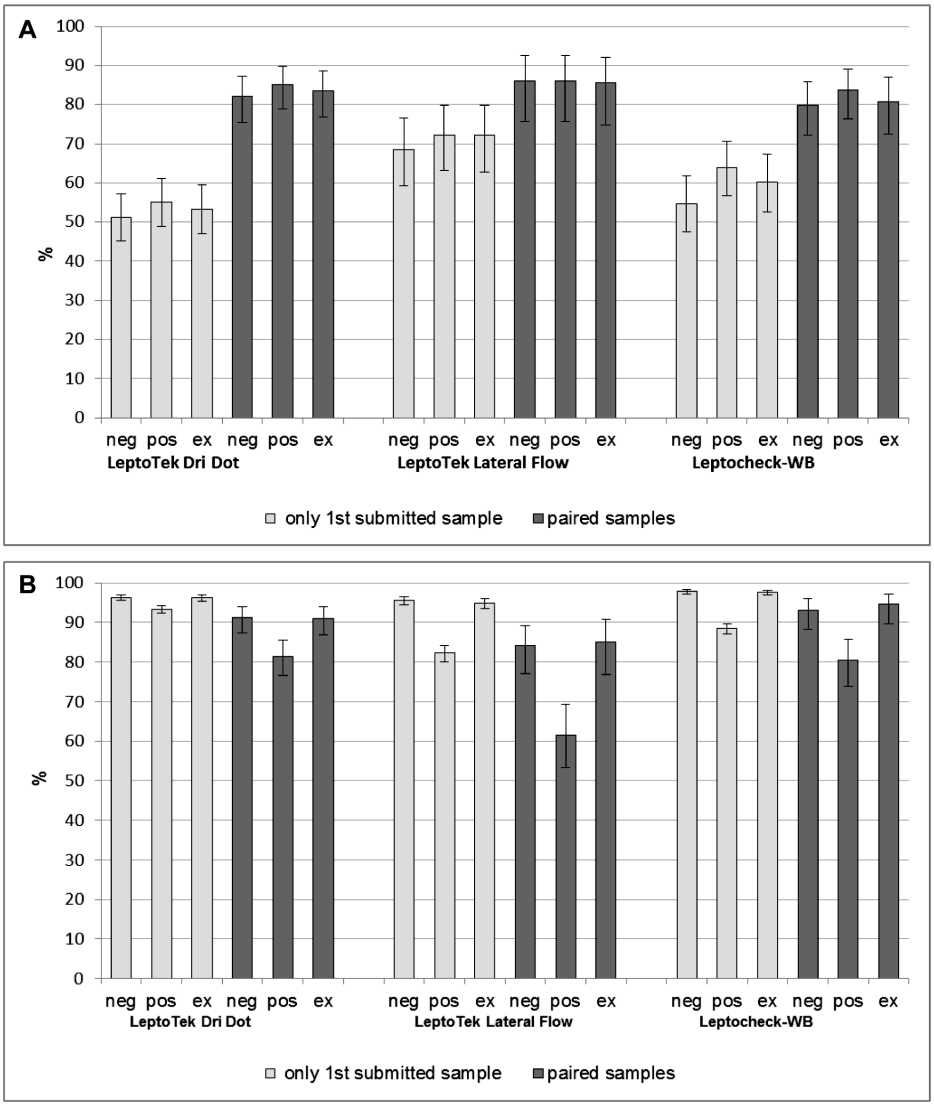


Figure 2. Sensitivity and specificity of the three RDTs of the 1st submitted sample and paired samples. Intermediate results are considered either negative (neg) or positive (pos), or are excluded (ex). Panel A: sensitivity, Panel B: specificity

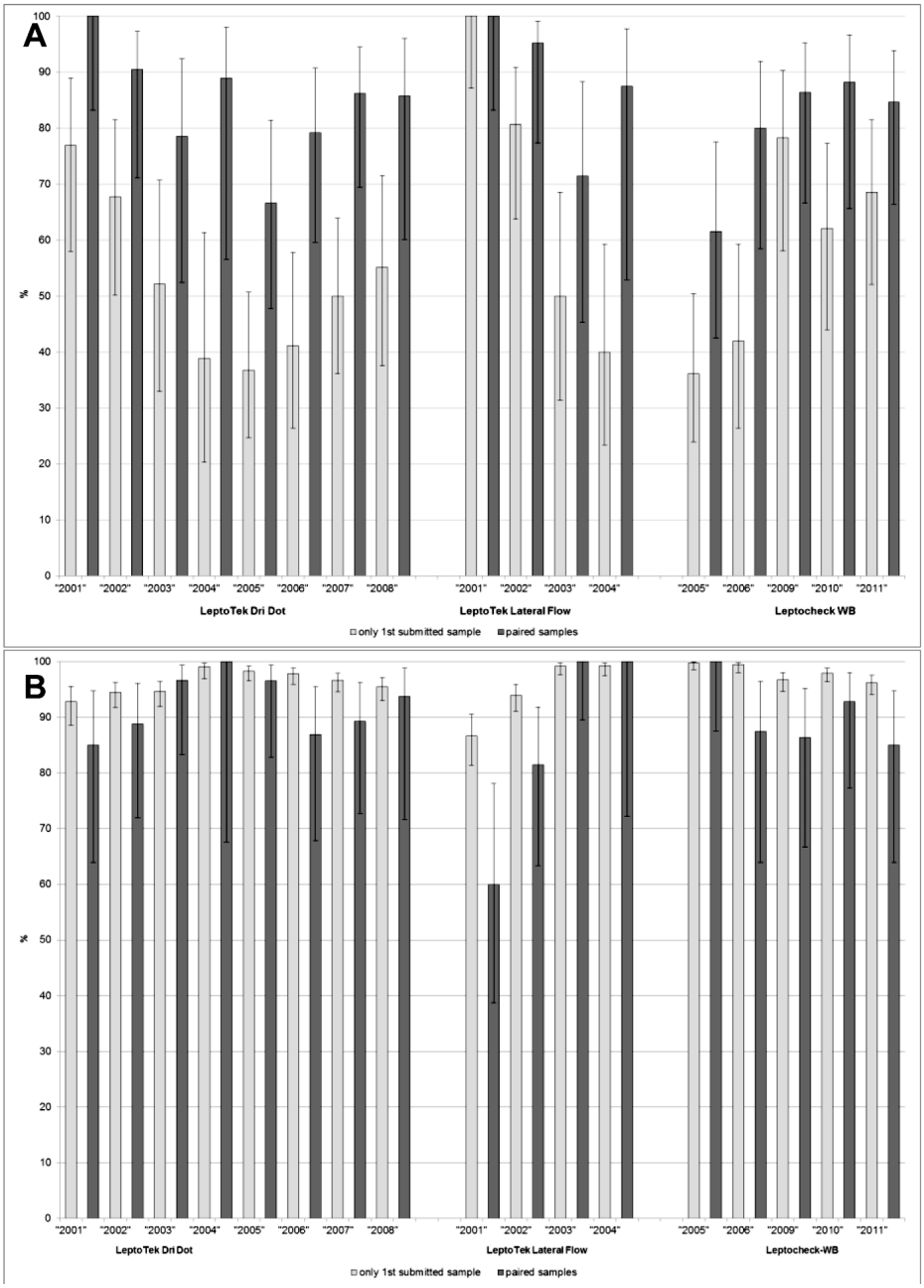


Figure 3. Sensitivity and specificity of the three RDTs of the 1st submitted sample and paired samples. Results are presented for each year.
Panel A: sensitivity, Panel B: specificity

ing cases: The LeptoTek Dri Dot showed a higher sensitivity for the paired samples in the Icterohaemorrhagiae infections (98%) compared to the other infections (81%) and non-classifiable serogroup infections (60%). The 1st submitted samples of the latter category also had a lower sensitivity (38%) compared to the Icterohaemorrhagiae group infections (67%).

The LeptoTek Lateral Flow showed a higher sensitivity in both the 1st submitted samples and the paired samples for the Icterohaemorrhagiae infections (85% respectively 100%) compared to for 'non-classifiable serogroups' (51% respectively 63%).

Leptocheck-WB showed a higher sensitivity in the 1st samples (68%) as well as the paired samples (95%) for the Icterohaemorrhagiae infections compared to the category 'non-classifiable serogroups' (1st submitted sample 38%, paired samples 65%).

Temporal consistency

To investigate the consistency of the diagnostic accuracy of these RDTs over the time period 2001 to 2011, the diagnostic accuracy based on the 1st submitted sample and paired samples for each year for each test was compared (Figure 3). Significant variation was observed in the following cases: for the 1st sample submitted, the sensitivity of the LeptoTek Dri Dot decreased from 77% in 2001 to 37% in 2005 combined with increasing specificity from 93% to 98%. During the same years the paired samples showed a decrease in sensitivity from 100% to 67%. Also the LeptoTek Lateral Flow showed on the 1st submitted sample a decreasing sensitivity from 100% in 2001 to 50% in 2003, whereas the specificity increased from 87% to 99%. For the paired samples, the specificity increased from 60% to 100%. On the contrary, based on the 1st submitted sample the Leptocheck-WB showed an increase in sensitivity, from 36% in 2005 to 78% in 2009, combined with a decreasing specificity from 100% to 97%.

DISCUSSION

This paper presents data of a prospective evaluation of three RDTs for leptospirosis, the LeptoTek Dri Dot, the LeptoTek Lateral Flow and the Leptocheck-WB, on a well-defined Dutch population. The overall sensitivity and specificity did not vary much between the tests, with sensitivity ranging from 75% to 78% and specificity ranging from 95 to 98%.

However, when based on first submitted sample only, the sensitivity of all tests depreciated substantially, with corresponding specificities remaining high. The sensitivity of the LeptoTek Dri Dot and the Leptocheck-WB was markedly lower, i.e. 51% and 55%, respectively while the sensitivity of the LeptoTek Lateral Flow test dropped less to a still appreciable 69%. This low sensitivity of first sample can be explained by the fact that these samples usually are collected at an early stage of disease when antibodies are not present yet at detectable levels[15]. Consistently, the sensitivity of the three tests increased to more than 80% when

results of a follow-up sample were included, supporting a significant increase of sero-diagnostic sensitivity when using paired samples as previously reported[10,15]. However, as the sensitivity increased with paired samples, the concomitant specificity reduced, with the largest reduction found for the LeptoTek Lateral Flow, i.e. from 96% to 84%.

Clinically this indicates that with around 500 suspected cases annually received at the NRL, comprising approximately 30 confirmed leptospirosis cases, RDTs used on the first sample alone, would lead to between half (LeptoTek Dri Dot, sensitivity of 51%) and a third (LeptoTek Lateral Flow) of the cases being missed. Yet, if paired samples are considered, then only 4 to 6 confirmed leptospirosis patients would be missed. This strongly advocates for clinicians to provide follow-up samples[15]. These paired samples, however, increase the number of false positives (from 17 to 33-75), which might contribute to an unneeded continuation of treatment with antibiotics.

It should be pointed out that in most situations, where leptospirosis is highly endemic, availability of only one acute phase sample is common practice and, hence, the diagnostic accuracy of tests on early acute samples is most relevant. In general, these RDTs showed disappointingly low sensitivities at the early stage of the disease, although associated with acceptable specificities of around 97%. From the subgroup analysis involving samples with known DPO, the LeptoTek Lateral Flow Test presents a favorable exception. Its sensitivity in the early acute phase was 62%, which is significantly higher than the sensitivity of the Leptocheck-WB (27%) and the LeptoTek Dri Dot (42%). From the literature, 62% is also higher than usually reported on the sensitivity of the MAT and ELISA in the earliest stage of the disease[10,15,19]. Apparently, this test is more capable to effectively detect 'early' antibodies and, hence, presents a respectable adjunct laboratory tool enabling a timely start of adequate care handling of patients.

This higher sensitivity in the early acute phase cannot be explained by the choice of antigen, which is most likely similar, since both lateral flow assays (LFAs) have been constructed using crude antigen probably derived from *Leptospira biflexa*, serovar Patoc, strain Patoc I[20]. However, differences in the diagnostic accuracy between the two LFAs might be caused by differences in the production, such as applying different amounts of antigen to the LFAs or providing different quantities of conjugate. Of note, visual inspection albeit subjective, judged the staining of the band in the LeptoTek Lateral Flow test stronger than that of the Leptocheck-WB (unpublished observation) facilitating an easier positivity score. Differences found in the diagnostic accuracy of the LeptoTek Dri Dot can easily be explained by the use of a different antigen that consists of crude antigen derived from *L. borgpetersenii*, serovar Hardjo type Bovis, strain Lely 607[21], although differences in production procedures might remain a valid explanation.

All RDTs showed a lower specificity when testing paired samples compared to the 1st submitted sample only. A possible explanation is that cases from whom follow-up samples were received more frequently present with persistent complaints due to chronic disorders such as autoimmune diseases that are notorious for causing cross-reactions in serological assays. However, be aware that in general, the more tests one does, the more likely the tests will be positive (which can lead to an increase in false positives). In this study, we have seen that for all three RDTS as sensitivity rises, specificity convergently decreases.

An unexpected high percentage of indeterminate results were found, considering the fact that the reading of the RDTs was done on a daily basis by a small group of well-experienced staff. This indicates that these tests are not always easily read. Although we found a high proportion of tests results to be indeterminate, especially for the LeptoTek Lateral Flow, this does not imply that such indeterminate results are of no value. In the sensitivity analyses we saw that scoring all indeterminate results as positive (instead of negative or excluded from analyses) resulted in an increase in the sensitivity of a test, as expected, but this corroborated with an unwanted reduction of test specificity, depending on the proportion of indeterminate results. Therefore, there may be practical consequences of a decision to score an indeterminate result as positive or negative. In case of a first sample giving an indeterminate result, a clinician might choose to regard this result as a negative result and not send in a follow-up sample. However, we have seen in those patients with follow-up samples, that in about 28% of the cases where the first sample gave an indeterminate LeptoTek Dri Dot or Leptocheck-WB result, the follow up sample gave a positive result and likely is a true positive, compared to only 10% of the first samples with a negative outcome. Hence, this implies that the indeterminate results incline more towards a final true case than negative results. Apart from the fact that a follow-up sample always should be issued after initial negative outcome, it is particularly advised to submit a follow-up specimen in case of an indeterminate result.

From the additional diagnostic accuracy of the RDTs by infecting serogroup, the sensitivity of all three RDTs was higher for infection with the Icterohaemorrhagiae group compared to infections with other serogroups. There is no conclusive explanation why infections with the other categories are associated with lower sensitivity. It may be that patients infected with Icterohaemorrhagiae present with more severe disease and may elicit strong humoral responses[22]. The finding that the test sensitivity depends on the causative leptospires associated with that fact that there is a wide diversity of geographic distribution of most *Leptospira* serovars suggests that the diagnostic accuracy of the various tests most likely will vary in different geographical locations. This explains, at least in part, the discrepant results of RDTs in previous studies performed in various regions[9,10] and reiterates that it is imperative to do a local evaluation and validation of tests prior to implementation.

The results revealed that the diagnostic accuracy of the RDTs varies through the years of our study. The sensitivity of the LeptoTek Lateral Flow and LeptoTek Dri Dot tended to decrease during the years while the Leptocheck-WB increased in sensitivity. Although it is unclear why this variability is present, it implies that one cannot rely on a constant performance of commercial RDTs, hence emphasizing the importance of continuous and thorough quality control of the RDTs by the manufacturer. Moreover, for the user this necessitates the evaluation of new purchases, preferably by using a standardized set of sera comprising a range of low to high 'reactors'.

The validity of our data is positively affected by the prospective nature of the evaluation as well as the use of fresh specimens. Furthermore, all participants suspected for leptospirosis were included in the study, allowing those who did not meet the case definition to serve as controls, hence evading the use of a less realistic, separate sample 'healthy controls'[23]. The case definition in this study was based both on culture and serology (MAT and IgM-ELISA). The MAT has a disappointing low sensitivity in the early phase of infection[15,24] and consequently, reliance on only the MAT as reference standard would result in a proportion of incorrect false positive scores for the RDTs with an erroneous lower specificity. The RDTs were performed by well-trained staff who were used to (optically) reading the tests results and performed prior to serologic tests and culture.

The study also presents with limitations. Only a subgroup of participants had their first day of illness documented and documentation on both treatment and hospitalization was not available. This information could have affected the test results, since it is known that the use of antibiotics reduces the immune response. Follow-up samples were not received from all participants. Therefore what is considered a false positive RDT result could actually have turned out to be leptospirosis cases, if a confirmatory sample had been received. With a high level of indeterminate tests, the study misses information on repeatability or reproducibility.

Conclusion and recommendations

The LeptoTek Lateral Flow presents in all scenarios with the best sensitivity and equally good specificity of all three RDT tests. All three tests, LeptoTek Dri Dot, LeptoTek Lateral Flow and Leptocheck-WB present useful antibody tests contributing to the diagnosis of leptospirosis. For sure, confirmation of clinical suspicion will contribute to increased local awareness of leptospirosis. Confirmation might also be beneficial for the clinical management of the patient. On the other hand, it should be noted that, especially in the early phase, a negative RDT and a high clinical suspicion still warrants antibiotic treatment since (untreated) leptospirosis is a potential fatal disease. Unfortunately, currently LeptoTek Dri Dot and LeptoTek Lateral Flow are not available due to manufacturer issues, presently leaving few options. The overall sensitivity of the tested RDTs did not exceed 80%, while their performance might depend on batch-to-batch and year-to-year variations as well as on varying ecological niches containing different circulating serovars. This latter drawback

might be extended with a reduced diagnostic accuracy due to past leptospiral infections or infections with other causative agents in high endemic areas, causing cross-reactions in these tests[9]. For these reasons, one should be cautious to only rely on an RDT result. Confirmation by reference tests is strongly recommended, and further conclusive studies are needed in endemic regions. From this study we have seen that rapid testing is not synonymous with easy testing. Reading of tests by eye is subjective and depends on the experience of the reader. At least it is of great importance that a test result, in case of doubt, is reported as such, indicating the need for a follow-up sample, especially evading the inclination of the reader to score a doubtful signal as a positive score.

ACKNOWLEDGMENTS

We thank all colleagues from the National Collaborating Centre for Reference and Research on Leptospirosis (NRL) for performing the RDTs.

Table S1 | STARD checklist for reporting studies of diagnostic accuracy

*Note: in this thesis the page numbers have changed.

Section and Topic	Item #		On page #*
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	1
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	5
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	6
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	6
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	6
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	6
Test methods	7	The reference standard and its rationale.	6-7
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	6-8
	9	Definition of and rationale for the units, cut-offs and/ or categories of the results of the index tests and the reference standard.	7-8
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	6
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	6
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	9
	13	Methods for calculating test reproducibility, if done.	na

Section and Topic	Item #		On page #*
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	6, 8
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	11
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	11
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	na
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	na
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	Table S3
	20	Any adverse events from performing the index tests or the reference standard.	na
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	11-14
	22	How indeterminate results, missing data and outliers of the index tests were handled.	12-13
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	na
	24	Estimates of test reproducibility, if done.	na
DISCUSSION	25	Discuss the clinical applicability of the study findings.	15-17

Table S2 | Availability of the three RDTs throughout the years.

Table S3. Results of the three RDTs.
The results are stratified for: 1st sample and follow up (FU) sample, DPO (0-4, 5-10, 11-20, >20), probable infecting serogroup (Icterohaemorrhagiae, Grippotyphosa, other, non-classifiable), years

test	timing	condition	n	sensitivity	ci lower	ci upper		n	specificity	ci lower	ci upper
LeptoTek Dri Dot	overall		259	75	69	79		2909	96	95	97
	1st		256	51	45	57		2903	96	96	97
	1st	indeterminate regarded positive	256	55	49	61		2903	93	92	94
	1st	indeterminate excluded	246	53	47	59		2818	96	95	97
	1st OR FU		167	82	76	87		286	91	87	94
	1st OR FU	indeterminate regarded positive	167	85	79	90		286	81	77	86
	1st OR FU	indeterminate excluded	151	83	77	89		256	91	87	94
	DPO 0-4		55	27	17	40		324	97	94	98
	DPO 0-4	indeterminate regarded positive	55	31	20	44		324	94	90	96
	DPO 0-4	indeterminate excluded	53	28	18	42		313	97	94	98
	DPO 5-10		141	55	47	63		469	96	94	98
	DPO 5-10	indeterminate regarded positive	141	60	51	67		469	93	90	95
	DPO 5-10	indeterminate excluded	135	58	49	66		452	96	94	97
	DPO 11-20		109	83	74	89		331	96	93	98
	DPO 11-20	indeterminate regarded positive	109	90	83	95		331	91	87	93
	DPO 11-20	indeterminate excluded	105	86	78	91		313	96	93	98
	DPO >20		163	74	66	80		571	96	95	98
	DPO >20	indeterminate regarded positive	163	79	72	84		571	94	92	96
	DPO >20	indeterminate excluded	155	77	70	83		557	96	95	98
	1st	Icterohaemorrhagiae	87	67	56	76					
	1st OR FU		65	98	92	100					
	1st	Grippotyphosa	27	41	25	59					
	1st OR FU		17	82	59	94					
	1st	other serogroups	44	57	42	70					
	1st OR FU		32	81	65	91					
	1st	not classifiable serogroup	98	38	29	48					
	1st OR FU		53	60	47	72					
	1st	2001	26	77	58	89		210	93	89	96
	1st OR FU		19	100	83	100		20	85	64	95
	1st	2002	31	68	50	81		397	94	92	96
	1st OR FU		21	90	71	97		27	89	72	96
	1st	2003	23	52	33	71		376	95	92	97
	1st OR FU		14	79	52	92		30	97	83	99
	1st	2004	18	39	20	61		238	99	97	100
	1st OR FU		9	89	57	98		8	100	68	100
	1st	2005	49	37	25	51		415	98	97	99
	1st OR FU		27	67	48	81		29	97	83	99
	1st	2006	34	41	26	58		415	98	96	99
	1st OR FU		24	79	60	91		23	87	68	95
	1st	2007	46	50	36	64		450	97	95	98
	1st OR FU		29	86	69	95		28	89	73	96
	1st	2008	29	55	38	72		402	96	93	97
	1st OR FU		14	86	60	96		16	94	72	99
LeptoTek Lateral Flow	overall		109	78	69	85		1295	95	94	96
	1st		108	69	59	77		1292	96	94	97
	1st	indeterminate regarded positive	108	72	63	80		1292	82	80	84
	1st	indeterminate excluded	104	72	63	80		1119	95	93	96
	1st OR FU		65	86	76	93		138	84	77	89
	1st OR FU	indeterminate regarded positive	65	86	76	93		138	62	53	69
	1st OR FU	indeterminate excluded	62	85	75	92		100	85	77	91
	DPO 0-4		21	62	41	79		125	98	93	99
	DPO 0-4	indeterminate regarded positive	21	67	45	83		125	78	70	84
	DPO 0-4	indeterminate excluded	20	65	43	82		100	97	92	99
	DPO 5-10		59	75	62	84		191	94	89	96
	DPO 5-10	indeterminate regarded positive	59	76	64	85		191	77	71	83
	DPO 5-10	indeterminate excluded	58	76	63	85		160	93	87	96
	DPO 11-20		54	81	69	90		150	93	88	96
	DPO 11-20	indeterminate regarded positive	54	87	76	94		150	83	76	88
	DPO 11-20	indeterminate excluded	51	86	74	93		134	93	87	96
	DPO >20		67	85	75	92		282	95	91	97
	DPO >20	indeterminate regarded positive	67	87	76	93		282	82	77	86
	DPO >20	indeterminate excluded	66	86	76	93		245	94	90	96
	1st	Icterohaemorrhagiae	40	85	71	93					
	1st OR FU		28	100	88	100					
	1st	Grippotyphosa	17	53	31	74					
	1st OR FU		8	75	41	93					
	1st	other serogroups	16	81	57	93					
	1st OR FU		10	100	72	100					
	1st	not classifiable serogroup	35	51	36	67					
	1st OR FU		19	63	41	81					
	1st	2001	26	100	87	100		210	87	81	91
	1st OR FU		19	100	83	100		20	60	39	78
	1st	2002	31	81	64	91		397	94	91	96
	1st OR FU		21	95	77	99		27	81	63	92
	1st	2003	24	50	31	69		382	99	98	100
	1st OR FU		14	71	45	88		33	100	90	100
	1st	2004	25	40	23	59		285	99	97	100
	1st OR FU		8	88	53	98		10	100	72	100

Leptocheck WB	overall		197	78	71	83		2560	98	97	98
	1st		183	55	47	62		2551	98	97	98
	1st	indeterminate regarded positive	183	64	57	71		2551	88	87	90
	1st	indeterminate excluded	166	60	53	67		2312	98	97	98
	1st OR FU		129	80	72	86		174	93	88	96
	1st OR FU	indeterminate regarded positive	129	84	76	89		174	80	74	86
	1st OR FU	indeterminate excluded	109	81	72	87		148	95	90	97
	DPO 0-4		38	42	28	58		337	97	95	99
	DPO 0-4	indeterminate regarded positive	38	47	32	63		337	86	82	89
	DPO 0-4	indeterminate excluded	36	44	30	60		299	97	94	98
	DPO 5-10		100	65	55	74		437	96	94	97
	DPO 5-10	indeterminate regarded positive	100	76	67	83		437	84	80	87
	DPO 5-10	indeterminate excluded	89	73	63	81		384	95	93	97
	DPO 11-20		87	72	62	81		263	98	95	99
	DPO 11-20	indeterminate regarded positive	87	77	67	85		263	89	85	93
	DPO 11-20	indeterminate excluded	83	76	66	84		241	98	95	99
	DPO >20		117	70	61	78		446	97	95	98
	DPO >20	indeterminate regarded positive	117	83	75	89		446	87	84	90
	DPO >20	indeterminate excluded	102	80	72	87		404	97	94	98
	1st	Icterohaemorrhagiae	56	68	55	79					
	1st OR FU		43	95	85	99					
	1st	Grippotyphosa	14	71	45	88					
	1st OR FU		9	89	57	98					
	1st	other serogroups	47	57	43	70					
	1st OR FU		37	76	60	87					
	1st	not classifiable serogroup	66	38	27	50					
	1st OR FU		40	65	50	78					
	1st	2005	47	36	24	50		392	100	99	100
	1st OR FU		26	62	43	78		27	100	88	100
	1st	2006	31	42	26	59		372	99	98	100
	1st OR FU		20	80	58	92		16	88	64	97
	1st	2009	23	78	58	90		461	97	95	98
	1st OR FU		22	86	67	95		22	86	67	95
	1st	2010	29	62	44	77		546	98	96	99
	1st OR FU		17	88	66	97		28	93	77	98
	1st	2011	35	69	52	81		475	96	94	98
	1st OR FU		26	85	66	94		20	85	64	95

Table S4 | Predictive value of indeterminate and negative test results.

indeterminate result in 1st sample	% (CI) patients with positve follow up sample and being true positive patient					negative result in 1st sample	% (CI) patients with positive follow up sample and being true positive patient				
LeptoTek Dri Dot	27.3	(	15.1	-	44.2)	LeptoTek Dri Dot	11.2	(	8.6	-	14.4)
Leptotek Lateral Flow	9.4	(	3.2	-	24.2)	Leptotek Lateral Flow	3.8	(	1.8	-	8.1)
Leptocheck WB	28.1	(	15.6	-	45.4)	Leptocheck WB	10.5	(	7.4	-	14.7)

indeterminate result in 1st sample	% (CI) patients with negative follow up sample and being true negative patients					negative result in 1st sample	% (CI) patients with negative follow up sample and being true negative patients				
LeptoTek Dri Dot	48.5	(	32.5	-	64.8)	LeptoTek Dri Dot	76.1	(	72.0	-	79.8)
Leptotek Lateral Flow	56.3	(	39.3	-	71.8)	Leptotek Lateral Flow	74.5	(	67.2	-	80.7)
Leptocheck WB	21.9	(	11.0	-	38.8)	Leptocheck WB	74.5	(	69.0	-	79.4)

REFERENCES

1. World Health Organization. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group, 2011.
2. Farr RW. Leptospirosis. *Clin Infect Dis* 1995; **21**(1):1-6.
3. Segura ER, Ganoza CA, Campos K, Ricaldi JN, Torres S, *et al*. Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. *Clin Infect Dis* 2005; **40**(3): 343-51.
4. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control; 2003.
5. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; 14(2): 296-326.
6. Hartskeerl RA. *Leptospira*. In: Liu D, editors. Molecular detection of human bacterial pathogens. CRC Press; 2011: 1169-87.
7. Wuthiekanun V, Amornchai P, Paris DH, Langla S, Thaipadunpanit J, *et al*. Rapid Isolation and Susceptibility Testing of *Leptospira* spp. Using a New Solid Medium, LVW Agar. *Antimicrob Agents Chemother* 2013; **57**(1): 297-302.
8. Ahmed A, Engelberts MF, Boer KR, Ahmed N, Hartskeerl RA. Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. *PLoS One* 2009; **4**(9): e7093.
9. Blacksell SD, Smythe L, Phetsouvanh R, Dohnt M, Hartskeerl R, *et al*. Limited diagnostic capacities of two commercial assays for the detection of *Leptospira* immunoglobulin M antibodies in Laos. *Clin Vaccine Immunol* 2006; **13**(10): 1166-9.
10. McBride AJ, Santos BL, Queiroz A, Santos AC, Hartskeerl RA, *et al*. Evaluation of four whole-cell *Leptospira*-based serological tests for diagnosis of urban leptospirosis. *Clin Vaccine Immunol* 2007; **14**(9): 1245-8.
11. Sehgal SC, Vijayachari P, Sugunan AP, Umapathi T. Field application of Lepto lateral flow for rapid diagnosis of leptospirosis. *J Med Microbiol* 2003; **52**(10): 897-901.
12. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, *et al*. Towards complete and accurate reporting of studies of diagnostic accuracy: The STARD Initiative. *Ann Intern Med* 2003; **138**(1): 40-4.
13. Hartskeerl RA. Leptospiroses [in Dutch]. In: van Steenbergen J, Timen A, Beaujan DJMA, eds. LCI-Guidelines Infectious Disease Control. Bilthoven: LCI, Coordinator Infectious Disease Netherlands; 2008.
14. Faine S, Adler B, Bolin CA, Perolat P. *Leptospira* and Leptospirosis. Melbourne, Australia: MediSci; 1999.
15. Goris MGA, Leeflang MMG, Boer KR, Goeijenbier M, van Gorp ECM, *et al*. Establishment of Valid Laboratory Case Definition for Human Leptospirosis. *Journal of Bacteriology and Parasitology* 2012; **3**(2).
16. Terpstra WJ, Ligthart GS, Schoone GJ. Serodiagnosis of human leptospirosis by enzyme-linked-immunosorbent-assay (ELISA). *Zentralbl Bakteriol* 1980; **247**(3): 400-5.
17. Terpstra WJ, Ligthart GS, Schoone GJ. ELISA for the detection of specific IgM and IgG in human leptospirosis. *J Gen Microbiol* 1985; **131**(2): 377-85.

18. Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. *Stat Med* 1998; **17**(8): 857-72.
19. Vijayachari P, Sugunan AP, Sehgal SC. Evaluation of Lepto Dri Dot as a rapid test for the diagnosis of leptospirosis. *Epidemiol Infect* 2002; **129**(3): 617-21.
20. Smits HL, Eapen CK, Sugathan S, Kuriakose M, Gasem MH, *et al*. Lateral-flow assay for rapid serodiagnosis of human leptospirosis. *Clin Diagn Lab Immunol* 2001; **8**(1): 166-9.
21. Smits HL, van der Hoorn MA, Goris MG, Gussenhoven GC, Yersin C, *et al*. Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis. *J Clin Microbiol* 2000; **38**(3): 1272-5.
22. Olszyna DP, Jaspars R, Speelman P, van Elzakker E., Korver H, *et al*. [Leptospirosis in the Netherlands, 1991-1995]. *Ned Tijdschr Geneeskd* 1998; **142**(22): 1270-3.
23. Rutjes AW, Reitsma JB, Vandenbroucke JP, Glas AS, Bossuyt PM. Case-control and two-gate designs in diagnostic accuracy studies. *Clin Chem* 2005; **51**(8): 1335-41.
24. Limmathurotsakul D, Turner EL, Wuthiekanun V, Thaipadungpanit J, Suputtamongkol Y, *et al*. Fool's gold: Why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. *Clin Infect Dis* 2012; **55**(3): 322-31.

CHAPTER 9

Serological laboratory tests for diagnosis of human leptospirosis in patients presenting with clinical symptoms

Marga G.A. Goris[1]
Kimberly R. Boer[1]
Meerjam Bouman-Strijker[2]
Rudy A. Hartskeerl[1]
Cees Lucas[2]
Mariska M.G. Leeflang[2]

1. Royal Tropical Institute, Department of Biomedical Research, Amsterdam, the Netherlands
2. Academic Medical Centre, University of Amsterdam, Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Amsterdam, the Netherlands

Protocol: Cochrane Database of Systematic Reviews 2011, Issue 11.
Review submitted

ABSTRACT

Background

The diagnosis of leptospirosis in clinically suspected patients relies on the detection of leptospires and anti-leptospiral antibodies. The reference standard is culture and the microscopic agglutination test (MAT). Detection of leptospires by culture can take weeks before a culture becomes positive and lacks accuracy. Detection of anti-leptospiral antibodies by MAT requires specialised laboratories. Serological tests, other than MAT, are available and can be performed routinely.

Objectives

To determine the diagnostic accuracy of serological tests for diagnosing leptospirosis in patients presenting with compatible clinical symptoms, for example fever or muscle pain, or both.

Search methods

We searched PubMed (1948 and onwards) and EMBASE (1947 and onwards) on October 2011 without applying language or date limits.

Selection criteria

This review includes papers that focus on patients with clinical symptoms who were suspected of leptospirosis and thus referred for diagnostic testing. The reference standard was the microscopic agglutination test (MAT) (detecting antibodies) or culture (detecting viable leptospires), or both.

Data collection and analysis

Three sets of two authors independently extracted data and assessed quality of included studies using the QUADAS-2 tool. Disagreements were solved by discussion or by consulting a third co-author. Meta-analysis to estimate sensitivity and specificity of serological tests was carried out using the HSROC model for meta-analyses. As possible sources of heterogeneity, we explored: incidence or endemicity (endemic versus non-endemic regions); reference standard; moment or time of testing (acute versus convalescent patients); setting of study (primary care patients or referral patients); brand of the test, and risk of bias (QUADAS-2).

Main results

Thirty-nine publications containing 53 separate studies with 36 different index tests belonging to 14 test types (complement fixation test (CFT), counter immunoelectrophoresis (CIE), dipstick, dot enzyme linked immuno assay (ELISA), flow through assay, intra dermal (ID) injection, immunofluorescence antibody test (IFA), IgG ELISA, IgM ELISA, Indirect Haemagglutination Assay (IHA), lateral flow, latex, microcapsule agglutination test (MCAT), microscopic slide agglutination test (MSAT) recruiting 9564 participants (3453 with lepto-

spirosis) were included. Ten studies evaluating serological tests at both the acute and convalescent stages of the disease showed a significant increase in sensitivity; 58% (95% CI 43%-71%) respectively 88% (95% CI 79%-94%) and a specificity of 91% (95% CI 81%-96%). A meta-analysis was carried out for the test types dipstick (22 studies), IgM ELISA (13 studies), latex tests (11 studies) and IHA (5 studies). As most tests did not have data available for the acute time period, comparisons across tests could only be done at convalescent phase or at any time point from symptoms to sample collection for diagnosis: dipstick, IgM ELISA and latex tests had no significant difference in the performance. The dipstick had the highest sensitivity and specificity (86.0% (95% CI 77.8% to 91.5%) respectively 91.1% (95% CI 88.0% to 93.5%). Latex tests showed a sensitivity of 82.1% (95% CI 75.2% to 87.4%) and specificity of 89.4% (95% CI 74.3% to 96.1%) and IgM ELISA showed a sensitivity of 80.6% (95% CI 69.9% to 88.2%) and specificity of 89.8% (95% CI 84.8% to 93.4%). The overall accuracy was high for the IHA tests with a sensitivity of 81.0% (95% CI 20.3% to 98.6%) and corresponding specificity of 97.3% (95% CI 87.2% to 99.5%), but presented with large confidence intervals.

Authors' conclusions

The meta-analysis showed similar performance of IgM ELISA, dipstick and latex tests at the non-acute time point. Other serological tests had a large confidence interval or the number of included studies was too low to allow meta-analysis.

PLAIN LANGUAGE SUMMARY

Serological diagnostic tests for leptospirosis

Leptospirosis is a bacterial infection caused by leptospires. This disease occurs worldwide but especially in (sub)tropical regions with high rainfall, close human contact with livestock or wild animals, and poor sanitation. Patients have non characteristic complaints such as fever, head- and muscle pain. The current leptospirosis management is prompt antibiotic therapy and patient support. Therefore early diagnosis is important. The laboratory confirmation of leptospirosis is based on the microscopic agglutination test (MAT), with or without culturing. The MAT is a serological test which has to be performed in specialised laboratories. There are other serological tests which can be done in routine laboratories. This Cochrane review describes how accurate these tests are for diagnosing leptospirosis. Studies evaluating serological tests in patients, who are suspected to have leptospirosis are summarized. Only studies using established methods to distinguish the patients with leptospirosis from those who do not have the disease are included.

We found 39 publications with 53 separate studies in which 36 different tests belonging to 14 test types were evaluated. A total of 9564 people participated in these studies. Ten studies tested patients at the acute and convalescent time point. At the acute time point the tests gave correct, positive results in 58% of the patients with leptospirosis, and at the convalescent

time point the tests gave correct, positive results in 88% of the patients. At both time points correct, negative results were obtained in 91% of the patients without leptospirosis. The test types IgM ELISA, dipstick and latex tests did perform similar at the non-acute time point. For the other test types evaluated, there are too few studies to know how accurate they are.

BACKGROUND

Target condition being diagnosed

Leptospirosis is one of the world's most wide-spread zoonoses, caused by pathogenic spirochaetes of the genus *Leptospira*[1]. Being spread by the urine of infected animals, the bacteria enter the human body via abraded skin, conjunctivae or mucous membranes, after which they disseminate throughout the body. The infection is commonplace in regions characterised by high rainfall, close human contact with livestock or wild animals, and poor sanitation. Due to increased rainfall and global warming, the disease is re-emerging, especially in urban areas where slums are rapidly growing due to increased urbanisation[2,3].

The benefits of antibiotic treatment of leptospirosis have been unclear, particularly for severe disease. Although antibiotic treatment may decrease the duration of clinical illness by 2-4 days, this was not statistically significant[4]. Also the benefit of antibiotic prophylaxis in reducing *Leptospira* seroconversion or clinical consequences of infection was unclear[5]. However, the current leptospirosis management is prompt antibiotic therapy and patient support[6]. Most patients develop a mild febrile illness with rather non-specific symptoms like fever, myalgia, and headache. A proportion of patients, however, develops rapidly progressing and severe complications, with a case fatality rate up to 70% in patients with severe pulmonary disorders[7]. Prompt differential diagnosis of leptospirosis is of great importance for the timely and adequate clinical management of these patients[8] as well as for ruling out other diseases. Leptospirosis is a notifiable disease in several countries, but it can only be notified in cases where a diagnosis has been made.

Due to the diverse symptoms mimicking various other infectious diseases that are often endemic and epidemic in the same situations (that is salmonellosis, yellow fever, hepatitis, hantavirus infections, and dengue), leptospirosis is difficult to diagnose on clinical grounds alone. Therefore, laboratory techniques are required to confirm clinical diagnosis. These techniques involve either demonstration of leptospires or their DNA in clinical samples, or by demonstrating an antibody response against leptospires[8,9]. Leptospiremia (presence of leptospires in the blood) occurs during the early acute stage of illness and usually disappears by the end of the first week when the immune response clears the blood. This immune response occurs approximately five to seven days after the onset of symptoms and results

in detectable levels of anti-*Leptospira* antibodies in the blood. In certain parts of the body, such as the cerebrospinal fluid (CSF) and kidneys, the bacteria may survive for several weeks before they are cleared from the body. Hence, after about five to 10 days the leptospires may appear in the patient's urine and CSF.

The laboratory confirmation of leptospirosis is based on microscopic agglutination test (MAT), with or without culturing. This is, in most studies, also used as the reference standard. MAT is a time consuming serological test by which agglutinating antibodies bind to live leptospiral bacteria, which can be seen using dark field microscopy. Agglutinating antibodies show serovar specificity. The MAT thus relies on the use of a panel of antigens that preferably represents locally circulating serovars. Therefore, the diagnostic performance of the MAT is strongly linked to the composition of the panel used for each locality. Considerable expertise is needed to maintain the panel of *Leptospira* and to perform the MAT, and therefore, execution of this test is limited to a few specialised laboratories.

Serological tests for the detection of antibodies are serum or whole blood based. In high-endemic areas, the interpretation of antibodies on a single sample may not always be possible since even healthy persons can have low titers due to continuous exposure to leptospires. Also, serological tests, including the MAT, have the disadvantage that a detectable antibody response generally occurs at around five to 10 days after onset of illness. Therefore, in these situations a rising titre in successive specimens is mandatory, that is, samples have to be taken one to two weeks apart to check for seroconversion or a titer rise. This has logistical challenges for both the patient and treating healthcare professional, as well as the laboratory.

Culturing leptospires is already possible during the first few days of illness. Leptospires can be cultured from blood, urine, cerebrospinal fluid, dialysate fluid, and (post mortem) tissue, often within 10 days post-onset of symptoms. Leptospires are slow growing fastidious bacteria. Cultures are usually maintained for at least four months before being discarded as negative. In the first two weeks, cultures are checked frequently for growth and absence of (fast growing) contaminating micro-organisms by dark field microscopy; and later on weekly or biweekly[10]. Culturing provides evidence for leptospirosis but lacks sensitivity and does not contribute to an early diagnosis.

Index test(s)

Various serological tests for the diagnosis of leptospirosis, as alternatives to the MAT, have been applied both as 'in house' and commercial tests over recent years. The most common tests are the enzyme linked immunosorbent assay (ELISA) and immunoglobulin (Ig) IgM dipstick assay. ELISAs detecting IgM class antibodies are generally more sensitive than agglutination type tests and detect the antibodies slightly earlier but may be subject to variations in specificity.

Clinical pathway

The clinical diagnosis of leptospirosis relies mainly on symptomology and exclusion of other diseases, globally only a handful of leptospirosis cases are referred for laboratory, for which advanced laboratories currently rely on MAT and culture for laboratory confirmed diagnosis of leptospirosis. In practice, most patients will be treated with antibiotics if the suspicion is high enough based on symptomology, either with or without a positive test result. However, a confirmed diagnosis is important, because leptospirosis is in many countries a notifiable disease for epidemic control (prevent additional cases) and to rule out other causes, such as malaria and typhus in many areas of the world where these are co-endemic, to ensure correct treatment for the patient.

Role of index test(s)

If a patient is suspected of having leptospirosis, the blood is sent to the laboratory for MAT and culture, and sometimes also for another serological test. Culture takes a long time and may not be very sensitive and MAT requires trained personnel. In places where the facilities are not sufficiently well equipped to conduct culture and MAT, other serological tests may be good replacements[11]. Also, for triage purposes[11], relying on serology other than MAT and therefore limit the MAT to confirmation of a positive outcome would make active case finding programs much cheaper.

After a negative first result, ideally, one should send in a second sample, because of the above described seroconversion. Additional information about the patient is also needed for example, most importantly, whether the patient is receiving treatment, which could affect the test results and the date of onset of disease. As well, in case that a second sample has not been tested, it will be useful to know how many cases would be missed when a confirmed diagnosis can only rely on one tested sample. False negative tests could result in a leptospirosis patient not receiving treatment, while false positive patients might not have been correctly treated for another disease.

Several challenges exist in confirming a diagnosis of leptospirosis, firstly, as mentioned, the symptoms are contiguous with other infectious diseases and therefore often misdiagnosed; secondly, in situations where the laboratories and reference standard tests are not available it is not possible to have a confirmed diagnosis. Thirdly, in many situations, it is not feasible to get multiple samples to submit to a reference laboratory. Patients do not return to the clinic for services once receiving treatment, an additional test is too expensive, doctors are unable to send additional samples or cannot link them to the initial sample. In only a small percentage of patients will a leptospirosis sample be sent to the reference laboratory and in an even smaller percentage will a second sample be available which reduces the sensitivity of the MAT and other serological tests. Therefore the index tests presented here can be used for both triage and replacement purposes.

Rationale

Many alternative serological tests have been developed to diagnose leptospirosis in situations in which MAT and culturing are not feasible. However, it is unknown how well these tests perform, being dependent on issues such as timing of the sampling and prevalence, and incidence of the disease in distinct countries[12].

The main aim of this systematic review is to better understand the role of available diagnostic tests, in which two options have been reviewed. Firstly, to assess whether other tests perform sufficiently well to replace MAT in acute and convalescent patients and in high- and low- incidence areas. Secondly is to determine whether it is feasible to use other serological tests than MAT to triage patients for leptospirosis and limit the application of MAT as a confirmation test, when available.

This review was restricted to determining the diagnostic performance of serological methods since these can be more routinely used in most laboratories and settings, while antigen detection requires either specialised training or sophisticated equipment. The tests were intended to be used for case-finding and to rule out suspect cases.

OBJECTIVES

To determine the diagnostic accuracy of serological tests for diagnosing leptospirosis in patients presenting with compatible clinical symptoms, for example fever or muscle pain, or both.

Secondary objectives

a) To determine the diagnostic accuracy of laboratory tests (i.e. ELISA, dipstick, agglutination test) for diagnosing human leptospirosis in acute patients (0 to 10 days post-onset) and convalescent patients (> 10 days post-onset).
b) To determine the best diagnostic laboratory test for diagnosing human leptospirosis in incidence-related subgroups (high incidence versus low incidence).
c) To determine whether one of the serological tests under consideration can be used either as replacement test for MAT or as a triage test before MAT.

METHODS

Criteria for considering studies for this review

Types of studies

Studies assessing the diagnostic accuracy of serological diagnostic tests for the triage and diagnosis of human leptospirosis were included. Studies were either prospective or retro-

spective and either case-control studies or consecutive patient series. Studies using one or more index tests and a reference standard were included with more than one two-by-two table; whilst studies reporting insufficient data for the construction of a two-by-two table were excluded from the final analysis.

Participants
Human patients presenting with clinical symptoms compatible with the diagnosis of leptospirosis were included.
In this systematic review, we included papers that focus on patients with clinical symptoms who were suspected of leptospirosis and thus referred for diagnostic testing. These were mostly patients with fever, living in leptospirosis-endemic areas, or having occupations with a higher risk of contracting leptospirosis. There were also papers that focus on the aforementioned laboratory tests in an asymptomatic population; these studies were not included in this review since the interpretation was different compared to patients with clinical symptoms.

Index tests
Serological laboratory tests for the detection of antibodies, like IgM ELISA, IgG ELISA, immunofluorescence antibody test (IFA), indirect hemagglutination (IHA), dipstick format tests, lateral flow tests and latex agglutination tests. The lateral flow and latex agglutination tests are so-called rapid diagnostic tests since the test result is usually available within 15 minutes.

Target conditions
The focus was on current clinical human leptospirosis, as defined by the reference standard described hereafter.

Reference standards
The reference standard was the microscopic agglutination test (MAT) (detecting antibodies) or culture (detecting viable leptospires), or both. Differences were expected in the reference standards used since the cut-off values and the antigens used in MAT varied in the studies from different geographical regions. Preferably, for each index test result we wanted to know whether it was performed in the acute phase or convalescent phase, and whether the MAT results were based on paired sera. Most studies provided these data for most patients. In this case, a fourfold titer rise or seroconversion would often be regarded as proof of having leptospirosis. Patients who did feel 'not too ill' and were tested MAT negative on the first sample often would not return for the second MAT measurement. Therefore, some studies used one single MAT titer as the reference standard; the exact cut-off titer for positivity depended on whether the region where the test was done was high-endemic or low-endemic. In endemic areas the cut-off titer was usually higher since many individuals were likely to have persistent antibodies due to passive infections[8].

Search methods for identification of studies

Electronic searches

In October 2011 PubMed (1948 and onwards) and EMBASE (1947 and onwards) through Ovid were searched. There were no specific electronic databases on this topic.

The search terms consisted of different terms for leptospirosis (synonyms) written in different ways. Terms were included in the search strategy which complemented the search result. Included terms for leptospirosis were combined with the search terms of each diagnostic test, which were determined in the same way as the terms for leptospirosis. Terms related to diagnostic accuracy were also included.

MeSH terms, free text words, and other important phrases were included in our search strategy. Search strategies with the expected time span of the search are given in Appendix 1.

Searching other resources

Grey literature

Conference proceedings and reports were searched for in the archive of the Royal Tropical Institute (KIT) and the National Institute for Public Health and the Environment (RIVM, Rijksinstituut voor Volksgezondheid en Milieu).

References lists

References of the retrieved articles were checked as well as articles through the 'Related articles' facility of PubMed.

Correspondence

The author was contacted if the full paper could not be retrieved or in cases of uncertainties.

Data collection and analysis

Selection of studies

The first selection, based on titles and abstracts, was done by four authors (MS, MG, ML, RH) since they have different expertise. Animal studies were removed as well as case reports, non-accuracy studies, and studies reporting the early development of a test since these do not match our research question. Discordant articles were discussed or included for the next step. Two authors ((MS and MG) or (MS and ML) or (MG and RH)) independently assessed eligible articles for inclusion with the use of a pre-designed inclusion form. Disagreements were solved by discussion or by consulting another author (either KB, ML or RH).

Data extraction and management

Since more than 12 studies were included, the extraction of the data took place by three sets of two authors working independently (MS-MG, MS-ML, MG-RH). Again, a pre-designed data-extraction form was used. Disagreements were solved by discussion or by consulting a third co-author. The following study characteristics were extracted:

- Study ID
- Year of publication
- Study region or country
- Setting where patients come from (serum bank, via clinician)
- Prevalence in underlying population
- Gender and age distribution
- Additional information about origin of patients (e.g. specific occupational groups, travelers)
- Number of tests evaluated
- Test names and cut-off values
- Day post-onset of the testing of patients
- Reference standard and cut-off values
- 2 x 2 tables

Assessment of methodological quality

The Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool was used to assess the risk of bias and concerns for applicability of the included studies[13]. Data were extracted using signaling questions and scored for risk of bias and concerns for applicability under the four main domains: participant selection, index test, reference standard, and participant flow. One specific question was added to the domain participant selection: "Are both single samples and paired samples included", since this reflects a study population as would be seen by a clinician in practice (Appendix 2). Results are presented in the text and graphically. We did focus on summary scores by comparing studies with low or high risk of bias for certain domains. We did not calculate a summary score estimating the overall quality of the compiled studies since the interpretation of such summary scores is potentially misleading[14,15]. Disagreements about study quality were resolved through consensus or by consultation with a third review author.

Statistical analysis and data synthesis

Studies reporting insufficient data for the construction of a two-by-two table were excluded from the final analyses. Studies calculating sensitivity in patients coming from a different population than the participants that were used to calculate specificity (two-gate design) were excluded as they often report an overestimated sensitivity and specificity[16]. The final analyses are based on two-by-two tables originating from clinically suspected patients who were equally suspected of leptospirosis before they were tested. The unit of analysis is the individual patient, instead of individual samples.

From each included study, data for acute diagnosis and for the convalescent stage was extracted for the two-by-two tables, if available. For both stages, if possible, the convalescent MAT titers or seroconversion are used as reference standard but the stages were analyzed separately. Also when the time point was not reported or overall results were presented,

data was extracted for the two-by-two tables. The data of the two-by-two tables was used to calculate sensitivity and specificity for each study. Individual study results are presented graphically by plotting the estimates of sensitivity and specificity (and their 95% confidence intervals) in both forest plots and the receiver operating characteristic (ROC) space.

This review includes several index tests, all providing a variation of results. ELISAs report an antibody titer, often expressed as 1:40 or 1:80. The variation in cut-off values is not so large, either 1:40, 1:80, or 1:160. Other rapid tests result in a coloring code, varying from 0 to ++. One + is often regarded as the cut-off value for test positivity. Because the index tests showed some variation in the test thresholds and because the reference standard was also used at different thresholds, we decided to use the HSROC model for meta-analyses. All analyses were done using Proc NLMIXED in SAS 9.3.

Because of this threshold variation in both index test and reference standard, it is impossible to come with one average sensitivity and specificity for every situation. We therefore took into account the interquartile range of the specificity and then estimated the sensitivity in this range. In the first instance, we summarized sensitivity and specificity for each test type separately, without formal testing of differences in accuracy between the test types. If sufficient data (more than 4 studies for each test type) were available, test types were compared directly by including test type as a covariate in the meta-analyses.

Investigations of heterogeneity

Heterogeneity was investigated in the first instance through visual examination of forest plots of sensitivities and specificities as well as through visual examination of the ROC plot of the raw data. If enough studies were available (at least three for each level of a covariate), the effects of covariates on sensitivity and specificity were more formally examined in the HSROC model.

The following sources of clinical and methodological heterogeneity were addressed:

- incidence or endemicity (endemic versus non-endemic regions)
- reference standard
- moment or time of testing (acute versus convalescent patients)
- setting of study (primary care patients or referral patients)
- brand of the test

Sensitivity analyses

To assess whether methodological quality influenced the results, we compared the overall analyses with the results of the analyses without studies that did not fulfil certain quality criteria. Although we did this for all quality criteria on which sufficient data were reported (less than 50% not reported), we did expect two quality criteria to be relevant to potential bias, these were: patient spectrum and appropriateness of the reference standard.

As expected, several studies compared two or more index tests in the same patients and against the same reference standard. These studies were analysed separately, where possible, and their results were compared with the overall analysis for each test with separate two-by-two tables.

RESULTS

Results of the search

The date of the search was 7 October 2011 for PubMed (10681 records) and 25 October 2011 for EMBASE (4203 records). After removal of duplicates 11823 records remained, each record corresponding to a publication. During the first screening process of titles and abstracts this was narrowed down to 304 potentially relevant publications. We were unable to find the full texts of 19 publications. After the inclusion procedure, 123 publications were eligible for data-extraction, of which 39 publications were included in this review (see Figure 1).

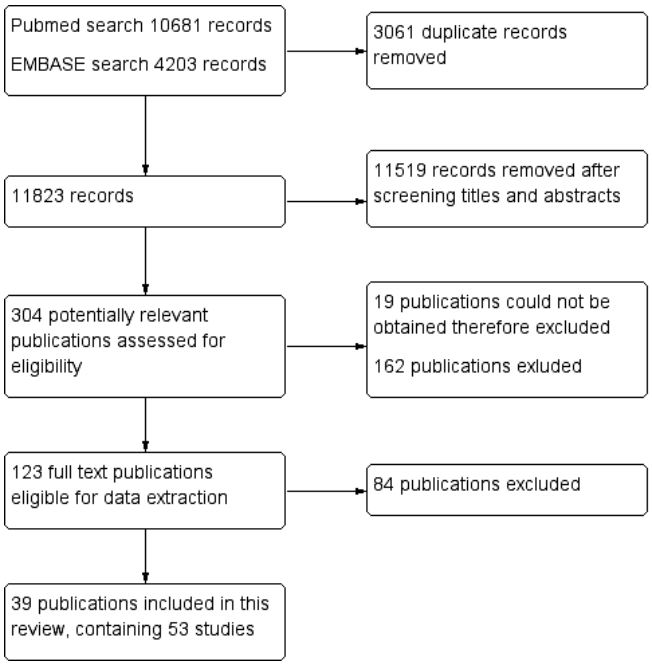


Figure 1 | Study Flow diagram showing the process of selection of studies for the review

These 39 included publications contained 53 separate studies and 92 two-by-two tables. The 53 studies included 9564 participants of whom 3453 were classified as having leptospirosis. The median prevalence in all studies was 45.4% (IQR 23.2% to 59.3%). Some publications contained more than one study, for example the paper by Smits 1999 contained 12 studies, all done by different laboratories and in different countries. Based on all 92 two-by-two tables, the median sensitivity was 82.7% (IQR 63.1% to 90.6%) and the median specificity was 90.1% (IQR 83.6% to 95.7%).

Overall, most studies (n=51) were not case-control design, with only one study a case-control design and in one study the design was unclear. Studies were conducted in India (n=13), Barbados (n=6), Thailand (n=5) and Hawaii (n=4) (Figure 2).

A total of 36 different index tests were analysed. These tests belong to one of the 14 test types presented (Table 1; Table 2). The test most frequently evaluated was the LEPTO dipstick (n=19). In 16 studies more than one test was evaluated, most evaluated two tests, except Effler 2002 who evaluated eight different tests.

The tests were done in the acute phase, or in the convalescent phase or at any time point following symptoms till diagnosis. Twenty-one analyses (two-by-two tables) were conducted in the acute phase, 18 in the convalescent phase, 36 that were a combination of both acute and convalescent samples, and for 17 analyses the exact time point was not reported. Ten studies evaluated one or more tests both in the acute phase and the convalescent phase and reported the data separately.

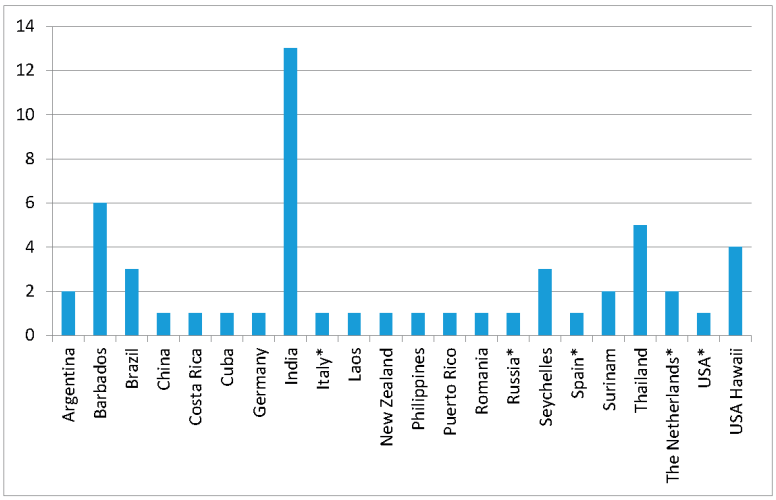


Figure 2 | Number of studies per country

*=non-endemic country

Table 1 | Test types

Test type	Number of studies	Percentage %
CFT	2	2,8
CIE	1	1,4
Dipstick	22	31,0
dot ELISA	1	1,4
Flow through assay	1	1,4
ID injection	1	1,4
IFA	3	4,2
IgG ELISA	2	2,8
IgM ELISA	14	19,7
IHA	5	7,0
Lateral Flow	2	2,8
Latex	11	15,5
MCAT	3	4,2
MSAT	3	4,2

Methodological quality of included studies

The QUADAS-2 tool was used to assess the quality in terms of the risk of bias and of concerns about applicability. The results are presented in Figure 3 and Figure 4.

A substantial proportion of studies were scored 'high risk of bias' in the domains 'patient selection', 'reference standard' and 'flow and timing'.

In the domain of 'patient selection', 19/53 studies scored a high risk of bias. This was mostly due to the fact that from a patient population either single samples or paired samples were submitted, instead of a combination of single samples and paired samples.

In the domain 'reference standard' 24/53 studies scored high risk of bias. The underlying reasons for this were because either other tests than MAT and culture were included in the reference standard, or from each patient a single sample with unknown DPO was tested, or it was unclear whether a single sample or paired samples were tested.

In the domain of 'flow and timing', 10/53 studies scored high risk of bias, due to studies in which not all entered study participants were included in their analysis.

A substantial proportion of studies 34/53 (>50%) scored an unclear risk of bias in the domain 'index test' because it was not reported whether the index test was performed blind.

Table 2 | Test types and test names

Test type	Test
CFT	CFT Patoc in house acc Sturda
	CFT in house with 3 antigens methanol
CIE	CIE in house Myers
Dipstick	LEPTO dipstick
	Dip-S-Tick
	Multi-Test Dip-S-Ticks
dot ELISA	IgM dot ELISA in house Sharma
Flow through assay	Flow through assay LipL41 in house
ID injection	Leptospirin ID injection
IFA	IFA Biognost
	IFA in house modification of ref Torten 1966
	IFA in house ref Torten 1966
IgG ELISA	IgG ELISA IVD *Leptospira* Microwell
	IgG ELISA in house Vanasco
IgM ELISA	IgM ELISA PanBio
	IgM ELISA Biolisa
	IgM ELISA InDx IVD Microwell
	IgM ELISA Serion
	IgM ELISA in house H1 antigen acc Pol
	IgM ELISA in house LipL41
	IgM ELISA in house OmpL1
	IgM ELISA in house Terpstra
	IgM ELISA in house EIACR Valverde
IHA	IHA MRL
Lateral Flow	LeptoTek Lateral Flow
	Lepto lateral flow in house
Latex	LeptoTek DriDot
	Latex Biosave
	Lepto Latex Test Crystal Diagnostics
	Lepto Latex in house
	Latex Shekatkar
	Latex in house Naigowit
	Latex LipL41 in house
MCAT	MCAT
MSAT	MSAT TR *Leptospira* antigen
	MSAT in house acc Andreescu

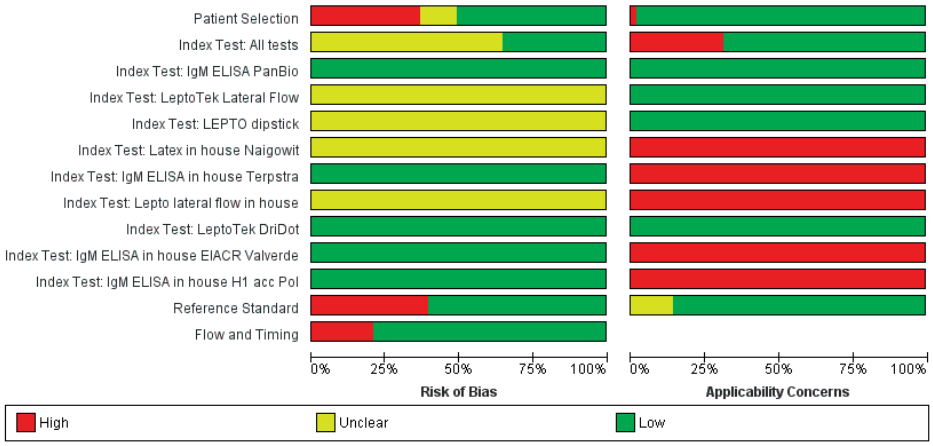


Figure 3 | Risk of bias and applicability concerns graph: review authors' judgments about each domain presented as percentages across included studies

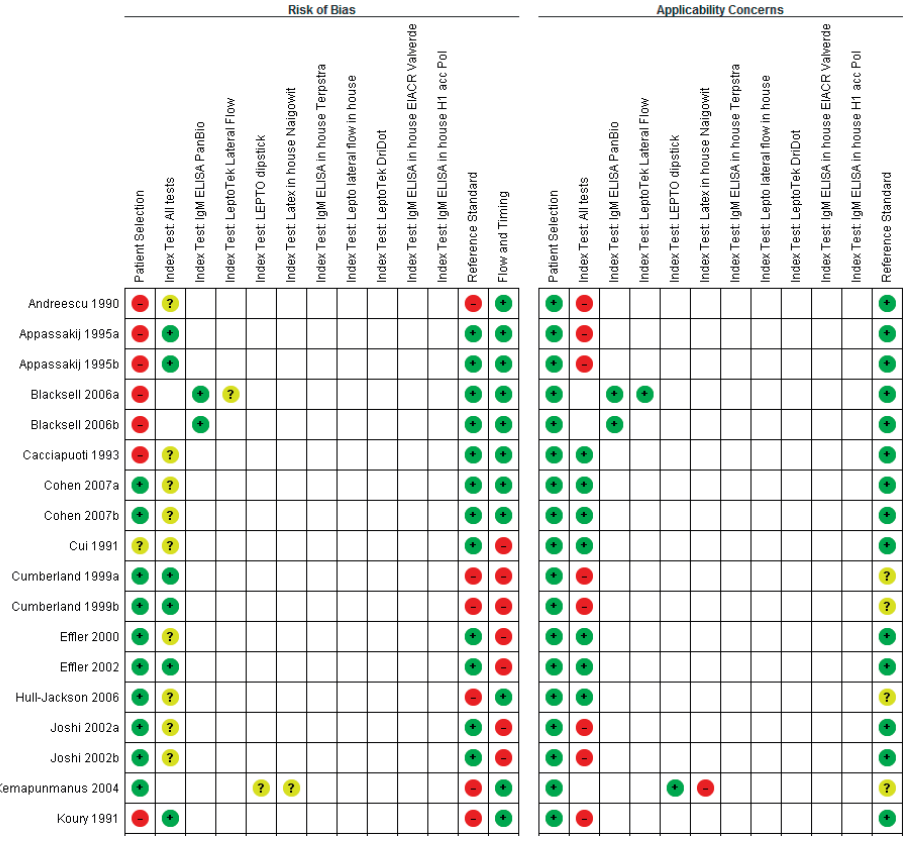


Figure 4 | Risk of bias and applicability concerns summary: review authors' judgments about each domain for each included study

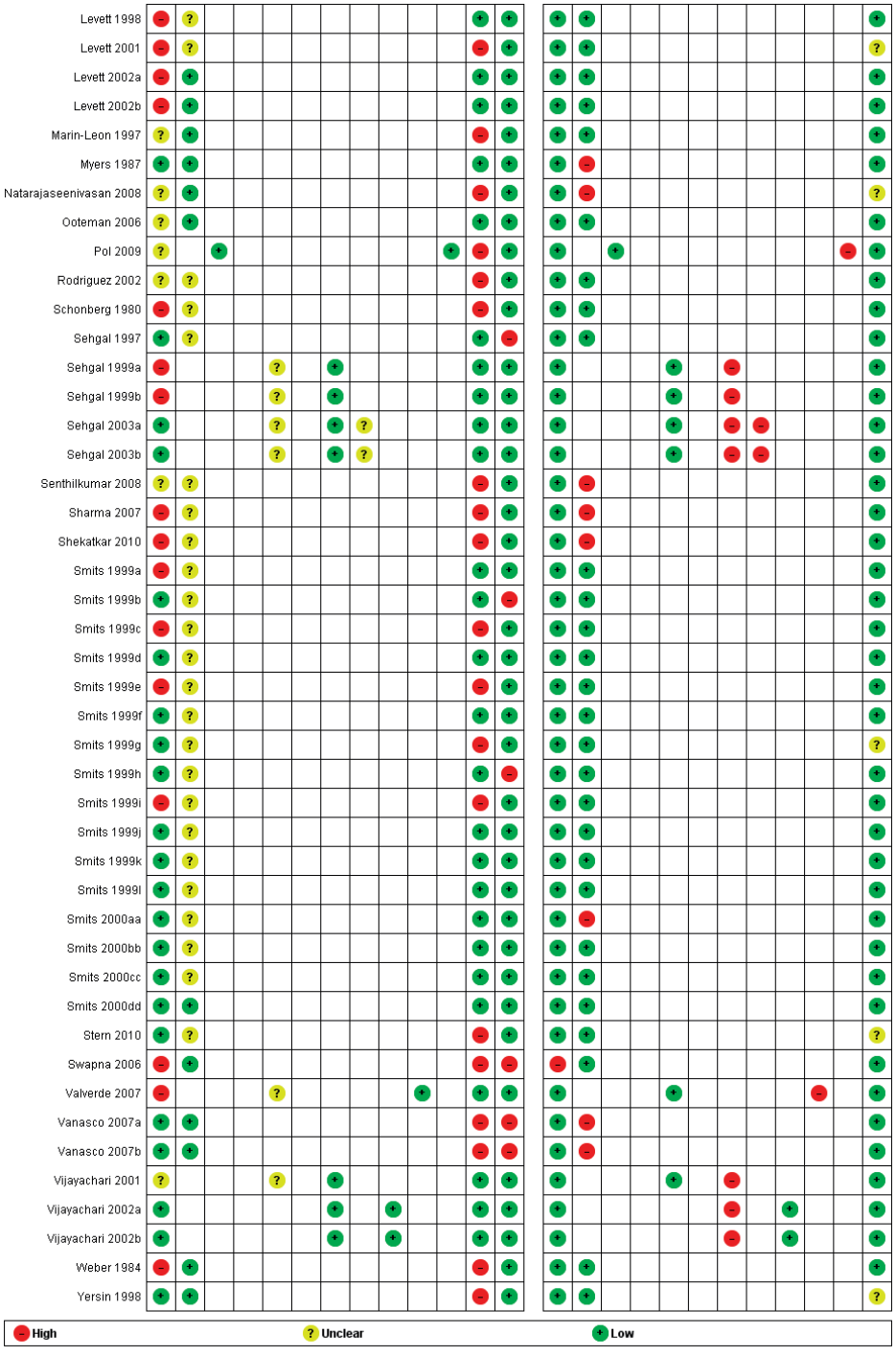


Figure 4 | Continued

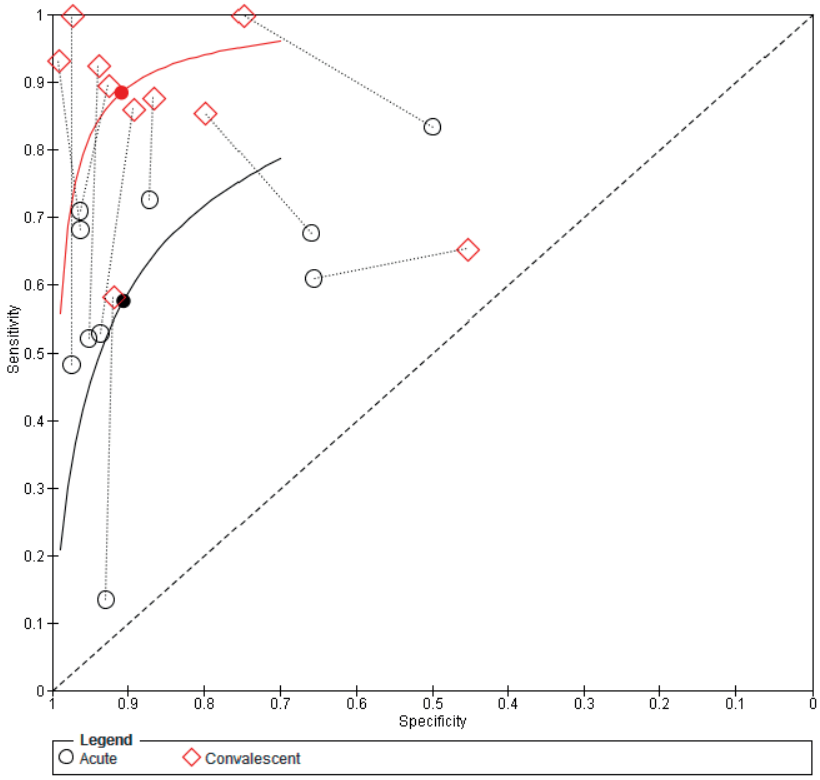


Figure 5 | Data points for studies investigating serological test accuracy in both the acute phase and the convalescent phase. One of the tests was randomly selected when a study evaluated more than one test.

Findings

Summary of findings is presented in Table 10.

Meta-analysis

Several analyses included 4 or more studies that reviewed a particular serological test and therefore the HSROC model could be conducted separately for each test type: dipsticks (22 studies), IgM ELISA (13 studies, in 3 of these more than 1 IgM ELISA was evaluated), latex tests (11 studies) and IHA (5 studies). Within each analysis, we investigated the following sources of heterogeneity: time point, endemicity (high- versus low- endemic area) and risk of bias assessment. The effect of time point was also investigated in studies that analyzed tests separately in acute phase and convalescent phase, for all test types (see below).

The effect of time point in studies that analysed both time points

Figure 5 shows the data points for studies that investigated serological test accuracy in both the acute phase and the convalescent phase. For each study that evaluated more than one

test, one of the tests was randomly selected. Figure 5 shows that serological tests have a (much) lower sensitivity in the acute phase than in the convalescent phase. This effect is consistent over all studies. The effect of time point on specificity varies, but in general the effect seems to be moderately decreasing. In three studies, the specificity increased in the convalescent analyses. Figure 5 also shows the hierarchical summary ROC curves for acute (black) and convalescent (red) samples. This is confirmed in the HSROC model, which showed that on average serological tests had significantly lower accuracy in the acute phase than in the convalescent phase (P=0.022). The summary DOR in the acute phase was 13.1 (95% CI 4.20 to 40.6), this coincides with a sensitivity of 57.5% (95% CI 42.7% to 71.1%) and a specificity of 90.6% (95% CI 80.5% to 95.8%). The summary DOR in the convalescent phase was 75.7 (95% CI 23.7 to 241), this coincides with a sensitivity of 88.4% (95% CI 79.4% to 93.8%) and a specificity of 90.9% (95% CI 81.4% to 95.8%). When all tests from all studies were included in the analyses, the effects remained roughly the same, with a DOR for acute of 10.3 (95% CI 5.03 to 20.9) and a DOR for convalescent at 61.8 (95%CI 29.3 to 131).

Dipstick (22 studies, 26 2x2 tables)

Data summary

Twenty-one studies evaluated one of three dipstick assays, whereas one study evaluated two dipstick assays: LEPTO Dipstick (n=19), Dip-S-Tick (n=3) or Multi-Test Dip-S-Tick (n=1) (Table 3). Fourteen different countries were included, with four studies from India, three from Thailand and two from Hawaii. Only three studies were done in a low-endemic areas. A forest plot of the available sensitivity and specificity is presented in Figure 6.

Effect of acute versus convalescent analyses.

Three studies evaluated dipstick assays at the acute as well as the convalescent time point: LEPTO Dipstick (n=2) and Multi-Test Dip-S-Tick (n=1). The results were not significantly different. There were also no differences with studies reporting the overall time point or no time point.

Overall meta-analysis dipsticks

Because all acute analyses were from studies that also contributed a 2x2 table for convalescent analyses in this set, we decided to remove the acute 2x2 tables (n=3) from the dataset. Meta-analysis was therefore done on studies that did not report the time point, used any time point or only convalescent phase. From the HSROC model follows that on average, dipstick tests used at these time points have a summary DOR of 62.9 (95% CI 35.3 to 112), with a sensitivity of 86.0% (95% CI 77.8% to 91.5%) and a specificity of 91.1% (95% CI 88.0% to 93.5%).

The effects of endemicity and country could not be investigated, as there were not enough data to make the models converge when endemicity was included as a covariate. Three studies were done in low-endemic countries. Removing these three studies, led to a hierarchical summary estimate of the DOR of 55.7 (95% CI 30.2 to 103). This is not different from the overall analyses including the low-endemic countries.

Table 3 | Publications Meta-Analysis Dipstick

	Study ID	Test	Timepoint	Country
1	Smits 1999a	LEPTO dipstick	overall/not reported	Barbados
2	Levett 2001	Dip-S-Tick	overall/not reported	Barbados
3	Valverde 2007	LEPTO dipstick	overall/not reported	Costa Rica
4	Rodriguez 2002	LEPTO dipstick	overall/not reported	Cuba
5	Sehgal 1999	LEPTO dipstick	acute**	India
5	Sehgal 1999	LEPTO dipstick	convalescent	India
6	Sehgal 2003	LEPTO dipstick	acute**	India
6	Sehgal 2003	LEPTO dipstick	convalescent	India
7	Smits 1999c	LEPTO dipstick	overall/not reported	India
8	Vijayachari 2001	LEPTO dipstick	overall/not reported	India
9	Smits 1999d	LEPTO dipstick	overall/not reported	New Zealand
10	Smits 1999e	LEPTO dipstick	overall/not reported	Philippines
11	Smits 1999f	LEPTO dipstick	overall/not reported	Puerto Rico
12	Smits 1999g	LEPTO dipstick	overall/not reported	Russia*
13	Smits 1999h	LEPTO dipstick	overall/not reported	Seychelles
14	Smits 1999i	LEPTO dipstick	overall/not reported	Surinam
15	Smits 1999j	LEPTO dipstick	overall/not reported	Surinam
16	Kemapunmanus 2004	LEPTO dipstick	overall/not reported	Thailand
17	Smits 1999l	LEPTO dipstick	overall/not reported	Thailand
18	Cohen 2007	Multi-Test Dip-S-Ticks	acute**	Thailand
18	Cohen 2007	Multi-Test Dip-S-Ticks	convalescent	Thailand
19	Smits 1999k	LEPTO dipstick	overall/not reported	The Netherlands*
20	Stern 2010	Dip-S-Tick	overall/not reported	USA*
21	Effler 2002***	LEPTO dipstick	overall/not reported	USA Hawaii
22	Smits 1999b	LEPTO dipstick	overall/not reported	USA Hawaii
23	Effler 2002***	Dip-S-Tick	overall/not reported	USA Hawaii

*not endemic

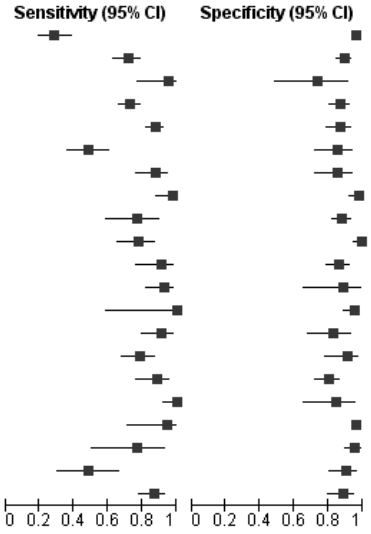
**excluded from meta-analysis

***this is one study with 2 different index tests

note that some rows have the same study number (e.g. Sehgal 1999 is two times in the table, both times numbered 5).

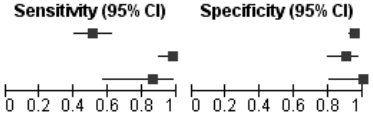
LEPTO dipstick

Study	TP	FP	FN	TN	Endemic	Time point	Country	Sensitivity (95% CI)	Specificity (95% CI)
Effler 2002	25	11	63	280	Yes	Overall/not reported	USA Hawaii	0.28 [0.19, 0.39]	0.96 [0.93, 0.98]
Kemapunmanus 2004	96	24	38	200	Yes	Overall/not reported	Thailand	0.72 [0.63, 0.79]	0.89 [0.84, 0.93]
Rodriguez 2002	21	5	1	14	Yes	Overall/not reported	Cuba	0.95 [0.77, 1.00]	0.74 [0.49, 0.91]
Sehgal 1999a	148	18	56	122	Yes	Acute	India	0.73 [0.66, 0.79]	0.87 [0.80, 0.92]
Sehgal 1999b	149	14	21	92	Yes	Convalescent	India	0.88 [0.82, 0.92]	0.87 [0.79, 0.93]
Sehgal 2003a	34	7	36	40	Yes	Acute	India	0.49 [0.36, 0.61]	0.85 [0.72, 0.94]
Sehgal 2003b	50	7	7	40	Yes	Convalescent	India	0.88 [0.76, 0.95]	0.85 [0.72, 0.94]
Smits 1999a	43	2	1	88	Yes	Overall/not reported	Barbados	0.98 [0.88, 1.00]	0.98 [0.92, 1.00]
Smits 1999b	26	20	8	147	Yes	Overall/not reported	USA Hawaii	0.76 [0.59, 0.89]	0.88 [0.82, 0.93]
Smits 1999c	49	1	14	99	Yes	Overall/not reported	India	0.78 [0.66, 0.87]	0.99 [0.95, 1.00]
Smits 1999d	31	15	3	95	Yes	Overall/not reported	New Zealand	0.91 [0.76, 0.98]	0.86 [0.79, 0.92]
Smits 1999e	49	2	4	16	Yes	Overall/not reported	Philippines	0.92 [0.82, 0.98]	0.89 [0.65, 0.99]
Smits 1999f	7	5	0	92	Yes	Overall/not reported	Puerto Rico	1.00 [0.59, 1.00]	0.95 [0.88, 0.98]
Smits 1999g	42	7	4	34	No	Overall/not reported	Russia	0.91 [0.79, 0.98]	0.83 [0.68, 0.93]
Smits 1999h	59	4	16	39	Yes	Overall/not reported	Seychelles	0.79 [0.68, 0.87]	0.91 [0.78, 0.97]
Smits 1999i	45	27	6	108	Yes	Overall/not reported	Surinam	0.88 [0.76, 0.96]	0.80 [0.72, 0.86]
Smits 1999j	44	4	0	22	Yes	Overall/not reported	Surinam	1.00 [0.92, 1.00]	0.85 [0.65, 0.96]
Smits 1999k	16	17	1	394	No	Overall/not reported	The Netherlands	0.94 [0.71, 1.00]	0.96 [0.93, 0.98]
Smits 1999l	13	5	4	105	Yes	Overall/not reported	Thailand	0.76 [0.50, 0.93]	0.95 [0.90, 0.99]
Valverde 2007	16	6	17	57	Yes	Overall/not reported	Costa Rica	0.48 [0.31, 0.66]	0.90 [0.80, 0.96]
Vijayachari 2001	80	9	12	69	Yes	Overall/not reported	India	0.87 [0.78, 0.93]	0.88 [0.79, 0.95]



Dip-S-Tick

Study	TP	FP	FN	TN	Endemic	Time point	Country	Sensitivity (95% CI)	Specificity (95% CI)
Effler 2002	45	14	43	277	Yes	Overall/not reported	USA Hawaii	0.51 [0.40, 0.62]	0.95 [0.92, 0.97]
Levett 2001	50	5	1	48	Yes	Overall/not reported	Barbados	0.98 [0.90, 1.00]	0.91 [0.79, 0.97]
Stern 2010	12	0	2	17	No	Overall/not reported	USA	0.86 [0.57, 0.98]	1.00 [0.80, 1.00]



Multi-Test Dip-S-Ticks

Study	TP	FP	FN	TN	Endemic	Time point	Country	Sensitivity (95% CI)	Specificity (95% CI)
Cohen 2007a	15	76	52	561	Yes	Acute	Thailand	0.22 [0.13, 0.34]	0.88 [0.85, 0.90]
Cohen 2007b	55	121	12	516	Yes	Convalescent	Thailand	0.82 [0.71, 0.90]	0.81 [0.78, 0.84]

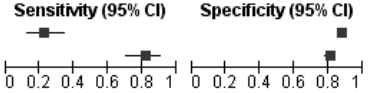


Figure 6 | Forest plot of tests: LEPTO dipstick, Dip-S-Tick, Multi-Test Dip-S-Ticks.

Accuracy could only be determined for one individual dipstick test, the LEPTO dipstick. The DOR was not significantly different from the overall DOR for the dipstick tests: DOR 64.1 (95% CI 23.5 to 105). The IQR for specificity runs from 85.1% to 95.5%. At a specificity of 85.1%, the sensitivity would be 94.5%, while at a specificity of 95.5%, the sensitivity will be 63.1%.

Sensitivity analyses for quality effects
Table 4 shows how the signalling questions and overall scores were divided.
None of the studies scored low risk of bias for all four domains. Seven studies scored low risk of bias for three domains (patient selection, reference standard, flow and timing) and unclear risk of bias for one domain (index test) (Sehgal 2003, Smits 1999 d,f,j,k,l,, Cohen 2007). These were analyzed separately, but showed no significant difference between the analyses, so the overall results are robust.

IgM ELISA (13 studies; 25 2x2 tables)
Data summary
Thirteen studies evaluated one or more IgM ELISA assays (Table 5). Four studies evaluated more than one test (indicated with a,b,c) and six studies evaluated tests both in the acute phase and in the convalescent phase. Five different in house assays were evaluated, with the assay according to the method of Terpstra being evaluated in 5 studies. Other assays are the IgM ELISA by PanBio (6 studies), the InDx IVD Microwell (1 study), Biolisa (1 study) and Serion (1 study). Six different countries were included, seven studies from India and two from Barbados. No IgM ELISAs were evaluated in a low-endemic country. A forest plot of the available sensitivity and specificity is presented in Figure 7.

Effect of acute analyses versus non-acute analyses
IgM ELISAs evaluated in the acute phase of disease had a significantly lower accuracy (DOR 16.0 (95% CI 5.73 to 44.6)) than IgM ELISAs that were evaluated in any phase or in the convalescent phase (DOR 36.8 (95% CI 18.1 to 74.7)). This is mainly due to a lower sensitivity; with a sensitivity of 64.7% (95% CI 51.9% to 75.8%) in the acute phase and 80.6% (95% CI 69.9% to 88.2%) in the convalescent phase, and a mean estimate of specificity of both acute and convalescent of around 90%.

Other effects
As there were no studies done in low-endemic countries, we could not investigate the effect of tests conducted in different endemicity areas. No significant differences in diagnostic accuracy were found for comparing country (India), or types of ELISA test, in-house testing (Terpstra) and PanBio and other tests. None of the investigated covariates had a significant effect on any of the parameters of the HSROC model. The PanBio test, however, did have a remarkably lower accuracy than the other tests: 14.0 versus 57.4 (P=0.05).

Table 4 | Signalling questions and overall scores Dipstick studies

				estimate	lower limit	upper limit
All studies for Dipstick N=22			DOR sens spec	62.9 0.860 0.911	35.3 0.778 0.880	112 0.915 0.935
Was a consecutive or random sample of patients enrolled?	20 'yes', 2 'unclear'	not analyzed				
Was a case-control design avoided?	only 1 case-control study	not analyzed				
Did the study avoid inappropriate exclusions?	All studies avoided inappropriate exclusions	not analyzed				
Could the selection of patients have introduced bias?	7 high risk; 13 low risk; 2 unclear	does removing high risk studies change results?	DOR sens spec	48.6 0.823 0.913	24.1 0.689 0.874	98.2 0.907 0.940
Are there concerns that the included patients and setting do not match the review question?	all low concern	not analyzed				
Were the index test results interpreted without knowledge of the results of the reference standard	all except 1 'unclear'	not analyzed				
If a threshold was used, was it pre-specified?	all 'yes' except 1 (unclear)	not analyzed				
Could the conduct or interpretation of the index test have introduced bias?	only 2 'low risk'	not analyzed				
Are there concerns that the index test, its conduct, or interpretation differ from the review question?	all low concern	not analyzed				
Is the reference standard likely to correctly classify the target condition?	8 were unclear; these scored high Risk of Bias as well	analyzed as such				

Table 4 | Continued

				estimate	lower limit	upper limit
Were the reference standard results interpreted without knowledge of the results of the index tests?	20 unclear; 2 yes	not analyzed				
Could the reference standard, its conduct, or its interpretation have introduced bias?	8 high risk; 14 low risk	does removing high risk studies change results?	DOR sens spec	58.5 0.840 0.918	25.8 0.703 0.884	132.6 0.921 0.942
Are there concerns that the target condition as defined by the reference standard does not match the question?	4 unclear concern; 18 low concern	not analyzed				
Was there an appropriate interval between index test and reference standard?	all appropriate interval	not analyzed				
Did all patients receive the same reference standard?	all avoided verification bias	not analyzed				
Were all patients included in the analysis?	18 explained missings; 4 did not	analysis of removing these 4?				
Could the patient flow have introduced bias?	3 high risk	does removing high risk studies change results?	DOR sens spec	81.5 0.893 0.907	42.2 0.830 0.868	157.4 0.935 0.935
Are both single samples and paired samples included?	14 included paired samples; 6 did not; 2 unclear	does removing 'single samples only' and 'unclear sampling' change results?	DOR sens spec	48.6 0.823 0.913	24.1 0.689 0.874	98.2 0.907 0.940
all seven 'high' quality studies			DOR sens spec	104.7 0.913 0.909	26.3 0.783 0.832	416.8 0.968 0.952

Table 5 | Publications Meta-Analysis IgM ELISA

	Study ID	Test	Timepoint	Country
1a	Levett 2002	IgM ELISA InDx IVD Microwell	acute	Barbados
1a	Levett 2002	IgM ELISA InDx IVD Microwell	convalescent	Barbados
1b	Levett 2002	IgM ELISA PanBio	acute	Barbados
1b	Levett 2002	IgM ELISA PanBio	convalescent	Barbados
2	Cumberland 1999	IgM ELISA in house Terpstra	acute	Barbados
2	Cumberland 1999	IgM ELISA in house Terpstra	convalescent	Barbados
3	Ooteman 2006	IgM ELISA PanBio	overall/not reported	Brazil
4	Valverde 2007	IgM ELISA in house EIACR Valverde	overall/not reported	Costa Rica
5a	Pol 2009	IgM ELISA PanBio	acute	India
5b	Pol 2009	IgM ELISAinhouse H1antigen accPol	acute	India
6	Swapna 2006	IgM ELISA PanBio	overall/not reported	India
7a	Natarajaseenivasan 2008	IgM ELISA in house LipL41	overall/not reported	India
7b	Natarajaseenivasan 2008	IgM ELISA in house OmpL1	overall/not reported	India
8	Sehgal 1999	IgM ELISA in house Terpstra	acute	India
8	Sehgal 1999	IgM ELISA in house Terpstra	convalescent	India
9	Sehgal 2003	IgM ELISA in house Terpstra	acute	India
9	Sehgal 2003	IgM ELISA in house Terpstra	convalescent	India
10	Vijayachari 2001	IgM ELISA in house Terpstra	overall/not reported	India
11	Vijayachari 2002	IgM ELISA in house Terpstra	acute	India
11	Vijayachari 2002	IgM ELISA in house Terpstra	convalescent	India
12	Blacksell 2006	IgM ELISA PanBio	acute	Laos
12	Blacksell 2006	IgM ELISA PanBio	convalescent	Laos
13a	Effler 2002	IgM ELISA Biolisa	overall/not reported	USA Hawaii
13b	Effler 2002	IgM ELISA PanBio	overall/not reported	USA Hawaii
13c	Effler 2002	IgM ELISA Serion	overall/not reported	USA Hawaii

*not endemic

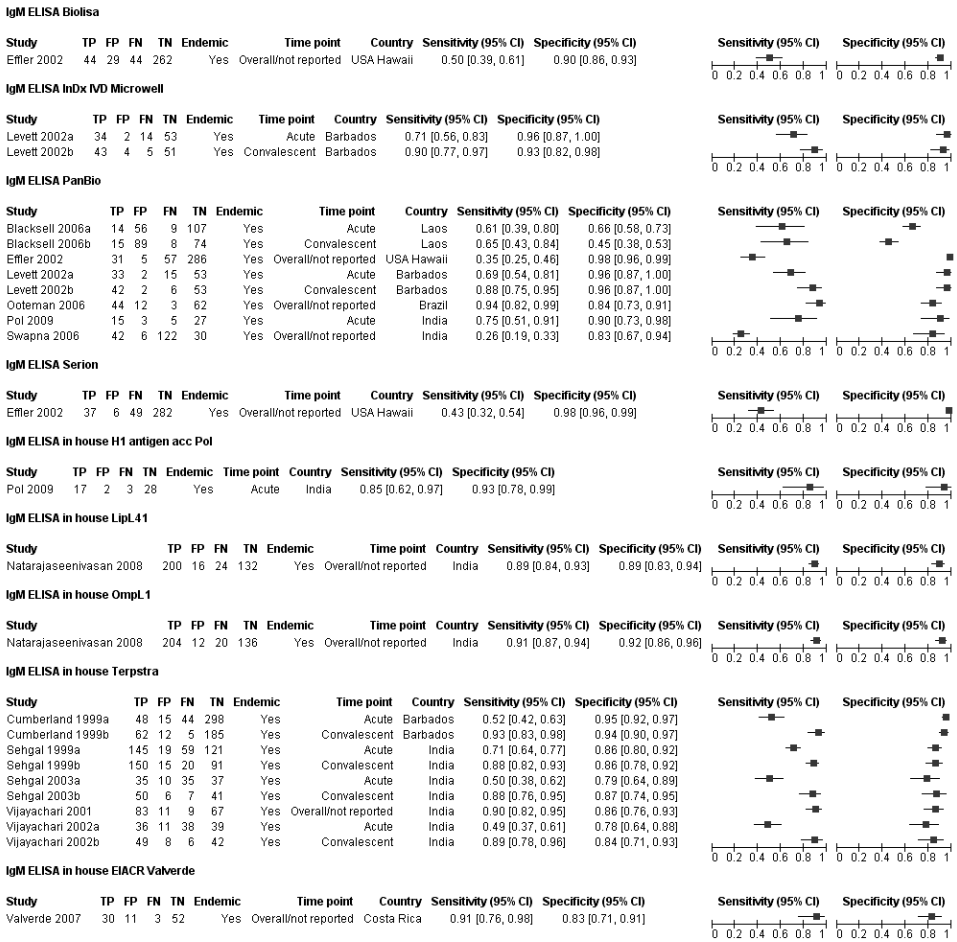


Figure 7 | Forest plot of tests: IgM ELISA Biolisa, IgM ELISA InDx IVD Microwell, IgM ELISA PanBio, IgM ELISA Serion, IgM ELISA in house H1 antigen acc Pol, IgM ELISA in house LipL41, IgM ELISA in house OmpL1, IgM ELISA in house Terpstra, IgM ELISA in house EIACR Valverde.

Sensitivity analyses for quality effects (limited to 2 x2 tables of non-acute studies) (n=16)

Table 6 shows how the signaling questions and overall scores were divided. Two studies scored low-risk of bias for all four domains (Sehgal 2003 and Vijayachari 2002) and one study scored low-risk of bias for three domains and unclear risk of bias for one domain (Ooteman 2006). However, this was not sufficient for separate analysis.

Table 6 | Signalling questions and overall scores IgM ELISA

				estimate	lower limit	upper limit
all studies for IgM ELISA n=13			DOR	57.4	24.5	134
			sens	0.857	0.722	0.932
			spec	0.906	0.847	0.944
Was a consecutive or random sample of patients enrolled?	12 'yes', 4 'unclear'	analyzed under roB				
Was a case-control design avoided?	only 1 case-control study	not analyzed				
Did the study avoid inappropriate exclusions?	All studies avoided inappropriate exclusions	not analyzed				
Could the selection of patients have introduced bias?	6 high risk; 6 low risk; 4 unclear	does removing high risk studies change results?	DOR	39.1	12.6	122
			sens	0.715	0.368	0.915
			spec	0.940	0.845	0.978
Are there concerns that the included patients and setting do not match the review question?	all low concern, except one	not analyzed				
Were the index test results interpreted without knowledge of the results of the reference standard?	12 'unclear', 4 'yes'	not analyzed				
If a threshold was used, was it pre-specified?	all 'yes'	not analyzed				
Could the conduct or interpretation of the index test have introduced bias?	15 low risk, 1 unclear	not analyzed				
Are there concerns that the index test, its conduct, or interpretation differ from the review question?	8 high concern, 8 low concern	not analyzed				
Is the reference standard likely to correctly classify the target condition?	4 were unclear, 12 yes	analyzed with RoB				

Table 6 | Continued

				estimate	lower limit	upper limit
Were the reference standard results interpreted without knowledge of the results of the index tests?	8 unclear; 7 yes; 1 no	analyzed with RoB				
Could the reference standard, its conduct, or its interpretation have introduced bias?	4 high risk; 12 low risk	does removing high risk studies change results?	DOR sens spec	35.8 0.805 0.896	16.1 0.663 0.804	79.6 0.897 0.948
Are there concerns that the target condition as defined by the reference standard does not match the question?	3 unclear concern; 13 low concern	not analyzed				
Was there an appropriate interval between index test and reference standard?	all appropriate interval	not analyzed				
Did all patients receive the same reference standard?	all avoided verification bias	not analyzed				
Were all patients included in the analysis?	10 explained missings; 6 did not	does removing of these 6 studies change results?				
Could the patient flow have introduced bias?	5 high risk	does removing high risk studies change results?	DOR sens spec	20.7 0.656 0.915	4.5 0.376 0.767	94.4 0.858 0.973
Are both single samples and paired samples included?	8 included paired samples, 5 did not; 3 unclear	does removing studies change results?	DOR sens spec	42.6 0.781 0.923	19.2 0.537 0.846	94.7 0.916 0.963

Latex tests (11 studies)

Data summary

Eleven studies evaluated one or more Latex assays (Table 7). All studies evaluated one test, either the LeptoTek DriDot (2 studies), the Lepto Latex Test Crystal (1 study), the Latex Biosave (1 study) or an in-house version (7 studies). A forest plot of the available sensitivity and specificity is given in Figure 8.

Table 7 | Publications Meta-Analysis Latex tests

	Study ID	Test	Timepoint	Country
1	Hull-Jackson 2006	LeptoTek DriDot	convalescent	Barbados
2	Vijayachari 2002	LeptoTek DriDot	convalescent	India
2	Vijayachari 2002	LeptoTek DriDot	acute	India
3	Shekatkar 2010	Latex Shekatkar	overall/not reported	India
4	Senthilkumar 2008	Latex LipL41 in house	overall/not reported	India
5	Smits 2000bb	Lepto Latex in house	overall/not reported	Seychelles
6	Cohen 2007	Lepto Latex Test Crystal	convalescent	Thailand
6	Cohen 2007	Lepto Latex Test Crystal	acute	Thailand
7	Smits 2000cc	Lepto Latex in house	overall/not reported	Thailand
8	Kemapunmanus 2004	Latex in house Naigowit	overall/not reported	Thailand
9	Smits 2000dd	Lepto Latex in house	overall/not reported	The Netherlands*
10	Effler 2002	Latex Biosave	overall/not reported	USA Hawaii
11	Smits 2000aa	Lepto Latex in house	overall/not reported	USA Hawaii

*not endemic

Unfortunately, none of the planned subgroup analyses were possible; two studies evaluated their test both in the acute phase and the convalescent phase of disease; this was insufficient to enable investigations of whether or not the time point influenced accuracy. One study used convalescent samples only; all others did not report the phase of disease or used samples from any time point. As only one study evaluated the test in a low-endemic country, we did not investigate the effect of endemicity on the accuracy of the tests. Three studies were done in India, three in Thailand, two in Hawaii and the remaining one each in Barbados, The Netherlands and Seychelles. These groups were too small to investigate the effect of country on accuracy.

The analyses were therefore done with the datasets for non-acute evaluations only regardless of endemicity and country. The overall DOR for the Latex tests was 38.9 (95% CI 13.9 to 109), which coincided with a specificity of 89.4% (95% CI 74.3% to 96.1%) and a sensitivity of 82.1% (95% CI 75.2% to 87.4%). The IQR for specificity runs from 83.5% to 94.9%. At a specificity of 83.5% the sensitivity would be 88.0% while at a specificity of 94.9%, the sensitivity will be 76.5%.

Sensitivity analyses for quality effects

Table 8 shows how the signaling questions and overall scores were divided. Only one study scored low risk of bias for all four domains (Vijayachari 2002). Risk of bias for the reference test could be analysed; 4 studies had a high risk of bias and 7 a low risk of bias for the reference test. Removing the high risk studies did not have a significant effect on the summary DOR.

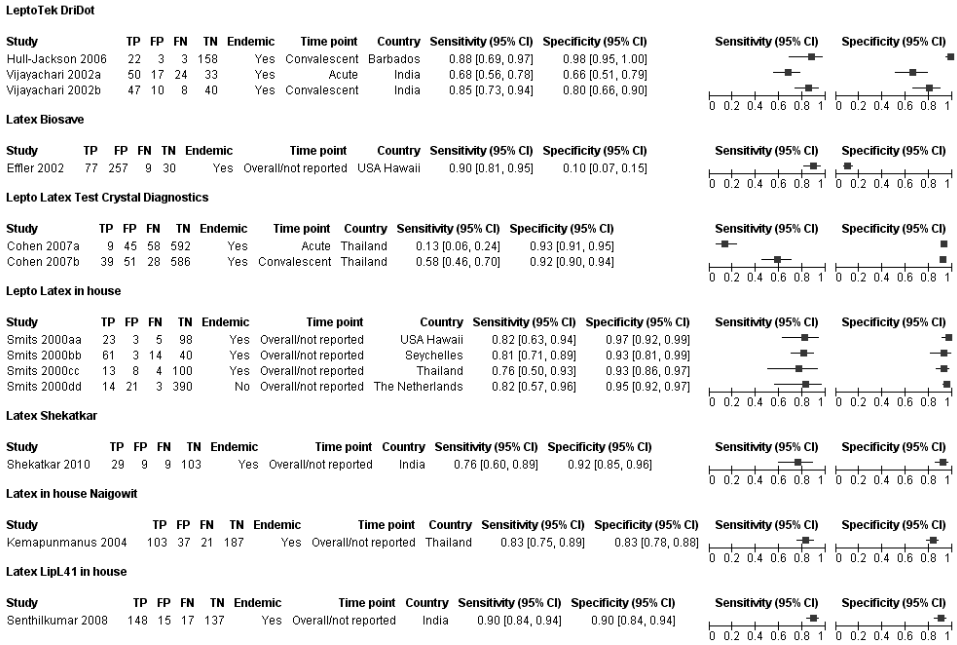


Figure 8 | Forest plot of tests: LeptoTek DriDot, Latex Biosave, Lepto Latex Test Crystal Diagnostics, Lepto Latex in house, Latex Shekatkar, Latex in house Naigowit, Latex LipL41 in house.

Indirect Haemagglutination Assay (IHA, 5 studies)

Data summary

Only five studies evaluated the IHA test from MRL (Table 9). Two of these studies were conducted in Barbados (both by the same author), two in Hawaii (both by the same author) and one in the Seychelles. None of the studies reported whether the study was conducted during the acute or convalescent or any phase of the disease. A forest plot of the available sensitivity and specificity is given in Figure 9.

The overall accuracy was high for the IHA tests: DOR 156 (95% CI 19.2 to 1270), which coincided with a specificity of 97.3% (95% CI 87.2% to 99.5%) and a sensitivity of 81.0% (95% CI 20.3% to 98.6%). The broad confidence interval for sensitivity reflects the HSROC plot (Figure 10). The sensitivity of the studies varies too much to assume that the average represented above is robust/accurate of IHA tests.

Sensitivity analyses for quality effects

This is not conducted, as there were only 5 studies available.

Table 8 | Signalling questions and overall scores Latex tests

				estimate	lower limit	upper limit
All studies for Latex tests N=11			DOR	38.9	13.9	109
			sens	0.821	0.752	0.874
			spec	0.894	0.743	0.961
Was a consecutive or random sample of patients enrolled?	10 yes; 1 unclear	not analyzed				
Was a case-control design avoided?	no case-control studies	not analyzed				
Did the study avoid inappropriate exclusions?	All studies avoided inappropriate exclusions	not analyzed				
Could the selection of patients have introduced bias?	1 high risk; 9 low risk; 1 unclear	not analyzed				
Are there concerns that the included patients and setting do not match the review question?	all low concern	not analyzed				
Were the index test results interpreted without knowledge of the results of the reference standard?	9 unclear; 2 yes	not analyzed				
If a threshold was used, was it pre-specified?	all yes except one (unclear)	not analyzed				
Could the conduct or interpretation of the index test have introduced bias?	2 low risk, 9 unclear	not analyzed				
Are there concerns that the index test, its conduct, or interpretation differ from the review question?	7 high concern; 4 low concern					
Is the reference standard likely to correctly classify the target condition?	3 unclear; 8 yes	analyzed with RoB				
Were the reference standard results interpreted without knowledge of the results of the index tests?	9 unclear; 2 yes	analyzed with RoB				

Table 8 | Continued

				estimate	lower limit	upper limit
Could the reference standard, its conduct, or its interpretation have introduced bias?	4 high risk; 7 low risk	does removing high risk studies change results?	DOR sens spec	26.0 0.798 0.868	5.50 0.674 0.533	123 0.882 0.974
Are there concerns that the target condition as defined by the reference standard does not match the question?	2 unclear concern; 9 low concern	not analyzed				
Was there an appropriate interval between index test and reference standard?	all appropriate interval	not analyzed				
Did all patients receive the same reference standard?	all avoided verification bias	not analyzed				
Were all patients included in the analysis?	9 explained missings; 2 did not	not analyzed				
Could the patient flow have introduced bias?	1 high risk	not analyzed				
Are both single samples and paired samples included?	9 included paired samples; 1 did not; 1 unclear	not analyzed				

Table 9 | Publications Meta-Analysis IHA

	study ID	Test	timepoint	Country
1	Levett 1998	IHA MRL	overall/not reported	Barbados
2	Levett 2001	IHA MRL	overall/not reported	Barbados
3	Yersin 1998	IHA MRL	overall/not reported	Seychelles
4	Effler 2000	IHA MRL	overall/not reported	USA Hawaii
5	Effler 2002	IHA MRL	overall/not reported	USA Hawaii

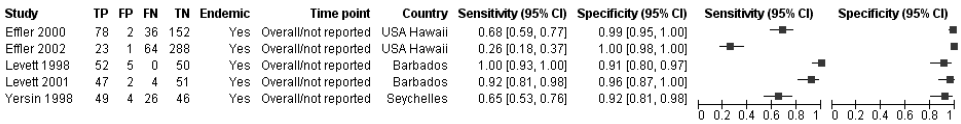


Figure 9 | Forest plot of IHA MRL

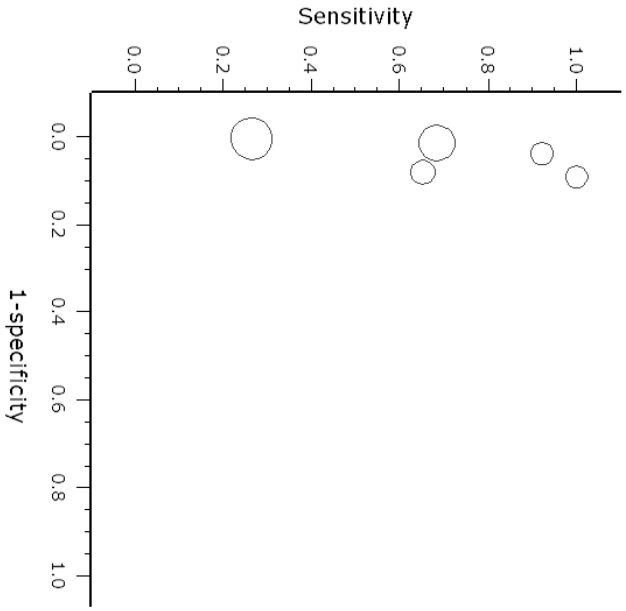


Figure 10 | HSROC plot IHA

Other tests, not included in the meta analysis for each test separately (17 studies)
A forest plot of the available sensitivity and specificity of these tests is given in Figure 11.

IgG ELISA (2 studies)
Two studies evaluated the IgG ELISA. *Leptospira* Microwell IgG ELISA from IVD was evaluated in India (Swapna 2006). This study included 200 participants of various risk groups to assess the seroprevalence. The sensitivity was 61% and the specificity 39%. An in house IgG ELISA was evaluated on participants suspected to have leptospirosis both at acute and convalescent stage in Argentina (Vanasco 2007a; Vanasco 2007b). At the acute stage, 224 participants were included, the sensitivity was 68% and specificity 96%. At the convalescent stage 261 participants were included; the sensitivity rose to 93% and the specificity to 99%.

Dot ELISA (1 study)
One study evaluated the in house dot ELISA (Sharma 2007). This study included 40 participants from a suspected leptospirosis outbreak. The sensitivity was 91% and the specificity 100%.

Lateral Flow (2 studies).
The lateral flow test was evaluated in 2 studies. In Laos 186 participants with unexplained fever in the acute stage were tested with LeptoTek Lateral Flow from Organon Teknika (Blacksell 2006a). The sensitivity was 48%, the specificity was 75%. An in house lateral flow

test was evaluated in India, in both the acute and convalescent stage (Sehgal 2003a; Sehgal 2003b). In the acute stage 117 participants with suspected leptospirosis were included, the sensitivity was 53% and specificity 94%. In the convalescent stage 104 participants were included, the sensitivity rose to 86%, the specificity declined to 89%.

Flow through assay (1 study)
One study evaluated an in house Flow through assay in India (Senthilkumar 2008) on 117 human serum samples clinically suspected for leptospirosis . The sensitivity was 89% and the specificity 77%. The timepoint was not reported.

Indirect Fluorescent Antibody assay (3 studies)
The IFA was evaluated in 3 studies. The IFA from Biognost was evaluated in Hawaii, USA (Effler 2002). This was a prospective study with consecutive enrolment of 379 participants with the overall results reported of acute and convalescent samples. The sensitivity was 38% and the specificity 85%. In 2 studies an in house IFA was evaluated. In Thailand 175 participants were included, tested at the acute and convalescent stage. In the acute stage the sensitivity was 48% and the specificity 97%. Overall results reported sensitivity 100% while the specificity remained 97% (Appassakij 1995a; Appassakij 1995b). In India 10 participants were included. The sensitivity in the acute stage was 83%, the specificity 50%. In the convalescent stage the sensitivity rose to 100% and the specificity to 75% (Joshi 2002a; Joshi 2002b).

Microcapsule Agglutination Test (3 studies)
The MCAT manufactured by Japan Lyophilization Laboratories was evaluated in 3 studies. In Italy 25 hospital in-patients with a pending clinical diagnosis of leptospirosis were included. The results of the paired samples showed a sensitivity and specificity of both 92% (Cacciapuoti 1993). In China 39 participants with a clinical diagnosis of leptospirosis were tested in the convalescent stage, the sensitivity was 100%, the specificity 0% (Cui 1991). In India 97 participants suspected of leptospirosis were tested in the acute stage, the sensitivity was 75%, the specificity 83% (Sehgal 1997).

Microscopic Slide Agglutination Test (2 studies)
The MSAT TR *Leptospira* antigen from Institut Pasteur was evaluated in 2 studies, both did not report the time point. In Spain 45 febrile participants working in a high prevalence area of leptospirosis were included. The sensitivity was 50% and specificity 97% (Marin-Leon 1997). The study performed in Germany included 110 suspected leptospirosis participants, the sensitivity was 54% and the specificity 78% (Weber 1984). An in house MSAT (Andreescu 1990) was evaluated in Romania. The timepoint was not reported. Probably 830 participants with suspected leptospirosis were included, the sensitivity was 95% and the specificity 88%.

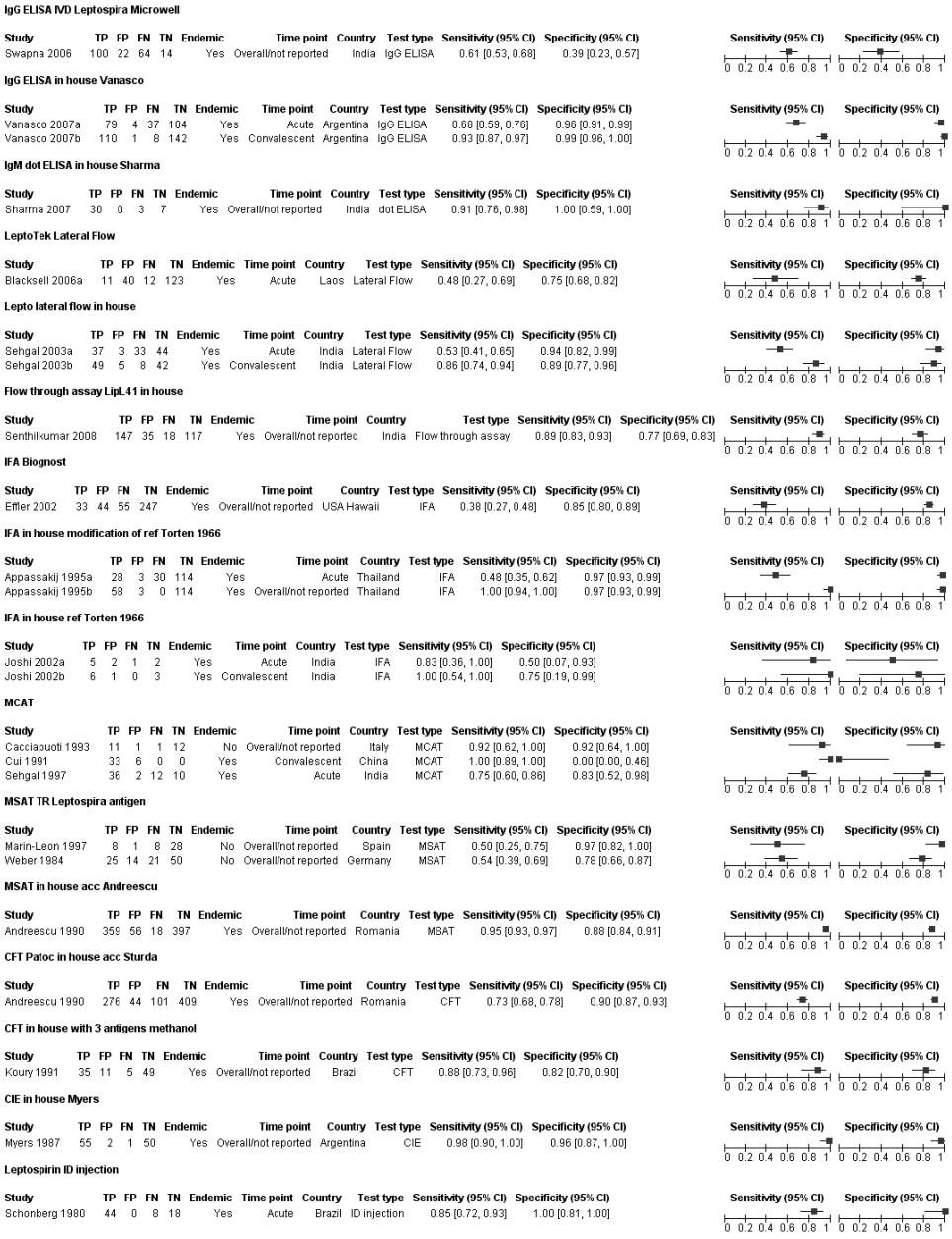


Figure 11 | Forest plot of tests: IgG ELISA IVD *Leptospira* Microwell, IgG ELISA in house Vanasco, IgM dot ELISA in house Sharma, LeptoTek Lateral Flow, Lepto lateral flow in house, Flow through assay LipL41 in house, IFA Biognost, IFA in house modification of ref Torten 1966, IFA in house ref Torten 1966, MCAT, MSAT TR *Leptospira* antigen, MSAT in house acc Andreescu, CFT Patoc in house acc Sturda, CFT in house with 3 antigens methanol, CIE in house Myers, Leptospirin ID injection.

Complement Fixation Test (2 studies)
CFT was evaluated in 2 studies. In both in-house tests were used and the timepoint not reported. In Romania probably 830 participants with suspected leptospirosis were included. The sensitivity was 73%, the specificity 90% (Andreescu 1990). In Brazil 100 participants with suspected leptospirosis during an outbreak were included, the sensitivity was 88%, the specificity 82% (Koury 1991).

Counter Immunoelectrophoresis (1 study)
One in house CIE was evaluated in Argentina with 108 participants with suspected leptospirosis included. The timepoint was not reported, the sensitivity was 98% and the specificity 96% (Myers 1987).

Intra Dermal injection (1 study)
Id injection was performed in 1 study in Brazil. At the acute timepoint, 70 participants clinically suspected of leptospirosis were included. The sensitivity was 85% and the specificity 100% (Schonberg 1980).

DISCUSSION

Summary of main results
The main aim of this systematic review was to better understand the role of available serological diagnostic tests for leptospirosis compared to MAT and/or culturing as the reference standard.

Of the 53 studies from 39 publications, data from 40 studies were included in a meta-analysis. The results of the meta-analyses were based on four test types i.e. dipstick, IgM ELISA, latex test and IHA (Summary of findings; Table 10). The number of studies addressing ten other test types (17 studies in total) was too low to allow meta-analysis (Table 2).

Of the tests reviewed in the meta-analysis, comparisons across tests can only be done in the convalescent time period as most tests do not have data available for the acute time period. At non-acute time points (i.e. at convalescent phase or at any time point from symptoms to sample collection for diagnosis), dipstick, IgM ELISA and latex tests did perform similar with no significant difference in the performance. At these time points the dipstick had the highest sensitivity and specificity (86.0% (95% CI 77.8% to 91.5%) respectively 91.1% (95% CI 88.0% to 93.5%). Latex tests showed a sensitivity of 82.1% (95% CI 75.2% to 87.4%) and specificity of 89.4% (95% CI 74.3% to 96.1%) and IgM ELISA showed a sensitivity of 80.6% (95% CI 69.9% to 88.2%) and specificity of 89.8% (95% CI 84.8% to 93.4%). The overall accuracy was high for the IHA tests with a sensitivity of 81.0% (95% CI 20.3% to 98.6%) and corresponding specificity of 97.3% (95% CI 87.2% to 99.5%), but presents with large confidence intervals.

In a hypothetical cohort of 1000 patients with a prevalence of 23.2% there will be 232 patients with leptospirosis. Latex tests will miss 28 to 54 patients, dipstick will miss 13 to 86 patients and IgM ELISA will miss 22 to 98 patients. Out of this cohort, 768 patients will not have leptospirosis. However the latex tests will have 39 to 127 false positives, dipstick 35 to 114 false positives and IgM ELISA 52 to 124 false positives. The IHA showed too much variation in the sensitivity (95% CI 20-99) to conclude to a solid 'average' sensitivity. In summary, a negative test result in one of the above serological tests does not rule out leptospirosis. This is especially true in the acute phase.

Ten studies evaluated serological tests at both the acute and convalescent stages of the disease; the summary DOR at the convalescent stage was significantly higher compared to the acute stage (p=0.022). The DOR in the acute stage was 13.1 (95% CI 4.20 to 40.6), coinciding with a sensitivity of 58% (95% CI 43%-71%) and a specificity of 91% (95% CI 81%-96%). In the convalescent phase the DOR (75.7 (95% CI 23.7 to 241)) coincided with a sensitivity of 88% (95% CI 79%-94%) and again a specificity of 91% (95% CI 81%-96%). This is as expected based on the humoral response which takes time to develop upon infection and gradually increases[9].

Leptospirosis is more prevalent in warm humid (sub)tropical areas. Since the majority of the studies were performed in high incidence areas the differences in diagnostic accuracy stratified by incidence could not be investigated. No other covariates or quality played a significant role in the accuracy of the test, although in many cases these were difficult to review due to small numbers of studies.

Strengths and weaknesses of the review

Strengths and weaknesses of included studies

Strengths

The used reference standard of the studies included in this review was the MAT and/or culture. These are internationally recognized as the reference standard for the diagnosis of leptospirosis[8]. The investigated patient population consisted of participants who were clinically suspected of having leptospirosis and therefore were representative of patients who will receive these tests in practice; the index tests were not performed on healthy persons since two-gate case control studies were excluded, except for one publication which used the index test for seroprevalence in a survey among risk groups.

Leptospirosis is often overlooked since it mimics many other febrile diseases and laboratory tests are therefore the basis of a confirmed case of leptospirosis, most relevant in high incidence areas. A substantial proportion of the studies included data from such areas.

Table 10 | Summary of findings

Index test type	Time point	Studies (n)	DOR (95% CI)	p	Sens % (95% CI)	Spec % (95% CI)	Comments			
All	all	53			82.7% (IQR 63.1%-90.6%)	90.1% (IQR 83.6-95.7%)	IQR is given here, since this is not based on meta data			
All*	acute	10	13.1 (4.20 - 40.6)	0.022	57.5 (42.7 -71.1)	90.6 (80.5 - 95.8)				
All*	convalescent	10	75.7 (23.7 - 241)		88.4 (79.4 - 93.8)	90.9 (81.4 - 95.8)				
							Median prevalence in all studies is 45.4 (IQR 23.2-59.3). Below are examples of the implications of the DOR. The lower prevalence of 23.2% is used since this might better reflect the population of suspected patients seen by clinicians. If prevalence 23.2% then 232 out of 1000 patients will have leptospirosis			
Latex	convalescent, overall or not reported	11	38.9 (13.9 - 109)		82.1 (75.2 - 87.4).	89.4 (74.3 - 96.1)	Sensitivity IQR	Specificity IQR	FN	FP
							88.0	83.5	28	127
							76.5	94.9	54	39
Dipstick	convalescent, overall or not reported**	22	62.9 (35.3-112)		86.0 (77.8-91.5)	91.1 (88.0 - 93.5)	Sensitivity IQR	Specificity IQR	FN	FP
							94.5	85.1	13	114
							63.1	95.5	86	35

Table 10 | Continued

Index test type	Time point	Studies (n)	DOR (95% CI)	p	Sens % (95% CI)	Spec % (95% CI)	Comments			
IgM ELISA	acute	7	16.0 (5.73 - 44.6)	0.046	64.7 (51.9 -75.8)	89.7 (77.4-95.7)				
IgM ELISA	convalescent, overall or not reported	12	36.8 (18.1 - 74.7)		80.6 (69.9 - 88.2)	89.8 (84.8-93.4)	Sensitivity IQR	Specificity IQR	FN	FP
							90.6	83.9	22	124
							57.6	93.3	98	52
IHA	overall or not reported	5	156 (19.2 - 1270		81.0 (20.3 - 98.6)	97.3 (87.2 - 99.5)	The broad confidence interval for sensitivity reflects the HSROC plot. The sensitivity of the studies varies too much to assume that the 'average' IHA tests indeed has sensitivity of 81%.			

*randomly 1 test per study, **acute only 3, so therefore not reported

Please note, this table for diagnostic tests can only be safely interpreted together with the original data presented in the main body of this review.

Weaknesses

- In this review, QUADAS 2[13] was used to assess the methodological quality of the included studies. A substantial proportion of the studies were scored 'high risk of bias' in the domains 'patient selections', 'reference standard' and 'flow and timing'. This was caused by the fact that either only single samples or paired samples were included from a suspected patient population. In practice, both single and paired samples should be submitted. A high proportion of the included studies did not report the time point of disease when the sample was taken or were unclear in that respect, therefore it was not possible to assess the diagnostic accuracy stratified by disease stage for each test. The disease stage at which a sample is taken is essential to interpret serological tests' results. Also, a substantial proportion of studies (34/53 (>50%)) scored an unclear risk of bias in the domain 'index test' because it was not reported whether the index test was performed blind. This illustrates the importance of pertaining to the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines[17]. Unfortunately most of the quality aspects could not be included in the meta-analysis due to small numbers of studies for each.
- In the meta-analyses heterogeneity was present, caused by the use of different test brands and in-house made tests. Therefore the summary estimate point might not reflect the real sensitivity and specificity in practice.
- None of the studies mentioned the possibility of batch to batch variation of the used tests[18]. This should be taken into consideration.
- It was not reported whether any of the laboratories performing the studies were accredited according ISO 15189[19], which would have assured the quality of the entire diagnostic process.
- There are concerns regarding the applicability of index tests used in the studies, as often in-house tests were used instead of commercial tests, which reduces the likelihood that these results could be reproduced elsewhere.
- In 8 studies another (extra) test was added to the reference standard.

Strengths and weaknesses of the review

Strengths

This review was performed by a team including a designated international leptospirosis reference laboratory which is knowledgeable of the subject and aware of developments in the field. It is therefore not expected that important studies were missed.

Weaknesses

Not all databases were searched, only PubMed and EMBASE. Studies in English, French, German and Spanish were included, other languages were excluded.

In this review MAT and/or culture were used as reference standard. However, the sensitivity of MAT on a single sample has been reported to be 6% in the acute phase and ≤80% at a

later stage[20,21], and that of culture between 10 and 50%. Therefore, false positive results with the index test might have actually been leptospirosis cases with a negative reference test[20]. Hence, presented specificities should be considered with care, taken into account that these probably present pessimistic values.

Applicability of findings to the review question

The aim of this review was to determine the diagnostic accuracy of alternative serological laboratory tests used to diagnose leptospirosis in patients with compatible clinical symptoms in both acute and convalescent stage of disease with 2 specific goals namely:

(i) Can other serological tests replace MAT and culture, and,

(ii) Can alternative serological tests been used for triage purposes

Unfortunately, available data and study designs do not allow a concrete answer to these questions.

Firstly, in most studies the day post onset of disease at which the samples were collected were not sufficiently specified, notably in the acute phase when humoral responses are most dynamic. Performance of a test on early acute samples (day 1-4) will differ from performance of the same test on late acute samples (day 5-10)[22-24].

Secondly, the low sensitivity of the reference test MAT and culture on single samples and in the acute phase interferes with the assessment of the specificity of alternative tests and hence on their diagnostic accuracy's[20,21]. MAT reaches an appreciably high sensitivity only when performed on paired samples. Only 10 studies presented results of both acute and convalescent stages but comprised six distinct test types. The pooled results of these 10 studies give a sensitivity of 57.5% in the acute phase and 88.4% in the convalescent phase respectively. This compares favorably with MAT performed on these stages which can be as low as 6% and 82 %, respectively[20]. This indicates one or more of these tests might be promising to replace the reference standard. However, data do not allow to identify the specific test(s) that could be used as alternative.

For triage purposes a test preferably has a high sensitivity at an early stage of the disease. Serological tests have a low sensitivity and culture results do not become available within a week, so these tests do not meet these requirements. However for the benefit of the individual acutely ill patient alternative serological tests might be a better option than the reference standard because of the higher sensitivity in the acute phase i.e. 57.5% and 64.7% for pooled alternative tests and for IgM ELISA respectively. As well, due to the low sensitivity, culture and MAT may not always confirm a positive triage test on a single sample. In general diagnostic tests based on serology and culture are valuable for confirmation afterwards. They might contribute to limiting over- and mistreatment with antibiotics but for early clinical decision taking their value is limited.

AUTHORS' CONCLUSIONS

Implications for practice

- All included studies used a reference test based on MAT and/or culture; therefore in the same geographical region both the reference tests and the index tests are performed on patients with clinical symptoms for leptospirosis. The diagnostic accuracy might differ when the index test is applied on patients without specific symptoms or other symptoms, or on a patient population from another geographical region. So therefore diagnostic accuracy of tests is particularly unknown in regions where leptospirosis has not been investigated. It is strongly recommended to locally validate tests prior to implementation.
- The meta-analysis did not show a significant difference between IgM ELISA, dipstick and latex tests at the non-acute time point while the diagnostic accuracy of IHA showed a range in confidence and cannot be said with certainty how effective it is compared to the other serological tests. In the acute phase of the disease the DOR coincided with a sensitivity of 58% (95% CI 43%-71%) and in the convalescent phase with a sensitivity of 88% (95% CI 79%-94%). This means that 42% respectively 12% of the patients with culture or MAT positive samples will be missed with these serological tests. At both stages the DOR coincided with a specificity of 91% (95% CI 81%-96%), which means that 9% of the samples that were negative on culture and/or MAT will result in a positive index serological test.
- As the more severely ill patients will be treated with antibiotics anyway, false positive results and false negative results will have mainly consequences for the notification of leptospirosis, where these numbers may lead to an underestimation of the total burden of disease in high prevalence countries (many diseased that will end up negative on serological testing) and to an overestimation in areas with a very low prevalence (here, the lower sensitivity will not weigh up against the 9% non-diseased that will be falsely positive). Other diseases with other possible diagnosis may be missed due to false positive results. Notification of leptospirosis is important, to inform public health professionals in order to avoid further possible outbreaks.
- In any case, follow up samples are important since the sensitivity is low in the acute phase and a negative test does not rule out leptospirosis. It is also important to relate the outcome of a test to the days post onset of disease.
- A perfect index test would be useful for the individual patient. However, from the public health perspective both MAT and culture will still be needed to get insight in the circulating serogroups and serovars in a certain area. When these can be linked to an animal reservoir, preventive measures to reduce leptospiral infections can be considered.
- The disadvantage of serology is that it will become positive in the convalescent phase. So it has a limited value for acute ill patients and therefore complementary tests based on antigen detection such as PCR should be considered for early diagnosis.
- Not all commercial index tests are currently available.

Implications for research

- In this review a high proportion of the studies did not report the time point when the sample was taken. For interpretation of the diagnostic accuracy the time point is essential and in future studies especially this should be reported. Authors are therefore encouraged to use the STARD guidelines in reporting the design and conduct of their studies.
- Additional research is needed in developing tests which have a higher diagnostic accuracy in the acute phase but also in the convalescent phase since the meta-analysis showed that with the current tests not all patients are detected. Antigen detection tests might play a role as well, since *Leptospira* antigen is present in the blood in the very early stage of disease. Ideally with both antigen and antibody detection techniques each performed at the correct time point, each leptospirosis patient should receive a proper diagnosis.

Acknowledgements

- We thank Dimitrinka Nikolova, Managing Editor, for all her help during the preparation process of the protocol. Agostino Colli and Christian Gluud, Cochrane Hepato-Biliary Group Editors, are also thanked for comments on the protocol.
- The Cochrane Hepato-Biliary Group Diagnostic Test Accuracy Reviews Editorial Team
- Peer Reviewers: Michael Sørensen, Danmark; Pascal Lapierre, Canada.
- Contact Editors: Dario Conte, Italy; Mirella Fraquelli, Italy.
- The Cochrane Diagnostic Test Accuracy Reviews Editorial Team
- Contact Editor: Prof Gianni Virgili, Italy.

REFERENCES

References to studies included in this review

Andreescu 1990
Andreescu N. A new prepatory method of thermically inactivated *Leptospira* Patoc antigen for rapid slide agglutination used as serosurvey test for human

Appassakij 1995 a,b
Appassakij H, Silpapojakul K, Wansit R, Woodtayakorn J. Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis. Am.J.Trop.Med.Hyg.1995;52:340–3.

Blacksell 2006 a,b
Blacksell SD, Smythe L, Phetsouvanh R, Dohnt M, Hartskeerl R, Symonds M, *et al*. Limited diagnostic capacities of two commercial assays for the detection of *Leptospira* immunoglobulin M antibodies in Laos. Clin.Vaccine Immunol. 2006;13:1166–9.

Cacciapuoti 1993
Cacciapuoti B, Ciceroni L, Arimitsu Y, Sato T, Seki M. Evaluation of a passive microcapsule agglutination test for the screening of human leptospirosis. Eur.J.Epidemiol. 1993;9:92–6.

Cohen 2007 a,b
Cohen AL, Dowell SF, Nisalak A, Mammen MP Jr, Petkanchanapong W, Fisk TL. Rapid diagnostic tests for dengue and leptospirosis: antibody detection is insensitive at presentation. Trop.Med.Int.Health 2007;12:47–51.

Cui 1991
Cui JJ, Xiao GX, Chen TZ, Zhu GF, Sato T, Seki M, *et al*. Further evaluation of one-point microcapsule agglutination test for diagnosis of leptospirosis. Epidemiol.Infect. 1991;106:561–5.

Cumberland 1999 a,b
Cumberland P, Everard CO, Levett PN. Assessment of the efficacy of an IgM-elisa and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. Am.J.Trop.Med.Hyg. 1999;61:731–4.

Effler 2000
Effler PV, Domen HY, Bragg SL, Aye T, Sasaki DM. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis in Hawaii. J.Clin.Microbiol. 2000;38:1081–4.

Effler 2002
Effler PV, Bogard AK, Domen HY, Katz AR, Higa HY, Sasaki DM. Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. J.Clin.Microbiol. 2002;40:1464–9.

Hull-Jackson 2006
Hull-Jackson C, Glass MB, Ari MD, Bragg SL, Branch SL, Whittington CU, *et al*. Evaluation of a commercial latex agglutination assay for serological diagnosis of leptospirosis. J.Clin.Microbiol. 2006;44:1853–5.

Joshi 2002 a,b
Joshi S, Bal A, Bharadwaj R, Kumbhar R, Kagal A, Arjunwadkar V. Role of IgM specific indirect immunofluorescence assay in diagnosing an outbreak of leptospirosis. Indian journal of pathology & microbiology 2002;45:75–7.

Kemapunmanus 2004
Kemapunmanus M, Sretrirutchai S, Khuntikij P, Pradutkanchana S, Pradutkanchana J. A prospective evaluation of four immunodiagnotic assays for human leptospirosis. Southeast Asian Journal of Tropical Medicine and Public Health 2004;35:863–7.

Koury 1991
Koury MC, Cisalpino EO, Rangel de HA. The use of methanol extract of *Leptospira interrogans* in complement fixation tests for leptospirosis. Canadian Journal of Microbiology 1991;37:455–8.

Levett 1998
Levett PN, Whittington CU. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis. J.Clin.Microbiol. 1998;36:11–4.

Levett 2001
Levett PN, Branch SL, Whittington CU, Edwards CN, Paxton H. Two methods for rapid serological diagnosis of acute leptospirosis. Clin.Diagn.Lab Immunol. 2001;8:349–51.

Levett 2002 a,b
Levett PN, Branch SL. Evaluation of two enzyme- linked immunosorbent assay methods for detection of immunoglobulin M antibodies in acute leptospirosis. Am.J.Trop.Med.Hyg. 2002;66:745–8.

Marin-Leon 1997
Marin-Leon I, Perez-Lozano MJ, De Villar-Conde E, Dastis-Bendala C, Vargas-Romero J, Pumarola-Sune T. Prospective evaluation of the macroagglutination slide test for *Leptospira*. Serodiagnosis and Immunotherapy in Infectious Disease 1997;8:191–3.

Myers 1987
Myers DM. Serodiagnosis of human leptospirosis by counterimmunoelectrophoresis. J.Clin.Microbiol. 1987;25:897–9.

Natarajaseenivasan 2008
Natarajaseenivasan K, Vijayachari P, Sharma S, Sugunan AP, Selvin J, Sehgal SC. Serodiagnosis of severe leptospirosis: Evaluation of ELISA based on the recombinant OmpL1 or LipL41 antigens of *Leptospira interrogans* serovar autumnalis. Annals of Tropical Medicine and Parasitology 2008;102:699–708.

Ooteman 2006
Ooteman MC, Vago AR, Koury MC. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. J.Microbiol.Methods 2006;65:247–57.

Pol 2009
Pol S, Bharadwaj R. Evaluation of high performance liquid chromatography purified leptospiral antigen for the diagnosis of leptospirosis. Japanese Journal of Infectious Diseases 2009;62:428–31.

Rodriguez 2002
Rodriguez I, Fernandez C, Llerena C, Victoria B, Rodriguez JE, Obregon AM. [Lepto-dipstick: results of its application to the fast diagnosis of human leptospirosis]. Rev.Cubana Med.Trop. 2002;54:44–7.

Schonberg 1980
Schonberg A, Caldas EM, Sampaio MB, Costa E, Plank SJ. Leptospirin - an intradermic test for the diagnosis of leptospirosis. Zentralbl.Bakteriol.A 1980;247:114–23.

Sehgal 1997
Sehgal SC, Vijayachari P, Subramaniam V. Evaluation of *Leptospira* micro capsule agglutination test (MCAT) for serodiagnosis of leptospirosis. Indian J.Med.Res. 1997;106:504–7.

Sehgal 1999 a,b
Sehgal SC, Vijayachari P, Sharma S, Sugunan AP. LEPTO Dipstick: a rapid and simple method for serodiagnosis of acute leptospirosis. Trans.R.Soc.Trop.Med.Hyg. 1999;93:161–4.

Sehgal 2003 a,b
Sehgal SC, Vijayachari P, Sugunan AP, Umapathi T. Field application of Lepto lateral flow for rapid diagnosis of leptospirosis. J.Med.Microbiol. 2003;52:897–901.

Senthilkumar 2008
Senthilkumar T, Subathra M, Phil M, Ramadass P, Ramaswamy V. Rapid serodiagnosis of leptospirosis by latex agglutination test and flow-through assay. Indian J.Med.Microbiol. 2008;26:45–9.

Sharma 2007
Sharma R, Tuteja U, Khushiramani R, Shukla J, Batra HV. Application of rapid dot-ELISA for antibody detection of leptospirosis. J.Med.Microbiol. 2007;56:873–4.

Shekatkar 2010
Shekatkar S, Harish BN, Parija SC. IgM Dot-ELISA assay using prevalent *Leptospira* strain for diagnosis of leptospirosis. International Journal of Collaborative Research on Internal Medicine and Public Health 2010;2:338–46.

Smits 1999 a,b,c,d,e,f,g,h,I,j,k,l
Smits HL, Ananyina YV, Chereshsky A, Dancel L, Lai A Fat, RF, Chee HD, *et al*. International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens. J.Clin.Microbiol. 1999;37:2904–9.

Smits 2000 aa,bb,cc,dd
Smits HL, van der Hoorn MA, Goris MG, Gussenhoven GC, Yersin C, Sasaki DM, *et al*. Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis. J.Clin.Microbiol. 2000;38:1272–5.

Stern 2010
Stern EJ, Galloway R, Shadomy SV, Wannemuehler K, Atrubin D, Blackmore C, *et al*. Outbreak of leptospirosis among adventure race participants in Florida, 2005. Clinical Infectious Diseases 2010;50:843–9.

Swapna 2006
Swapna RN, Tuteja U, Nair L, Sudarsana J. Seroprevalence of leptospirosis in high risk groups in Calicut, North Kerala, India. Indian J.Med.Microbiol. 2006;24:349–52.

Valverde 2007
Valverde J MD, Leon B, Taylor L, Visona K. Development of a Lepto-IgM EIACR test to diagnose leptospirosis disease in Costa Rican patient samples. Investigacion Clinica 2007;48:295–304.

Vanasco 2007 a,b
Vanasco NB, Lottersberger J, Schmeling MF, Gardner IA, Tarabla HD. [Diagnosis of leptospirosis: evaluation of a solid-phase enzyme immunoassay in different stages of the disease]. Rev.Panam.Salud Publica 2007;21:388–95.

Vijayachari 2001
Vijayachari P, Sugunan AP, Umapathi T, Sehgal SC. Evaluation of darkground microscopy as a rapid diagnostic procedure in leptospirosis. Indian J.Med.Res. 2001;114:54–8.

Vijayachari 2002 a,b
Vijayachari P, Sugunan AP, Sehgal SC. Evaluation of Lepto Dri Dot as a rapid test for the diagnosis of leptospirosis. Epidemiol.Infect. 2002;129:617–21.

Weber 1984
Weber A, Brem S. Serologic detection of leptospiral antibodies with a commercially available slide agglutination test. Arztliche Laboratorium 1984;30:361–4.

Yersin 1998
Yersin C, Bovet P, Merien F, Wong T, Panowsky J, Perolat P. Human leptospirosis in the Seychelles (Indian Ocean): a population-based study. Am.J.Trop.Med.Hyg. 1998;59:933–40.

Additional references

1. Farr RW. Leptospirosis. *Clin Infect Dis* 1995; **21**(1): 1-6.
2. Ko AI, Galvao RM, Ribeiro Dourado CM, Johnson WD, Jr., Riley LW. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. *Lancet* 1999; **354**(9181): 820-5.
3. McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. *Curr Opin Infect Dis* 2005; **18**(5): 376-86.
4. Brett-Major DM, Coldren R. Antibiotics for leptospirosis. *Cochrane Database Syst Rev* 2012; **2**: CD008264.
5. Brett-Major DM, Lipnick RJ. Antibiotic prophylaxis for leptospirosis. *Cochrane Database Syst Rev* 2009; (3): CD007342.
6. Slack A. Leptospirosis. *Aust Fam Physician* 2010; **39**(7): 495-8.
7. Gouveia EL, Metcalfe J, de Carvalho AL, *et al*. Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. *Emerg Infect Dis* 2008; **14**(3): 505-8.
8. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control; 2003.
9. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; **14**(2): 296-326.
10. Faine S, Adler B, Bolin CA, Perolat P. *Leptospira* and Leptospirosis. MediSci; 1999.
11. Bossuyt PM, Irwig L, Craig J, Glasziou P. Comparative accuracy: assessing new tests against existing diagnostic pathways. *BMJ* 2006; **332**(7549): 1089-92.
12. Durski KN, Jancloes M, Chowdhary T, Bertherat E. A global, multi-disciplinary, multi-sectorial initiative to combat leptospirosis: Global Leptospirosis Environmental Action Network (GLEAN). *Int J Environ Res Public Health* 2014; **11**(6): 6000-8.
13. Whiting PF, Rutjes AW, Westwood ME, *et al*. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Ann Intern Med* 2011; **155**(8): 529-36.
14. Whiting P, Rutjes AW, Dinnes J, Reitsma JB, Bossuyt PM, Kleijnen J. A systematic review finds that diagnostic reviews fail to incorporate quality despite available tools. *J Clin Epidemiol* 2005; **58**(1): 1-12.
15. Juni P, Witschi A, Bloch R, Egger M. The hazards of scoring the quality of clinical trials for meta-analysis. *JAMA* 1999; **282**(11): 1054-60.

16. Rutjes AW, Reitsma JB, Di Nisio M, Smidt N, van Rijn JC, Bossuyt PM. Evidence of bias and variation in diagnostic accuracy studies. *CMAJ* 2006; **174**(4): 469-76.
17. Bossuyt PM, Reitsma JB, Bruns DE, *et al*. Towards complete and accurate reporting of studies of diagnostic accuracy: The STARD Initiative. *Ann Intern Med* 2003; **138**(1): 40-4.
18. Goris MG, Leeflang MM, Loden M, *et al*. Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis. *PLoS Negl Trop Dis* 2013; **7**(7): e2290.
19. Beastall GH. Adding value to laboratory medicine: a professional responsibility. *Clin Chem Lab Med* 2013; **51**(1): 221-7.
20. Goris MGA, Leeflang MMG, Boer KR, *et al*. Establishment of Valid Laboratory Cases Definition of Human Leptospirosis. *Journal of Bacteriology and Parasitology* 2011; **S5 001**: 1-8.
21. Limmathurotsakul D, Turner EL, Wuthiekanun V, *et al*. Fool's gold: Why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. *Clin Infect Dis* 2012; **55**(3): 322-31.
22. Gussenhoven GC, van der Hoorn MA, Goris MG, *et al*. LEPTO dipstick, a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human sera. *J Clin Microbiol* 1997; **35**(1): 92-7.
23. McBride AJ, Santos BL, Queiroz A, *et al*. Evaluation of four whole-cell *Leptospira*-based serological tests for diagnosis of urban leptospirosis. *Clin Vaccine Immunol* 2007; **14**(9): 1245-8.
24. Sehgal SC, Vijayachari P, Sharma S, Sugunan AP. LEPTO Dipstick: a rapid and simple method for serodiagnosis of acute leptospirosis. *Trans R Soc Trop Med Hyg* 1999; **93**(2): 161-4.

Appendix 1 | Search strategy

Database: MEDLINE (PubMed)
Time span: 1948 to the date of search
Search strategy:
#1 "Leptospirosis" [Mesh] OR "leptospiraceae"[MeSH] OR leptospirosis [tw] OR leptospir* OR Weil disease [tw] OR Weil's disease [tw] OR stuttgart disease [tw] OR infectious icterus [tw] OR leptospirose [tw] OR canicola [tw] OR leptospires OR leptospiraceae[tw] OR mud fever[tw] OR grippotyphosa OR icterohaemorrhagiae OR hardjo OR icterohaemorrhagica OR spirochetosis[tw]
#2 "Enzyme-Linked Immunosorbent Assay"[Mesh] OR Enzyme-Linked-Immuno-Sorbent-Assay [tw] OR "serologic tests"[TIAB] OR Serodiagnosis[tw] OR EIA[tw] OR immunosorbent assay[tw] OR ELISA*[tiab]
#3 "Microbiological Techniques"[Mesh] OR "isolation and purification"[subheading] OR culture[tw] OR cultured[tw] OR cultures[tw] OR culturing[tw])
#4"microscopy"[MeSH] OR microscopy[tw] OR microscope[tw] OR darkfield[tw] OR dark-field[tw] OR microscopic[tw] OR direct observation[tw] OR phase contrast[tw] OR electron[tw] OR electrons[tw]
#5 "staining and labeling"[Mesh] OR "coloring agents"[Mesh] OR "fluorescent antibody technique"[Mesh] OR "fluorescence"[Mesh] OR "staining and labeling"[TIAB] OR "coloring agents"[Pharmacological Action] OR "fluorescent dyes"[Pharmacological Action] OR Staining[tw] OR Stained[tw] OR Stain OR Stains[tw] OR Counterstain[tw] OR Counterstains[tw] OR silver deposition[tw] OR silverstaining[tw] OR immunofluorescence[tw] OR immuno-fluorescence[tw] OR immunofluorescent[tw] OR immuno-fluorescent[tw] OR fluorescence[tw] OR fluorescent OR fluorochrome[tw] OR immunohistochemistry[tw] OR immuno-histochemistry [tw] OR immunohisto-chemistry[tw] OR IFAT[tw] OR IFA[tw]
#6 "agglutination tests"[MeSH] OR "agglutination"[MeSH] OR agglutination[tw] OR Microscopic-Agglutination[tw] OR MAT OR agglutinating[tw] OR agglutinated[tw]
#7 "counterimmunoelectrophoresis"[MeSH] OR "Epitopes"[Mesh] OR ("antigens"[TIAB] NOT Medline[SB]) OR counter current immuno electrophoresis[tw] OR countercurrent immuno electrophoresis[tw] OR CCIE[tw] OR antigen-binding[tw] OR cross-linking[tw] OR countercurrentimmunoelectrophoresis[tw]
#8Microcapsule agglutination[tw] OR Micro capsule agglutination[tw] OR MCAT[tw]
#9 "polymerase chain reaction"[MeSH] OR DNA[Mesh] OR "indicators and reagents"[MeSH] OR "indicators and reagents"[Pharmacological Action] OR polymerase chain reaction[tw] OR PCR[tw] OR DNA[tw] OR reagents[tw] OR reagent [tw]
#10 Lateral flow[tw] OR flow test[tw] OR lateral test[tw] OR flow tests[tw] OR lateral tests[tw]
#11 latex agglutination
#12 granular[tw] OR agglutination card[tw] OR slide agglutination[tw]OR heat stable antigen[tw] OR derivative[tw] OR slide[tw]) OR macroscopic agglutination[tw] OR MSAT
#13 indirect haemagglutination[tw] OR IHAT[tw] OR IHA test[tw] OR IHA tests[tw] OR haemagglutination[tw] ORhemagglutination[tw]
#14 Complement Fixation Tests[MeSH]

Appendix 1 | Continued

#15 dipstick[tw] OR dipsticks[tw]
#16 rapid tests[tw] OR rapid test[tw]
#17 Screening[tw] OR "diagnosis, differential"[MeSH] OR differential diagnosis[tw] OR "early diagnosis"[MeSH] OR early diagnosis[tw] OR "diagnostic errors"[MeSH] OR "sensitivity and specificity"[MeSH] OR ("sensitivity and specificity"[TIAB] NOT Medline[SB} OR diagnostic errors[tw] OR false negative reactions[tw] OR false positive reactions [tw] OR observer variation[tw] OR sensitivity [tw] OR specificity[tw]
#18 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 OR 9 OR 10 OR 11 OR 12 OR 13 OR 14 OR 15 OR 16 OR 17
#19 1 AND 18
Database: EMBASE (OvidSP)
Time span: 1947 to the date of search
Search strategy:
#1 (exp Leptospirosis/ OR exp leptospiraceae/ OR leptospir*.ti,ab. OR Weil's disease.ti,ab. OR infectious icterus.ti,ab. OR canicola.ti,ab. OR grippotyphosa.ti,ab. OR icterohaemorrhagiae.ti,ab. OR hardjo.ti,ab. OR spirochetosis.ti,ab.) NOT exp animals/ NOT Humans/
#2 Enzyme-Linked Immunosorbent Assay/ OR Enzyme-Linked-Immuno-Sorbent-Assay.ti,ab. OR Enzyme linked immunosorbent assay.ti,ab. OR Serodiagnosis.ti,ab. OR EIA.ti,ab. OR immunosorbent assay.ti,ab. OR ELISA*.ti,ab.
#3culture.ti,ab.
#4exp microscopy/ OR microscop*.ti,ab. OR darkfield.ti,ab. OR dark-field.ti,ab. OR direct observation.ti,ab. OR phase contrast.ti,ab.
#5 exp fluorescent antibody technique/ OR Fluorescence/ OR Staining.ti,ab. OR Stained.ti,ab. OR Stain.ti,ab. OR immunofluorescent.ti,ab. OR fluorescence.ti,ab. OR exp "staining and labeling"/ OR fluorescent.ti.ab. OR IFAT.ti.ab. OR IFA.ti.ab.
#6 exp agglutination tests/ OR Agglutination Test.ti,ab. OR Agglutination Tests.ti,ab. OR MAT.ti,ab. OR agglutinat*.ti,ab.
#7 exp immunoelectrophoresis/ OR immunoelectrophoresis.ti,ab. OR exp Antigens, Bacterial/ OR exp Antigens, Surface/ OR exp Epitopes/
#8 Microcapsule agglutination.ti,ab. OR micro capsule agglutination.ti,ab. OR MCAT.ti,ab.
#9 exp polymerase chain reaction/ OR exp DNA/ OR exp Reagent Kits, Diagnostic/ OR polymerase chain reaction.ti,ab. OR PCR.ti,ab. OR reagents.ti,ab. OR reagent.ti,ab.
#10 Lateral flow.ti,ab.
#11 latex agglutination test.ti,ab.
#12 slide agglutination test.ti,ab. OR macroscopic agglutination.ti,ab.
#13 exp Hemagglutination/ OR Hemagglutination Inhibition Tests/ OR indirect haemagglutination. ti,ab. OR IHA Test.ti,ab.OR haemagglutination.ti,ab. OR indirect hemagglutination.ti,ab. OR hemagglutination.ti,ab.
#14 Complement Fixation Tests/
#15 dipstick.ti,ab. OR dipsticks.ti,ab

Appendix 1 | Continued

#16 rapid tests.ti,ab. OR rapid test.ti,ab.
#17 diagnosis, differential/ OR early diagnosis/ OR exp Diagnostic Errors/ OR Screening.ti,ab. OR differential diagnosis.ti,ab. OR early diagnosis.ti,ab.OR sensitivity.ti,ab. OR specificity.ti,ab.
#18 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 OR 9 OR 10 OR 11 OR 12 OR 13 OR 14 OR 15 OR 16 OR17
#19 1 AND 18

Appendix 2 | QUADAS-2 key

Domain 1. Patient Selection **Risk of Bias:** Could the selection of patients have introduced bias?	
Low	if Q1 Yes and Q2 Yes and Q3 Yes and Q4 Yes
High	if Q1 No and/or Q2 No and/or Q3 No and/or Q4 No
Unclear	if it is not Low or High
Q1. Was a consecutive or random sample of patients enrolled?	
Yes	if a consecutive or random sample of patients was enrolled
No	if a selected group of patients was enrolled
Unclear	if there was insufficient information on enrolment
Q2. Was a case-control design avoided?	
Yes	if it was no case-control study or when it was case-control study in which cases and controls entered the study the same way.
No	if it was a case-control study in which cases and controls entered the study via different routes
Unclear	if there was insufficient information
Q3. Did the study avoid inappropriate exclusions?	
Yes	if there were no inappropriate exclusions
No	if there were inappropriate exclusions
Unclear	if there is insufficient information on exclusions
Q4. Are both single samples and paired samples included?	
Yes	if both single samples and paired samples are included
No	if either only paired or only single samples are included
Unclear	if it not clear whether both single and paired samples are included
Applicability: Are there concerns that the included patients and setting do not match the review question?	
Low	if participants are suspected of leptospirosis and the spectrum of the participants is representative of the participants who will receive the test in practice.
High	if the participants are not suspected of leptospirosis
Unclear	if there is insufficient information on the participants

Appendix 2 | Continued

Domain 2. Index Test **Risk of Bias:** Could the conduct or interpretation of the index test have introduced bias?	
Low	· if Q1 Yes and Q2 Yes · if Q1 No/Unclear and the index test is read by machine (OD) and Q2 Yes
High	· if Q2 No · if Q1 No and Q2 No · if Q1 No and the index test is read by eye and Q2 Yes
Unclear	If Q1 unclear and index test is read by eye
Q1. Were the index test results interpreted without knowledge of the results of the reference standard?	
Yes	if there is a statement that the index test results were interpreted blind to the results of the reference test.
No	if this does not appear to be the case
Unclear	if this information is not reported
Q2. If a threshold was used, was it pre-specified?	
Yes	if the threshold value are pre-specified before start of the study
No	if the threshold values were not pre-specified
Unclear	if there is insufficient information to make a judgment
Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	
Low	if index test is performed according to manufacturer's instructions
High	if the index test is an in-house test
Unclear	if insufficient data are reported
Domain 3. Reference Standard **Risk of Bias:** Could the reference standard, its conduct, or its interpretation have introduced bias?	
Low	if Q1 Yes and Q2 Yes/No/Unclear; we assume that reading of MAT is not influenced by the results of the index test
High	if Q1 No/Unclear and Q2 Yes/No/Unclear
Q1. Is the reference standard likely to correctly classify the target condition?	
Yes	if MAT or MAT in combination with culture is used, AND if paired samples or convalescent samples are included
No	if MAT is not used
Unclear	if · other tests are added · 1 sample per participant is tested with unknown DPO · unclear whether 1 sample or paired samples are tested

Appendix 2 | Continued

Q2. Were the reference standard results interpreted without knowledge of the results of the index tests?	
Yes	if there is a statement that the reference standard results were interpreted blind to the results of the index test.
No	if this does not appear to be the case
Unclear	if this information is not reported
Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	
Low	If MAT is used (antibody detection)
High	If reference standard is based on antigen detection only (culture/PCR). This was not the case for any of the studies
Domain 4. Flow and Timing **Risk of Bias:** Could the patient flow have introduced bias?	
Low	Q1 Yes and Q2 Yes and Q3 Yes
High	Q3 No (we did not have other scenario's)
Q1. Was there an appropriate interval between index test and reference standard?	
Yes	if the index test was performed on the same sample which also underwent reference standard
No	if this was not the case
Unclear	if this information is not reported
Q2. Did all patients receive the same reference standard?	
Yes	if all participants had the same reference standard
No	if this was not the case
Unclear	if this information is not reported
Q3. Were all patients included in the analysis?	
Yes	if all participants entered in the study are included in the analysis
No	if not all participants in the study are included in the analysis
Unclear	if it not clear whether all participants were accounted for

CHAPTER 10

Summarizing discussion

SUMMARIZING DISCUSSION

Leptospirosis is a neglected and underreported disease since it presents with a broad variety of (a)specific clinical symptoms and laboratory confirmation is challenging (Chapter 1). Case detection plays a pivotal role in the adequate treatment of the individual patient as well as in the understanding of the epidemiology and is essential for research on pathogenesis. Poor diagnosis contributes to a vicious circle of under-diagnosis, under-reporting, poor surveillance and unawareness.

Since 1924, KIT has worked continuously on leptospirosis and performs the majority of the human diagnostics for the whole Netherlands' population, which yields comprehensive information. As such KIT has a unique position in the world. In Chapter 2 historical passive surveillance reports of this entire national cohort from 1925 till 2008 were analyzed. While the total incidence showed a small decrease, the number of imported (especially from tropical countries) infections increased from 1% in the 1950's to more than 50% of the cases in 2008. Remarkably, a substantial number (almost 15%) of the infections were attributed to injury such as traffic accidents and concomitant water exposure. Thus far, there is only scarce attention for accidents as a cause of infection. In Germany the number of cases due to accidents was much lower[1]. It is not clear why accidents in the Netherlands are a more dominant cause. This could be because of unawareness of accidents as a cause of leptospirosis. Another plausible reason might be that (traffic) accidents with water contact are more common in the Netherlands because of the abundance of surface waters. The lack of global information on this subject does not allow a straightforward conclusion. Clinicians should be aware that accidents can imply water contact which in turn holds the risk for acquiring leptospirosis. Results presented in Chapter 2 suggest that males are more prone to leptospirosis and to a more severe form of leptospirosis than females. Similar gender–associated differences in incidence and morbidity for leptospirosis have been reported by the group of Jansen *et al.*[2] and are known for several other infectious diseases e.g. tuberculosis and sepsis[3,4]. Gender associated differences in leptospirosis are based on statistical evaluation of patients data but active research focusing on the issue is missing so far. Remarkably, another study of Jansen *et al.* on an outbreak among strawberry pickers in Germany showed that the same proportion of males and females were infected, but unfortunately the authors did not report whether there was a difference in severity of the disease between males and females[5].

The incidence of leptospirosis is associated with environmental temperature[6]. Leptospirosis is more common in (sub)tropical countries than in temperate climates. Hence, one would expect that global warming, which also extends into temperate regions, would cause concomitant increase of leptospirosis cases. However, the historical incidence is trending downwards and effects of global warming were not clear till recently. In 2014, a marked increase of autochthonous infections in the Netherlands have been observed and described in Chapter 3. This observation extended to other countries in Europe, including mainland France (M.

Picardeau, personal communication). Currently an overview is being prepared with seven European countries on the increasing trend of leptospirosis in Europe. In the review period 1925-2008, the main serogroups present in the Netherlands were Icterohaemorrhagiae, Grippotyphosa and Sejroe. In addition to these 'traditional' serogroups we found infections caused by the Sejroe/Hebdomadis/Mini group and Javanica in 2014. This might imply an increase of incidence with a concomitant increase of serogroups, possibly associated with new or other natural hosts. This is consistent with a hypothetical effect of climate warming on the continent, since at a global scale a higher environmental temperature is associated with a higher incidence and a wider variety of serovars/serogroups in a wider range of hosts[7,8]. Further studies are needed to elucidate whether the marked increase of the incidence of autochthonous infections presents an exceptional occurrence or whether it is a trend and has to do with climate changes. Current research and data implies indeed climate effect[9].

The difficulty of diagnosing leptospirosis and as a consequence the neglected nature of leptospirosis has for a long time discouraged studies on the burden of leptospirosis. The Leptospirosis Burden Epidemiology Reference Group (LERG) of the WHO estimated that there are over a million cases globally resulting in almost 60.000 deaths each year and a deduced 2.9 million DALY's[10,11]. These numbers are mainly based on hospital-based surveillance studies and therefore represent the more severe cases and hence underestimate the total number of leptospirosis cases.

Little is known about persistent complaints after acute leptospirosis. It is a common perception that leptospirosis is a self-limiting disease with complete recovery and only leptospiral uveitis was recognized as a sequel[12]. Little attention therefore has been paid to long term effects. In general, long term complaints are not seen by the hospital medical specialists because the patient does not associate these other complaints with the previous acute disease and/or visits general practitioners with generally milder complaints. Hence, clinicians will not be aware of sequels. During the outbreak of Hardjo cases in the 1980's, which are usually relative mild cases, we noticed in several occasions persistent complaints after acute disease and it was decided to further investigate this phenomenon by approaching general practitioners. For this purpose a simple questionnaire was designed and replies were evaluated. Chapter 4 presents the data together with an assessment of the burden of acute disease. It was found that one third of the patients showed persistent complaints, mostly depression compatible symptoms. One fifth of the patients with sequels had complaints for more than two years. To assess whether complaints were due to leptospirosis and not to other common causes, findings were corrected for data on the health status of the general population in the Netherlands which were retrieved from the national Central Bureau for Statistics (CBS). It was concluded that a significant part of sequels could indeed be attributed to leptospirosis, indicating that leptospirosis can cause long-term sequels which can hamper individuals in their daily activities.

Unawareness of leptospirosis leads to missing cases and incomplete reports. Therefore, worldwide not much is known about the consequences for both public and veterinary health, generally referred to as One Health[13]. A comprehensive multidisciplinary study on the epidemiology of the haemorrhagic fevers leptospirosis and dengue in Central America led to strengthening of laboratories in the region. In a short period of time, this resulted in Costa Rica in improved case detections and as a consequence in the identification of three new serovars, i.e. Arenal, Corredores and Costa Rica[14,15]. In Chapter 5 we described the analysis of two of these new serovars and the consequences for the public health. All three, so far unknown serovars, were isolated from severely ill patients and likely did not present single cases but signify the tip of the iceberg. This substantiates the common notion that major proportions of leptospirosis cases in countries with a lacking alertness actually are not recognized and as such contribute to underreporting and further unawareness. Moreover, in Latin America, leptospirosis cases are mostly misdiagnosed as dengue[16] which, in contrast to leptospirosis, cannot be treated with antibiotics. This implies that a large proportion of febrile patients in the region does not receive adequate antibiotic treatment. This potentially contributes to an increased morbidity[17] and worse, in a large proportion (10-30%) may result in an unneeded fatal outcome. This situation in Costa Rica specifically and Latin America in general, is not unique but very common in socio-economical deprived regions in the world where leptospirosis happens to be highly prevalent and which covers over 5 billion of the world population.

Leptospirosis in dogs can present comparable to humans, with a similar spectrum of clinical disease. As with other species, many infections are subclinical[18]. Dogs as an animal reservoir form a hidden public health problem. Studies in Ireland and the USA showed that 7-9% of the dogs shed leptospires in their urine[19,20] and hence can infect each other and their owners. Also in our study presented in Chapter 6, a high proportion of healthy dogs had antibodies to pathogenic serovars, indicating subclinical or unnoticed infections. Horses can suffer from equine recurrent uveitis (ERU); an inflammatory disease of all parts of the uvea caused by persistent intraocular leptospiral infections[21]. With a reported prevalence of around 8%, it is the most common cause for impaired vision and blindness in horses worldwide[22]. In Chapter 6 we showed that almost 80% of healthy horses had antibodies to a variety of leptospiral serovars. Antibodies against serovar Copenhageni were most prevalent in both dogs and horses, indicating that the main reservoir of this serovar, the brown rat, contaminates biotopes of dogs as well as horses which leads to similar levels of exposure.

Leptospirosis has a complex and dynamic epidemiology. More than 250 pathogenic serovars have been identified[23], each adapted to one or more animal hosts. Spatial and temporal variation occurs in the serovar distribution and clinical manifestations, reflecting also in variations of laboratory diagnostic parameters. Therefore, we re-assessed the case definition used in the Netherlands to the current situation, as described in Chapter 7. The availability

of a valid laboratory case definition, which includes the reference standard test (MAT), is a prerequisite to correctly identify cases. Based on leptospirosis patients of whom leptospires were cultured, cut off titers for MAT and IgM-ELISA were determined as 1:160 and 1:80; respectively, with a concordant 100% specificity. As expected [24], serology does not contribute to diagnosis in the early acute phase of disease when the antibodies are below detection level. The MAT, which is considered the gold standard[25], displayed a poor sensitivity, notably at the early acute stage of illness. Moreover, IgM-ELISA not only gave a higher sensitivity, but was also earlier positive in the acute phase of disease and as such represents a test with a better diagnostic value than the reference standard MAT. In our routine practice in the Netherlands however, MAT and IgM-ELISA are performed in parallel and hence provide a mutual quality check on their performance. Moreover, MAT and IgM ELISA provide partly complementary results, increasing the sensitivity of the final diagnostic outcome which becomes particularly evident in the convalescent phase. The most important and most remarkable observation was the marked increase in sensitivity in serological testing of paired samples compared to single samples. Especially during the first 10 days of disease, the sensitivity increased towards 100%. While general recommendations indicate 2 weeks between first and follow up samples[25], the shorter time interval of less than 10 days reveals an important gain of testing of paired samples already in the acute phase. The highest sensitivity of MAT was 82% at days 11-20 post-onset. So, when relying on MAT on single samples alone, two out of 10 patients will be missed. The low sensitivity of MAT is confirmed by another independent study[26], disputing its status as the gold standard.

For the early acute phase when leptospires still are present in blood, leptospiral antigen or nucleic acid detection tests are useful. Culturing takes too long to be beneficial for the individual patient and mainly serves surveillance purposes. For early confirmation of clinically suspected leptospirosis, several validated real time PCR tests are available[27,28]. These have a high diagnostic accuracy, notably in this early acute phase and as such complement serodiagnostic testing in the acute phase of disease. Our case definition analyses was done on samples reflecting the situation in the Netherlands and as such may seem to be of 'confined' applicability. However, globally defined laboratory case definitions are scarce or poorly established. In this regard, this study is of enormous global importance as it serves as an example of a feasible approach to establish the case definition, notably for social-economic deprived regions where leptospirosis is highly prevalent but diagnostic capacities are limited. As indicated above, continuous variation in leptospirosis dynamics as well as dynamics of other infectious diseases imply that determination of the laboratory case definition should be a dynamic process rather than a single exercise. As a typical example supporting this statement, we recently adjusted the cut off value for IgM-ELISA from 1:80, as reported in our laboratory case definition in Chapter 7, to 1:160. This was done as a response on our notification that hantavirus infections, which are increasing in the Netherlands and neighboring countries[29], can cross react with titers up to 1:80 in ELISA (unpublished observation).

This adapted case definition was applied in Chapter 8 where 3 rapid diagnostic tests were prospectively evaluated. Their overall sensitivity did not exceed 80%. As expected, the sensitivity of the first submitted samples was lower compared to the results which included a follow up sample. Indeterminate results were observed with all 3 tests, and prompted us to strongly recommend reporting these as such and subsequently request for a follow up sample as a common practice to limit the reporting of inaccurate results. Observed batch-to-batch variation warranted the need for establishing a proper quality control system by both manufacturer and end-user. Although rapid diagnostic tests are relatively easy to perform, the interpretation has to be done with caution. Diagnostic accuracy not only depends on subjective interpretation and varying quality parameters, but also on local prevalence of leptospirosis and other, cross-reacting diseases[30]. Therefore, prior to local implementation of these tests, it is advisable to validate these with the reference tests. Also in the course of time, regular evaluation and validation is essential due to the above mentioned variations in batches as well as the possibility of emerging new serovars into a geographical area or cross reactions with (upcoming) other diseases.

The Cochrane systematic review of serological tests described in Chapter 9 confirms these findings. Alternative serological tests are not sufficiently sensitive to replace the reference standard or to serve as a triage test. However, in the early phase of disease meta-analysis of the alternative serological tests showed a higher sensitivity compared to MAT. Since the MAT is a complicated test, these alternative serological tests can be used for diagnosis, provided that one is aware of their limitations. Again, local validation is important, especially when a test is newly introduced into a geographical region to be used to test a suspected population for leptospirosis.

FUTURE DIRECTIONS

Nowadays techniques such as whole genome sequencing (WGS) using next Generation Sequencers (NGS) and molecular typing arrays are becoming available and affordable. These have the potential to detect and characterize the bacterium directly in clinical samples and link these to the possible reservoir, since leptospiral DNA might also allow typing to serovar level using *rfb* loci. This emerging technology thus has consequences for the diagnostics and the typing of leptospires. Moreover, in principle NGS allows the detection of many pathogens in a single test on a patient's specimen. In potential, this enables the transfer from a single disease diagnosis and surveillance towards a multiple (syndromic) disease approach.

NGS and meta-genomic approaches do have limitations. To decide on the application of this technology it is imperative to take into account the stage of the disease since DNA or RNA is present in the blood only in the acute stage of disease with often a shorter time window as for leptospirosis. Therefore, serological diagnostic techniques will still be needed

for patients tested at a later stage of disease. Compared with the conventional culturing and serological typing techniques, WGS and molecular typing arrays are expected to yield faster and high throughput results not only of patients' specimens, but particularly of reservoir and environmental samples and therefore contribute to a better understanding of the epidemiology. This may lead to better awareness and a rationale for preventive measures. Further research is needed to explore, validate and implement the possibilities and added value of WGS in all aspects of leptospirosis.

A case definition adapted to each geographical area instead of global criteria, will improve the laboratory diagnosis for the individual patient and give a better insight in incidence and prevalence. This case definition is needed to assess the diagnostic accuracy of the available diagnostic tests as well as newly developed tests, whether they can replace the conventional tests. In situations where implementation of laboratory confirmation remains problematic, the design of tailor-made clinical algorithms, taking into account 'similar' other locally prevalent infectious diseases, is highly recommended.

REFERENCES

1. Jansen A, Schoneberg I, Frank C, Alpers K, Schneider T, Stark K. Leptospirosis in Germany, 1962-2003. *Emerg Infect Dis* 2005; **11**(7): 1048-54.
2. Jansen A, Stark K, Schneider T, Schoneberg I. Sex differences in clinical leptospirosis in Germany: 1997-2005. *Clin Infect Dis* 2007; **44**(9): e69-e72.
3. Davila S, Hibberd ML, Hari Dass R, *et al*. Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. *PLoS Genet* 2008; **4**(10): e1000218.
4. Hubacek JA, Stuber F, Frohlich D, *et al*. Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide binding protein in sepsis patients: gender-specific genetic predisposition to sepsis. *Crit Care Med* 2001; **29**(3): 557-61.
5. Desai S, van Treeck U, Lierz M, *et al*. Resurgence of field fever in a temperate country: an epidemic of leptospirosis among seasonal strawberry harvesters in Germany in 2007. *Clin Infect Dis* 2009; **48**(6): 691-7.
6. Andre-Fontaine G, Aviat F, Thorin C. Waterborne Leptospirosis: Survival and Preservation of the Virulence of Pathogenic *Leptospira* spp. in Fresh Water. *Curr Microbiol* 2015; **71**(1): 136-42.
7. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS. Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int J Syst Bacteriol* 1999; **49 Pt 2**: 839-58.
8. Nalam K, Ahmed A, Devi SM, *et al*. Genetic affinities within a large global collection of pathogenic *Leptospira*: implications for strain identification and molecular epidemiology. *PLoS One* 2010; **5**(8): e12637.
9. Rood E, Goris MGA, Picardeau M, Bakker M, Hartskeerl RA. Leptospirosis trends and climate induced outbreaks in the Netherlands: an emerging public health threat in Western Europe? 9th European Congress on Tropical Medicine and International Health; 2015; Basel, Switzerland; 2015.
10. Costa F, Hagan JE, Calcagno J, *et al*. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Negl Trop Dis* 2015; **9**(9): e0003898.
11. Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLoS Negl Trop Dis* 2015; **9**(9): e0003843.
12. Hartskeerl RA, Wagenaar JFP. Leptospirosis. In: Kasper DL, Hauser SL, Jameson JL, Fauci AS, Longo DL, Loscalzo J, eds. Harrison's principles of internal medicine. 19 ed; 2015.
13. Halliday JE, Allan KJ, Ekwem D, Cleaveland S, Kazwala RR, Crump JA. Endemic zoonoses in the tropics: a public health problem hiding in plain sight. *Vet Rec* 2015; **176**(9): 220-5.
14. Valverde M de L, Ramirez JM, Montes de Oca LG, Goris MGA, Ahmed N, Hartskeerl RA. Arenal, a new *Leptospira* serovar of serogroup Javanica, isolated from a patient in Costa Rica. *Infect Genet Evol* 2008; **8**(5): 529-33.
15. Valverde M de L, Goris MGA, Gonzalez V, *et al*. New serovars of *Leptospira* isolated from patients in Costa Rica: implications for public health. *J Med Microbiol* 2013; **62**(Pt 9): 1263-71.

16. Flannery B, Pereira MM, Velloso LdF, *et al*. Referral pattern of leptospirosis cases during a large urban epidemic of dengue. *Am J Trop Med Hyg* 2001; **65**(5): 657-63.
17. Watt G, Padre LP, Tuazon ML, *et al*. Placebo-controlled trial of intravenous penicillin for severe and late leptospirosis. *Lancet* 1988; **1**(8583): 433-5.
18. Ellis WA. Animal leptospirosis. *Curr Top Microbiol Immunol* 2015; **387**: 99-137.
19. Rojas P, Monahan AM, Schuller S, Miller IS, Markey BK, Nally JE. Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. *Eur J Clin Microbiol Infect Dis* 2010; **29**(10): 1305-9.
20. Harkin KR, Roshto YM, Sullivan JT, Purvis TJ, Chengappa MM. Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. *J Am Vet Med Assoc* 2003; **222**(9): 1230-3.
21. Wollanke B, Rohrbach BW, Gerhards H. Serum and vitreous humor antibody titers in and isolation of *Leptospira interrogans* from horses with recurrent uveitis. *J Am Vet Med Assoc* 2001; **219**(6): 795-800.
22. Hartskeerl RA, Goris MGA, Brem S, *et al*. Classification of *Leptospira* from the eyes of horses suffering from recurrent uveitis. *J Vet Med B Infect Dis Vet Public Health* 2004; **51**(3): 110-5.
23. Adler B, de la Pena MA. *Leptospira* and leptospirosis. *Vet Microbiol* 2010; **140**(3-4): 287-96.
24. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; **14**(2): 296-326.
25. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control; 2003.
26. Limmathurotsakul D, Turner EL, Wuthiekanun V, *et al*. Fool's gold: Why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. *Clin Infect Dis* 2012; **55**(3): 322-31.
27. Ahmed A, Engelberts MF, Boer KR, Ahmed N, Hartskeerl RA. Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. *PLoS One* 2009; **4**(9): e7093.
28. Slack A, Symonds M, Dohnt M, Harris C, Brookes D, Smythe L. Evaluation of a modified Taqman assay detecting pathogenic *Leptospira* spp. against culture and *Leptospira*-specific IgM enzyme-linked immunosorbent assay in a clinical environment. *Diagn Microbiol Infect Dis* 2007; **57**(4): 361-6.
29. Goeijenbier M, Hartskeerl RA, Reimerink J, *et al*. The hanta hunting study: underdiagnosis of Puumala hantavirus infections in symptomatic non-travelling leptospirosis-suspected patients in the Netherlands, in 2010 and April to November 2011. *Euro Surveill* 2014; **19**(32).
30. Blacksell SD, Smythe L, Phetsouvanh R, *et al*. Limited diagnostic capacities of two commercial assays for the detection of *Leptospira* immunoglobulin M antibodies in Laos. *Clin Vaccine Immunol* 2006; **13**(10): 1166-9.

CHAPTER 11

Nederlandse samenvatting

Dankwoord

Curriculum vitae

PhD Portfolio

NEDERLANDSE SAMENVATTING

Leptospirose: epidemiologie, klinische aspecten en diagnose

Leptospirose is een van de meest wijd verspreide zoönotische ziekten in de wereld. De klinische verschijnselen van de ziekte zijn niet specifiek en komen dus overeen met vele andere ziekten, en kunnen sterk variëren. Typische manifestaties zijn o.a. koorts, spierpijn, nierfunctiestoornissen en geelzucht. Een diagnose kan alleen met zekerheid worden bevestigd door laboratoriumonderzoek. Echter, beschikbare laboratoriumtesten zijn op hun beurt ook lastig en kunnen veelal alleen in gespecialiseerde laboratoria worden uitgevoerd. Dit verklaart dat de ziekte sterk wordt onder-gediagnosticeerd, onder-gerapporteerd en daardoor niet herkend wordt. Leptospirose wordt veroorzaakt door leptospiren, lange en dunne spiraalvormige bacteriën die behoren tot het genus *Leptospira*. Er zijn zowel pathogene leptospiren, die een gastheer nodig hebben om te leven, als niet-pathogene leptospiren die in staat zijn zich in de omgeving te handhaven. In totaal zijn er ongeveer 300 verschillende types, die op basis van serologische kenmerken serovars worden genoemd. Besmettelijke leptospiren leven in het urogenitale stelsel van hun gastheer en worden uitgescheiden met de urine in het milieu. In het milieu kunnen de leptospiren voor een lange tijd overleven, afhankelijk van gunstige, namelijk warme en vochtige, omstandigheden. Infectie van de toevallige gastheer gebeurt door direct contact met het reservoir of indirect door de verontreinigde omgeving. Indirecte transmissie is de belangrijkste route van de infectie; leptospirose is vooral endemisch in (sub) tropische gebieden waar omgevingsomstandigheden gunstig zijn om te overleven. Recente studies geleid door de Wereld Gezondheidsorganisatie schatten dat er wereldwijd meer dan 1.000.000 ernstige gevallen van leptospirose zijn, met ongeveer 60.000 doden per jaar.

Deze en andere epidemiologische, klinische en diagnostische aspecten van leptospirose worden in Hoofdstuk 1 uitgebreid beschreven.

Het doel van dit proefschrift is om (i) de epidemiologische parameters van leptospirose bij mens en dier te onderzoeken, en (ii) de waarde van diagnostische laboratoriumtesten te onderzoeken.

De sectie 'Epidemiologie en klinische aspecten' begint met Hoofdstuk 2, waarin de trends in het voorkomen van leptospirose bij mensen in Nederland in de periode 1925-2008 worden geanalyseerd. Sinds 1924 heeft het Koninklijk Instituut voor de Tropen (KIT), waar dit promotieonderzoek grotendeels werd uitgevoerd, continu gewerkt aan leptospiroseonderzoek en voert het grootste deel van de humane laboratoriumdiagnostiek voor Nederland uit. Resultaten van dit onderzoek in het gehele nationale cohort van 1925 tot 2008 laten een kleine daling zien van de autochtone incidentie, terwijl het aantal geïmporteerde (vooral uit tropische landen) infecties steeg van 1% in 1950 tot meer dan 50%

van de gevallen in 2008. Opvallend is dat een aanzienlijk aantal (bijna 15%) van de infecties kon worden toegeschreven aan ongelukken, zoals verkeersongevallen en gelijktijdige blootstelling aan water. Leptospirose komt meer voor bij mannen dan vrouwen en ook lijken mannen een meer ernstige vorm van de ziekte te krijgen.

In Hoofdstuk 3, wordt de plotselinge toename van leptospirose in 2014 in mensen en ook in honden in Nederland onderzocht. Deze toename is ook geobserveerd in andere Europese landen. Er is ook een grotere variatie in infecterende serogroepen gevonden. Deze bevindingen zijn consistent met een hypothetisch effect van klimaatsopwarming. Verdere studies zijn nodig om te onderzoeken of de sterke toename van het aantal gevallen van autochtone infecties een uitzonderlijke gebeurtenis of een trend weergeeft. Lopend onderzoek en data impliceren inderdaad een klimaateffect.

Om voor de Wereld Gezondheidsorganisatie de wereldwijde ziektelast van leptospirose te kunnen inschatten (in termen van DALY's: verloren gegane gezonde levensjaren), zijn gegevens nodig over duur en ernst van ziekte en restverschijnselen. Er is weinig bekend over de aanhoudende klachten na doorgemaakte leptospirose. Het is een algemene perceptie dat leptospirose spontaan geneest met volledig herstel terwijl slechts uveitis wordt erkend als gevolgschade. In Hoofdstuk 4 worden resultaten gepresenteerd van onderzoek verkregen middels een vragenlijst voorgelegd aan huisartsen; zij zijn degenen die patiënten regelmatig, ook na de ziekte, zien. Om te beoordelen of klachten aan leptospirose te wijten konden zijn en niet aan andere voorkomende oorzaken, zijn bevindingen gecorrigeerd met gegevens over de gezondheidstatus van de algemene bevolking in Nederland die werden verkregen van het Centraal Bureau voor de Statistiek. Het bleek dat een derde van de patiënten aanhoudende klachten vertoonden, vooral met depressie compatibele symptomen. Een vijfde van de patiënten met restklachten had deze langer dan twee jaar. Conclusie van dit onderzoek is dat leptospirose restklachten veroorzaakt, die langdurig kunnen aanhouden en individuen kunnen hinderen in hun dagelijkse activiteiten.

Hoofdstuk 5 beschrijft de typering van twee nieuwe leptospiren serovars van patiënten in Costa Rica en de implicaties van deze bevindingen voor de regionale volksgezondheid. Deze serovars konden worden gevonden dankzij een versterking van lokale laboratoriumcapaciteit en werden geïsoleerd uit ernstig zieke patiënten. Het is waarschijnlijk dat meer gevallen gevonden kunnen worden als beter wordt gezocht en gediagnosticeerd. In deze regio wordt leptospirose veelal verward met dengue, hetgeen adequate behandeling in de weg staat.
In Hoofdstuk 6 wordt onderzoek beschreven naar agglutinerende antistoffen tegen pathogene leptospiren in gezonde honden en paarden om mogelijke blootstelling aan leptospiren en het bestaan van subklinische infecties te verkennen. In honden varieerde de aanwezigheid van antilichamen tegen leptospiren tussen de 32% en 87%, terwijl bij bijna 80% van de gezonde paarden antilichamen tegen verschillende leptospiren serovars kon worden aangetoond.

Antilichamen tegen serovar Copenhageni waren het meest voorkomend in zowel honden en paarden, wat aangeeft dat het belangrijkste reservoir van dit serovar, de bruine rat, de biotopen van honden en paarden besmet.

Leptospirose heeft een complexe en dynamische epidemiologie met vele pathogene serovars, elk aangepast aan een of meer dierlijke gastheren. Ruimtelijke en temporele variatie in de serovar distributie en klinische verschijnselen, wordt ook weerspiegelt in de variaties van de laboratorium diagnostische parameters. De sectie 'Diagnostiek' begint met Hoofdstuk 7, waarin het proces van de formulering van een juiste laboratorium patiëntdefinitie voor menselijke leptospirose is beschreven. Deze definitie, voorwaarde voor een correcte identificatie van leptospirose onder patiënten met symptomen, werd bepaald door gebruik te maken van het Nederlandse cohort van patiënten met een bewezen leptospirose op basis van positieve kweek. Bij deze groep patiënten is de optimale afkapwaarde voor positiviteit van twee serologische testen, de MAT en de ELISA, vastgesteld. De afkapwaarden voor MAT en IgM-ELISA werden bepaald op 1: 160 en 1:80; respectievelijk, met een overeenstemmende 100% specificiteit. Een belangrijke en opmerkelijke waarneming was de sterke toename van de gevoeligheid bij de serologische testen van gepaarde monsters in vergelijking met enkele monsters. Vooral tijdens de eerste 10 dagen van de ziekte kon de gevoeligheid verhoogd worden richting 100%, terwijl er volgens de algemene aanbevelingen 2 weken tussen de afname van het eerste en het opvolgende monster geadviseerd wordt.

Deze laboratorium patiëntdefinitie wordt toegepast in Hoofdstuk 8 waarin drie snelle serologische diagnostische testen voor leptospirose prospectief worden geëvalueerd. De gevoeligheid van deze testen was niet meer dan 80%. Zoals verwacht, was de gevoeligheid van de monsters vroeg in de ziekte lager in vergelijking met de resultaten van vervolgmonsters. Waargenomen "batch-to-batch" variatie rechtvaardigt de noodzaak voor een systeem van adequate kwaliteitscontroles, zowel bij de fabrikant als eindgebruiker. Hoewel de snelle diagnostische tests relatief eenvoudig zijn uit te voeren, moet de (subjectieve) interpretatie behoedzaam gedaan worden. Voordat deze sneltesten lokaal geïmplementeerd kunnen worden, is het raadzaam deze te valideren met referentietesten.

Hoofdstuk 9 presenteert een zgn. Cochrane systematische review en meta-analyse van de diagnostische nauwkeurigheid van de serologische laboratoriumtesten voor de diagnose van leptospirose bij patiënten met klinische symptomen. Serologische testen anders dan de MAT zijn niet gevoelig genoeg om de referentiestandaard van MAT met kweek te vervangen of om te dienen als triagetest. Echter, in de vroege fase van de ziekte kunnen alternatieve serologische testen een hogere gevoeligheid laten zien dan de MAT. Aangezien de MAT een ingewikkelde test is, kunnen deze alternatieve serologische testen worden toegepast voor diagnose, mits men zich bewust is van hun beperkingen. Ook lokale validatie is belangrijk, vooral als de test wordt geïntroduceerd in een andere geografische omgeving met een andere epidemiologie.

Tenslotte wordt in Hoofdstuk 10 de bevindingen van de studies in dit proefschrift bediscussieerd en samengevat met het oog op toekomstig onderzoek op het gebied van leptospirose.

DANKWOORD

Veel mensen hebben meegeholpen om dit proefschrift mogelijk te maken. Hier wil ik iedereen graag voor bedanken: de medeauteurs, de promotoren Eric van Gorp en Paul Klatser, de copromotoren Kimberly Boer en Rudy Hartskeerl en de overige leden van de promotiecommissie.

In het bijzonder wil ik Rudy Hartskeerl bedanken voor zijn inzet, visie, wijsheid en dagelijkse begeleiding. Ook alle (oud)collega's van de leptospirose groep ben ik zeer erkentelijk voor de goede en prettige samenwerking, met name het huidige team in deze intensieve periode: Ahmed Ahmed, Hans van der Linden, Hans de Ronde, Jane de Meza en Jiri Wagenaar.

De afgelopen jaren heb ik met veel plezier gewerkt op KIT Biomedical Research, dank aan alle collega's voor jullie samenwerking, adviezen, hartelijkheid, betrokkenheid en gezelligheid.

Lieve familie en vrienden, jullie durfden er bijna niet meer naar te vragen, maar het boekje is af! Dank voor jullie interesse, aanmoediging en support.

CURRICULUM VITAE

Marga Goris (Loo 1962) graduated from pre-university education in 1981. After finishing higher vocational education in medical laboratory techniques (OLAN, now Hogeschool Arnhem Nijmegen), she worked for five years as laboratory technologist at the clinical laboratory of Rijnstate Hospital in Arnhem. She joined Doctors without Borders (MSF) in 1989 and worked in projects in Pakistan, Sudan, Ethiopia and Kenya. In 1993 she obtained a MSc in Applied Parasitology and Medical Entomology at the Liverpool School of Tropical Medicine. She started working at the Royal Tropical Institute at the department KIT Biomedical Research in 1994, at the Rapid Diagnostic group where she was involved in the development of rapid diagnostic tests for infectious diseases. She moved to the Leptospirosis group and since 2000 she is coordinator of the WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis. The Leptospirosis group is the diagnostic and reference center for leptospirosis in the Netherlands. Furthermore the group performs research ranging from improved diagnostic and leptospires typing tools to epidemiology and pathogenesis and provides courses and tailor-made training. She visited laboratories in Central America to support the correct implementation of standard methods and diagnostic tests for leptospirosis and to assess the quality. In Indonesia she trained staff of the microbiology laboratory of Dr. Kariadi Hospital in Semarang to become a leptospirosis reference laboratory. She was involved in collaborations with the Academic Medical Center in Amsterdam about innate immunity in leptospirosis and with Erasmus Medical Center in Rotterdam about the etiology of rodent borne diseases. During her time at KIT, Marga has voluntarily supported medical programs in South Sudan run by SSMR.

PHD PORTFOLIO

Name PhD student	Marga Goris
Promotors	Prof. dr. E.C.M. van Gorp & Prof. dr. P.R. Klatser
Co-promotors	Dr. R.A. Hartskeerl & Dr. K.R. Boer
Education	1992-1993 Master of Science "Applied parasitology and medical entomology", Liverpool School of Tropical Medicine, United Kingdom

In-depth courses

2002 Epidemiologisch onderzoek: opzet en interpretatie (EMGO)

2003 Clinical Epidemiology, Academic Medical Center, Amsterdam
Practical biostatistics, Academic Medical Center, Amsterdam

2005 Write it right, NWO Talent classes, The Hague
Reference Manager basis, Academic Medical Center, Amsterdam

2008 Developing a Cochrane Systematic review, Cochrane collaboration Amsterdam

2009 Project Management, University of Amsterdam

2010 Scientific writing, Free University
Developing a Cochrane Diagnostic Tests Accuracy Review, Cochrane Collaboration, Amsterdam
Basic Safety and Security Course Light, Centre for Safety and Development

2013 Accreditatie volgens ISO 15189. Wat gaat er veranderen?', NVKC

2014 Short Course on Training of Trainers, KIT, Amsterdam

Presentations

Oral

2007 5th Meeting of the International Leptospirosis Society, Quito, Ecuador
2009 6th Scientific Meeting International Leptospirosis Society "LeptoCON" Cochin, India
2012 European meeting of leptospirosis Eurolepto 2012, Dubrovnik, Croatia
2013 8th Scientific Meeting of the International Leptospirosis Society (ILS), Fukuoka, Japan

2014 3rd International congress about Leptospirosis, Syphillis and Borreliosis, Habana, Cuba
2015 2nd Scientific Meeting on Leptospirosis and other rodent-borne haemorrhagic fevers of the European Leptospirosis Society (ELS2015), Amsterdam (*Key note presentation*)
2015 9th International Leptospirosis Society Scientific Meeting (ILS 2015), Semarang, Indonesia

Poster
2005 4th Scientific Meeting of the International Leptospirosis Society, Chiang Mai, Thailand
2009 6th Scientific Meeting International Leptospirosis Society "LeptoCON" Cochin, India
2011 VII Reunion de la International Leptospirosis Society, Merida, Yucatan, Mexico

Attended conferences and symposia
2002 3rd Scientific Meeting of the International Leptospirosis Society, Bridgetown, Barbados.
2004 Second Scientific Meeting 'Leptospirosis Habana 2004', Cuba
2005 4th Scientific Meeting of the International Leptospirosis Society, Chiang Mai, Thailand
2007 5th Meeting of the International Leptospirosis Society, Quito, Ecuador
2009 6th Scientific Meeting International Leptospirosis Society "LeptoCON", Cochin, India
2011 VII Reunion de la International Leptospirosis Society, Merida, Yucatan, Mexico
2012 Symposium on the occasion of inauguration prof. dr. PR Klatser, Amsterdam
2012 European meeting of leptospirosis Eurolepto 2012, Dubrovnik, Croatia
2013 8th Scientific Meeting of the International Leptospirosis Society (ILS), Fukuoka, Japan
2013 Symposium on the occasion of inauguration prof. dr. ECM van Gorp, Rotterdam
2014 3rd International congress about Leptospirosis, Syphillis and Borreliosis, Habana, Cuba
2015 3rd International One Health Congress, Amsterdam
2015 2nd Scientific Meeting on Leptospirosis and other rodent-borne haemorrhagic fevers of the European Leptospirosis Society (ELS2015), Amsterdam, the Netherlands
2015 9th International Leptospirosis Society Scientific Meeting (ILS 2015), Semarang, Indonesia

Teaching activities

2000-2006	EPI-info course to Msc students, Amsterdam
2000-2006	International course on laboratory methods for the diagnosis of leptospirosis, Amsterdam,
2001-2008	International course on laboratory methods for the diagnosis of leptospirosis, Havana, Cuba
2005	Advanced Course Leptospirosis in Laboratory from Department of Microbiology, Dr. Kariadi Hospital/Faculty of Medicine, Diponegoro University, Semarang, Indonesia
2007-2015	Supervision MSc thesis (PM, MS, TD, DL, VK, EK, RP, BY)
2012-2015	Immunology, theory and practical to Students Jazan University Saudi Arabia. Amsterdam
2013	Antistof, het verborgen goud, serologie cursus VUMC
2014	International workshop at Instituto Pedro Kouri, Havana, Cuba
2015	Expert/Lecturer Technical Cooperation Programme for the Joint FAO/ IAEA Division in Vienna for the training course: “Early Detection of Animal Diseases in Post Flooding Environment, with Emphasis on Water Borne and Vector Borne Diseases”. Teaching subjects: *Leptospira* infections in animals (including the zoonotic context) and diagnostic methods

Miscellaneous

Vice President European Leptospirosis Society
Executive member International Leptospirosis Society
Reviewer PLoS One
Reviewer Journal of Epidemiology and Global Health
Reviewer Asian Pacific Journal of Tropical Medicine
Reviewer American Journal of Tropical Medicine & Hygiene
Reviewer Comparative Immunology, Microbiology & Infectious Diseases
Reviewer The Veterinary Journal
Reviewer Anais da Academia Brasileira de Ciências (Journal of Brazilian Academy of Sciences)
Reviewer PLoS Neglected Tropical Diseases
Reviewer Memórias do Instituto Oswaldo Cruz

Reviewer BMC Infectious Diseases
Reviewer Epidemiology and Infection
Reviewer Emerging Infectious Diseases
Reviewer BMC Veterinary Research
Reviewer The Lancet Infectious Diseases
Reviewer Transboundary and Emerging Diseases
Reviewer Transactions of the Royal Society of Tropical Medicine & Hygiene
Reviewer WHO South-East Asia Journal of Public Health
Reviewer Microbiology
Reviewer BMC Research Notes
Reviewer International Journal of Microbiology
Reviewer Pathogens and Global Health
Reviewer New Microbes and New Infections
Reviewer Veterinary Quarterly

Publications

Suspected leptospiral meningitis in adults: report of four cases and review of the literature
van Samkar A, van de Beek D, Stijnis C, **Goris M**, Brouwer M
The Netherlands Journal of Medicine, December 2015 Volume 73 Number 10

Global burden of leptospirosis: Estimated in terms of Disability Adjusted Life Years
Torgerson P, Hagan J, Costa F, Calcagno J, Kane M, Martinez-Silveira M, **Goris M**, Stein C, Ko A, Abela-Ridder B
PLoS Negl Trop Dis. 2015 Oct 2;9(10):e0004122

Markers of endothelial cell activation and immune activation are increased in patients with severe leptospirosis and associated with disease severity
Goeijenbier M, Gasem M, Meijers J, Hartskeerl R, Ahmed A, **Goris M,** Isbandrio B, Schuller S, Osterhaus A, Martina B, van Gorp E, Nally J, Wagenaar J
J Infect. 2015 Oct;71(4):437-46

Letter to the editor: Trends in human leptospirosis in Denmark, 1980 to 2012
Goris M, Hartskeerl R
Euro Surveill. 2015;20(9)

Leptospirosis in Sub-Saharan Africa: a systematic review
de Vries S, Visser B, Nagel I, **Goris M**, Hartskeerl R, Grobusch M
Int J Infect Dis. 2014 Nov;28:47-64

Preliminary Investigations on the Distribution of *Leptospira* Serovars in Domestic Animals in North-west Morocco
Benkirane A, Noury S, Hartskeerl R, **Goris M**, Ahmed A, Nally J
Transbound Emerg Dis. 2014 Jul 26

Leptospirosis serodiagnosis by the microscopic agglutination test
Goris M, Hartskeerl R
Curr Protoc Microbiol 2014;32:12E.5.1-12E.5.18

The hanta hunting study: underdiagnosis of Puumala hantavirus infections in symptomatic non-travelling leptospirosis-suspected patients in the Netherlands, in 2010 and April to November 2011
Goeijenbier M, Hartskeerl R, Reimerink J, Verner-Carlsson J, Wagenaar J, **Goris M**, Martina B, Lundkvist A, Koopmans M, Osterhaus A, van Gorp E, Reusken C
Euro surveillance 19 (32), 2014

Towards the burden of human leptospirosis: duration of acute illness and occurrence of post-leptospirosis symptoms of patients in the Netherlands
Goris M, Kikken V, Straetemans M, Alba S, Goeijenbier M, van Gorp E, Boer K, Wagenaar J, Hartskeerl R
PLoS One. 2013 Oct 3;8(10):e76549

Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis
Goris M, Leeflang M, Loden M, Wagenaar J, Klatser P, Hartskeerl R, Boer K
PLoS Negl Trop Dis. 2013 Jul 11;7(7):e2290

New serovars of *Leptospira* isolated from patients in Costa Rica: implications for public health
Valverde de M, **Goris M**, González V, Anchia M, Díaz P, Ahmed A, Hartskeerl R
J Med Microbiol. 2013 Sep;62(Pt 9):1263-71

Human leptospirosis trends, the Netherlands, 1925-2008
Goris M, Boer K, Duarte T, Kliffen S, Hartskeerl R
Emerg Infect Dis. 2013 Mar;19(3):371-8

Rodent-borne hemorrhagic fevers: under-recognized, widely spread and preventable-epidemiology, diagnostics and treatment
Goeijenbier M, Wagenaar J, **Goris M**, Martina B, Henttonen H, Vaheri A, Reusken C, Hartskeerl R, Osterhaus A, Van Gorp E
Crit Rev Microbiol. 2012 Jun 7

Establishment of valid laboratory case definition for human leptospirosis.
Goris M, Leeflang M, Boer K, Goeijenbier M, van Gorp E, Wagenaar J, Hartskeerl R
Journal of Bacteriology and Parasitology 2012 3:2

An unusual cause of a usual presentation
Goeijenbier M, Nur E, **Goris M**, Wagenaar J, Grunberg K, Nurmohammed S, Martina BE, Osterhaus AD, van Gorp E
The Netherlands Journal of Medicine 2011 June, vol 69 no 6

Potent innate immune response to pathogenic *Leptospira* in human whole blood
Goris M, Wagenaar J, Hartskeerl R, van Gorp E, Schuller S, Monahan A, Nally J, van der Poll T, van 't Veer C
PLoS One. 2011 Mar 31;6(3):e18279

Agglutinating antibodies against pathogenic *Leptospira* in healthy dogs and horses indicate common exposure and regular occurrence of subclinical infections
Houwers DJ, **Goris M**, Abdoel T, Kas J, Knobbe S, van Dongen A, Westerduin F, Klein W, Hartskeerl R
Vet Microbiol. 2011 Mar 24;148(2-4):449-51

Challenges in determining the pathogenicity status of *Leptospira* isolates with phenotypic methods: The need for a polyvalent approach
Mgode G, Machang'u R, Collares-Pereira M, Vieira M, **Goris M**, Engelberts M, Hartskeerl R
African Journal of Microbiology Research Vol. 4(23), pp. 2528-2533, 4 December, 2010

Coagulation disorders in patients with severe leptospirosis are associated with severe bleeding and mortality
Wagenaar J, **Goris M,** Partiningrum D, Isbandrio B, Hartskeerl R, Brandjes D, Meijers J, Gasem M, van Gorp E
Trop Med Int Health. 2010 Feb;15(2):152-9

Murine typhus and leptospirosis as causes of acute undifferentiated fever, Indonesia
Gasem M, Wagenaar J, **Goris M**, Adi M, Isbandrio B, Hartskeerl R, Rolain J, Raoult D, van Gorp E
Emerg Infect Dis. 2009 Jun;15(6):975-7

Soluble ST2 levels are associated with bleeding in patients with severe Leptospirosis
Wagenaar J, Gasem M, **Goris M**, Leeflang M, Hartskeerl R, van der Poll T, van 't Veer C, van Gorp E
PLoS Negl Trop Dis. 2009 Jun 2;3(6):e453

Long pentraxin PTX3 is associated with mortality and disease severity in severe Leptospirosis
Wagenaar J, **Goris M**, Gasem M, Isbandrio B, Moalli F, Mantovani A, Boer K, Hartskeerl R, Garlanda C, van Gorp E
J Infect. 2009 Jun;58(6):425-32

Arenal, a new *Leptospira* serovar of serogroup Javanica, isolated from a patient in Costa Rica
Valverde de M, Ramírez J, Montes de Oca L, **Goris M**, Ahmed N, Hartskeerl R
Infect Genet Evol. 2008 Sep;8(5):529-33

What role do coagulation disorders play in the pathogenesis of leptospirosis?
Wagenaar J, **Goris M**, Sakundarno M, Gasem M, Mairuhu A, de Kruif M, Ten Cate H, Hartskeerl R, Brandjes D, van Gorp E
Trop Med Int Health. 2007 Jan;12(1):111-22

Seroepidemiology of leptospirosis in southern Vietnamese children
Thai K, Binh T, Giao P, Phuong H, Hung le Q, Van Nam N, Nga T, **Goris M**, de Vries P
Trop Med Int Health. 2006 May;11(5):738-45

An outbreak of leptospirosis in seals (Phoca vitulina) in captivity
Kik M, **Goris M**, Bos J, Hartskeerl R, Dorrestein G
Vet Q. 2006 Mar;28(1):33-9

New *Leptospira* serovar Sokoine of serogroup Icterohaemorrhagiae from cattle in Tanzania
Mgode G, Machang'u R, **Goris M**, Engelberts M, Sondij S, Hartskeerl R
Int J Syst Evol Microbiol. 2006 Mar;56(Pt 3):593-7

Serological and molecular characterization of *Leptospira* serovar Kenya from captive African giant pouched rats (Cricetomys gambianus) from Morogoro Tanzania
Machang'u R, Mgode G, Assenga J, Mhamphi G, Weetjens B, Cox C, Verhagen R, Sondij S, **Goris M**, Hartskeerl R
FEMS Immunol Med Microbiol. 2004 Jun 1;41(2):117-21

Impact of proficiency testing on results of the microscopic agglutination test for diagnosis of leptospirosis
Chappel R, **Goris M**, Palmer M, Hartskeerl R
J Clin Microbiol. 2004 Dec;42(12):5484-8

Machang'u R, Mgode G, Assenga J, Mhamphi G, Weetjens B, Cox C, Verhagen R, Sondij S, **Goris M**, Hartskeerl R
Serological and molecular characterization of *Leptospira* serovar Kenya from captive African giant pouched rats (Cricetomys gambianus) from Morogoro Tanzania
FEMS Immunol Med Microbiol. 2004 Jun 1;41(2):117-21

Classification of *Leptospira* from the eyes of horses suffering from recurrent uveitis.
Hartskeerl R, **Goris M**, Brem S, Meyer P, Kopp H, Gerhards H, Wollanke B.
J Vet Med B Infect Dis Vet Public Health. 2004 Apr;51(3):110-5

Leptospira interrogans serovar Valbuzzi: a cause of severe pulmonary haemorrhages in the Andaman Islands
Vijayachari P, Sehgal S, **Goris M**, Terpstra W, Hartskeerl R
J Med Microbiol. 2003 Oct;52(Pt 10):913-8.

Simple dipstick assay for the detection of *Salmonella typhi*-specific IgM antibodies and the evolution of the immune response in patients with typhoid fever
Hatta M, **Goris M**, Heerkens E, Gooskens J, Smits H
Am J Trop Med Hyg. 2002 Apr;66(4):416-21

Evaluation of a simple and rapid dipstick assay for the diagnosis of typhoid fever in Indonesia
Gasem M, Smits H, **Goris M**, Dolmans W
J Med Microbiol. 2002 Feb;51(2):173-7

Latex based, rapid and easy assay for human leptospirosis in a single test format
Smits H, Chee H, Eapen C, Kuriakose M, Sugathan S, Gasem M, Yersin C, Sakasi D, Lai-A-Fat R, Hartskeerl R, Liesdek B, Abdoel T, **Goris M**, Gussenhoven G
Trop Med Int Health. 2001 Feb;6(2):114-8

Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis
Smits H, van der Hoorn M, **Goris M**, Gussenhoven G, Yersin C, Sasaki D, Terpstra W, Hartskeerl R
J Clin Microbiol. 2000 Mar;38(3):1272-5

Development and evaluation of a rapid dipstick assay for serodiagnosis of acute human brucellosis.
Smits H, Basahi M, Díaz R, Marrodan T, Douglas J, Rocha A, Veerman J, Zheludkov M, Witte O, de Jong J, Gussenhoven G, **Goris M**, van Der Hoorn M
J Clin Microbiol. 1999 Dec;37(12):4179-82

International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens
Smits H, Ananyina Y, Chereshsky A, Dancel L, Lai-A-Fat R, Chee H, Levett P, Masuzawa T, Yanagihara Y, Muthusethupathi M, Sanders E, Sasaki D, Domen H, Yersin C, Aye T, Bragg S, Gussenhoven G, **Goris M**, Terpstra W, Hartskeerl R
J Clin Microbiol. 1999 Sep;37(9):2904-9

LEPTO dipstick, a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human sera
Gussenhoven G, van der Hoorn M, **Goris M**, Terpstra W, Hartskeerl R, Mol B, van Ingen C, Smits H.
J Clin Microbiol. 1997 Jan;35(1):92-7

Leish-KIT, a stable direct agglutination test based on freeze-dried antigen for serodiagnosis of visceral leishmaniasis
Meredith S, Kroon N, Sondorp E, Seaman J, **Goris M**, van Ingen C, Oosting H, Schoone G, Terpstra W, Oskam L
J Clin Microbiol. 1995 Jul;33(7):1742-5

Prevalence and clinical presentation of glucose-6-phosphate dehydrogenase deficiency in Pakistani Pathan and Afghan refugee communities in Pakistan; implications for the use of primaquine in regional malaria control programmes
Bouma M, **Goris M**, Akhtar T, Khan N, Khan N, Kita E
Trans R *Soc Trop Med Hyg. 1995 89: 62-64*

Incrimination of Phlebotomus (Larroussius) orientalis as a vector of visceral leishmaniasis in western Upper Nile Province, southern Sudan
Schorscher J, **Goris M**
Trans R Soc Trop Med Hyg. 1992 Nov-Dec;86(6):622-3