ROYAUME DU MAROC
Université Mohammed V - RABAT -
جامعة محمد الخامس - الرباط -
Faculté des sciences
كلية العلوم

CENTRE D'ETUDES DOCTORALES - SCIENCES ET TECHNOLOGIES

N° d'ordre 3233

THESE

En vue de l'obtention du : ***DOCTORAT***

Centre de Recherche : Biotechnologies Végétale Et Microbienne, Biodiversité Et Environnement
Structure de Recherche : Botanique et Valorisation des Ressources Végétales et Fongiques
Discipline : Microbiologie
Spécialité : Biologie Moléculaire

Présentée et soutenue le :17/07/2019 par :

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Diagnosis of human leptospirosis by real-time PCR and serological methods in Morocco

JURY

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Année Universitaire : 2018-2019

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Résumé

Cette étude a été entreprise, pour la première fois au Maroc, au Laboratoire de Bactériologie et d'Epidémiologie de l'Institut National d'Hygiène à Rabat. Entre 2004 et 2016, soixante-sept sérums issus de 67 patients présentant des signes cliniques de la leptospirose ont été analysés au laboratoire pour un diagnostic de routine et de confirmation. Pour ce faire, les tests SAT, ELISA IgM, ELISA IgG et qPCR ont été utilisés pour le diagnostic de cette maladie. Parmi tous les sérums suspects, 48 (71,6%) et 39 (58,2%) se sont révélés positifs respectivement pour les tests ELISA IgM et SAT. Parmi les 67 sérums inclus, seuls 17 sont suspectés d'être positifs par ELISA IgG, et un sérum a un résultat positif (5,88%), contre 15 (88,24%), 10 (58,82%) et 3 (17,64%) par SAT, ELISA IgM et PCR en temps réel respectivement. Dans notre étude, la PCR en temps réel a été utilisée sur 36 sérums et seuls trois sérums (8,33%) étaient positifs, comparés à 15 (41,66%) et 10 (27,77%) des tests SAT et ELISA IgM respectivement. Considérant l'approche genre, 46 patients sont des hommes (68,68%) et 21 des femmes (31,34%). L'incidence la plus élevée a été observée à Sidi Kacem, 27 cas (40%) suivi d'El Jadida avec 9 cas (13%).La majorité des patients présentaient un ictère et de la fièvre, et un sérum a été utilisé pour le diagnostic. Les cliniciens n'ont pas pris en compte le second sérum, ni l'heure de l'échantillonnage ni les informations épidémiologiques.
Mots clés : Léptospirose, Maroc, diagnostic, SAT, ELISA IgM, qPCR

Summary

This study was undertaken at the laboratory of Bacteriology and epidemiology in the National Institute of Hygiene in Rabat.
From 2004 to 2016, sixty-seven sera related to 67 patients had clinical signs similar to leptospirosis were sent to the laboratory of Bacteriology for routine diagnosis and confirmation. The ELISA IgM and SAT techniques were used for all samples and 48 (71.6%) were positive when using the SAT test, while 39 (58.2) were positive when using ELISA IgM.The ELISA IgG technique was used on 17 isolates. It was observed that only one treatment gave positive result (5.88%) compared to SAT which obtained 15 positive results (88.24%) and ELISA IgM which achieved 10 (58.82%) positive results.The qPCR technique was used in 36 isolates. The technique gave positive results for three serotypes (8.33%) compared to the SAT method which obtained 15 positive results (41.66%) and the ELISA IgM method which gave 10 positive results (58.82%). The number of males in this study was forty-six (68.68%), while the number of females was twenty-one (31.34%). It was noted that the majority of the injuries were in the Sidi Kacem area, with 27 cases (40%) followed by Al Jadida area with nine cases (13%).
Key words: Leptospirosis, Morocco, diagnosis, real time PCR, ELISA IgM, SAT,

Acknowledgements

This thesis has been performed in the Laboratory of Botanique and Valorisation of Plant Resources and Fungal at the Faculty of Sciences Rabat, Mohammed V University, under the supervision of Professor **Moustapha ARAHOU,** and in the Laboratory of Epidemiology at the National Institute of Hygiene, Rabat under the supervision of Assistant Professor **Zakaria MENNANE.**

First at all I would like to thank my supervisor, Professor **Moustapha ARAHOU** (PES) in the Faculty of Sciences Rabat, Mohammed V University, for all support and collaboration.

The work in this thesis would not have been made possible without the contributions made by my co-supervisor Assistant Professor **Zakaria MENNANE** (PA), in the National Institute of Hygiene, Rabat, many thanks for all his support.

Many thanks to Professor **Rachida HASSIKOU** (PES), the Faculty of Sciences Rabat, Mohammed V University, for agreeing to chair the committee of my thesis, and for her valuable guidance, advice, and encouragement throughout the process of developing and implementing the research and writing this thesis.

I would like to thank Professor **Laila SBABOU** (PH), the Faculty of Sciences Rabat, Mohammed V University, to report this work and for her valuable comments that helped me to improve this thesis.

I would like to thank Professor **Abdellatif BOUR** (PES), the Faculty of Sciences, Kenitra, to report this work and for his remarks in order to ameliorate my work.

Many thanks to Dr **Réda CHAROF** (DB), the National Institute of Hygiene, Rabat for his kind willingness to attend and judge in this work and all his support.

I would also like to express my gratitude to all employers in the National Institute of Hygiene in Rabat and the Laboratory of Epidemiology for all their collaboration, without their help, the studies would not have happened.

I would like to thank all fellow PhD students, staff and everyone I had the pleasure of working alongside who made my time here enjoyable.

I would like to thank all my friends who had helped and supported me during this study. Many thanks to all my family in my country Yemen, my parents, my wife and my children for their full support, encouragements, and sacrifice.

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Cette étude a été entreprise, pour la première fois au Maroc, au Laboratoire de Bactériologie et d'Epidémiologie de l'Institut National d'Hygiène à Rabat. Entre 2004 et 2016, soixante-sept sérums issus de 67 patients présentant des signes cliniques de la leptospirose ont été analysés au laboratoire pour un diagnostic de routine et de confirmation. Pour ce faire, les tests SAT, ELISA IgM, ELISA IgG et qPCR ont été utilisés pour le diagnostic de cette maladie. Parmi tous les sérums suspects, 48 (71,6%) et 39 (58,2%) se sont révélés positifs respectivement pour les tests ELISA IgM et SAT. Parmi les 67 sérums inclus, seuls 17 sont suspectés d'être positifs par ELISA IgG, et un sérum a un résultat positif (5,88%), contre 15 (88,24%), 10 (58,82%) et 3 (17,64%) par SAT, ELISA IgM et PCR en temps réel respectivement. Dans notre étude, la PCR en temps réel a été utilisée sur 36 sérums et seuls trois sérums (8,33%) étaient positifs, comparés à 15 (41,66%) et 10 (27,77%) des tests SAT et ELISA IgM respectivement. Considérant l'approche genre, 46 patients sont des hommes (68,68%) et 21 des femmes (31,34%). L'incidence la plus élevée a été observée à Sidi Kacem, 27 cas (40%) suivi d'El Jadida avec 9 cas (13%).La majorité des patients présentaient un ictère et de la fièvre, et un sérum a été utilisé pour le diagnostic. Les cliniciens n'ont pas pris en compte le second sérum, ni l'heure de l'échantillonnage ni les informations épidémiologiques.

Mots clés : Léptospirose, Maroc, diagnostic, SAT, ELISA IgM, qPCR

الخلاصة

الهدف من هذه الدراسة هو تقييم التشخيص بطريقة التفاعل التسلسلي للبوليمريز (qPCR) في تشخيص داء اللولبية النحيفة في الإنسان في مختبر البكتريولوجي والأوبئة في المعهد الوطني للصحة وذلك لأول مرة في المملكة المغربية.

في الفترة من 2004 إلى 2016 استلم المعهد الوطني للصحة في الرباط سبعة وستين مصلا لسبعة وستين مريضا يشتبه بإصابتهم بداء اللولبية النحيفة. لغرض التشخيص تم استعمال التقنيات التالية:

SAT, ELISA IgM, ELISA IgG و qPCR.

تم استعمال تقنيتي ELISA IgM وSAT على جميع العينات وكانت (71.6%)48عينة موجبة عند استعمال الفحص SAT في حين كانت (58.2)39 عينة موجبة عند استعمال تقنية ELISA IgM.

تم استعمال تقنية ELISA IgGعلى 17 مصلا ولوحظ أن مصلا واحدا فقط أعطى نتيجة ايجابية (5.88%) مقارنة بتقنية SAT التي استطاعت الحصول على 15 نتيجة ايجابية (88.24%) و ELISA IgM التي استطاعت الحصول على 10 (58.82%) نتائج ايجابية.

تم استعمال تقنية qPCR على ستة وثلاثين مصلا وأعطت هذه التقنية نتيجة ايجابية لثلاثة امصال (8.33%) مقارنة بطريقة SAT التي استطاعت الحصول على 15 نتيجة ايجابية (41.66%) وطريقة ELISA IgM التي أعطتعشرة نتائج ايجابية (58.82%).

كان عدد الذكور في هذه الدراسة ستة وأربعون (68.68%) في حين كان عدد الاناث واحد وعشرين (31.34%). لوحظ أن غالبية الإصابات كانت في منطقة سيدي قاسم إذ بلغت سبعة وعشرين حالة (40%) تلتها منطقة الجديدة بتسعة حالات(13%).

الكلمات الرئيسية: داء اللولبية النحيفة, المملكة المغربية, التشخيص qPCR, ELISA IgM, SAT

Résumé détaillé

Cette étude sur le diagnostic de la leptospirose humaine par PCR en temps réel et les méthodes sérologiques au Maroc a été élaborée en partenariat entre la Faculté des Sciences de Rabat -FSR- (Equipe de Botanique et valorisation des ressources végétales et fongique du Centre de Biotechnologies Végétale et Microbienne, Biodiversité et Environnement et l'Institut National d'Hygiène -INH- (Laboratoire des maladies épidémiques).

L'introduction générale apporte les informations générales de base sur la leptospirose avec sa taxonomie et classification, épidémiologie et charge de morbidité, réservoirs de la maladie, transmission, facteurs de risque, manifestations cliniques ainsi que la prévention et la vaccination contre la maladie.

La synthèse bibliographique donne un aperçu bibliographique sur le diagnostic de la leptospirose au laboratoire avec les examens direct à savoir la culture, l'étude microscopique et l'amplification par PCR et les examens indirects basés sur les examens sérologiques. L'auteur clôture sa revue bibliographique par une partie sur le traitement de la maladie aussi bien que les mesures de prévention à prendre. En effet, la leptospirose est une zoonose qui provoque de nombreux symptômes cliniques et peut entraîner la mort si elle n'est pas traitée. Cette maladie sévit dans de nombreux pays du monde, en particulier dans les pays tropicaux et en développement. Le diagnostic de la leptospirose est compliqué et dépend de la phase de la maladie. Cependant, les tests sérologiques sont largement utilisés en raison de leur sensibilité et de leur disponibilité.

Ce travail traîtede la bactériologie, la Biologiehumaine, l'Immunologie et la Biologiemoléculaire. Le principal objectif est l'évaluation des différentsmoyens et outils de diagnostic de la leptospirose humain qui sontsouventbasés sur des tests sérologiques. Il s'agitensuite de comparer ces tests de routine à un test basé sur une technique moléculairedite : la qPCR ou la PCR en temps réel. Cetteapproche est une étapecruciale pour décider du choix du meilleur test qui doitêtre à la fois le plus simple, le plus pratique et le plus fiable pour assurer un diagnostic préalable, et par conséquence, un traitementefficace. Par ailleurs, cetteétudeconstitue le point de départ des études futures en raison de ses adaptations importantes

Pour ce faire, une revue de literature publiée a étéélaborée sur les tests de laboratoireutilisés pour le diagnostic de la leptospirose humaine, tout en se focalisant sur les tests de diagnostic standard qui sont de plus en plus utilisés, à savoir : culture, MAT, ELISA et la PCR. Le travail expérimental est basé sur un échantillonnage de sérums de 50 patients originaires de Béni Mellal, Meknès, Agadir, Rabat, Taza, Salé, Tanger, ElJadida
et Sidi Qacem. Ces sérums ont été analysés par des tests d'étude sérologique par testd'agglutination sur lame (SAT) et par dosage immuno-enzymatique (ELISA)

Une analyse sur la conformité des résultatsobtenus par ces tests a été effectuée en utilisant soixante-sept échantillons de sérumissus de patients présentant des signescliniques de la leptospirose.

Plusieurs tests sérologiques son tutilisés pour confirmer le diagnostic et évaluer leur efficacité, à savoir:MAT: Microscopic Agglutination le test ELISA (Enzyme-Linked Immuno sorbent Assay), le test IgM

Cette étude a été entreprise, pour la première fois au Maroc, au Laboratoire de Bactériologie et d'Epidémiologie de l'Institut National d'Hygiène à Rabat.
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pas pris en compte le second sérum, ni l'heure de l'échantillonnage ni les informations épidémiologiques.

En conclusion, on peut avancer que les tests SAT et ELISA IgM peuvent être utilisés pour le diagnostic de la leptospirose humaine au Maroc. Ces tests sont faciles, simples, ne nécessitent pas de personnel expérimenté, peuvent être utilisés dans n'importe quel laboratoire et sont moins chers que les qPCR.

Cette étude est très importante en raison de ses adaptations importantes pour des études futures. Les résultats de ce travail sont d'un grand intérêtscientifique;leur exploitation sera judicieuse.

List of publications

1. **Waleed Al-orry, Moustapha Arahou, Rachida Hassikou, Aicha Qasmaoui, Réda Charof, Zakaria Mennane.**Diagnosis of Human Leptospirosis in Morocco by IgM ELISA and Slide agglutination test (SAT). International Journal of Innovation and Applied Studies. 2016;14(4):1015-1018.
2. **Waleed Al-orry, Moustapha Arahou, Rachida Hassikou, Aicha Qasmaoui, Réda Charof, Zakaria Mennane.** Leptospirosis: Transmission, Diagnosis and Prevention. International Journal of Innovation and Applied Studies. 2016; 15(3): 467-467.
3. **Waleed Al-orry, Moustapha Arahou, Rachida Hassikou, Aicha Qasmaoui, Réda Charof, Zakaria Mennane.**Human Leptospirosis in Morocco, 2004-2010: Serological Study by Slide Agglutination Test (SAT). Asian Journal of Applied Sciences.2016;4(3):680-684.
4. **Waleed Al-orry, Moustapha Arahou, Rachida Hassikou, Aicha Qasmaoui, Réda Charof, Zakaria Mennane.** A Review of Laboratory Diagnosis and Treatment of Leptospirosis. International journal of pharmacy and pharmaceutical sciences. 2016;8(12):7-13.
5. **Waleed Al-orry, Moustapha Arahou, Rachida Hassikou, Aicha Qasmaoui, Réda Charof, Zakaria Mennane.** Serodiagnosis of Human Leptospirosis by Enzyme-Linked-Immunosorbent -Assay (ELISA). Australian Medical Journal. 2017;10(1)30-34.
6. **Waleed Al-orry, Moustapha Arahou, Rachida Hassikou, Aicha Qasmaoui, Soumaya Chaiboub,Réda Charof, Zakaria Mennane.** First real time PCR in Morocco for human leptospirosis using TaqMan probs Targeting the LipL32 Gene. Archives of clinical infectious diseases. 2019 (Febraury;14(1):1-6.

List of presentations and posters

1- **Waleed Al-orry, M. Arahou, R. Hassikou, A. Qasmaoui, R. Charof, and Z. Mennane.** Risk factors and prevention of leptospirosis. Journées Ibn Roched d'hygiènehospitalière. *Faculté de Médecineet de Pharmacie Casablanca, décembre*2016.(Poster)

2- **Waleed Al-orry, M. Arahou, R. Hassikou, A. Qasmaoui, R. Charof, and Z. Mennane.** Diagnosis of Human Leptospirosis in Morocco by IgM ELISA and Slide agglutination test (SAT).*The second conference of Biology at The University of Sidi Mohamed Ben Abdellah, Fes, Morocco*. 2017. (Oral)

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Abbreviations

CSF	: Cerebrospinal fluid
°C	: Degree Celsius
DELM	: Direction de l'Epidémiologie et de Lutte contre les Maladies
ELISA	: Enzyme-linked immunosorbent assay
EMJH	: Ellinghausen- McCullough-Johnson-Harris medium
IgM	: Immunoglobulin M
IgG	: Immunoglobulin G
LA	: latex agglutination
ml	: Millilitre
MAT	: Microscopic Agglutination Test
Min	: Minute
PCR	: Polymerase chain reaction
pH	: Minus log of the hydrogen icon concentration *et al*.and others
PBS	: Phosphate buffer saline
qPCR	: quantitative real time PCR
SAT	: Slide Agglutination Test
WHO	: World Health Organisation

General Introduction

Leptospirosis is a zoonotic disease transmitted from animals to humans by pathogenic serovars of the genus *Leptospira*[1]. The disease has wide distribution in tropical regions comparing with developed countries [1]. The annual incidence is 1.03 million cases and 58,900 deaths due to leptospirosis. Majority of cases occur in adult males with age of 20–49 years [2]. The major problem due to leptospirosis has been its severe life-threatening manifestations related to pulmonary hemorrhage syndrome and acute kidney injury due to Weil's disease [2]. The infection may occur through either direct contact with urine of infected animal or through indirect contact via soil or water contaminated with urine from an infected animal. Indirect contact is much more common, and can be associated with occupational, recreational, or avocational activities[3]. The main responsible agent of infection is pathogenic *Leptospira interrogans* which contains more than 250 pathogenic serovars [4]. All mammalian species can be reservoirs of infection but the main reservoir is the rat which is responsible for the most serious manifestation related to serovar Icterohaemorrhagia [5].

In Morocco, leptospirosis was mentioned for the first time by Melnotte and Farjot who reported 7 cases of ictero-haemorrhagic spirochaetes in Fes in 1927. Between 1950 and 1962 research on leptospirosis was conducted under the direction ofDr. Blanc with the participation of Mailloux and Kolochine Erber [6]. From 2001 to 2010, a total of 399 cases by DELM were reported [7]. In 2010, one case of human leptospirosis was reported in Al-Jadida[8]. In 2011, Facial palsy associated with rare leptospirosis in Fes was reported [9]. In 2014 a study in Casablanca was conducted among poultry workers (23.3%), in fishing workers (11.6%) and abattoir workers (7.4%) [10]. Majority of cases in Morocco occur in September and October due to rainfall as well as Males have higher incidence than Females andthe median age of confirmed cases is 36 years [7]. Mohammed, Haraji, et al (2011) indicated the epidemiology risk factors that were: wet environmental living conditions, lack of protective footwear, infestation of dwelling with rats, history of unprotected contact with dairy stagnant water and working in farm lands [7].

In Morocco, no known studies have reported the diagnosis of leptospirosis by Real-time PCR. The diagnosis of leptospirosis at the National Institute of hygiene, Rabat, Morocco usually conducted using serological tests (ELISA IgM and SAT). This study is the first study by qPCR for the diagnosis of human leptospirosis. PCR is superior for the diagnosis, it can be used during the first week of illness, thus making it ideal for the rapid detection of organisms involved in acute infections [11]. Serological methods are easy, fast, not expensive and can be used easily in developing countries and in Resource Poor Settings where majority of cases, but they have low sensitivity during the first week of illness [11]. MAT the reference serological test has low sensitivity during the acute phase andrequires paired sera in order to allow the detection of rising titers. Rapid diagnosis of leptospirosis is of extreme importance, because antibiotic treatment is more effective in altering the course of this life-threatening disease when initiated early [11]. To overcome all these limitations PCR have been used, which is considered sensitive and specific for the rapid detection of Leptospira during the first days of illness [12].

Therefore, the aim of this thesis was to compare and evaluate PCR real time with serological tests (ELISA IgM, ELISA IgG and SAT), and our research question was can qPCR diagnose human leptospirosis better than serological methods or serological methods are adequate for the diagnosis?

Thesis structure

Chapter 1 is a literature review.

Chapter 2 evaluated ELISA IgM and SAT for the diagnosis of human leptospirosis, the objective of this study was to compare ELISA IgM with SAT for the diagnosis of human leptospirosis.

Chapter 3 descripted qPCR for the diagnosis, the aim was to compare and evaluate qPCR with serological tests.

Chapter 1: Literature review

1. Introduction

Leptospirosis is an infectious zoonosis common worldwide, but most prevalent in developing countries. It is caused by the pathogenic serovars of the genus *Leptospira*, which are shed in the urine of infected animals.

Humans become ill after exposure to infected urine or via contact with water, soil or food that has been contaminated [13].

Currently, there are more than 200 pathogenic serovars divided into 25 serogroups within the genus *Leptospira*[14],[15]. The main transmission routes are via injured skin, mucus membranes and by long contact with contaminated water or soil [16].

Leptospires can persist for weeks or months in the environment under favorable conditions such as temperatures of 28°C to 32°C and a neutral or slightly alkaline pH. Therefore, the highest incidence occurs in developing countries with tropical and temperate climates [17].

The clinical signs of this disease vary from asymptomatic or mild to severe manifestations ; this depends on the infecting serovar, the location and the immune status of the patient. The common signs include bleeding, malaise, fever, headache, jaundice and myalgia[18].

Weil's disease, pulmonary hemorrhage syndrome and liver or renal failure might develop and lead to death if untreated[19].

Because its resemblances to other diseases, leptospirosis can be difficult to distinguish from other febrile conditions (such as malaria, typhoid fever, influenza, rickettsiosis, aseptic meningitis, hepatitis and hemorrhagic fever). Diagnosis is therefore critical for prompt and appropriate treatment [20].

2. Leptospira (The organism)

The genus *Leptospira* includes saprophytic, intermediate and pathogenic species and belongs to the family *Leptospiraceae*, order *Spirochaetales*. Saprophytic leptospires (e.g. *L. biflexa*) are free-living bacteria found in water and soil. Unlike pathogenic *Leptospira* spp. saprophytic leptospires do not infect animal hosts [21].

Leptospires are thin (6-25 μm long and 0.1 to 0.2 μm in diameter) and have a distinctive double membrane structure as found in other spirochetes. They are highly motile, obligate aerobes with an optimum growth at a temperature of 28 to 30°C, pH

range 6.8 to 7.4, and can survive in a moist conditions (e.g. soils, mud, swamps, streams and rivers) and in organs and tissues of live or dead animals or in diluted milk [22].

Leptospires have unique hooked ends (Figure1); two periplasmic flagella with polar insertions are sited in the periplasmic space and are responsible for motility [18]. Motility plays a critical role in invasion of host animals and in migration to tissues [23].

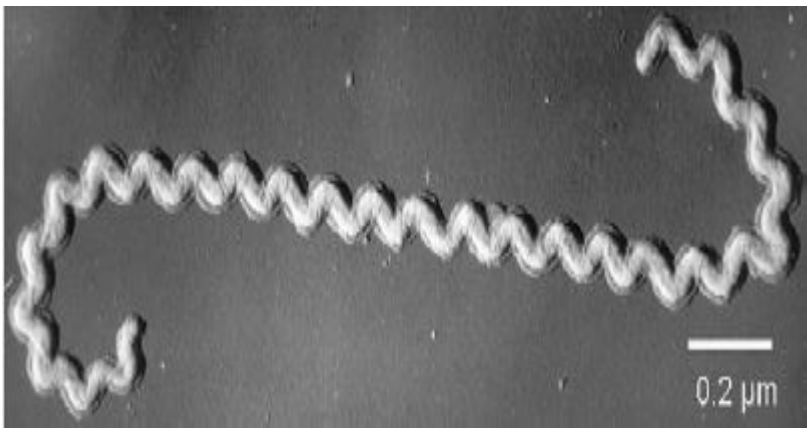

Figure 1*:*Photomicrograph of Leptospira spp. photo reproduced from [24].

3. Historical Aspects

- ✓ In 1882 Adolf Weil described clinical syndrome characterized by splenomegaly, jaundice and nephritis, referred to as Weil´s disease, which became synonymous with leptospirosis. Although the etiology of the disease at that time was unknown, it seemed to be infectious in wildlife and was associated with outdoor water occupations. However, an apparently identical syndrome occurring in sewer personnel was reported several years earlier [18].
- ✓ In 1907 Stimson demonstrated by silver staining the existence of clumps of spirochetes in the kidney of a man who died of yellow fever. The spirochetes had hooked ends and Stimson named them *Spirochaeta interrogans* because of their resemblance to a question mark [18].
- ✓ In 1914 Wolbach and Binger reported the first isolation of Leptospira from freshwater [25].
- ✓ In 1915 Leptospira was isolated from the blood of miners workers in Japan by Inada and Ido, where the disease was common in coal miners [26].

Inada and Ido firstly named this bacteria *Spirochaeta icterohaemorrhagica japonica*; however, this was changed to *Spirochaeta icterohaemorrhagiae* before the primary publication [24].

Within a few months Inada and colleagues had succeeded in propagating the bacteria in vitro in a medium made from emulsified guinea-pig kidney and indicated a preference for growth at 25°C with loss of viability at 37°C [24].

- ✓ In 1917 the relationship between rats and Weil's disease were described; Ido and his group noted and cultured spirochetes from the kidneys and urine of many species of house and wild rats, and identified them as *Spirochaeta icterohaemorrhagiae*[27].
- ✓ In 1917 the genus Leptospira was defined by Noguchi [28].
- ✓ In 1957 in the 7th edition of Bergey's Manual the genus Leptospira was divided into two species; L. icterohaemorrhagiae including all pathogenic strains and L. biflexa comprising saprophytic strains. L. icterohaemorrhagiae was further subdivided into serotypes but L. biflexa was not [24].
- ✓ In 1979 the family Leptospiraceae was officially proposed [29].
- ✓ In 1982, the subcommittee on the classification of Leptospira accepted the notion of two species of Leptospira with L. interrogans comprising the pathogenic serovars and L. biflexa containing the saprophytic serovars [30],[24].
- ✓ In 1987 the advent of a much more objective and rational molecular classification brought major changes to the taxonomy of Leptospira. Based on DNA–DNA relatedness *L. interrogans* was divided into seven species; however, new isolations have added several additional species of both pathogenic and saprophytic *Leptospira*[31],[24].

4. Disease names

There are many different names for leptospirosis, including : "7-day fever", "harvest fever", "field fever", "canefield fever", "mild fever", "rat catcher's yellows", "Fort Bragg fever" and "pretibial fever". It has traditionally been known as "black jaundice" and it is called "nanukayami fever" in Japan. Weil's disease or Weil's syndrome is also recognised as spirochaetosis icterohaemorrhagica [32].

5. Taxonomy and classification

The family *Leptospiraceae* was defined in 1979 to contain two genera ;*Leptospira* and *Leptonema*. Now it contains three genera of spirochaetes: *Leptospira*, *Leptonema* and *Turneriella*[24].

5.1 Serological classification (phenotypic classification)

Prior to 1989, the genus *Leptospira* was divided into two species: *L. interrogans* sensu lato including all pathogenic strains and *L. biflexa* sensu lato including the saprophytic strains isolated from the environment [30].

Both *L. interrogans* and *L. biflexa* are divided into many serovars, and the closely related serovars are arranged in serogroups. More than 200 serovars arranged into 25 serogroups have been described under the species *L.interrogans*, while *L.biflexa* has 65 serovars arranged in 38 serogroups [33]. Further serovars have been isolated, but have yet to be validly published and the list of serovars is updated periodically [34].

The serovar is the basic taxon used to classify *Leptospira* and is defined on the basis of structural heterogeneity in the carbohydrate component of the lipopolysaccharide (LPS) [35]. Serogroups which are identified using the micro agglutination test (MAT), have no official taxonomic status [35]. The serogroups of *Leptospira* and common serovars are shown in Table 1.

Table 1: Serogroups and some serovars of *L. interroganssensu lato*[36]

Serogroup	Serovar(s)
Icterohaemorrhagiae	icterohaemorrhagiae, copenhageni, lai, zimbabwe
Hebdomadis	hebdomadis, jules, kremastos
Pyrogenes	pyrogenes
Bataviae	bataviae
Grippotyphosa	grippotyphosa, canalzonae, ratnapura
Canicola	canicola
Australis	australis, bratislava, lora
Pomona	pomona
Javanica	javanica
Sejroe	sejroe, saxkoebing, hardjo
Panama	panama, mangus
Cynopteri	cynoptri
Djasiman	djasiman
Sarmin	sarmin
Mini	mini, Georgia
Tarassovi	tarassovi
Ballum	ballum, aroborea
Celledoni	celledoni
Louisiana	louisiana, lanka
Ranarum	ranarum
Manhao	manhao
Shermani	shermani
Hurstbridge	hurstbridge

5.2 Differentiation of pathogenic and saprophytic leptospires

Pathogenic leptospires can not be distinguished from saprophytic according to morphological characteristics. To classify saprophytic and pathogenic leptospires the commonly tests used are: growth at low temperature (13ºC) and resistance to 8-Azaguanine (225 µg/ml) [34].

The growth of pathogenic serovars is restricted by the purine analogue 8-azaguanine and at 13ºC, whereas saprophytic serovars can grow in the presence of this compound. Pathogenic serovars have a generation time of about 20 h. Therefore, they are considered to be slow-growing bacteria compared with saprophytic leptospires which grow more rapidly (generation time of around 5 h) [35].

5.3 Genotypic classification (Classification based on DNA relatedness)

This classification does not correspond to the previous two species (*L. interrogans* and *L. biflexa*), the pathogenic and nonpathogenic serovars are found in the same species, as well as one serogroups (table 2) can be found in different species [36]. This identification is particularly complicated because in some cases serovars within the same serogroup may be distributed between different species [36]. However, molecular classification gives a clear identification of distinct subtypes (e.g. Hardjoprajitno and Hardjobovis, grouped previously under serovar Hardjo, now belong to *L. interrogans* and *L. borgpetersenii)* [37]. Twenty species have been described in the genus *Leptospira* based on this classification, including nine pathogenic species (*L. interrogans, L. kirschneri, L. kmetyi, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. alexanderi*, and *L. alstoni*), six saprophytic species (*L. biflexa, L. wolbachii, L. meyeri, L. vanthielii, L. terpstrae*, and *L. yanagawae*), and five intermediate species (*L. inadai, L. broomii, L. fainei, L. wolffii,* and *L. licerasiae*) [38].

Table 2 : Genomospecies of Leptospira and distribution of serogroups [36]

Species	Serogroups
L. interrogans	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin
L. noguchii	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona
L. santarosai	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri
L. meyeri	Ranarum, Semaranga, Sejroe, Mini, Javanica
L. wolbachii	Codice
L. biflexa	Semaranga, Andamana
L. fainei	Hurstbridge
L. borgpetersenii	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
L. kirschneri	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis,Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae
L. weilii	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
L. inadai	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica
L. parva	Turneria
L. alexanderi	Manhao, Hebdomadis, Javanica, Mini

6. Epidemiology and burden of disease in humans and animals

Leptospirosis occurs worldwide (table 3) excluding the polar regions [39]. However, the highest incidence occurs in humid countries for several reasons :

1- The warm, wet and humid climatic environments encourage the survival and growth of the bacteria in moist soil and fresh water for long periods.
2- The close contact between the reservoir animal hosts and humans.
3- Human practices such as walking barefoot and working in wet fields without protection leading to indirect transmission from contaminated environment [36],[37].

In tropical areas, different species of wild mammals often infected by diverse ranges of leptospiral serovars [40]. A region with a richly varied fauna will therefore support a greater variety of strains than a region with few animal hosts [36]. On the other hand, leptospiral diversity seems to be restricted on islands and in urban settings where the major potential reservoir animals are limited to rats and domestic or production animals [40].

Morbidity and mortality are high in the poorest regions of the world and in areas where surveillance is not routinely performed (such as in urban slams where poor sanitation are frequent, leptospirosis may reach high levels) [41], [42].

The worldwide incidence of leptospirosis is still not well established ; a study estimated the annual incidence of leptospirosis at 1.03 million cases and 58300 deaths [41]. The World Health Organization (WHO) [33], has estimated the annual incidence of human leptospirosis between 0.1–1 cases/100000 in temperate non-endemic areas, and 10–100 cases/100000 in humid tropical endemic regions, where the number of severe cases may reach 500000.

Pathogenic leptospires are widespread in nature reflecting maintenance in the kidneys of many wild and domestic reservoir hosts. The leptospiral life cycle includes shedding in the urine, persistence in the environment and acquisition of a new host [24].

Infected animals may have leptospires persistently colonising the proximal renal tubules and excrete the bacteria intermittently for months or years or even for life [17]. A study of seemingly healthy dogs in Kansas, USA confirmed such an event of 500 dogs considered without regard to health status. Of 500 dogs considered without regard to health status, forty one were shown by PCR to have leptospiruria but only four had clinical signs consistent with leptospirosis [40].

Leptospirosis is also an animal health problem, due to reproductive failure, reduced milk and meat production, reduced growth and clinical illness, which lead to economic losses in the livestock industries. Young animals are usually more susceptible to leptospirosis than adults, frequently suffering severe outbreak and high mortality rates [43].

Table 3 : Annual incidence of leptospirosis worldwide [19]

Countries with the highest incidence				**Other countries**	
Rank	Country	Annual incidence per million population	Countries probably endemic	Country	Annual incidence per million population
1	Seychelles	432.1	India	Belarus	3.4
2	Trinidad and Tobago	120.4	Malaysia	Bulgaria	3.7
3	Barbados	100.3	Bangladesh	Chile	1.6
4	Jamaica	78	Vietnam	Colombia	1.6
5	Costa Rica	67.2	Laos	Czech Republic	1.8
6	Sri Lanka	54	Nepal	France	3.9
7	Thailand	48.9	Cambodia	Germany	0.7
8	El Salvador	35.8	Indonesia	Greece	3
9	New Zealand	26	Myanmar	Honduras	3.1
10	Uruguay	25	China	Hungary	3.1
11	Cuba	24.7	Iran	Ireland	2.2
12	Nicaragua	23.3	Suriname	Italy	0.7
13	Croatia	17.3	Haiti	Lithuania	2.2
14	Russia	17.2	Peru	Mexico	1
15	Ukraine	15.3		Netherlands	1.9
16	Dominican Republic	13.8		Panama	1.3
17	Brazil	12.8		Paraguay	1.9
18	Ecuador	11.6		Serbia and Montenegro	1.5
19	Argentina	9.5		Singapore	2
20	Romania	9.4		South Korea	2.8
21	Australia	8.9		Spain	0.3
22	Portugal	6.8		UK	0.6
23	Denmark	6		USA	0.1
24	Latvia	5.6		Venezuela	3.8
25	Slovenia	5.4			
26	Philippines	4.8			
27	Slovakia	4.4			
28	Taiwan	4.1			

7. Is leptospirosis an endemic or an epidemic disease?

Leptospirosis is endemic in many countries in the world (table 3); it has a seasonal distribution, increasing with increased rainfall or higher temperature. However, the disease can occur throughout the year. Epidemics are associated with changes in human behaviour, animal or sewage contamination of water, changes in animal reservoir density, or follow natural disasters such as cyclones and floods [44].

8. Reservoirs of infection

Leptospira has been isolated from nearly all mammalian species, and several natural carriers (maintenance hosts) of pathogenic leptospires; including wild farm and pet animals[36]. However, the most important source of infection for humans is the rat which is responsible for the more severe icteric manifestation of leptospirosis [14].

Rats were considered as a primary source of infection, and they can shed the bacteria throughout their lifespan without clinical manifestations [44]. Fur-bearing animals (silver foxes, mink and nutria) are also potential sources of human leptospirosis [15]. Although bats, pinnipeds, poikilothermic animals (such as frogs and toads) may play a role in the circulation of leptospirosis in the environment, they may not be significant reservoirs of human infection [3].

Leptospira serovars are usually adapted to one or more mammalian species; e.g. dogs are the reservoir hosts for serovar Canicola, pigs for Bratislava and Pomona, horses can maintain Bratislava and cattle are the principal reservoir hosts for Hardjo [13]. Rodents and insectivores are reservoir hosts for many *Leptospira* serovars. Rats are important hosts for serovars Icterohaemorrhagiae and Copenhageni [13].

Additional mammalian species, both wild and domestic can be reservoirs [39]. One host can be a carrier of several serovars, whereas one serovar may be found in many distinct hosts [45].

9. Transmission

Leptospirosis is primarily an infection of animals, humans are accidental victims [33]. Human infection results from exposure to infected urine of carrier mammals or by indirect contact via water, or soil contaminated with infected urine (Figure 1.2) which is the most common source for human infection [20],[40].

Other modes of transmission, include handling infected animals tissues, ingestion of contaminated food and water [15], long periods of exposure to contaminated water or

soil [13],[16], and drinking contaminated water (an outbreak of 33 people was reported in Italy due to drinking contaminated water from a fountain) [46].

Rare routes of transmission in people include rodent bites and laboratory accidents [13]. Environmental conditions strongly affect the transmission of this disease; the transmission is facilitated by the survival of pathogenic serovars in moist environments outside their mammalian host. In one study, *L. interrogans* serovar Canicola was reported to remain motile for 110 days in distilled water (pH 7.2), and when incubated in a semi-solid medium composed of distilled water and 0.5% purified agarose (pH7.2) leptospires survived for 347 days [47].

Leptospira may penetrate the body via cuts and abrasions or mucous membranes (e.g. the conjunctival, oral, or genital surfaces). Pathogenic leptospires make their way into the bloodstream and persist there during the leptospiremic stage of the illness and invade all tissues and organs. They are then cleared by the host's immune response to the infection. However, they may settle in the convoluted tubules of the kidneys and be excreted in the urine for a period of a few weeks to several months [44],[3].

Low numbers of *Leptospira* can cause severe manifestations or death; levels of 10^2-10^{10} are enough to cause severe symptoms (uncomplicated, renal failure, myocarditis, and multi-organ failure), while levels $\geq 10^4$ may lead to death. [48], [49].

In animals, sexual contact or artificial insemination can lead to the infection [16]. *Leptospira* was reported in the semen of bulls infected either naturally or experimentally, demonstrating the possibility of bovine leptospirosis transmission by natural coition or by artificial insemination [39],[50].

9.1 Human-to-human transmission

Leptospirosis can be transmitted from human to human in rare cases [44]. Leptospires have been isolated from human breast milk; in one case serovar Hardjo was maybe transmitted from an infected mother to her infant by breastfeeding [3].

Transmission might be occur by sexual intercourse and blood transfusion, however in blood transfusion antibodies appear after a week or so after the onset of disease and usually clear leptospires from the blood [15],[44].

Although excretion of leptospires in human urine for weeks or months, humans are not observed as a source of disease transmission [40].

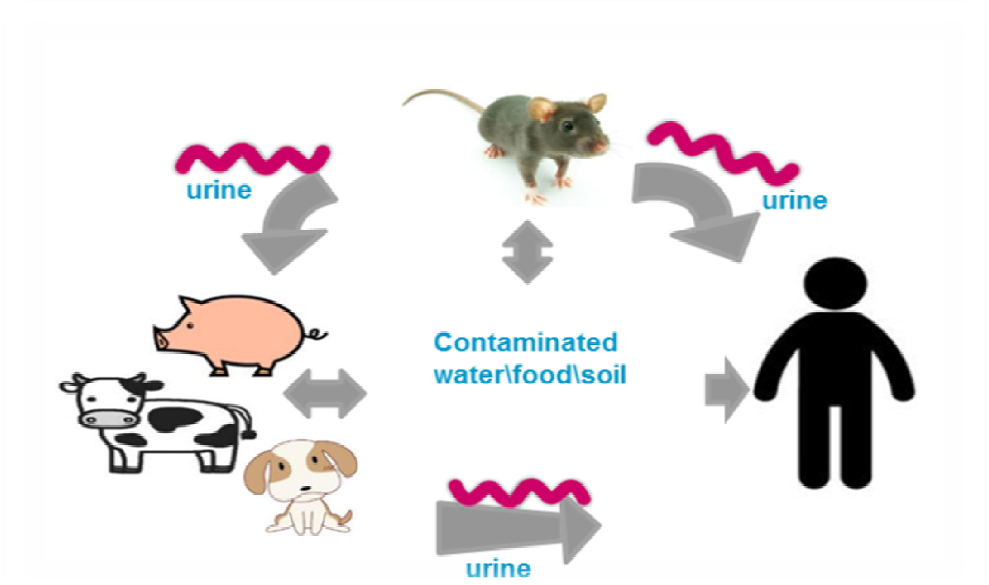

Figure 2 : Transmission of leptospirosis [11]

10.Risk factors

There are certain groups of humans in a population that are more likely to be exposed, as a result of either occupational or recreational activities [15].

10.1 Occupational exposures

Occupational risks can be divided into two groups : occupations involving direct animal contact and occupations including indirect contact. The first includes abattoir workers, farmer/dairy farmers, veterinarians, meat inspectors, rodent control workers, pet shop owners, butchers, animal shelter workers, and gamekeepers. Occupations involving indirect contact include sewer workers, miners, military personnel, septic tank cleaners, fish farm workers, canal and river workers, watercress farmers, flood relief workers, gravel pit workers, street dweller/urban slums, plumbers, construction and demolition site workers [51],[52].

The major risk factors in occupational exposures are: wounds, smoking, eating at work, cleaning the offal and having a borehole for personal water use [53].

Other factors include contact with water contaminated with animal urine in the field (e.g. workers in waterlogged agricultural fields) [54]. The infection were reported by serovar Icterohaemorrhagiae among rice mill workers [55], and banana workers

(banana workers accounted for two-thirds of the informed leptospirosis cases in a tropical region of Queensland, Australia) [56].

In India a seroprevalence study indicated that agriculture workers had high incidence, followed by sewage workers, animal handlers, forest workers, and butchers [57]. In Malaysia, garbage collectors and town cleaners were shown to have higher incidence [58].

Using conventional cultivation methods (by cattle or buffaloes) for dry and wet farming may lead to the infection, due to the fact that these occupations include activities likely to result in exposure of cuts and abrasions to soil and water contaminated with the urine of rodents and other animals attracted to food sources [56],[59].

In Nepal, leptospirosis among farmers was reported, where rearing cattle and/or buffaloes is a common practice [59]. However, most of these occupational infections can be avoided by using suitable personal protective equipment such as rubber boots, gloves and protective eyewear [51].

While professional exposure plays an important role in transmission in tropical and rural countries, it is less common in most developed nations, due to a wider understanding of the hazards and the use of suitable protective measures. In such settings most cases occur following recreational activities [39].

10.2 Recreational exposures

Recreational exposure includes all freshwater sports where leptospirosis may be acquired from prolonged exposure to contaminated water. The importance of this type of exposure has increased recently, particularly in developed countries [60].

Swallowing while swimming in contaminated water is a risk factor for infection [3], and outbreaks due to this practice were reported (such as the outbreak among United States military personnel in Okinawa, Japan) [61].

In some activities (e.g. caving) the pH of water is favorable for the survival of Leptospira, leading to an increased risk of infection [62]. Indeed, many participants in international events have become ill after having returned home [24], especially, following activities in tropical endemic regions where the combination of multiple skin abrasions with immersion occurs [62]. In Germany, one male had severe leptospirosis after returning home from an iron man contest in the Philippines [63]. In

another case, an American man had leptospirosis after he returned from caving in Sarawak, Malaysia despite taking doxycycline daily for malaria prophylaxis [62].

Significant risks associated with recreational exposure occurring in water sports were reported (such as swimming, rafting, canoeing, and kayaking). In addition, leisure risks (e.g. gardening, animals breeding at home, fishing, bathing and hunting) may lead to infection [60],[64],[65].

10.3 Rainfall and flooding

Leptospirosis is considered a disease of the environment, and outbreaks have been associated with heavy rainfall [66], and typhoons [67], which increase the risk of leptospirosis by bringing the bacteria and their animal hosts into closer contact with humans. Heavy rains not only spread these bacteria to new places but also lead to their survival for long periods of time [66],[68].

Rainfall, flooding and natural disasters can affect animal population density. Rodents and other animals can proliferate due to the scattering of garbage, debris, food and the stimulation of vegetation growth resulting in an increase in food availability [68].

In Argentina, flooding has emerged as the major risk factor for leptospirosis ahead of occupational exposure. In Mumbai, India, an eight-fold increase in disease incidence was noted after severe flooding in 2005. In Manila, Philippines, a large outbreak of leptospirosis was reported after tropical storms and severe flooding in October 2009. A higher seroprevalence of infection has also been associated with heavy rainfall and flooding in China, France, Brazil, Trinidad, Tobago, French Polynesia and Salvador [68],[37],[42].

10.4 Poor sanitation and inadequate waste disposal

Poor sanitation and waste management can lead to an increase in the incidence of many infectious diseases, especially in developing countries. The presence of garbage, waste and sewage encourages the proliferation of rodents and may upsurge the risk of leptospirosis [68]. Therefore, urban slum residents in areas with poor sanitation are at high risk of leptospirosis [42].

10.5 Age

Leptospirosis occurs in all people of all age, but it is more frequent among youths and young adults in the middle age (20-29) followed by the 30-39 age bracket and less frequent in adults more than 75 years of age. This is likely related to professional and recreational activities [69],[59], [70].

Students and children ≤12 may also contract the disease [59],[64],[71]. But, children often contract milder forms of leptospirosis than adults [67].

10.6 Sex

Leptospirosis seems to be more frequent in males than females, related to higher exposure of males to risk factors (such as: animal rearing, working in fields and other related occupations) [59],[64].

10.7 Other risk factors

In normal daily activities significant exposure might occur ; for example exposure to flood or sewer water and mud at the workplace is a risk factor for acquiring leptospirosis during daily activities [42]. Residential risk factors (e.g. animals at home, rats in the house, poor housing conditions and live in a floodable area) can also lead to infection [64].

11. Clinical manifestations

The clinical manifestations of leptospirosis are highly variable and nonspecific depending on both host and pathogen factors. The highest majority of infections are subclinical or very mild (accounting for 90% of cases) These are usually self-limiting and patients do not seek medical attention [36],[52]. However, leptospirosis might progress to severe clinical manifestations in 5-10% of patients (figure 3) and can be fatal especially if treatment is not initiated early [49].

The main common symptoms are: febrile illness, chills, headache, myalgia, abdominal pain, nausea/vomiting, photophobia, anuria/oliguria, stiff neck, skin rash, anemia and conjunctival suffusion [72],[70],[67].These symptoms can emulate other tropical diseases (e.g. dengue and malaria) that have similar signs, especially during the acute stage of the illness [73].

Icterus (jaundice) which is a relatively common symptom in leptospirosis, may found in many other diseases such as various forms of hepatitis [44]. Therefore, it is very difficult to make the diagnosis clinically, and the suspicion is further increased if there is a history of occupational or recreational exposure to infected animals or to contaminated environment [44].

In animals, leptospirosis may be asymptomatic, mild or severe and acute or chronic. The infection tends to be milder in the reservoir host, and severe when the serovar is not adapted to the host species[13]. Infected animals are usually symptom-free and continue shedding leptospires in the environment through their urine [40].

Abortions and neonatal deaths are the most outcome of animal chronic infection (abortions generally occur 2 to 12 weeks after infection in cattle and 1 to 4 weeks after infection in pigs). The economic importance appears when food producing animals are infected [13].

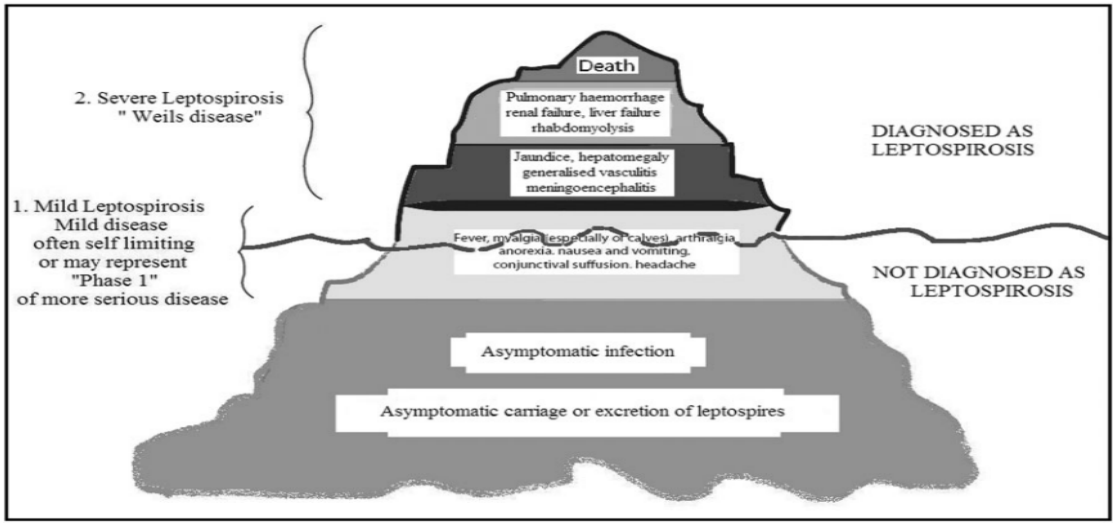

Figure 3 : Clinical features of leptospirosis [52]

11.1 Incubation Phase

In humans, the incubation stage (phase) from exposure to the onset of symptoms usually lasts 7 to 12 days ; however, it can be as short as 3 days or as long as one month[61].

In animals, the incubation phase can be as short as a few days with clinical signs appearing after 5 to 15 days in infected dogs, or may be longer when clinical signs are the result of chronic low-level damage to the kidneys or liver. The disease is rare in cats, possibly due to the likelihood of prolonged incubation phase in this species[13].

11.2 Clinical symptoms

11.2.1 Anicteric febrile illness

Anicteric leptospirosis is the most common syndrome, namely a self-limiting disease that occurs in 85% to 90% of the cases [74]. Leptospirosis is biphasic, with fever recurring after a remission of three to four days. The first septicemic stage is leptospiremic (also called acute phase or febrile stage) and lasts for 4-7 days. During

this period leptospires can be found in the blood and in cerebrospinal fluid (CSF)[33].

The major symptoms in this phase are fever, headache, severe myalgia, chills with rigors, prostration and sometimes circulatory collapse [75]. Myalgia is frequently located in the lower limbs and it is very severe and intense [33].

After the acute phase, the majority of patients have a period of one to three afebrile and asymptomatic days, then followed by the second phase called the immune stage (also called convalescent phase). The immune phase, is characterized by leptospiruria and correlates with the appearance of IgM antibodies in the serum and leptospires in the urine [4]. This phase continues for 4-30 days or longer (table 4).

The immune phase continues for 4-30 days or longer (table 4), and conjunctival suffusion, fever and acute myalgia are the most specific findings in this phase. Aseptic meningitis may occur in 25% of all leptospirosis cases [36].

Mortality is usually very low in anicteric leptospirosis, but death can occur, resulting from massive pulmonary hemorrhage [36].

11.2.2 Icteric leptospirosis (Weil´s Disease)

The combination of jaundice, renal failure, and hemorrhage is known as Weil's disease ; it is the most severe pattern of leptospirosis [76]. Icteric leptospirosis occurs when the septicemic phase progresses to acute icteric illness with renal failure. Nearly 5-10% have jaundice which develops early in the course of the disease [44].

Usually serovars of Icterohaemorrhagiae serogroups are responsible for icteric leptospirosis [77]. However, this phase may occur in infection by other serovars [69].

In México serovar Panama was predominant in confirmed icteric and anicteric cases, followed by Pomona, Bratislava and Icterohaemorrhagiae [69]. Weil's disease was also reported due to serovars related to *L. santarosai* species [78], and serogroupBataviae [79].

Jaundice is the most significant clinical feature of severe leptospirosis. Meningitis and fever may occur in the first day and during the second day fever (38ºC), and severe myalgia may persist. Dyspnea, jaundice, massive haemoptysis, pulmonary hemorrhage and oliguria may occur in the 3rd day [77].

Usually, at the end of the second week, the patient is deeply jaundiced and these symptoms lead to death if untreated [77]. Oliguria maybe occur as early as in the fifth day of illness and in the eighth day anuria occurs due to renal function decline [79].

Severe bleeding, cardiac and pulmonary complications are frequent ; these complications lead to death, and the mortality rate may reach 15-40% [33],[24],[4]. Weil's disease manifestations was reported in children from 1 month to 12 years age, but, it may be less fatal than in adults [71],[79].

Table 4 : Leptospirosis: Anicteric and Icteric manifestation [4]**.**

	Anicteric		**Icteric**	
Leptospirosis	First stage (3-7) days (Septicemic)	Second stage (0-30) days (Immune)	First stage (3-7) days (Septicemic)	Second stage (0-30) days (Immune)
Clinical findings	Fever myalgia headache conjunctival suffusion abdominal pain vomiting	Meningitis uveitis rash fever	High fever Jaundice Hemorrhages Renal failure	
Leptospires Present	Blood CSF	Urine	Blood CSF	Urine

11.2.3 Other Complications

Uveitis has been considered a rare complication of leptospirosis in South India [80]. Pancytopenia might be accompanied by other potentially fatal complications, such as pulmonary hemorrhage, myocarditis and acute respiratory distress syndrome (ARDS) [81]. Other symptoms might also occur, such as skin rash, conjunctival suffusion, subconjunctival hemorrhage [67], facial palsy [9] and acalculous cholecystitis [82].

11.2.4 Leptospirosis in pregnancy

Leptospires can be isolate from amniotic fluid, placenta and cord blood [83] ; however only a few cases of leptospirosis in pregnancy have been reported [44]. The hazards of leptospirosis during pregnancy include intrauterine infection with foetal death and abortion, stillbirth, premature labour [33].

Leptospires can be secreted in the milk of lactating mothers, who during the septicemic stage should be regarded as potentially infectious for breast fed infants [33].

11.2.5 Recovery Phase

Most leptospirosis patients recover completely with appropriate supportive care. Patients with severe renal failure, who require dialysis usually recover their renal function by the 21st day [79].

Mild renal impairment may persist, and some patients may suffer from chronic post leptospirosis signs ; in a recent study of laboratory-confirmed leptospirosis patients in the Netherlands, 30% of patients experienced persistent complaints after severe leptospirosis (PCAC) characterized by fatigue, myalgia, malaise, headache and weakness. Of patients with PCAC, 21% described that their complaints lasted for more than 24 months [3].

Headache may persist for six weeks, and patients recover from jaundice within 10 weeks [70]. Patients with pulmonary haemorrhage may recover after 11-20 days of hospitalization [84].

11.2.6 Death

Nearly 58900 deaths occur annually due to leptospirosis worldwide [41]. While the highest morbidity occurs among males with 20-29 years of age, the highest mortality occurs in old males with 50-60 years of age [41]. Mortality seems to be negligible in children under 15 years of age [77].

The case fatality rate ranges from 5 to 25% due to severe icteric disease, renal failure and the virulence of the infecting serovar [77]. Therefore, mortality is high in countries where the predominance of serovar Icterohaemorrhagiae (this serovar causes a higher mortality of 13.1% and an increased frequency of jaundice 62.9% compared to other serovars) [77].

12.Prevention and vaccination

12.1 Prevention

Due to large number of serovars, infection sources and the wide differences in transmission conditions, the control of leptospirosis is complicated and depends on the local conditions [44].

Removal of leptospires from the environment is impractical. Prevention can be achieved by avoidance of high-risk exposures, avoiding contact with animal urine and covering wounds with waterproof dressings, controlling animal reservoir populations e.g. rats and dogs, adoption of protective measures, immunization and use of chemoprophylaxis after excess rainfall in local populations in endemic areas [85].

Therefore, the hazards will still exist and minimisation of leptospirosis is the best control for managing risk [86], and avoidance of high-risk exposure still the most effective preventive measure [44].

Leptospires are sensitive to many environmental influences, and although they can survive in the environment for weeks to months under suitable conditions they are rapidly killed by disinfectants and desiccation [44].

12.2 Vaccination

12.2.1 Human Vaccination

Measures for occupational hygiene (such as protective clothing and avoidance of splash from urine or water) are often useful, but they hard to implement because they impede work or are unacceptable to both workers and employers. Therefore, prevention of leptospirosis without vaccination is quite difficult [18].

Although leptospirosis is difficult to eliminate from the environment, vaccines have been used to reduce the risk especially in the workplace and some closed-herd housed situations and individuals in high-risk occupations [86].

One of the first reports of human leptospirosis immunization involved the vaccination of thousands of miners in Japan using a culture derived from *L. interrogans* serovar Icterohaemorrhagiae [3].

Vaccines usually composed of crude antigens consisting of leptospires killed by phenol or formaldehyde that give unfavorable side effects [24].

A number of disadvantages may be observed, including a short duration of efficacy and incomplete protection against all serovars [35].

Vaccines against leptospirosis are serovar specific; therefore, they may only provoke immune responses to the serovars included in the vaccine, and do not provide cross-protective immunity against heterologous leptospiral serovars [36], [44].

Therefore, Protective antibodies are produced only against the serovars included in the vaccine [44]. For this reason, vaccines must contain serovars representative of those present in the population to be immunized [36].

A vaccine containing serovar Icterohaemorrhagiae has been used in employees in high-risk occupations, and vaccines have been developed for human use in some countries such as Cuba, China and Russia [44],[86].

The Pasteur Institute developed a killed, whole-cell vaccine derived from *L. interrogans* serovar Icterohaemorrhagiae and this vaccine was used for Parisian sewer workers. This vaccine is available under the name Spirolept®. The

recommended vaccination involves two booster doses after the initial immunization followed by re-immunization every two years [16].

Although commercial human vaccines have been produced in some countries these vaccines do not induce long-term protection against infection and do not provide cross-protective immunity against heterogenous leptospiral serovars

Although commercial human vaccines have been produced in some countries these vaccines do not induce long-term protection against infection and do not provide cross-protective immunity against heterogenous leptospiral serovars [44].

Due to vaccines may not prevent the infection in humans therefore, infection in humans involves controlling infection in animals [86].

12.2.2 Animal vaccination

Good animal health can reduce the infection of leptospirosis. The majority of human cases of leptospirosis that are acquired from production animals originate from unvaccinated animals; therefore, farmers can vaccinate their stock to protect themselves [86].

Vaccination may prevent the infectious cycle of the disease, and therefore prevention of infection in humans; in New Zealand, about 90% of dairy farmers vaccinate their breeding stock to protect themselves [86].

However, vaccination before infection protects the animal against the infection, infected animals will not stop shedding after vaccination [41].

Vaccination will be most effective if animals are in good health, and young animals must have completed a full vaccination course by the age of 3-6 months, before exposure to infection [44], [86].

Although commercial leptospiral vaccines are available globally for cattle, pigs and dogs, vaccination has showed to be only partially effective, due in part to the potential presence of local serovars others than those included in the vaccines [18].

Animal vaccines are available in developed countries where pigs and cattle are widely immunized, but in developing countries vaccines are not available [18], [36].

Serovars canicola, grippotyphosa, icterohaemorrhagia hardjo and pomona can be found in most bovine and porcine vaccines. Serovars canicola and icterohaemorrhagiae can be found in canine vaccines [36].

A successful vaccination program requires continued epidemiological studies to assess the incidence of different serovars in a given population [18], [36].

13.Laboratory diagnosis and treatment of leptospirosis

Leptospirosis is generally underreported; due to nonspecific symptoms, lack of awareness, challenging diagnostics and poor access to health care [87]. Due to variety of clinical and often "flu like" symptoms, human leptospirosis is often undiagnosed or misdiagnosed as other illnesses with febrile syndromes (such as aseptic meningitis, influenza, hepatic disease and hantavirus infections) [4],[1]. This is also the case in animals, where most cases are difficult to diagnose clinically, due to non-specific or unapparent clinical signs with host-adapted serovars. Therefore, diagnosis of leptospirosis in humans and animals cannot be made without laboratory confirmation [22].

Early diagnosis is essential, because antibiotic treatment is most effective when it is initiated early in the course of the disease. However, adequate laboratory tests for early diagnosis are still lacking [88]. It is not necessary to confirm the diagnosis or wait for the result of the tests before starting treatment. The clinical assessment and epidemiologic history are more important for early recognition and treatment to prevent complications of the severe manifestations and mortality [89].

Leptospirosis of humans and animals is investigated by direct and indirect laboratory methods [90]:

- ✓ Direct methods are detection of the organism or its components in body fluid or tissues; by isolation of the bacteria in cultures, identification in tissue and body fluids (e.g. immunofluorescence staining, immunochemistry, immunoperoxidase staining, silver staining and methods of Polymerase Chain Reaction) [4],[8]. Direct visualization of leptospires in blood or urine by dark field microscopic examination has been used for direct diagnosis [90].
- ✓ Detection of leptospira antibodies (serological diagnosis) is an indirect evidence [33]. These methods are either methods detecting serum antibodies without discriminating on serovars (such as various ELISA tests) or methods

reliably identifying the infecting serovars (such as the Microscopic Agglutination Test) [83].

The collection of appropriate specimens and selection of tests for the diagnosis depends on the timing of collection and the duration of symptoms [36]. In humans, the first phase of the biphasic illness occurs before antibodies develop, and leptospira may be found in the blood and cerebrospinal fluid. Early cases must be diagnosed with tests that detect the organism, its antigens or DNA. However, in many cases leptospirosis is diagnosed by serology (especially the MAT or ELISAs) due to its availability [92]. Diagnostic testing should be requested for patients in whom there is a high index of suspicion for leptospirosis, based either on signs and symptoms or on occupational and recreational activities or environments contaminated with animal urine [93].

Early diagnosis of leptospirosis is critical, due to the risk of severe complications of the disease which requires intense care therapy (such as pancreatitis, lung and intracranial hemorrhages) [94].

14. What kind of sample, what test and when?

The choice for the use of a diagnostic test depends on a number of factors, including its diagnostic accuracy, financial feasibility technical or practical feasibility and the need for an early and/or rapid result [95]. Infection by pathogenic leptospires may be divided in two stages (Figure 4); the first stage is leptospiraemia or the acute phase, which lasts from 3 to 10 days. During this phase, leptospires are be found in blood in a decreasing number until 15 days after the onset of symptoms. It is important that samples must be collected up to 2 days after initiating antibiotherapy to detect leptospires [39]. The chances of detect leptospira or its DNA from blood or other tissues or body fluids is usually high during this stage [33]. However, The short of leptospiremia during the acute phase of the disease can lead to absence of detection of leptospiral antigen or its DNA in confirmed cases of leptospirosis [95].

The second phase (convalescent) or immune stage called leptospiruria, usually occurs during the second week after the onset of symptoms [39]. This stage is be characterized by excretion of the bacteria in the urine and the appearance of antibodies in the blood.Therefore, leptospires are be cleared from the bloodstreams and the titers of IgM class antibodies be increased [16]. Antibodies generally reach maximum levels within two to three weeks, then gradually recede [36]. But may

remain detectable for two to ten years in humans, and for similar periods or for lifetime at low levels in animals (particularly in reservoir hosts) [22].

Generally, during the first week of illness, only PCR from blood samples can be performed. From 6^{th} to 10^{th} day it is possible to perform ELISA IgM if PCR is unavailable (PCR with serological tests was recommended). From the 11^{th} day the PCR should be performed from samples of CSF or urine and also MAT or ELISA IgM can be performed [96]. The definitive diagnostic test is detect leptospires from clinical specimens either by culture which is insensitive and slow, by immunohistochemical staining or by detecting the presence of leptospiral DNA through PCR techniques [97].

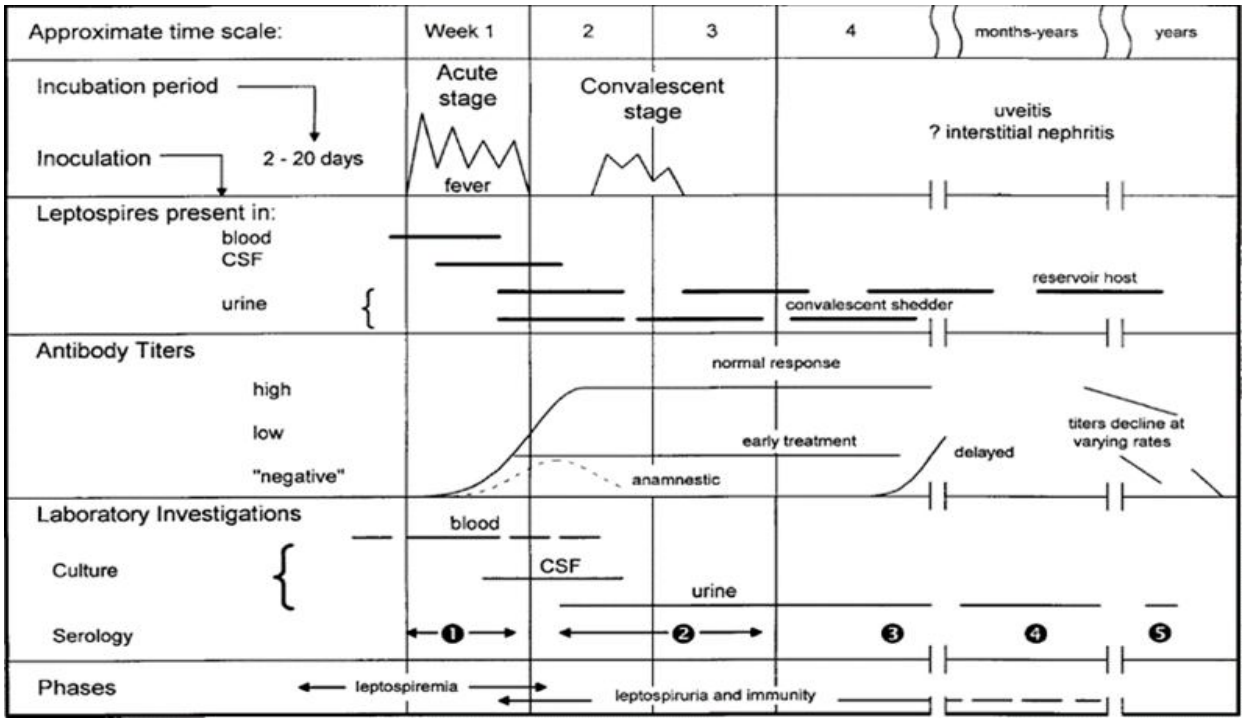

Figure 4: Schematic representation of the biphasic nature of leptospirosis and relevant diagnostic investigations at different stages of disease. This figure was from [1].

15.Leptospirosis diagnosis

15.1 Direct examination for leptospires

15.1.1 Dark field microscopic examination (DFM)

Leptospires may be detected by direct microscopic observation of clinical specimens [88]. Dark field microscopic examination (DFM) of body fluids (e.g. blood, urine, CSF and dialysate fluid), tissues removed for surgical or experimental reasons from animals or necropsy specimens, tissues from carcasses or abortion products can be used to rapidly demonstrate the presence of leptospires [36],[17].

Although this technique has advantages for early diagnosis and it is useful in situations where laboratory resources are limited[88], it has low sensitivity and specificity and provides no information on the infecting serovar [16].

The result of DFM is affected by the timing of sample collection; the bacterial load in blood ranges from 10^2-10^6 Leptospira per millilitre during the first week of illness [95], and approximately 10^4 leptospires/ml is the detection threshold for one cell per field to be visible [88]. Therefore, the concentration is too low to allow the detection by direct microscopy, due to the organism is only present in blood during a short period during the acute phase of the disease, and shedding in urine is intermittent [54]. The risk of false positives is frequent, due to misinterpretation of fibrin or protein threads, cell debris and other artefacts can be high even for experts.

Thus, result of direct examination may be supported by other laboratory irrespective of positive results [88].

However, DFM seems to be inexpensive test, it requires a dark-field microscope which is rarely available in resource-limited settings [95].

To increase the sensitivity of direct microscopic examination in veterinary specimens, a variety of staining methods have been conducted, including immunofluorescence staining [4],[5].

15.1.2 Culture

Leptospires can be isolated from whole blood (within 7 days of onset), cerebrospinal fluid (CSF) (4-10 days from onset), and from urine (after the 7th day and only if inoculated into special media within 2 hours of voiding). Clinical or autopsy specimens (e.g. punch biopsy of kidney) should be submitted fresh or frozen [93].

However, it requires special media, isolation of leptospires from clinical specimens is the strongest evidence for confirmatory the diagnosis, and a definite proof of infection. By culture serovars can be identified and local isolates can be used as antigens in MAT and in vaccine development [33].

Samples for culture must be collected prior to the administration of antibiotics [72], should be stored and transported at ambient temperatures (low temperatures are detrimental to pathogenic leptospires) [98].

Usually, a few drops of blood are be inoculated into several tubes, each containing five ml of a suitable medium. After incubated at 30°C, cultures should be checked regularly for a period of 4–6 months [15]. Before cultures can be discarded as negative, incubation for up to 13 weeks at 30^{o}C with weekly examination by DFM is necessary [18].

Growth of leptospires is often slow on primary isolation, but pure subcultures in liquid media usually grow within 10 to 14 days [36]. Subculture should be made within 48 hours to minimize the inhibitory effect of the selective agents on leptospires [33].

A commonly used medium for culture is Ellinghausen- McCullough-Johnson-Harris medium (EMJH), which is available commercially from several manufacturers. This media contains 1% bovine serum albumin and polysorbate 80 as a source of long-chain fatty acids. Agar may be added at low concentrations (0.1 to 0.2%) and growth on media solidified with agar has been reported [36]. Several liquid media containing rabbit serum were described [36]. Due to EMJH is expensive and technically demanding, it is stocked in few laboratories[22],

In urine samples, fresh midstream urine is collected and inoculated immediately [15]. The highest success rate of culturing from urine is 14-28 days after infection when significant leptospiruria is seen, and the optimum growth pH is 6.8-7.4 [22].

Because the organisms die or lyse rapidly in acid urine, humans urine and acidic animals urine should be processed immediately by centrifugation, and the sediment resuspended with phosphate-buffered saline after wards to neutralize the pH [4],[1].

Intermittent shedding of bacteria in both humans and animals and problems with contamination may lead to poor sensitivity in culture from urine [99].

Leptospira spp. stain poorly with the Gram stain and may not be observed by microscopy, unless special stains or methods are employed. Silver staining or immune gold-silver staining is sometimes useful as an adjunct technique to detect Leptospira [13].

Culture provides valuable information and material, it is only positive in a minority of cases, and it is rarely performed in routine clinical practice, since this may take several months and requires considerable expertise [100].

Although, culture is not considered useful as a routine test for diagnosis of individual patients, it remains important for epidemiological purposes and definitive [26],[24].

15.1.3 Polymerase Chain Reaction (PCR)

Isolation of leptospires from clinical specimens requires several weeks for growth, and current serological tests exhibit low sensitivity at the acute phase which limits their contribution to early diagnosis [88]. To overcome all these limitations polymerase chain reaction (PCR) has been used and was considered sensitive and specific for the rapid detection of Leptospira in clinical samples. Evaluation of PCR based studies for the identification and as a rapid detection of Leptospira DNA, has been reported elsewhere, either in humans or in animals [13],[28],[29],[30].

PCR detects DNA in blood during the first 5-10 days after the onset of the disease, and allows detection of leptospires in culture negative blood if the patient has received an effective antimicrobial drug but have not cleared nonviable organism [72]. Leptospiral DNA has been amplified from serum, urine, aqueous humor, CSF, water and a number of organs post mortem [31],[32], depending on primer sets and disease stage at the time of analysis [105].

The main advantage of PCR is confirming the diagnosis during the early acute stage (leptospiremic) of the illness before the appearance of immunoglobulin M (IgM) antibodies, when treatment is likely to have the greatest benefit [90].

Therefore, both conventional and real-time PCR are useful for early diagnosis and should be made available for clinicians for the early diagnosis and prompt treatment of the disease [13],[5].

To improve the sensitivity of the diagnosis PCR was recommend to be used in combination with serological tests [105].

PCR assay can differentiate between pathogenic and non-pathogenic species [97], and have a higher specificity than conventional methods, such as culture and dark-field microscopy [104]. Real-time PCR (either using SYBR Green or Taqman technology) has the advantage that it gives a result much faster than conventional methods and is less prone to contamination [95].

Although, PCR has high specificity and sensitivity with the capability of detecting as few as 10 organisms in a sample, its sensitivity declines over the course of the disease [5],[25]. As well as, leptospires antigens or DNA may not be detected in blood in some cases of leptospirosis; maybe because of a weak or short leptospiremia during the acute stage, late of sampling or because of antibiotic administration which rapidly eliminates the bacteria from blood [39].

A limitation of PCR is the current inability to identify the infecting serovar (while this is not significant for individual patient management) [3].

15.2 Serological diagnosis (Indirect Methods)

Serologic methods are largely have been used, and most cases of leptospirosis are diagnosed serologically, due to the lower probability of leptospires detection in blood [74],[106],[35]. Using serum or plasma for the serological tests gives equivalent results, however, serum should be used preferentially [39]. Antibodies may be detected in the blood 5-7 days after the onset of symptoms [36], and due to reactions persists for months or years, persistent antibodies allow retrospective diagnosis [74].

IgM antibodies start appearing early in the course of the disease and reach detectable levels within one week or as early as on third or fourth day of illness, however, rarely IgM may persist at low level for several years [33]. IgM antibodies decline slowly over months and become undetectable within six months (Figure 5) [33].

IgG antibodies appear later than IgM and reach peak level after few weeks of illness and may persist at low level for years [33].

Enzyme-linked immunosorbent assay (ELISA) and microscopic agglutination test (MAT) are the most commonly available serological tests. However, MAT is the definitive serological investigation in leptospirosis [38],[1].

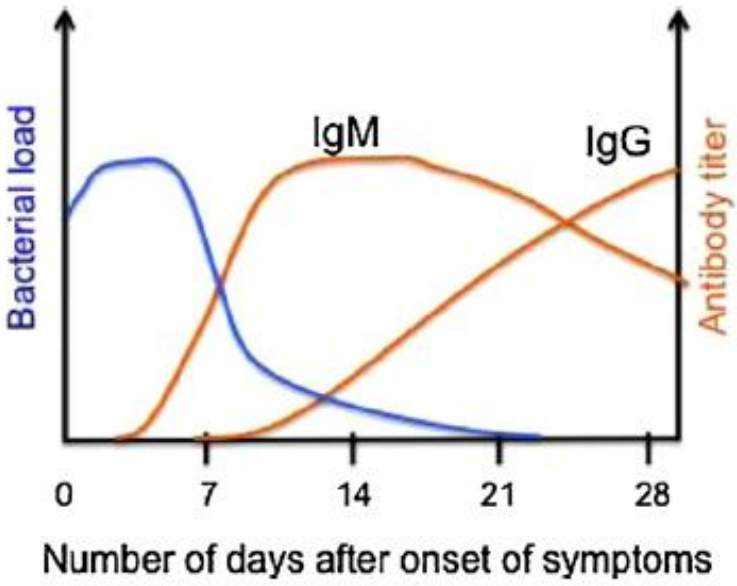

Figure 5: IgM and IgG antibodies, figure from [39].

15.2.1 Microscopic Agglutination Test (MAT)

MAT or Martin and Pettit test was developed almost one century ago at the Pasteur Institute after the first isolation of Leptospira. It remains the gold standard for the serological diagnosis of leptospirosis, which provides an indication of the serovar or serogroup responsible for an infection [15,35].

The test remains the corner stone of sero-diagnosis of this disease and a helpful tool in understanding the epidemiology of leptospirosis [54]. MAT is the most appropriate test to employ in epidemiological sero surveys; as it can be applied to sera from any animal species and the range of antigens used can be expanded or decreased as required [36]. It has been used as the test of choice in outbreaks and sporadic cases, and has also been useful in retrospective studies for confirming and identifying the prevalent serovar [37].

Due to MAT can detect both class M and G antibodies, it cannot differentiate between current or past infections [72]. Its sensitivity is 41% during the 1st week, 82% during

the 2nd to 4th week and the sensitivity can be 96% beyond the 4th week of illness [72]. However, at the acute phase this sensitivity is low compared to ELISA and slide agglutination test (SAT) [14]. But the test have superior specificity, because the presence of heterologous antibodies is not interfering in the results [83].

MAT require expertise pathogen containment level 2 (PC2) laboratory safety and also it is time-consuming, due to live Leptospira culture used as the antigen [22],[87]. In this test, serial dilutions of serum should be incubated with cultures of specific Leptospira serovars (antigen). After incubation, the serum/antigen mixtures are examined by dark field microscopy for agglutination and to determine the titers. A serum is considered as positive if at least 50% of leptospires are agglutinated compared to a control antigen without serum [15],[1].

The range of antigens used should include serovars representative of all serogroups, also all locally common serovars and panels of live leptospires belonging to different serovars must be maintained in the laboratory [1],[21]. At the Leptospirosis National Reference Center in Pasteur Institute, twenty-four strains have been used including non-pathogenic strain *Leptospira biflexa* strain Patoc1 which has for particularity to cross-react with several antigens of pathogenic serogroups. A smaller panel of antigens may lead to non-detection of serogroups not presents in the panel and gave false negative, according to French guidelines for biological procedures the test should be performed with a minimum of nine antigens [15],[21].

MAT is a complex test to control, perform and interpret, interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups especially in acute-phase samples [3]. As well as the repeated weekly subculture of large numbers of strains presents hazards for laboratory workers and laboratory-acquired infections may occur. In addition, because of its sensitivity is low during the acute phase, it is ideally performed on paired sera samples (acute and convalescent), which is difficult in actual practice [36].

Many laboratories and hospitals do not have the facilities required to perform the MAT [35], therefore, it is restricted to a few reference laboratories, and a number of rapid screening tests for antibody detection in acute infection have been developed [5],[41].

15.2.1.1 Titers of the MAT

The standard criterion for a positive MAT are a fourfold increase between paired sera in antibody titer (between acute and convalescent sera samples) or titer of 400-800 or more in a single sera [97],[108]. In endemic areas, a titer of 1/100 or 1/200 is considered low, while high titer is usually >1/400 (some consider 1/800 or 1/1600 as diagnostic criteria). In non-endemic areas, 1/100 titer is taken as diagnostic criteria [109]. The titer cut-off of 1 :48 is recommended to determine exposure to leptospires but not for clinical disease [87].

15.2.1.2 Interpretation of diagnostic MAT

It is difficult to confirm the acute infection from a single serum sample [88]. The modified Faine's criteria that include ELISA IgM and other rapid tests, along with culture and MAT for diagnosis of leptospirosis is the more practical guideline for Indian institutions (Table 5). If MAT is available as a single test, positive rapid tests plus high titers in MAT can confirm the diagnosis of current leptospirosis, while a negative rapid test with positive MAT might suggest past infection [110].

Table 5: Interpretation of MAT with ELISA and SAT [14].

ELISA/SAT	MAT	Interpretation
+	Single high titer	Current infection
+	-	Current infection
-	Single high titer	Past infection
+ -	Seroconversion/ 4 fold rise in titer	Current infection

In a non-endemic area, any level of antibodies however low may signify leptospirosis, especially if the titer rises in a second specimen taken after 3 to 7 days. If the titer remains below 100 even on repeated testing it may be assumed that it was due to previous leptospirosis, and not to current illness [36],[3].

In endemic areas, the diagnosis will be confirmed if the titer rises on retesting, but will be negated if it is unchanged, assuming that the infecting serovar was included among the antigens for the MAT [74].

15.2.3 Enzyme-linked immunosorbent assay (ELISA)

Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed ; conventional serological methods such as enzyme-linked immunosorbent assay (ELISA) is widely used for the diagnosis of leptospirosis, and several IgM ELISA are available on the market. However pathogenic species have been used, saprophytic strain *L. biflexa* the most strain used [34],[44].

The specificity and the sensitivity of ELISA are quite variable, may due to differences in the studied population (previous exposure to pathogenic or environmental leptospires)[39].

ELISA is easy, safe and can detect IgM and IgG [112], can be used in humans and animals [46],[47], and provides results in 2–4 hours [95]. Therefore, it has been recommended for the rapid diagnosis of leptospirosis in endemic areas [115].Using human sera collected at the acute phase of infection, IgM ELISA is more sensitive than the MAT [116]. ELISA IgM is usually positive from day 6-8 earlier than the MAT [72], allowing the diagnosis to be confirmed and treatment to be initiated while it is likely to be most effective [39]. In addition, it is less expensive than MAT, can be performed in any routine laboratory, does not require trained personnel and IgM and IgG can be detected separately [37]. Therefore, ELISA has been used as alternative test to MAT for screening for leptospiral infection in both humans and animals [22].

Although, patient's serum may be positive 5 days after onset of symptoms, this period may be increased in cases where antibiotic treatment has been initiated [15]. Therefore, when the result of the IgM-ELISA for the first sample is negative and the clinical findings are suggestive or the agglutination test is positive, the second sample for IgM-ELISA should be examined [117].

Maybe, IgM ELISA presents high sensitivity, but its specificity with crude antigens of pathogenic and nonpathogenic leptospires is not always adequate for routine tests; it has been shown that specific antibodies are detectable in patients who have been free of active disease for long periods [105]. Specificity of ELISA is affected by the antigen used in the assay, by the presence of antibodies due to previous exposure (e.g. in endemic regions) and by the presence of other diseases [3]. Finally, ELISA gives

no indication on the infecting serovar/serogroup and it should be confirmed by MAT, PCR or culture [39].

15.2.3 Slide agglutination test (SAT)

Several attempts have been made to simplify and reduce the time, skill and expense required for serological testing [118]. Ideal test should be safe, rapid and simple, has good sensitivity rates and be indicated to the diagnosis of the acute form of leptospirosis [118].SAT test was developed at the first time in 1958 by Galton *et al*. for the diagnosis of leptospirosis [119], Stoenner and Davis have modified the preparation of plate antigens for leptospirosis diagnostic, and reported that this antigen could be used in rapid tests, obtaining similar sensitivities with the MAT in human, porcine and bovine sera [120]. SAT is inexpensive, can be performed more quickly (take less than 5 minutes), more easily than ELISA and MAT, and can be used by the less well-equipped laboratories. It seems to be a convenient test for the initial diagnosis of leptospirosis and presenting high sensitivity at the acute phase of the illness [121].

The main disadvantages of this test are that it is not suitable for epidemiological studies, identification of strains, assessment of the probable infecting serogroup and confirmation of illness for public health surveillance [118]. However, SAT can be performed to provide physicians with preliminary and rapid results [117].

15.2.4 Other serological tests

Other serological tests have been developed; such as complement fixation, indirect fluorescent antibody, sensitized erythrocyte lysis, latex agglutination (LA), macroscopic slide agglutination, microcapsule agglutination, and Patoc slide agglutination [72]. Nevertheless, these tests even if some are marketed, are rarely used and lack specificity or sensitivity [39].

16. Treatment and prophylaxis

Antibiotic therapy should be started as soon as the diagnosis of leptospirosis is suspected, regardless of the phase of the disease or duration of symptoms [89].

The majority of patients especially in endemic areas do not develop a clinical syndrome. Of those with symptoms, 90% present with a flu-like, self-remitting disease that can easily run undetected. Five to nine percent develop a moderate clinical syndrome requiring hospitalisation and 1–5% develop the severe form of leptospirosis usually manifested as the icterohaemorrhagic Weil’s syndrome and

pulmonary haemorrhagic form [122]. If a clinical diagnosis is made, the patient should be treated with doxycycline or penicillin, and the severe form of the disease is best undertaken in hospital [123].

Antibiotic treatment is effective within 7 to 10 days after the infection, and should be given immediately on diagnosis or suspicion [4]. Patient should be observed for evidence of renal failure and treated if necessary with hemodialysis. Patients with Weil's disease having hemorrhagic manifestation may require whole blood or platelet transfusion and patients with severe signs require to be observed in intensive care unit [4].

Usefulness of antimicrobial treatment is controversial ; penicillin and doxycycline are widely used for the treatment in humans. To be effective, treatment should be started early during the acute stage of illness [87]. The drug of choice is benzyl penicillin by injection in the doses of five million units per day for five days, patients who are hypersensitive to penicillin can be given erythromycin 250 mg four times daily for five days. Tetracyclines and Doxycycline are effective but contraindicated in patients with renal insufficiency, in children and pregnant or breast-feeding women [58],[59]. Erythromycin has also been found effective in severe leptospirosis [4]. Injection of Hydrocortisone 100 mg every 8 hourly is also given in severe cases [126]. Antibiotic therapy should be completed for 7 days except for azithromycin dihydrate which could be given for 3 days [89].

Doxycycline (hydrochloride and hyclate) has been used as a chemo prophylactic agent for short time exposure, but it cannot be recommended for routine continuous use or for a long-term occupational exposure [126].

The duration of prophylaxis depends on the degree of exposure and the presence of wounds. Individuals should continue to monitor themselves for fever and other flu-like symptoms and should continue to wear personal protective measures, since antibiotic prophylaxis is not 100% effective (Table 6) [6],[61].

Table 6: Antimicrobial agents recommended for treatment and chemoprophylaxis of leptospirosis [123].

Indication	Compound	Dosage
Chemoprophylaxis	Doxycycline	200 mg orally once-weekly
Treatment of mild leptospirosis	Doxycycline Ampicilline	100 mg twice-daily 500 mg every 6 h
Treatment of moderate to severe leptospirosis	Penicillin G Ceftriaxone	1.5 MU intravenously every 6 h 1g intravenously every 24 h

17.Diagnosis conclusion

Diagnosis of human leptospirosis is very complicated especially at the acute phase of the illness. In this review, the diagnostic tests currently available for leptospirosis were descript. The diagnostic method depends on the course of the disease, therefore, tests used should be chosen carefully ; during the first week PCR, Dark field microscopy and culture from the blood can be used. While serological tests can be used during the second week of the illness, (they may be positive from the 5ed day of the illness). The main problem is during the first week at the acute phase, when low sensitivity. PCR from blood samples has good sensitivity, but it is not available in most laboratories and in developing countries. MAT is the reference test but it is a laborious technique, time consuming and limited to reference laboratories. However, MAT can be used to confirm the diagnosis and to identify the infecting serovar. Positive culture is a definitive test for any disease, but for leptospirosis culture needs 13 weeks to give the result and cannot use for the diagnosis, it can be used in epidemiological investigations and to confirm the diagnosis in retrospective studies. DFM is not recommended, due to its low sensitivity and specificity. Other serological tests such as ELISA IgM, lepto dipstick test and SAT are widely used, due its availability and good sensitivity at the acute phase of the disease, but the second serum should be obtained to confirm the diagnosis if the first serum is negative and the clinical sings were observed.

Chapter 2:
Diagnosis of human Leptospirosis in Morocco by Slide Agglutination test (SAT) and ELISA IgM

1. Objective

To compare ELISA IgM and SAT for the diagnosis of human leptospirosis.

2. Material and Methods

2.1 Patients and serum samples

During 2004 to 2010, fifty sera specimens from 50 patients were referred to the National Institute of Hygiene, Rabat, Morocco (table 7).

A total of 50 sera included in this study; 1 (2%) was from Beni Melal, 21(42%) were from Meknes, 1(2%) was from Agadir, 2(4%) were from Rabat, 2 (4%) were from Taza, 2 (4%) were from Sale, 2(4%) were from Tanger, 9(18%) were from El Jadida and 10 (20%) were from Sidi Kacem.

The majority of cases (21sera) in this study were from Meknes (tables7,8).

Single sera was used and the results of ELISA IgM in this study were compared with the results of SAT conducted previously [127]. Clinicians suspectleptospirosis due to some signs occurred similarto this disease, particularly fever and jaundice. Sera were sent to the laboratory of Bacteriology in the National Institute of Hygiene in Rabat for diagnosis, however, other information such as epidemiology conditions, occupations and the date of sampling were not indicated in all sera.

Table 7 : Number of sera suspected with leptospirosis in different regions in Morocco

Regions	No. of cases
Beni Melal	1
Meknes	21
Agadir	1
Rabat	2
Taza	2
Sale	2
Tanger	2
El Jadida	9
Sidi Kacem	10
Total	50

Table 8 : Number of sera included in this study

Year	No. of sera samples
2004	23
2005	5
2006	1
2007	-
2008	2
2009	8
2010	11
Total	50

2.2 Slide Agglutination test (SAT)

Leptospira antigen purchased from Bio-Rad (Marnes-la-Coquette, France) was used. Test was performed as previously described [118]. Antigenic suspension was homogenized immediately after the use. Volumes of 15µl of each undiluted serum to be tested were added to 55µl of antigenic suspension on a glass slide. After they mixed with sticks, they placed 4 minutes in room temperature, and the agglutination was observed under direct light.

2.3 ELISA IgM

Detection of IgM antibodies was determined using a commercially available leptospira IgM ELISA kit from nal von minden, Germany. Sera and controls were diluted 1:100 and performed according to the manufacturer's instructions. Each sera had an absorbance ratio greater than that of the cutoff calibrator was defined as positive (results in Units [U] = Patient (mean) absorbance value$_x$10/Cut-off). The result considered positive if U›11, negative if U‹9 and if U between 9-11 it is recommended to repeat the test with a fresh sample in 2 to 4 weeks.

3. Results

While 33 sera were positive by Slide Agglutination Test, thirty-one sera were positive by ELISA IgM, (table 9). Positivity of ELISA and SAT were 62% and 66% respectively.

Table 9: Results and the positivity of ELISA IgM and SAT

The test	Positive	Negative	Positivity
ELISA IgM	31	19	62%
SAT	33	17	66%

The number of males was more than females (thirty-one (62٪) were males and 19 (38٪) were females), (table 10).

Table 10: Males and females included in this study

Males (%)	Females (%)
31 (62%)	19 (38%)

4. Discussion

Limitation of ELISA for the diagnostic of leptospirosis was observed [128]; IgM class antibodies may remain detectable for several months or even years particularly in endemic areas, as well as some ELISA test systems are less specific than the MAT, and weak crossreactions due to the presence of other diseases may be observed. Therefore, ELISA results should be confirmed by MAT the gold serological test for leptospirosis [129], or testing a second sample at the convalescent phase for seroconversion or a significant rise in titre should be required in cases with an initial negative result [130].

Although all limitation, ELISA IgM can be used in the first week and its sensitivity seems to be higher than the MAT (the international standard). However, the sensitivity will increased in the second week (table 11) when the IgM antibodies have had time to develop[121].

Usually, nonpathogenic *Leptospira biflexa* serovar Patoc (strain Patoc I) used as antigen; it has been indicated that sera from patients with leptospirosis cross-reacted with antigens from this nonpathogenic strain [88]. However, antigens isolated from one or preferably multiple locally dominant pathogenic species, could improve sensitivity and specificity of the assay instead of the standard bacterial isolates [131].

Another ELISA contained mixture of some antigens was used by Angela P. Brandão et al [121]. who indicated that the sensitivity of SAT and ELISA IgM in the first week were 57% and 53% respectively comparing with 34% for MAT the gold standard serodiagnostic method. While in the second week, the sensitivity for both SAT and ELISA IgM were 83%. Due to our study is a retrospective study, we cannot obtain another sera, as well as the time of sampling was not considered by the clinicians from the past and at the time of this article.

In our study, males had high incidence than females, this consisted with other studies indicated that adults and males had a greater risk for leptospirosis than children and females, related to the effect of occupation factor [16].

SAT is easy, safe, inexpensive, very convenient for developing countries and less-equipped laboratories [121],[117],[128],[132], and also can be used for human and animal leptospirosis [118]. Although, sensitivity of SAT seems to be more than ELISA IgM at the acute phase [117],[121].

PCR was recommended to use in combination with serological tests [133]. PCR can detect leptospirosis from blood samples during the first week and had more sensitivity and considered that PCR based diagnosis of leptospirosis should be made available for clinicians for the early diagnosis and prompt treatment of the disease [94].

According to our results, the highest incidence was observed in 2004, due to leptospirosis outbreak occurred in Meknes region, seventy seven cases of human leptospirosis were declared by the Ministry of Health [134],[135], out of them 23 sera were sent to the National Institute of Hygiene in Rabat. All of eight cases reported in 2009 were from El Jadida, Haraji, 2011 indicated an outbreak occurred in 2009 in this region [136]. Of 11 cases in 2010, ten of them were from Sidi Qacem and only one was from El Jadida. High incidence in Sidi Qacem was reported, however the epidemiological information is unavailable [137].

Table 11: Sensitivity of ELISA IgM and SAT according to other studies

Duration of illness in days	Sensitivity		Reference
	ELISA %	SAT%	
2-3 4-5 6-7	28 54 77.8	ND	[138]
0-6 7-14 15-60 4-5mo 6-8mo	53 83 99 92 74	57 83 99 50 17	[121]
ND	63.6	91	[127]
3-8 9-14 >14	79.3 100 88.8	72.4 100 77.7	[133]
2-7 8-13 14-19	62.1 91.7 100	ND	[132]

ND, not determined

5. Conclusion

Leptospirosis may become positives by serological tests after the 5th day. At this stage the diagnosis can be done according to clinical and epidemiological criteria or PCR if it is available. PCR is expensive and unavailable in developing countries where are the most cases. ELISA and SAT can be used for human leptospirosis; these techniques can measure IgM antibodies and become positive by the 5th day. They seem to be useful for current infection and require one sample comparing to MAT, which need two samples for the diagnosis. However, the second sample should be obtained if the first was negative and clinical signs occurred.

In this study, although clinical manifestations were observed, poor positivity was noted by ELISA IgM and SAT (however SAT was higher slightly than ELISA IgM), might due to one serum used, or other factors such as patients may treated before the raise of antibodies or they were under medication.

We strongly recommend the clinicians, if the first serum is negative by serological tests (ELISA IgM and SAT), the second serum in the second week of the illness

should be obtained, or conduct the diagnosis in combination with PCR and serological tests.

6. Limitations

1- No availability to compare our results with gold serological test (MAT).

2- No possibility to obtain the second serum at the convalescent phase.

3- Date of sampling was not indicated by clinicians.

4- Epidemiology factors and occupations were not considered in all sera referred.

Chapter 3:

First real time PCR in Morocco for human leptospirosis using TaqMan probs Targeting the LipL32 Gene

1. Objective

The aim of this study is to evaluate qPCR as a diagnostic method for human leptospirosis at The National Institute of Hygiene, Rabat

2. Methods

2.1 Sera from patients suspected with leptospirosis

Sixty-seven single sera related to 67 patients from different regions during 2004 to 2016 were used in this study (table 15). All sera were referred to The National Institute of Hygiene in Rabat, Morocco for routine diagnosis and confirmation. The main symptoms reported were icterus, abdominal, myalgia, and fever. The clinicians did not consider epidemiological information and time of sampling of patients included in this study.

2.2 DNA extraction

A commercial kit QIAamp DNA Mini Kits, QIAGEN, Germany, was used to extract the DNA. Total DNA was extracted using 200µl of patient's sera and was eluted in a final volume of 200µl as described previously [139],[140], and according to manufacturer's instructions as follows:

20µl Proteinase K were mixed with 200µl lysis buffer and 200µl sera sample (if the sample volume is less than 200µl, appropriate volume of PBS was added). 200µl ethanol (96–100%) were added and were centrifuged at 6000 x g (8000 rpm) for 1 min. AW1 and AW2 buffers were added and were centrifuged at 6000 x g (8000 rpm) for 1 min and at full speed (20,000 x g; 14,000 rpm) for 3 min respectively. Finally, DNA was eluted with 150µl of AE buffer and then were centrifuged at 6000 x g (8000 rpm) for 1 min. Eluted DNAs were stored at -20°C until use.

2.3 Primer designs

Forward and reverse primers and the TaqMan probe were designed to amplify *lipL32* sequences from pathogenic Leptospira serovars. Genesig Standard Kit for leptospirosis qPCR from Genesig Company was used. GenBank species included in this kit can be shown in the appendix1. Forward and revers primers sequences are not provided by the company.

2.4 Real Time PCR (qPCR)

Real-time quantitative PCR was performed on the CFX96 real-time PCR detection system (Bio-Rad, USA). The amplification mixture consisted of 10µl mastermixe, one µl primer/probe mix, four µl RNAse/DNAse free water and 5µl template DNA in a total volume of 20µl. For DNA amplification, the program was used according to the manufacturer's instructions (appendix 2).

2.5 ELISA IgM and IgG

IgM and IgG antibodies were determined by a commercially leptospira IgM ELISA kit and leptospira IgG ELISA kit from nal von Minden, Germany. All sera and controls were diluted 1:100 and were conducted according to the manufacturer's instructions and as previously reported [141].

2.6 Slide agglutination test (SAT)

Leptospira antigen purchased from Bio-Rad (Marnes-la-Coquette, France) was used according to the manufacturer's instructions and as previously descript [127],[118]; Antigenic suspension was homogenized and volumes of 15µl of each undiluted sera were added to antigenic suspension on a glass slide, and the agglutination was observed under direct light during four minutes.

3. Results

3.1 ELISA IgM and SAT

Of 67 sera included, thirty-nine (58.2%) and forty-eight (71.6%) were positive by ELISA IgM and SAT respectively (table 12). Of them, sixty-one sera were diagnosed previously by SAT and ELISA IgM [127],[137],[141].

Table 12: Positivity of SAT and ELISA IgM in patients suspected with leptospirosis

Number of serum samples	**No. (%) of sera positive by the following:**	
67	SAT	ELISA IgM
	48 (71.6)	39 (58.2)

3.2 ELISA IgG

Of 17 sera subjected to ELISA IgG, only one serum had positive result (table 13). The positive serum by ELISA IgG was also positive by ELISA IgM and SAT, and had negative result by qPCR.

Table 13: Positivity of ELISA IgG in patients suspected with leptospirosis

Number of serum samples	**No. (%) of sera positive by the following:**			
17	SAT	ELISA IgM	ELISA IgG	Real-time PCR
	15(88.24)	10(58.82)	1(5.88)	3(17.64)

3.3 qPCR

Thirty-six sera were subjected to qPCR, and only 3 sera had positive results (table 14 and figure 6). Positivity of PCR was 8.33% comparing to SAT (41.66%) and ELISA (27.77%), Of 36 sera tested by qPCR, nineteen sera had negative results by ELISA IgM and SAT [141],[137]. All negative sera by ELISA IgM and SAT were also negative by qPCR, and the three positive sera by PCR were positive by ELISA IgM and SAT.

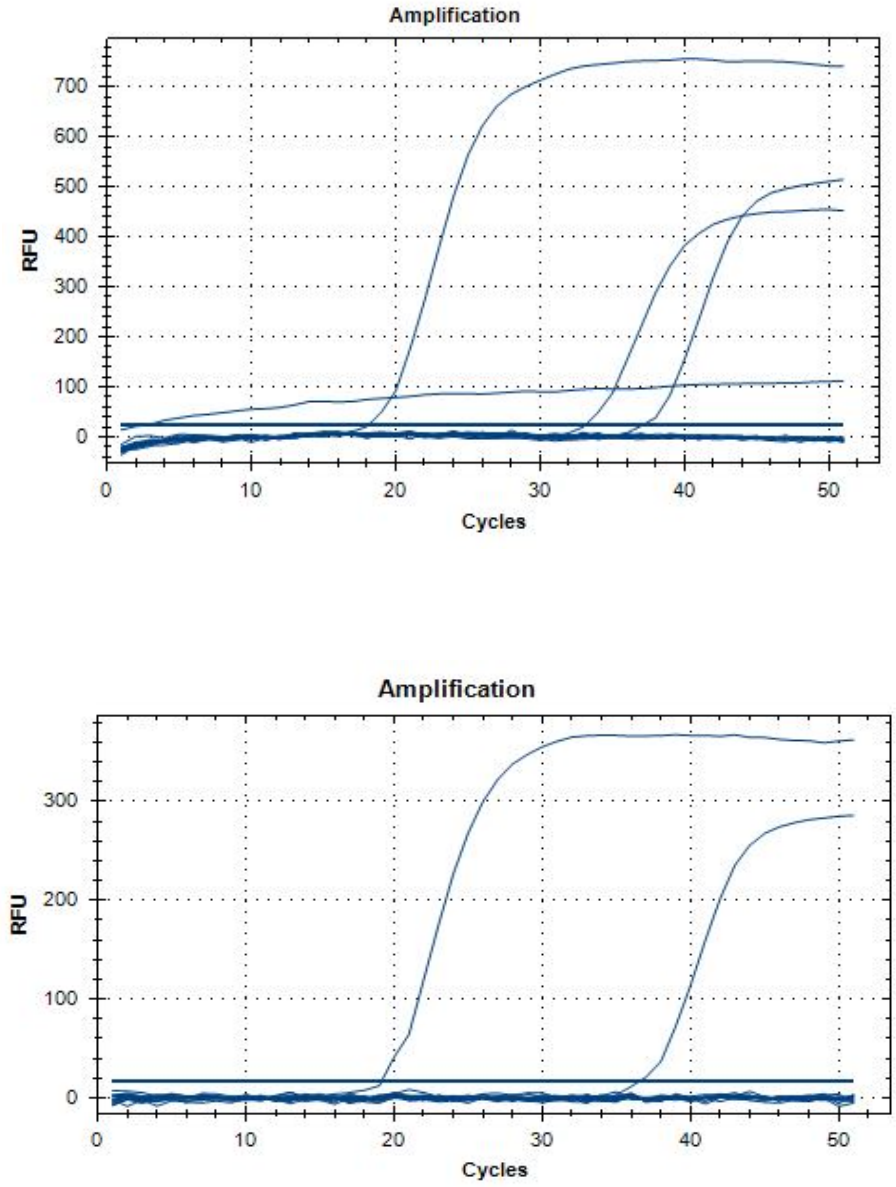

Figure 6: Detection of *lipL32* gene through DNA extracted from patients sera samples

Table 14: Positivity of SAT, ELISA IgM and qPCR

Sera	**No. (%) of sera positive by the following:**		
36	SAT	ELISA IgM	PCR real time
	15(41.66)	10(27.77)	3(8.33)

3.4 Geographical distribution of cases

During 2004 to 2016, sera were sent from nine regions (figure 7), high incidences were observed in Sidi Kacem followed by Meknes (table 15). During this period, sera related to males were 46 (68.68%) comparing to 21 (31.34%) related to females.

Figure 7: Geographical distribution of cases included in this study

(photo from Google Maps)

Table 15: Cases of suspected patients with leptospirosis during 2004 to 2016

Region	Beni Mellal	Meknes	Agadir	Rabat	Taza	Sala	Tanger	El Jadida	Sidi Kacem
Number (%)	1(2%)	21(31%)	1(2%)	2(3%)	2(3%)	2(3%)	2(3%)	9(13%)	27(40%)

4. Discussion

Since 2004, ELISA IgM and SAT have been routinely used in our laboratory for the diagnosis of human leptospirosis [141]. However, the sensitivity of serological tests increased in the second week of the illness [121], serological methods still have used due to its facility, sensitivity and availability [117].

SAT and ELISA IgM seem to be more superior at the acute phase than MAT the reference serological method [121],[117], and they were suggested for the diagnosis of leptospirosis [121],[133].

PCR has more sensitivity at the first days of illness; due to its ability to detect 2-20 genomic of leptospires from serum and 10 genomic from urine [142],[140]. However, low positivity during the course of the disease was observed [133],[142],[11],[143].

In Morocco, MAT was reported for the diagnosis [8], however the test is not available in Morocco and was conducted in the Pasteur Institute of Paris [8]. Real-time PCR was reported in animal leptospirosis in one study [144]. The laboratory of Bacteriology at The National Institute in Rabat is the first laboratory in Morocco has the ability to conduct real-time PCR for human leptospirosis.

Positivity of SAT and ELISA IgM for all 67 sera were 48 (71.6%) and 39 (58.2%) respectively. Although, unavailability to conduct the reference serology test (MAT), ELISA IgM and SAT can detect leptospirosis at the acute and convalescent stages, and these tests have high sensitivity as reported [121],[11],[117],[145],[146]. Therefore, we suggest that patients who had negative results by ELISA IgM and SAT might had not leptospirosis.

Claudia de Abreu Fonseca et al. indicated that SAT and ELISA IgM have adequate sensitivity at the acute phase, moreover SAT can detect leptospira antibodies before ELISA IgM [133]. Brandão a P et al. reported that positivity of SAT, ELISA IgM and MAT at the acute phase were 57%, 53% and 34% respectively, however the positivity reach 99% in all tests from the 15^{th} day of illness [121].

ELISA IgG had the lowest positivity (5.88%) comparing with SAT (88.24%), ELISA IgM (58.82%) and real-time PCR (8.33%). The lowest positivity of ELISA IgG might due to sampling conducted before development of IgG antibodies. P. Cumberland et al. reported that IgG antibodies appeared after IgM, and maximum titers of IgG can be detected at convalescent phase of illness [147].

In our study, of 36 sera subjected to qPCR only 3 sera had positive results. Low positivity was observed in qPCR comparing with ELISA IgM and SAT. Although, the date of sampling was not reported by the clinicians, sera that were negative by qPCR and positive by ELISA IgM and SAT might due to late of sampling conducted after leptospires were cleared from the blood. Khalid Perwez et al. reported that PCR was superior at the first week of illness comparing to ELISA IgM, but low positivity was observed from the 8^{th} day, and during 13-15 day ELISA IgM was superior comparing to PCR [146].

Claudia de Abreu Fonseca et al. have compared PCR with ELISA IgM, and reported that ELISA IgM had more sensitivity, however PCR was most sensitive in initial sera samples presenting no specific antibodies detectable by any of the serological methods tested [133]. Therefore, it was suggested that the positivity of the diagnosis increased when PCR was used with serological tests [145],[133].

In addition to late of sampling, several reasons may lead to low positivity in PCR; such as substances (e.g. urea, creatinine and haemoglobin derivates) which may inhibit DNA amplification with leptospiral primers [133]. As well as, absence of the organisms in blood [12], the microbial counts that were low to be detected which may be about five cells [12],[143], and degradation of DNA during prolonged sample storage, also if samples were thawed more times [12],[48].

Moreover, using serum for the diagnosis may lead to less positivity; Uraiwan Kositanont et al. observed that the positive rate for DNA detection in buffy coat (peripheral white blood cells) was higher comparing with plasma and serum [148]. Robyn A. Stoddard et al. also indicated the same findings [142]. For this reason, serum seems to be not optimal specimen for real-time PCR [142], however when comparing with blood, serum gave high positivity than whole blood [48],[143].

Our findings revealed that males had higher leptospirosis than female, this may due to occupation factor [11],[1]. Majority of cases were observed in Sidi Qacem region followed by Meknes; the high incidence in Sidi Qacem was indicated [141],[137], and

high incidences in Meknes region was related to outbreak occurred in 2004 as it reported previously [141],[136].

Due to limitations of resources, blood cultures were not performed on patient's samples and MAT therefore, we were unable to assess the sensitivity of the tests used in this study. Other limitations should be recognized such as time of sampling, second sample and infecting serovars. We recommend to conduct the diagnosis from buffy coat and urine specimens; urine is a useful sample for the testing of leptospirosis because the bacteria are only present in the blood for about the first week after onset of symptoms, but they can be detected in urine for several weeks [1]. As well as multiplex PCR (mPCR) should be considered when it will be available; using two sets of primers in mPCR can increase the sensitivity and specificity of the test [149].

5. Conclusion

Our study consists with other studies that PCR is not useful for the diagnosis during the course of leptospirosis, and ELISA IgG cannot be used for early diagnosis. ELISA IgM and SAT are useful, do not need high experiences, rapid, as well as their availability in low-income countries and in less well-equipped laboratories, and seem to be useful for the diagnosis of human leptospirosis.

6. Appendix

AM937000.1	DQ286418.1	AY776294.1	AY609332.1	AY609329.1	AY609327.1
AY609325.1	AY609324.1	AY609321.1	AY461909.1	AY461908.1	AY461907.1
AY461905.1	AY461903.1	AY461902.1	AY568679.1	AY442332.1	AB094433.2
AE016823.1	DQ343231.1	AY776293.1	AY609333.1	AY609328.1	AY609326.1
AY461920.1	AY461910.1	AY461906.1	AY461904.1	AY461901.1	AY423075.1
DQ092412.1	AF181553.1	AJ580493.1	AB094437.2	AB094434.2	AY223718.1
AY609323.1	AY461919.1	AY461918.1	AY461917.1	AY461916.1	AY461915.1
AY461914.1	AY461913.1	AY461912.1	AY461911.1	AF181556.1	AF121192.1
CP000350.1	CP000348.1	DQ286417.1	AY609331.1	AY461930.1	AY461899.1
AY461895.1	AY461893.1	AF181554.1	EU526390.1	EU526389.1	EU293442.1
EU293441.1	DQ320625.1	DQ286415.1	AY609330.1	AY609322.1	AY461929.1
AY461900.1	AY461898.1	AY461897.1	AY461896.1	AY568680.1	DQ286416.1
AY461894.1	AY776292.1	AY461928.1	AY461927.1	AY461926.1	AY763509.1
AY461925.1	AY461923.1	AY461922.1	AY461921.1	AF181555.1	

Appendix 1: GenBank accession numbers of *Leptospira* serovars used in the design of the primers and probe of the *LipL32* qPCR

	Step	Time	Temp
Cycling x50	Enzyme activation	2 mins	95 °C
	Denaturation	10 Secs	95 °C
	Data collection	60 Secs	60 °C

Appendix 2: Amplification protocol for qPCR used in this study

7. Acknowledgments

We would like to thank Dr. Mohamed Benhafid from the department of Virology at The National Institute of Hygiene, Rabat for providing the kit of DNA extraction (QIAamp DNA Mini Kits).

General conclusion

1- Serological tests have good positivity for the diagnosis of human leptospirosis.

2- SAT is easy, safe, fast, and cheaper as well as can be used in Morocco and in less equipped laboratories.

3- ELISA has advantages that it can detect IgM or IgG.

4- qPCR has less positivity comparing with serological tests in this study.

Recommendations

1- Date of sampling and epidemiological information should be considered for the diagnosis of human leptospirosis.

2- If the date of sampling was not indicated, the diagnosis should be conducted by PCR and serological tests.

3- The second serum during the second week of illness (convalescent phase) should be recognized and tested especially if the first one was negative.

4- Survey study in endemic areas (e.g. Sidi Qacem)should be conducted to investigate the disease in humans and animals.

5- MAT and culture should be used to assess the sensitivity of tests that have used in this study, and comparing our results by MAT and culture if they will be available(stored DNA samples used in this study are available for this purpose).

6- Study the prevalence of infection in different occupational groups in Morocco e.g. abattoir workers, fishers, miners, sewage workers and farmers.

7- Urine samples from patients suspected with leptospirosis should be considered in future studies and for the diagnosis by real time PCR.

8- Identification of emerging serovars in each region to provide information for vaccines against leptospirosis in Morocco.

9- Investigation of the possible presence of leptospirosis in patients who have unknown fever and patients with renal failure in different dialysis centers in Morocco.

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