Leptospirosis in the Seychelles : geographic, molecular and epidemiological investigations of a zoonotic disease in a tropical insular environment

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Leptospirosis in the Seychelles: geographic, molecular and epidemiological investigations of a zoonotic disease in a tropical insular environment

La leptospirose aux Seychelles: investigation d'une maladie zoonotique en environnement insulaire tropical par des approches géographique, moléculaire et épidémiologique

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Table of Contents

List of Abbreviations

CFR - Case Fatality Rate

CSF – Cerebrospinal fluid

DALY – Disability-Adjusted Life Year

EID(s) – Emerging Infectious Disease(s)

ELISA – Enzyme-linked immunosorbent assay

EMJH – Ellinghausen and McCullough Johnson and Harris medium

HIV/AIDS – Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome

IHA – Indirect Haemagglutination Assay

IHC – Immunohistochemistry

LAMP – Loop-mediated isothermal Amplification

LPS – Lipopolysaccharide

MALDI-ToF MS – Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

MAT – Microscopic Agglutination Test

MERS – Middle Eastern Respiratory Syndrome

MLST – Multiple Locus Sequence Typing

MoU – Memorandum of Understanding

PCR – Polymerase Chain Reaction

PFGE – Pulsed- Field Gel Electrophoresis

PNLP - Programme National de Lutte contre le Paludisme in the Comoros

POC – Point-of-Care

RDT – Rapid Diagnostic test

RN – *Rattus norvegicus*

RR – *Rattus rattus*

rRNA – ribosomal RNA

RVF – Rift Valley Fever

SAA – Seychelles Agricultural Agency

SARS-CoV – Severe Acute Respiratory Syndrome novel Coronavirus

SIDS – Small Island Developing States

SPDL – Soil and Plant Diagnostic Laboratory

SPHL – Seychelles Public Health Laboratory

VNTR – Variable Number Tandem Repeat

WGS – Whole Genome Sequencing

WHO – World Health Organisation

List of Figures

List of Tables

List of Annexes

Abstract

Leptospirosis is an emerging neglected disease representing a heavy burden in the tropics, especially in tropical islands such as Seychelles, which record among the highest human incidence worldwide. This thesis aims at exploring the eco-epidemiology of leptospirosis in Seychelles by (i) using rats as markers of environmental exposure to *Leptospira* infection, (ii) describing the molecular epidemiology of the disease in humans and animals in a *One Health* framework, and (iii) identifying occupational and behavioural risk factors while comparing the current situation to that described 25 years ago. The combination of fine spatial distribution, molecular and clinical epidemiology complement each other in providing a comprehensive picture of the continuum involving reservoirs and human hosts within a shared environment.

Habitat fragmentation and proximity to nutritional sources are found good predictors of *Leptospira*-laden *Rattus* spp. Geospatial analyses determined a selection of other important variable factors that are strongly correlated with *Leptospira* infection in *Rattus* spp., including altitude or distance to surface water (negative correlation), urbanization and heavy rainfall (positive correlation). Results of these analyses can guide policy makers and especially urban planners to best implement landscape structures for conservation or pest control goals leading to reduced exposure of humans to rat-borne diseases.

Rattus norvegicus is found significantly more infected than *Rattus rattus*. Therefore, increased infection in urbanized/fragmented habitats may result at least in part from *Rattus* spp distribution, as *R. norvegicus* is mostly found in urban areas. Most importantly, genotyping of *Leptospira* in human acute cases and rats suggests that these rodents are involved in only a third of human acute infections, while most human cases originate from yet to be identified reservoir(s).

An annual incidence of 54.6 (95% CI 40.7-71.8) per 100,000 confirms the major medical and public health importance of the disease in the country. The disease affects mainly men (96%) and displays a case fatality rate of 11.2%, mostly associated with severe forms (acute renal failure, hepatic failure and pulmonary haemorrhage). Farming and gardening related activities, proximity to cattle and cats, thrombocytopaenia, leukocytosis, elevated bilirubin and high values for renal function tests are predictors of leptospirosis. The geographical distribution of human cases poorly overlaps districts of high prevalence in rats in keeping with a restricted role of rats in human disease.

The comparison of figures reported herein and in previous studies published 25 years ago reveals changes in behaviour and exposure, and shows that the development of health care has lowered the case fatality despite still high disease incidence in the country. A low level of knowledge on leptospirosis is reported, urging the need for implementing health education campaigns. Altogether, the data presented in this thesis strongly supports the implementation of a research program aiming at discovering alternative reservoir(s) to provide a full understanding of the epidemiological situation, which will allow fine tuning preventive measures for an efficient control of a disease that is still recognised as the infectious disease causing the highest mortality in the country.

Résumé.

La leptospirose est une maladie négligée émergente touchant plus particulièrement les régions tropicales, et plus encore les îles tropicales telles que les Seychelles, qui enregistrent des incidences humaines parmi les plus élevées au monde. Cette thèse a pour objectifs d'explorer l'éco épidémiologie de la leptospirose aux Seychelles (i) en utilisant les rats comme marqueurs d'exposition environnementale, (ii) en décrivant l'épidémiologie moléculaire de la maladie chez l'homme et l'animal à travers une approche "One Health", et enfin (iii) en identifiant les comportements et professions à risque tout en en comparant la situation actuelle à celle décrite il y a 25 ans. La combinaison d'approches géographique, moléculaire et clinique vise à dresser un tableau complet de la situation épidémiologique de cette maladie aux Seychelles en intégrant les réservoirs animaux, l'homme et l'environnement qu'ils occupent.

La fragmentation de l'habitat et la proximité de ressources alimentaires apparaissent comme de bons prédicteurs d'infection chez les rats. Les analyses géo-spatiales permettent de mettre en évidence d'autres variables corrélées négativement (altitude ou distance à un point d'eau douce) ou positivement (niveau d'urbanisation, pluviométrie) au statut d'infection chez les rats. Ces résultats pourraient être pris en compte dans les politiques d'aménagement du territoire mises en place dans des buts de conservation des habitats ou de contrôle des rongeurs, afin de réduire l'exposition de l'homme à des pathogènes maintenus dans l'environnement par les rats.

Si le niveau d'urbanisation est positivement corrélé avec le statut d'infection, ce patron pourrait au moins en partie résulter de la distribution des deux espèces *Rattus norvegicus* et *Rattus rattus*. En effet la première espèce, retrouvée essentiellement en milieu urbain, est nettement plus infectée que la deuxième que l'on retrouve partout sur l'île. Néanmoins, la comparaison des leptospires retrouvés chez les rats et chez les cas humains graves indique que les rats ne sont impliqués que dans un tiers des transmissions à l'homme, la majorité des cas humains étant causée par des leptospires dont le(s) réservoir(s) reste(nt) à identifier.

Une incidence annuelle de 54,6 (95% IC 40,7-71,8) pour 100 000 habitants confirme l'importance médicale majeure de cette maladie dans le pays. La maladie touche très majoritairement les hommes (96%) et présente un taux de mortalité élevé (11,2%), essentiellement associé à des formes sévères (dysfonctions rénales et hépatiques, hémorragie pulmonaire). Les activités agricoles et le jardinage, la proximité d'élevages et de chats, une thrombocytopénie, une leucocytose, un taux de bilirubine élevé et des valeurs élevées aux tests de fonction rénale sont de bons prédicteurs de leptospirose. La distribution géographique des cas humains ne correspond pas

à celle des districts hébergeant des populations de rats aux prévalences d'infection élevées, en cohérence avec un rôle restreint des rats dans la leptospirose humaine.

La comparaison des données présentées ici avec celles publiées il y a 25 ans révèle un changement dans les comportements et les expositions, et montre qu'une meilleure prise en charge hospitalière a vraisemblablement contribué à faire diminuer le taux de mortalité lié à la leptospirose, même si celle-ci reste élevée. Un faible niveau de connaissance de la maladie en population générale souligne l'importance de mettre en place des campagnes de sensibilisation. Les données produites dans le cadre de cette thèse stimulent par ailleurs la mise en place d'études complémentaires visant à mettre en évidence le(s) réservoir(s) complémentaire(s) afin d'apporter toute la lumière sur la situation épidémiologique. Une compréhension plus complète des chaînes de transmission permettra d'adapter les mesures de prévention afin de limiter le fardeau que représente cette maladie aux Seychelles, aujourd'hui encore reconnue comme la maladie infectieuse causant le plus de décès dans le pays.

General Introduction

Emerging infectious diseases

Infectious diseases have been a constant threat to human survival throughout recorded history whether from the Black Death (bubonic/pneumonic plague), the Spanish Flu epidemic, HIV/AIDS pandemic, and emerging epidemics of SARS-CoV (Severe Acute Respiratory Syndrome novel Coronavirus), MERS (Middle Eastern Respiratory Syndrome), up to recent outbreaks of Dengue, Chikungunya and Ebola fevers. The described evolutionary history of pathogens is such that they emerge to cause outbreaks and epidemics, constantly adapting with periodic emergence and finally becoming endemic with the impending threat of future outbreaks (Fauci, Touchette, & Folkers, 2005; Jones et al., 2008; Morens & Fauci, 2013; van Doorn, 2014).

The definition of what is an emerging infectious disease needs to be traced in history to mankind's complacency and their grandiose statements of victory against infectious diseases upon the advent of antibiotics and shortly followed by the eradication of smallpox through vaccination. In a review in 1998 by Cohen, a description of emerging and "resurgent" infectious disease and the reasons for their occurrence was described (Cohen, 1998) following a landmark publication in 1992 about the threat of such diseases (Institute of Medicine & Committee on Emerging Microbial Threats to Health, 1992). This definition of Emerging Infectious Diseases (EIDs) is the subject of debate, evolving to emerging and re-emerging infectious diseases, followed by classifications into three groups, i.e. *i*) novel diseases originating from wildlife, *ii*) mutants involved in previously treatable diseases, and *iii*) diseases emerging in a new geographic location (Engering, Hogerwerf, & Slingenbergh, 2013; Institute of Medicine (US) Forum on Microbial Threats., 2009).

Daszak *et al*. describe emergence as being associated with a range of underlying causal factors that are embedded in a complex continuum of interactions involving zoonotic parasites (where parasites denote viruses, eukaryotic or prokaryotic infectious agents), wildlife, domestic animals and human populations as shown in Fig. 1 (Daszak, Cunningham, & Hyatt, 2000).

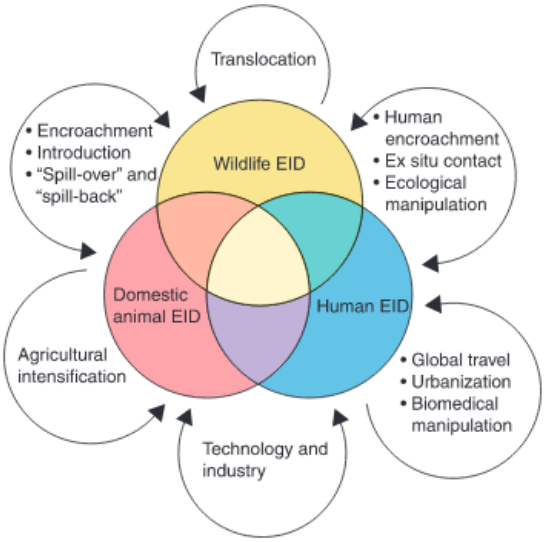

Fig. 1. The host-parasite ecological continuum (Daszak et al., 2000).

The emergence of novel human pathogens has mostly resulted from the ever-increasing interactions of humans (e.g. through trade, travel, agriculture, etc.) to various environments amplifying the exposure to a wider range of infectious diseases that can be transmitted from animal reservoirs (Engering et al., 2013; Lindahl & Grace, 2015). In addition, the growing phenomenon in recent times of multidrug-resistance in various human pathogens due to irrational antimicrobial usage and delay in developing diagnostics, is an example of how previously treatable diseases are re-emerging and present a significant public health challenge (Casadevall, 2017; Prestinaci, Pezzotti, & Pantosti, 2015).

The World Health Organization (WHO) includes disease X, which conceptually represents an unknown pathogen that could potentially cause a serious international epidemic, with an updated blue-print list of priority diseases, namely SARS, MERS, Rift Valley Fever (RVF) and Zika (Hui & Peiris, 2019; "WHO | List of Blueprint priority diseases," 2019). The list also includes Lassa fever and Chikungunya, which are under watch, and a further two are included in view of their public health importance: monkeypox and leptospirosis ("WHO | List of Blueprint priority diseases," 2019).

The amplification effect that tropical islands have in causing explosive infectious disease epidemics of global public health importance has been recently reviewed by Cao-Lormeau *et al*. in a synthetic presentation of the origin and distribution of arboviral outbreaks of Dengue, Chikungunya and Zika that have occurred in the last decade on islands in the Indian and Pacific oceans, as well as in the Caribbean (Cao-Lormeau, 2016). Although known since the mid-20th

century, these three arboviruses were not considered a global public health concern except for Dengue. This was until 2005 when a particularly virulent strain (with mutation of the membrane fusion glycoprotein E1, A226V) of Chikungunya virus (family *Togaviridae*, genus *Alphavirus*), different from the above two listed arboviruses Dengue and Zika (family *Flaviviridae*, genus *Flavivirus*), emerged in Indian Ocean islands (Schuffenecker et al., 2006). This was due to Chikungunya virus adaptation to *Aedes albopictus*, an abundant mosquito species in the SWIO islands, as well as to a lack of herd immunity in the human populations inhabiting these islands. The following years saw the dramatic impact of Chikungunya virus infection (which causes symptoms such as fever, joint pain, rash, polyarthralgia), in the Indian subcontinent, Asia, and Central Africa, with autochthonous transmission even reported in Europe (Cao-Lormeau, 2016). The long-term severe sequelae of the disease in terms of chronic arthralgia, destructive arthritis and fulminant hepatitis was shown by Renault *et al* in Reunion Island where an attack rate of 35% and 244,000 suspected cases was reported (Renault et al., 2007). Likewise, Zika virus first circulated in Micronesia and the French Polynesian islands before affecting Brazil where the disease was associated with severe forms such as neurologic disorders and a 20-fold increase in Guillain-Barré syndrome, and eventually spreading to neighbouring countries, which further highlighted the role that tropical islands play in causing global outbreaks (Cao-Lormeau, 2016).

Zoonoses

The word zoonosis (plural, zoonoses) derives from the Greek word "zoo" denoting animals and "sis" denoting a state or condition (Lipkin, 2015). In the present thesis, the term "animal" will use the veterinarian definition that includes all non-human animals. Taylor *et al*. have listed approximately 1415 known human pathogens out of which more than 60% are zoonoses (Taylor, Latham, & Woolhouse, 2001), whereas Jones *et al* have shown that 60.3% of the emerging infectious diseases are zoonoses, with 71.8% originating from wildlife (Jones et al., 2008). This emphasizes the importance of zoonoses in infectious disease emergence at the human-animal interface.

Indeed, zoonotic diseases that are either endemic to humans or enzootic in wild or domestic animals and leading to frequent **cross-species transmission** have been reported to cause approximately a billion cases of illness and millions of deaths per annum, whereas emerging zoonoses have cost hundreds of billions of US dollars in recent decades (Karesh et al., 2012). **Anthropogenic practices** (e.g. incursions into natural sites and changes in agropastoral practices) and increased risks (through **global travel** and **trade**) are components that synergistically increase

the disease burden of zoonoses globally, particularly in tropical areas (Halliday et al., 2015; Karesh et al., 2012).

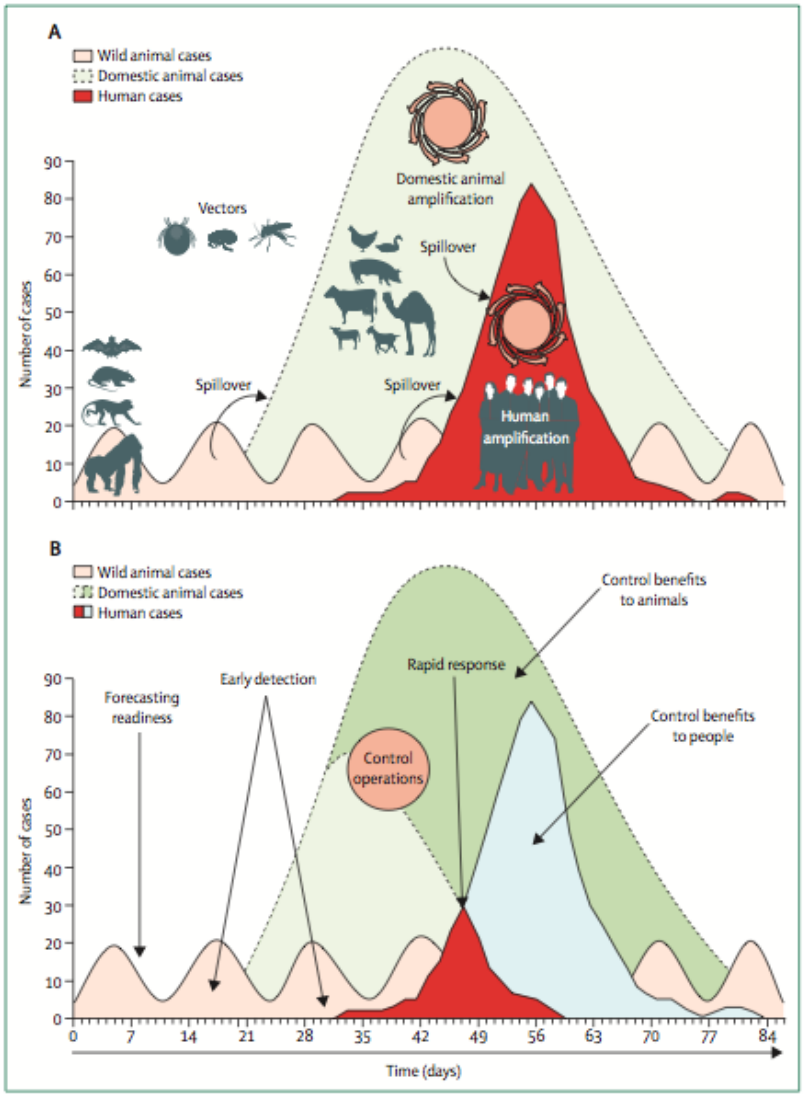

Fig. 2: Clinical relevance of disease ecology (**A**) Transmission of infection and amplification in people (bright red) occurs after a pathogen from wild animals (pink) moves into livestock to cause an outbreak (light green) that amplifies the capacity for pathogen transmission to people. (**B**) Early detection and control efforts reduce disease incidence in people (light blue) and animals (dark green). Spillover arrows show cross-species transmission (Karesh et al. 2012).

Emerging zoonoses are mainly of mammalian and avian origin (Taylor et al., 2001). Prediction of human infections due to emerging zoonoses can be done with an understanding of the dynamic relationship between the environment, wildlife/livestock and their microbiome, which can help in early detection and intervention to reduce disease incidence in humans and animals

(Han, Schmidt, Bowden, & Drake, 2015; Morse et al., 2012). The use of mathematical modelling to predict and thus better target prevention and control strategies, as well as the filling of research gaps can be achieved if sufficient data gathered from human, animal and environmental compartments are associated with climatic surveillance in order to pinpoint the interactions that can potentially cause disease emergence (Leighton, Koffi, Pelcat, Lindsay, & Ogden, 2012; Morse et al., 2012).

Bats and rodents are two examples of wild animal species with high potential for harbouring emerging zoonoses (Allocati et al., 2016; Han et al., 2015). The peculiar biology of bats including aggregation behaviour, torpor and longevity, has been proposed to contribute to an increased risk of emerging infectious diseases that is magnified by a unique flight ability among mammals conferring an increased potential for pathogen dispersal and contact to a diversity of environments and animal species (Calisher, Childs, Field, Holmes, & Schountz, 2006). Zoonotic diseases directly originating from bats have been described although these events are still limited in number (Nathwani et al., 2003; Weir, Annand, Reid, & Broder, 2014), while several diseases which could potentially spillover into humans have been proposed (Allocati et al., 2016; Joffrin, Dietrich, Mavingui, & Lebarbenchon, 2018; Wood et al., 2012).

Rats are known sources of zoonoses contributing to consequent human disease morbidity and mortality. The proximity of rats to humans that occurs in urban settings has been shown to provide ideal environments and resources for rat proliferation and for increased potential for transmission of zoonoses like leptospirosis (Pantimay, 2016; Byers, 2019). However, the eco-epidemiology of rat-associated zoonoses is complex due to the numerous interactions that can occur between rats, humans, zoonoses and the environment. Nevertheless common determinants of human disease can be identified that can lead to the prevention and control strategies against zoonoses transmitted by rats (Byers, Lee, Patrick, & Himsworth, 2019; Himsworth, Parsons, Jardine, & Patrick, 2013). However, the complexity of the ecology of these rodents, including their territorial behaviour, may lead to counterproductive preventive measures (Lee et al., 2018).

One Health

The emergence and re-emergence of zoonotic diseases within the "global village" we live in has been attributed to anthropogenic changes primarily related to land use and agriculture (Brown, 2004; Sofia, 2011). Expansion of the One Medicine concept proposed by Virchow in

1855 following his studies on the zoonoses trichinosis, to include human and animal health has eventually lead to the development of the One Health concept (Greger, 2007).

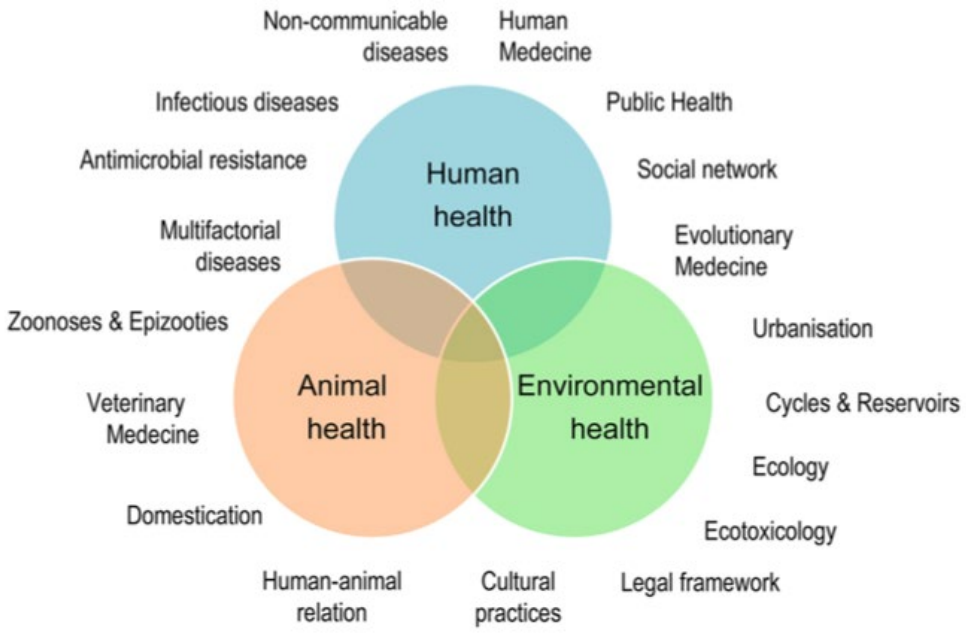

Fig. 3: One Health Concept (Destoumieux-Garzon et al, 2018)

The One Health approach that was jointly adopted as a concept by FAO, OIE and WHO in Hanoi in 2010 has been seen as a relevant framework in providing solutions to address the challenges presented by the emerging and re-emerging epidemics and pandemics of epizootics and zoonoses (Food and Agriculture Organization of the United Nations. World Organization for Animal Health. World Health Organization, 2010). A recent review highlighted the fact that developing countries suffering the highest burden of emerging zoonoses have least developed programmes following the One Health approach contrary to developed countries (Bidaisee & Macpherson, 2014). Similarly, while infectious diseases have been identified as a threat to wildlife conservation, decades later little has changed at policy level to improve transversal integration of One Health approaches. It is hoped that such approaches, which would help in improving the management and mitigation measures against emerging epizootics, will be integrated in the future (Cunningham, Daszak, & Wood, 2017).

A list of barriers against the effective interdisciplinary implementation of the One Health approach was recently published (Fig. 3) in a bid to inspire integrated operational research leading

to innovative strategies in human and animal health (Destoumieux-Garzón et al., 2018). The authors noted that the marginalised fields of wildlife, social, legal, economic sciences, plant health, and ethics are yet to be well integrated in this multidisciplinary approach. These integration efforts have led to recent developments looking at the converging field of EcoHealth (Harrison, Kivuti-Bitok, Macmillan, & Priest, 2019) and the burgeoning field of Planetary Health (Lancet, 2019) in considering the human impact on ecosystems.

Disentangling the impact of anthropogenic activities on animal and human health is challenging and can be achieved through non-oriented strategies that are being facilitated by the development of high throughput screening methods. Alternatively, this can be addressed using diseases models with environmental, direct or vectorial transmission. For instance, Rift Valley Fever has been repeatedly used as a model of vectorial disease to test the importance of climatic oscillations on vector abundance and eventually on disease emergence (Pachka et al., 2016; Sang et al., 2017; Sindato et al., 2016). Similarly, leptospirosis is a relevant model of environmental disease as it is caused by a highly prevalent and diversified pathogen hence allowing testing a number of hypotheses. In addition, this disease is of major medical importance specifically in some tropical island-states, which provides the investigation of this zoonosis both academic and medical importance. Leptospirosis was consequently examined in this context in this thesis, in the island state of Seychelles, which suffers from a particularly heavy burden of the disease.

Introduction on Leptospirosis

Viral zoonoses such as the ongoing Ebola outbreaks in Congo (Grady, 2019), usually make the news headlines. However, among zoonoses, leptospirosis is considered as one of the most prevalent infectious disease worldwide (Levett, 2001). It is estimated to affect over 1 million persons annually causing nearly 60,000 deaths (Costa, Hagan, et al., 2015) and a 2.9 million Disability-Adjusted Life Years (DALYs) lost per annum. A DALY is a human health metric measurement for disease burden that is defined by the WHO as "*a measurement of the gap between current health status and an ideal health situation, where the entire population lives to an advanced age, free of disease and disability*". Males are most affected with this disease with a global burden of 2.33 million DALYs and the most exposed regions are the resource-poor tropical regions of the world (Torgerson, 2015).

Leptospirosis is also considered as a re-emerging disease (Hartskeerl, Collares-Pereira, & Ellis, 2011), and due to its importance has also been recognized by the WHO as being amongst

the world's neglected tropical diseases with epidemic-prone potential causing significant public health impact (World Health Organization, 2015; World Health Organization, ICONZ - Integrated control of neglected zoonotic diseases, & United Kingdom. Dept for International Development Research in Use, 2011). Due to the complexity of the disease, WHO initiated in 2010 a global multidisciplinary approach to identify priority areas for research (Durski, Jancloes, Chowdhary, & Bertherat, 2014), which had as goal to eventually translate into focussed public health interventions aiming at lowering the global burden of the disease.

Leptospirosis is transmitted to humans and domestic animals through direct or indirect contact with infected urine excreted by reservoir/carrier hosts (Bharti et al., 2003) and has been associated primarily with rats. Leptospirosis is found ubiquitously in animals and it is primarily the renal carrying animal excreter that contaminates the environment with leptospires (Ellis, 2015). Leptospirosis prevalence is higher in tropical environments where the usually humid and hot conditions may be more conducive to *Leptospira* survival (Pappas, Papadimitriou, Siozopoulou, Christou, & Akritidis, 2008). Much interest has recently been shown for example towards the soil and similarly aqueous environments in elucidating their role in the maintenance and transmission of leptospires (Lall et al., 2018; Mendoza & Rivera, 2019; Sato et al., 2019). Prevalence is maximal on tropical islands for unknown reasons, although reduced species diversity typical of insular ecosystems may boost pathogen transmission (Derne, Fearnley, Lau, Paynter, & Weinstein, 2011a; Wong, Katz, Li, & Wilcox, 2012).

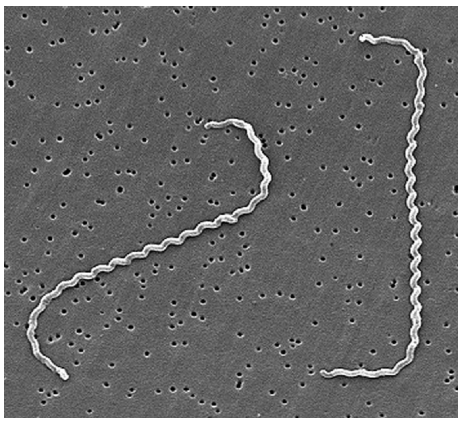

Fig. 4. Scanning electron micrograph showing spirochaete shape of Leptospires from Weyant *et al*. (1999)

Leptospirosis is caused by infection with pathogenic *Leptospira* species that are classified as spirochaetes and distinguished morphologically among this phylum by having hooked ends

(Fig. 4.). An extremely wide spectrum of human disease can be caused by *Leptospira* species ranging from mild subclinical infections to severe syndromes of multi-organ involvement resulting in high mortality (Levett, 2001).

Several textbooks (e.g. *Leptospira* and Leptospirosis, 2015) have comprehensively described various aspects of leptospirosis, however the following section summarises some salient introductory points.

Historical Aspects of Leptospirosis

According to most textbooks, Adolf Weil first described the syndrome corresponding to the severe form (with mainly jaundice and haemorrhage as symptoms) of leptospirosis in 1886 (Weil, 1886), although for historical accuracy Landouzy described a similar disease in 1883 (Landouzy, 1883), without recognition. Previous to this, a class of diseases that came to be known as the "yellow fever of the tropics" encompassed a range of diseases including yellow fever, hepatitis and leptospirosis. Icteric leptospirosis with renal failure was first reported over 100 years ago by Adolf Weil in Heidelberg (Weil, 1886), who is the origin of the name of the severe form of the infection: Weil's disease.

The word ***Leptospira*** comes from the Greek *leptos* meaning "fine or thin" and the Latin *spira* meaning "coil". Icterus comes from the Latinised (hence the addition of –us) Greek word ikteros, which to the ancient Greeks meant both jaundice and a yellow bird. The latter came about by the belief that observing a yellow bird would heal those affected by jaundice. Leptospirosis is also known colloquially by various names throughout the world such as nanukayami fever (Japan), which means 7-day fever and which explains the symptoms of the disease (Ido, Ito, & Wani, 1918), or canefield fever (Australia), mud fever (Germany) and rat disease (France), which associate professional, environmental and reservoir host to the disease (Haake & Levett, 2015a).

The search for the causative agent of *Weil's disease* as it was so named was launched during an era of acceptance of the theory that pathogenic microorganisms were the causative agents for diseases (Kobayashi, 2001). The causative agent of leptospirosis was discovered in 1915 by Inada and Ido, who went on to play an early and prominent role in the aetiological, pathogenic, diagnostic, therapeutic and epidemiological aspects of leptospirosis (Kobayashi, 2001).

Taxonomy and Nomenclature

The spirochaetal bacteria *Leptospira* genus belongs to the family *Leptospiraceae*, which also includes *Leptonema* and *Turneriella*. The genus name *Leptospira* was first proposed by Noguchi in 1918 and currently includes pathogenic, intermediate and saprophytic species (Levett, 2001). This classification has come to question recently with *Leptospira* in the intermediate category being isolated or detected from ill patients while a phylogenetically distinct clade (called Clade C) has been classified together with the pathogenic lineages (Ganoza et al., 2006; Lehmann, Matthias, Vinetz, & Fouts, 2014). Leptospires are also divided into serovars based on antigenic classification. Serovar names are written with an initial capital letter and are not italicized. An example of a correct nomenclature for a serovar is *Leptospira interrogans* serovar Icterohaemorrhagiae. Serovars that are antigenically similar are grouped together into serogroups, although these do not have any taxonomy standing, but are useful for serological diagnosis and epidemiology (Levett, 2001).

Pathogenic and saprophytic *Leptospira* can be differentiated by their phenotypic characteristics, e.g. saprophytic *Leptospira* will grow at 13°C and in the presence of 8-azaguanine, whereas pathogenic *Leptospira* do not grow in these conditions (World Health Organization, 2003).

Molecular phylogeny

The genotypic classification of leptospires has replaced the phenotypic serological classification (Levett, 2001; Musso & La Scola, 2013). The molecular classification of *Leptospira* species is based on phylogenetic analyses of DNA sequence data. The species of *Leptospira* cluster into three groups, comprising pathogens, saprophytes and an intermediate group (see Fig.5.). Recently, a polyphasic stepwise isolation and classification scheme has been used to recover and identify 26 *Leptospira* spp. from tropical freshwater and soil, 12 being novel species. Techniques employed by the authors included plating positive liquid cultures on EMJH agar followed by picking of single colonies which were identified sequentially by melting temperature analysis of PCR product within the 16S rRNA gene, followed by pairwise comparisons of MALDI-ToF MS database and finally with WGS analyses (Thibeaux et al., 2018).

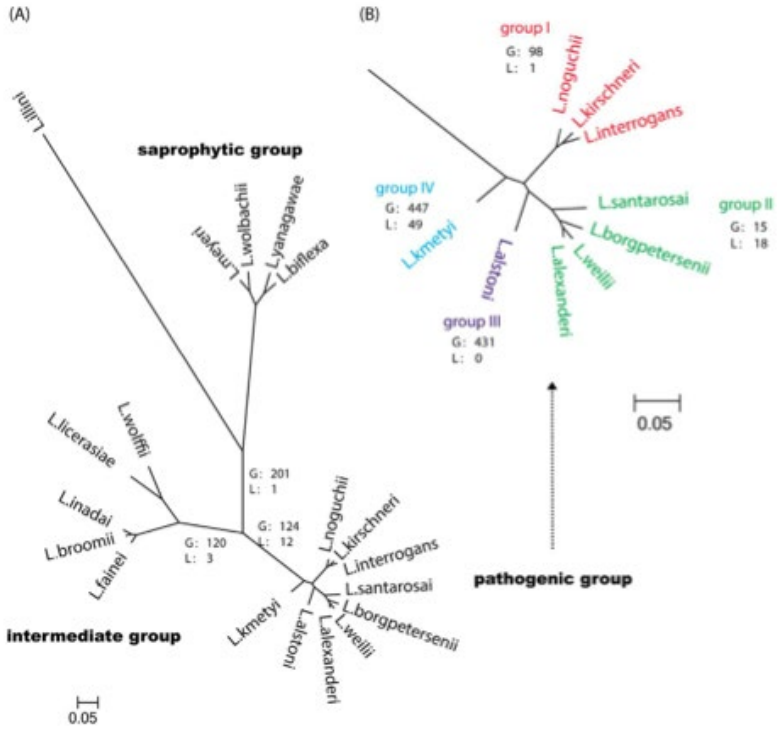

Fig. 5. Phylogenetic analysis based on the maximum likelihood of the concatenated core genes of the *Leptospira genome* with *Leptonema illini* as the outgroup. (**A**) All spirochete species genomes were included. (**B**) The enlarged pathogenic group from the phylogenetic tree. Numbers before and after slash showed the numbers of gains and losses, respectively (G: gain; L: loss). There were gene gain and loss events in the evolution from the root to the lineages. Scale bar indicated an evolutionary distance of 0.05 amino acid substitutions per position (Xu et al., 2016).

The growing diversity of *Leptospira* strains isolated from diverse origins has recently led to DNA-based barcoding methods leading to the proposal of new standards of classification and nomenclature to replace the classical DNA-DNA hybridisation species identification and serological techniques for serovar identification (Guernier, Allan, & Goarant, 2018; Vincent et al., 2019)

General Morphology

Although over 200 *Leptospira* serovars have been described, all members of the genus have similar morphology. *Leptospira* are spiral-shaped, highly motile bacteria that are 6-20 µm long and 0.1 µm in diameter with a wavelength of about 0.5 µm. One or both ends of the spirochaete are usually hooked. Live *Leptospira* are best observed by darkfield microscopy rather

than light microscopy. The bacteria have a number of degrees of freedom; when ready to proliferate via binary fission, the bacterium noticeably bends in the place of the future split (Levett, 2001; Cameron, 2015).

Genome

The genome of *Leptospira* spp. is relatively large (>3.9 Mb) in comparison with other spirochetes, like *Treponema pallidum* (1.1 Mb) and *Borrelia burgdorferi* (1.5 Mb). Leptospires contain 16S and 23S rRNA genes as well as only one 5S rRNA gene which is spread out on the large chromosome (Levett, 2001; Mathieu Picardeau, 2015).

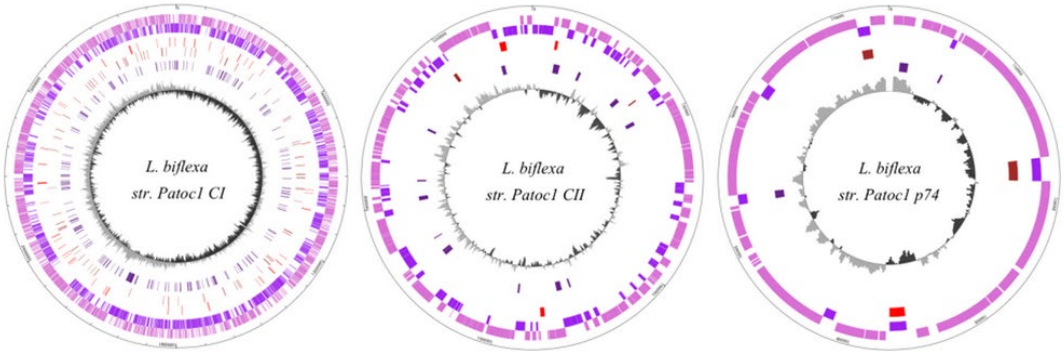

Fig. 6. Circular maps of the three *L. biflexa* replicons (Picardeau *et al*. 2008)

Two circular replicons are present in several *Leptospira* spp. (see Fig. 6. above) where the larger circular chromosome (cI, >3.6 Mb) codes for largely essential housekeeping functions while the smaller second replicon (cII, 278 -350 kb) carries essential genes such as *met*F (encoding methylenetetrahydrofolate reductase) and *asd* (encoding aspartate semialdehyde dehydrogenase) (Mathieu Picardeau, 2015). Whole genome sequencing has identified additional circular replicons (p74, 74 kb) in *L. biflexa* and *L. mayottensis* (Mathieu Picardeau, 2015; Cordonin et al., 2019).

Several insertion sequences such as IS1500, IS1502 and IS1533 of varying copy number between *Leptospira* serovars have been identified, and these play a role in transposition and genomic rearrangements within the bacteria (Levett, 2001; Mathieu Picardeau, 2015). Sequencing of *Leptospira* genomes has allowed the identification of several other IS elements, e.g. ISlin1, which belongs to a diverse range of IS families, including IS110, IS3, and IS4 (Mathieu Picardeau, 2015). The number of insertion sequences in *L. borgpetersenii* (mostly from the IS110 family) is much higher than in *L. biflexa*, *L. licerasiae*, and *L. interrogans* (Mathieu Picardeau, 2015). The considerable genome reduction characteristic of *L. borgpetersenii* serovar Hardjo may be the result of genomic deletions or rearrangements mediated by such IS elements, which could have in turn

led to lower environmental survival together with increased host specificity, in this case to cattle (D. M. Bulach et al., 2006; Mathieu Picardeau, 2015).

Treatment

Leptospirosis is usually easily treated with a broad spectrum of antibiotics and prompt therapy can avert patients from developing severe disease, whereas the majority of cases are mild and resolve spontaneously. Patients presenting with signs and symptoms, leptospirosis-associated risk factors and exposure are those that would constitute a suspected case based on clinical examination. Unfortunately, due to the mostly flu-like symptoms of leptospirosis, severe patients presenting late with increasing complications usually face an impaired treatment effectiveness. Although there has been guidance to clinicians for early antimicrobial therapy for patients with such a profile in spite of negative rapid diagnostic tests (Levett, 2001; Haake & Levett, 2015b), the jury on the effectiveness of such practice being lifesaving is still out. Recent studies have shown that delaying antimicrobial administration does not have any effect in treating severe leptospirosis (Herrmann-Storck et al., 2010; Tubiana et al., 2013) contrary to what has been observed by others (McClain, Ballou, Harrison, & Steinweg, 1984; Spichler et al., 2008). The situation becomes even more confusing for clinicians treating severe cases during viral epidemics such as dengue, which mimic leptospirosis symptoms and which further complicates the treatment with reports of higher mortality due to misdiagnosis (Flannery et al., 2001).

Early onset of the disease in adult outpatients is easily treated with daily oral doxycycline or azithromycin, whereas weight-adjusted doses of azithromycin or amoxicillin is indicated for pregnant women and children. Doxycycline has been shown to reduce shedding of leptospires in urine, decreasing illness duration and has also proved useful as prophylaxis of choice for patients at high risk of exposure. In hospitalised patients treatment usually involves intravenous penicillin-based (penicillin or ampicillin) or cephalosporin (ceftriaxone or cefotaxime) (Haake & Levett, 2015b; Levett, 2001).

Transmission and Reservoir hosts

Leptospirosis is an epidemic-prone infection that can be transmitted from contaminated water (Fig. 7). Transmission occurs through contact of the skin and mucous membranes with water, damp soil or vegetation (such as sugar cane), or mud contaminated with rodent urine (Ko, Goarant, & Picardeau, 2009). The occurrence of flooding after heavy rainfall facilitates the spread of the

organism caused by infected rodents that shed large amounts of leptospires in their urine (Haake & Levett, 2015a).

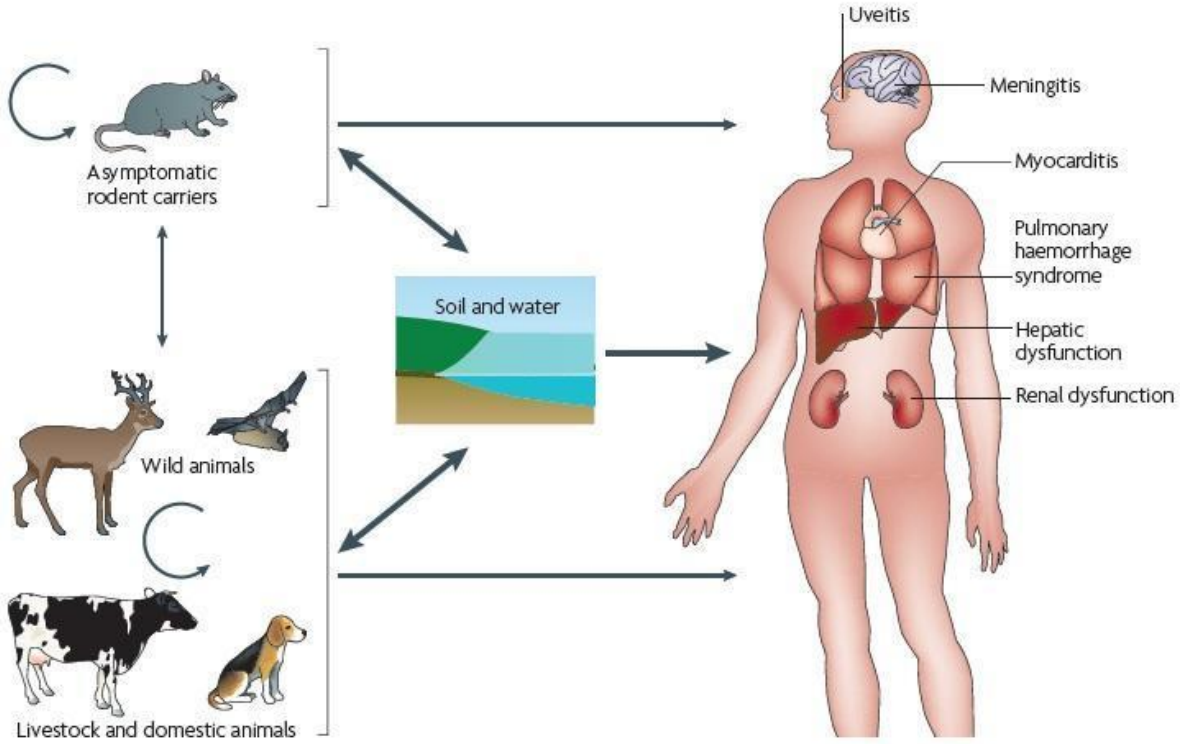

Fig. 7. The cycle of leptospiral infection. (Ko et al., 2009)

Reservoir hosts (asymptomatic rodent carriers, wild animals, livestock and domestic animals) are considered to play an important role in maintaining the leptospires in the environment (Ko et al., 2009). Leptospires have shown some degree of host specificity, to the extent that by isolating a particular serovar/lineage, one can be able to infer their relationship to a particular host most likely to harbour it (Adler & de la Peña Moctezuma, 2010; Bharti et al., 2003; Gomard et al., 2016).

Laboratory diagnosis

The diagnosis of leptospirosis in humans is not easy in view of the generalised signs and non-specific symptoms manifestation. Laboratory diagnosis of leptospires can be accomplished by direct detection of the organism or its components in body fluid or tissues, by isolation of leptospires in cultures or by detection of specific antibodies. The collection of appropriate specimens and selection of laboratory investigations depends upon the timing of collection and the

duration of symptoms (Fig. 8) (Haake & Levett, 2015a; Levett, 2001; Musso & La Scola, 2013; M. Picardeau, 2013).

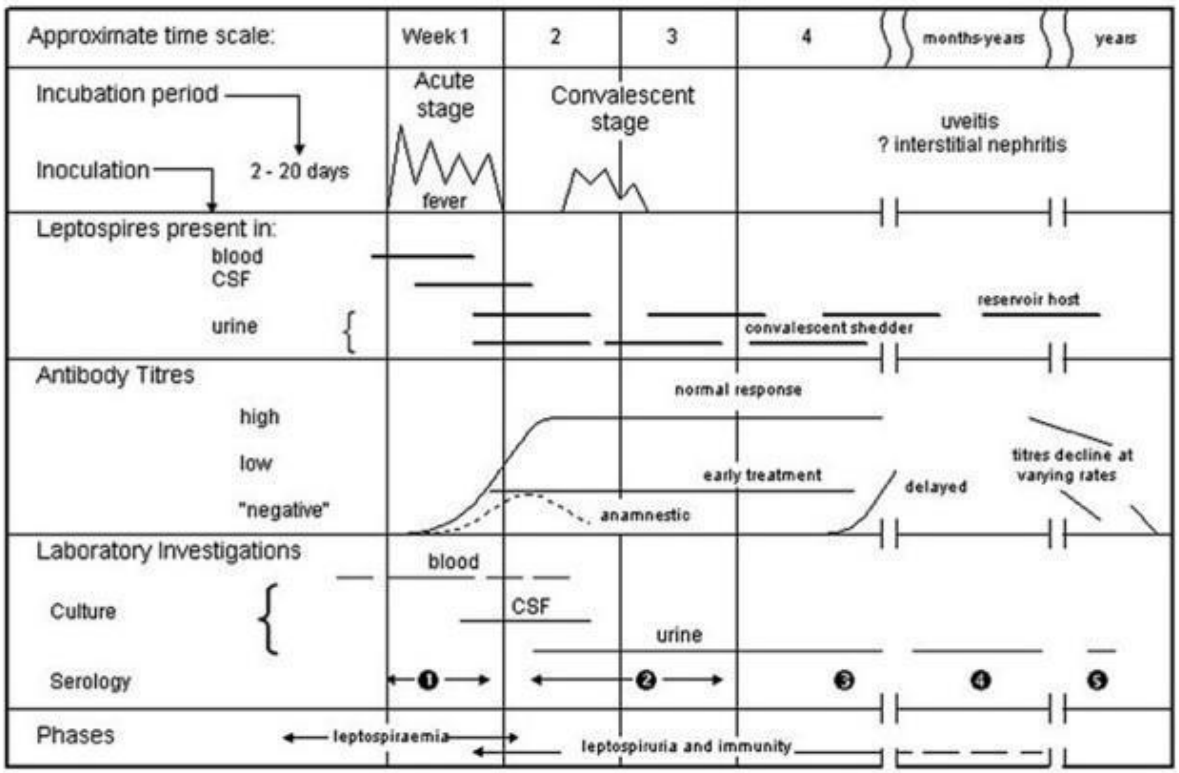

Fig. 8. Biphasic nature of leptospirosis and relevant investigations at different stages of the disease. Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase sample which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information, such as the presumptive infecting serogroup (Levett, 2001).

Similarly, to humans, the diagnosis of leptospirosis in animals relies on laboratory procedures demonstrating leptospires in tissues or antibodies in serum, as mild and imperceptible signs and symptoms complicate clinical diagnosis (Ellis, 2015).

Microscopy

Several microscopy techniques are in use for the detection of leptospires in the laboratory. These include direct observation by dark-field microscopy and by staining viewed by light or immunofluorescence microscopy (Levett, 2001; Ahmad, Shah, & Ahmad, 2005).

Dark-field microscopy can be done on a variety of body fluids such as whole blood, serum sediment, urine sediment, sediment of cerebrospinal fluid, sediment of dialysate fluid, and

sediment of supernatant of minced tissues from suspected patients. The disadvantage of dark-field microscopy is that it is both insensitive and lacks specificity. This can lead to false positives as artefacts (e.g. fibrin and protein threads) may be easily confused with leptospires unless there is a large number of leptospires (10^4 leptospires/ml) for one cell to be viewed per field as well as false negatives even with experienced microscopists (Ahmad et al., 2005; Levett, 2001). Lastly, as leptospires are slow growing, microscopy takes too long to be effective as a diagnostic tool.

Detection of leptospires in tissue

Currently available methods to detect leptospires in tissue include silver staining, Warthin-Starry stain, Immunohistochemistry (IHC) and quantitative buffy coat, all of which are useful in confirming the cause of death in suspicious cases of leptospirosis (Ahmad et al., 2005; Levett, 2001). These methods are used to increase the sensitivity of direct microscopic examination and include immunofluorescence staining of bovine urine, water, and soil and immunoperoxidase staining of blood and urine (Ahmad et al., 2005).

Culture

Although bacterial culture of leptospires from blood, (midstream) urine, cerebrospinal fluid, dialysate fluid (haemodialysis) and (post mortem) tissue is confirmatory, the procedure is very laborious and time-consuming. Growth of *Leptospira* is encouraged by undertaking serial dilutions to dilute out growth inhibitors. Tubes containing EMJH culture medium are inoculated and incubated at 28°-30°C and checked (by dark field microscopy) weekly for growth for a duration of six months (Ahmad et al., 2005; Ellis, 2015). Special care is taken to prevent contamination by ensuring materials and media are sterile. Care is also taken to filter water used for media preparation to prevent contamination from ubiquitous saprophytic leptospires (Ahmad et al., 2005; Ellis, 2015). A series of alternative media have been recently published including culture in semi solid (Wuthiekanun et al., 2013) and liquid complex media developed following genomic analyses (Dhayabaran, Chidambaram, & Krishnaswamy, 2019).

Serology

Serological analysis is the method of choice for laboratory diagnosis of leptospirosis in resource-limited tropical countries, which additionally harbour the highest incidence of the disease (Musso & La Scola, 2013; M. Picardeau, 2013). Serological tests for leptospirosis can be divided into those, which are genus-specific, and those, which are serogroup-specific. As IgM antibodies are detectable in the blood only 5-7 days after the onset of symptoms, significant limitations apply

to early diagnosis using any serological test and the testing of a second convalescent sample (usually 1-2 weeks after the acute sample was taken) should be considered mandatory (M. G. A. Goris & Hartskeerl, 2005; Musso & La Scola, 2013; World Health Organization, 2003).

Rapid Immunochromatographic tests

Several rapid diagnostic test formats for the detection of acute cases of leptospirosis using *Leptospira* genus-specific IgM antibodies are currently available or in development. These rapid diagnostic tests (RDTs) like Leptocheck-WB (Niloofa et al., 2015) are intended for use at the point-of-care (POC) tests or in resource-poor laboratories. These tests include dipstick formats, latex agglutination, lateral flows and dual path platform (Haake & Levett, 2015a; Mathieu Picardeau et al., 2014).

Enzyme-linked immunosorbent assay (ELISA)

ELISA prepared by using antigen prepared from cultures of *L. biflexa* is commonly used for the detection of IgM antibodies, although the use of pathogenic species has also been implemented (M. Goris et al., 2012; Mathieu Picardeau et al., 2014). Several commercial ELISA kits for the detection of *Leptospira* antibodies are available. Although recombinant antigens have been developed, these have not been widely evaluated yet, which is crucial and should take into account the diversity of pathogenic *Leptospira* prevailing in a given environmental setup. Specificity of IgM detection by ELISA is affected by the antigen used in the assay, by the presence of antibodies due to previous exposure (in endemic regions), and by the presence of other diseases (Ahmad et al., 2005; World Health Organization, 2003).

Microscopic Agglutination Test

The use of agglutination tests was described soon after the isolation of the organism and the microscopic agglutination test remains the definitive confirmatory serological investigation in both humans and animals (Ahmad et al., 2005; Ellis, 2015). The microscopic agglutination test (MAT) is considered the "gold standard" or cornerstone of serodiagnosis because of its unsurpassed diagnostic (serovar/serogroup) specificity in comparison with other currently available tests (Palaniappan, Ramanujam, & Chang, 2007; Thongboonkerd, 2008; World Health Organization, 2003). In this method, live antigens from fresh cultures are mixed in incremental dilutions to patient sera, and the reaction is read by dark-field microscopy, where the titre is noted

when 50% of agglutination is observed compared to a control antigen without serum (M. Picardeau, 2013; World Health Organization, 2003). MAT is unfortunately not available in resource-poor settings, is labour intensive and additionally a confirmed result is only after paired acute and convalescent sera is obtained. The challenge is to get the convalescent sera which are usually more than 4 weeks after the onset of the disease, as patients have already left the health facility or deceased by that time period thereby limiting the confirmatory power of the test. Demonstration of a four-fold rise in titre from the acute to convalescent sera is deemed serological confirmation of an acute infection in humans (Ahmad et al., 2005; M. G. A. Goris & Hartskeerl, 2005; World Health Organization, 2003).

Molecular diagnostic tests

Leptospirosis may be misdiagnosed more often using serodiagnosis, due to the nature of the presentation of the disease and the limitations of the tests to detect the presence of antibodies (Levett, 2001; Palaniappan et al., 2007). Molecular tests provide rapid confirmatory tests allowing a definitive diagnosis during acute illness prior to antibodies becoming detectable (i.e. before 5-7 days post onset of symptoms) and when treatment may be most effective (Musso & La Scola, 2013).

Leptospiral DNA has been amplified by Polymerase Chain Reaction (PCR) from serum, urine, aqueous humor, CSF, and a number of organs post mortem. Conventional PCR and other assays such as LAMP and NASBA have been published and many quantitative PCR assays have been described, which target a number of different genes (A. Ahmed, Engelberts, Boer, Ahmed, & Hartskeerl, 2009; Palaniappan et al., 2005; Smythe et al., 2002; Stoddard, Gee, Wilkins, McCaustland, & Hoffmaster, 2009).

The use of PCR to detect acute cases, which fall within the 5-7 days post-onset of symptoms, has rapidly increased the detection rate of leptospirosis cases. A positive PCR result provides confirmation of the presence of leptospires in the blood, which aids the physician in the prompt clinical management of cases and hence save lives.

Assays developed for diagnostic use can be considered in two broad categories, targeting either housekeeping genes, such as *rrs*, *gyrB*, or *secY*, or pathogen-specific genes such as *lipL32, lig*, or *lfb1* (A. Ahmed et al., 2009). PCR was shown to be more sensitive than culture, although serological analyses by MAT still detected more cases, as shown in the large case-control

evaluation of two quantitative assays in Thailand, which has a high leptospirosis prevalence (Haake & Levett, 2015a).

A limitation of PCR-based diagnosis of leptospirosis is the current inability of PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has both epidemiological and public health values (Blanco, dos Santos, Galloway, & Romero, 2016). Molecular epidemiology of circulating pathogenic *Leptospira* strains helps greatly in understanding the transmission of the disease and its maintenance in reservoir hosts, which in turn helps the public health interventions to curb the occurrence of cases.

Serovar identification requires isolation of the infecting strain from patients or carrier animals, however techniques for direct serovar identification are being developed, e.g. High Resolution Melt (Naze, Desvars, Picardeau, Bourhy, & Michault, 2015).

Genotyping

Genotyping of *Leptospira* strains has been done by various methods such as Multiple Locus Sequence Typing (MLST) (N. Ahmed et al., 2006; Boonsilp et al., 2013; Varni et al., 2014), Pulsed Field Gel Electrophoresis (PFGE) (Galloway & Levett, 2010; Mende et al., 2013), Multiple Locus Variable Number Tandem Repeat (VNTR) or (MLVA) (Koizumi et al., 2015; Salaün, Mérien, Gurianova, Baranton, & Picardeau, 2006), and High Resolution Melt (HRM) analyses (Esteves et al., 2018; Naze et al., 2015; Peláez Sánchez, Quintero, Pereira, & Agudelo-Flórez, 2017).

Genotyping by MLST using the Ahmed *et al.* (2006) method (Scheme #3) was the originally universally accepted method. It consists of sequencing six housekeeping genes (*adk*, *icdA*, *lipL32*, *lipL41*, *rrs* and *secY*) and comparing their sequences to online leptospirosis database PubMLST. Each sequence profile for a particular gene is allocated an allele number and the whole allelic profile leads to a sequence type (ST) (N. Ahmed et al., 2006). A 7-loci MLST scheme was later proposed which was proposed by Thaipadungpanit et al. (2007) and modified by Boonsilp *et al*. (2013). It consists of *glmU*, *pntA*, *sucA*, *tpiA*, *pfkB*, *mreA* and *caiB* (Scheme#1) (Boonsilp et al., 2013; Thaipadungpanit et al., 2007). The last MLST scheme (Scheme#2) proposed by Varni *et al*. (2014) consists of a hybrid of the previous two schemes and uses the polymorphism of *adk*, *glmU*, *icdA*, *lipL32*, *lipL41*, *mreA* and *pntA* genes, analysed as having being able to resolve strain type and provide a higher level of intra-species discrimination (Varni et al., 2014). Interestingly the latter scheme was able to establish congruence between allelic profile and serogroup in Argentinian *Leptospira* isolates which presents the possibility of inferring *Leptospira* serogroups

using this technique (Varni et al., 2014). The development of genomics has recently allowed describing a core genome useful for high resolution typing (Guglielmini et al., 2019).

Latest research and diagnostic techniques

Despite advances in diagnostics and treatments of many infectious diseases, leptospirosis remains a neglected disease where comparatively little research is being undertaken for the development of better diagnostic techniques and treatment. In addition, the paucity of genetic tools for these slow growing bacteria limits reverse-genetics approaches. Therefore, there are still many gaps in our knowledge of *Leptospira* pathogenicity, and genetic and molecular approaches for identifying environmentally regulated or *in vivo* expressed/induced genes are necessary (Palaniappan et al., 2007). The relatively new field of integrated genomics and proteomics, however, is helping to elucidate *Leptospira* pathogenicity at the genome level (Palaniappan et al., 2007; Mathieu Picardeau, 2015; Thongboonkerd, 2008). In recent years, numerous reports have been published on the use of transcriptomics to conduct genome-wide screening of extracellular pathogens such as *Helicobacter pylori*, *Yersinia enterocolitica*, *Borrelia burgdorferi* and other intracellular pathogens (Palaniappan et al., 2007), and recent studies on leptospires have been reported (Mathieu Picardeau, 2015).

Bioinformatics and transcriptomic studies in pathogenic leptospires have predicted that there are 226 genes that could be exploited in candidate vaccines (Palaniappan et al., 2007). Global approaches using proteomic studies have also led to the identification of novel genes that are involved in host–bacterium interactions (Palaniappan et al., 2007). Further studies into functional aspects by applying both proteomic and transcriptomic tools to leptospires grown at different environmental conditions are enhancing our knowledge on the pathogenicity of *Leptospira* spp. and will provide us with clues that may lead to novel vaccine candidates (Palaniappan et al., 2007; Mathieu Picardeau, 2015). A classical proteomic approach has been applied for the identification and characterisation of leptospiral outer membrane proteins (Thongboonkerd, 2008).

The study of leptospiral genetics has been slowed by the lack of an efficient transformation system (Mathieu Picardeau, 2008). Genetic manipulation of pathogenic *Leptospira* is difficult because of the restriction-modification system in *Leptospira* spp. (Palaniappan et al., 2007). It was thought that the development of a shuttle vector using the temperate bacteriophage LE1 from *L. biflexa* would be an answer but this has not proved to be successful thus far (Mathieu Picardeau, 2008). The use of RP4-based broad-host-range plasmids to transfer DNA from a donor *Escherichia*

coli strain to recipient strains belonging to both saprophytic and pathogenic *Leptospira* species has been published (Mathieu Picardeau, 2008). This approach is expected to accelerate reverse genetics approaches in pathogenic strains of leptospirosis towards better understanding of its pathogenesis. However, it is important to mention that *Leptospira* exhibit a significant level of gene redundancy (D. Bulach & Adler, 2018) which challenges reverse genetic approaches.

Comparative genomics is a powerful approach for elucidating changes in genetic constitution that occur in a given phenotype, including strain differences in virulence modes and/or antibiotic resistance (Fouts et al., 2016; Moreno et al., 2018; Nascimento et al., 2004). Over 150 draft genomes have been completed and are accessible online (https://www.patricbrc.org/view/Taxonomy/171). Comparative genomics is providing us with increased knowledge about speciation, host restriction, and differences in genotype, which in turn will help us to establish a strategy to control this important zoonotic disease (Fouts et al., 2016). However, since spirochetes based on 16S RNA analyses have diverged from other bacteria in ancient evolutionary times (Saier, 2000), they exhibit several peculiar cellular structures as well as genes of unknown function, which somehow impairs classical comparative genomics approaches.

The immunoproteomics approach has been used to identify potential leptospiral immunogens, and uses leptospiral proteins as the antigens and patients' sera as the sources of primary antibodies (Mathieu Picardeau, 2015; Thongboonkerd, 2008). Both classical and immunoproteomics approaches, together with other complementary proteome profiling tools, will make the search for potential leptospiral immunogens to be used in diagnostic and vaccine development more feasible (Mathieu Picardeau, 2015; Thongboonkerd, 2008).

An assay that is said to be comparatively faster and more economical than PCR is the LAMP assay which does not require specialised personnel or equipment for the detection of leptospires and which have good prospect for application in field screening and surveillance (Suwancharoen, Sittiwichianwong, & Wiratsudakul, 2016; Tubalinal, Balbin, Villanueva, Domingo, & Mingala, 2018).

Global Epidemiology of Leptospirosis

Leptospirosis is among the leading zoonotic causes of morbidity worldwide and accounts for numbers of deaths, which approach or exceed those for other causes of haemorrhagic fevers. Highest morbidity and mortality were estimated to occur in resource-poor countries, which include

regions where the burden of leptospirosis has been underappreciated (Fig. 9).

During outbreaks and in high-exposure risk groups, disease incidence may reach over 100 per 100000. Additionally, a recent outbreaks in Brittany (France) (Guillois et al., 2018) and New York City (USA) ("Leptospirosis Outbreak in NYC Has Public Health Officials on Alert," 2017) highlighted the fact that even developed countries can be affected by leptospirosis.

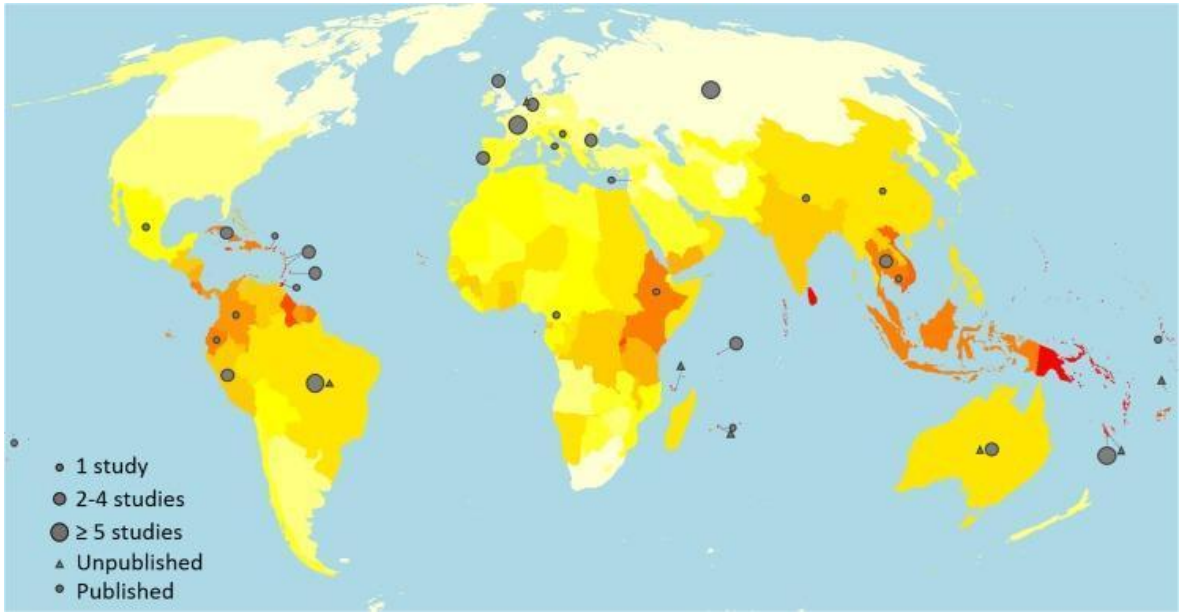

Fig. 9. Map showing estimated annual global morbidity rates of leptospirosis by country or territory. Annual disease incidence is represented as an exponential colour gradient from white (0–3), yellow (7–10), orange (20–25) to red (over 100), in cases per 100,000 population. Circles and triangles indicate the countries of origin for published and grey literature quality-assured studies, respectively. (Source: Costa *et al*., 2015)

Leptospirosis is an infection that can have fatal consequences in altogether benign situations as shown by the high profile death of Holmes, an accomplished Olympic rower for the UK from the 1980s, felt unwell in the days after a race in 2010 (Quarrell, 2010), developed a fever and was subsequently diagnosed with Weil's disease. Similarly, a woman died in 2008 within 48 hours after reportedly being scratched by a wild rat while she was gardening, which an inquest confirmed as Weil's disease (Britten, 2008). This illustrates the fact that being an environmental pathogen; *Leptospira* can infect all category of people at any time if engaged in risky activities.

Leptospirosis in insular tropical countries

The insularity of tropical island states poses challenges in addressing infectious diseases that continental states may not encounter. The United Nations Office of the High Representative for the Least Developed Countries, Landlocked Developing Countries and Small Island Developing States defines "small island developing states", or **SIDS**, as "a distinct group of developing countries facing specific social, economic and environmental vulnerabilities"("The Challenge of Small Island Developing States: Barbados, Mauritius, Samoa and beyond | ACS-AEC," 2017).

Table 1. List of SIDS by geographical division

Caribbean			
Anguilla	British Virgin Islands	Guyana*	Saint Kitts and Nevis*
Antigua and Barbuda*	Cayman Islands	Haiti*	Saint Lucia*
Aruba	Cuba*	Jamaica*	Saint Vincent and the
Bahamas*	Curacao	Martinique	Grenadines*
Barbados*	Dominica*	Montserrat	Suriname*
Belize*	Dominican Republic*	Netherlands Antilles	Trinidad and Tobago
Bermuda	Grenada*	Puerto Rico	Turks and Caicos Islands
	Guadeloupe		United States Virgin Islands
Pacific			
American Samoa	Kiribati*	Northern Mariana Islands,	Timor-Leste*
Cook Islands	Marshall Islands*	Commonwealth of the (USA)	Tonga*
Federated States of	Nauru*	Palau*	Tuvalu*
Micronesia*	New Caledonia	Papua New Guinea*	Vanuatu*
Fiji*	Niue	Samoa*	
French Polynesia		Solomon Islands*	
Guam			
AIMS			
Bahrain*	Guinea-Bissau*	Mauritius*	Seychelles*
Cabo Verde*	Maldives*	São Tomé and Principe*	Singapore*
Comoros*			

[1] * UN members, others are non-UN members/associate members of the Regional Commissions

SIDS countries are across the globe in the **Caribbean**, the **Pacific**, Atlantic and Indian Oceans, and the Mediterranean and South China Sea (**AIMS**) (Table 1.). In addition to common difficulties faced by developing countries, SIDS have an additional series of challenges to cope with that require special assistance from the international community. These challenges were highlighted in the 1994 Barbados Programme of Action (BPOA) and the Mauritius Strategy of Implementation (MSI) of 2005, both of which stated that the difficulties SIDS face in the pursuit of sustainable development are particularly severe and complex. Recognition of these issues was reinforced in September of 2014 when Member States of the United Nations officially adopted the Small Island Developing States Accelerated Modalities of Action, known as the SAMOA pathways ("The Challenge of Small Island Developing States: Barbados, Mauritius, Samoa and beyond | ACS-AEC," 2017).

A report has placed Seychelles as a world leader in leptospirosis incidence (Table 2.), which highlights the importance of this disease in small island state countries. We should notice that four countries reported with the highest incidence in this publication (Pappas et al., 2008), are

SIDS (highlighted in red in Table 2), strengthening the peculiarity of these ecosystems in maintaining a high level of transmission.

Table 2. Rank of countries reported annual incidence rates (Pappas *et al*., 2008) with SIDS highlighted in red.

Countries with the highest incidence			Countries for which no data are available, probably endemic	Other countries	
Rank	Country	Annual incidence per million population		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

The challenges that SIDS face are varied (Table 3.), but all conspire to constrain their development processes. They typically do not have a wide base of available resources and thus do not benefit from cost advantages that this could potentially generate. Two of the issues identified that relate to infectious diseases are (i) high population densities impacting biodiversity and ecosystems, and (ii) low resilience to frequent outbreaks/epidemics and natural hazards posed by environmental change ("The Challenge of Small Island Developing States: Barbados, Mauritius, Samoa and beyond | ACS-AEC," 2017).

Table 3. Types of health emergencies or outbreaks experienced by SIDS between January 2015 and the 31st October 2016 (as reported by WHO offices) (World Health Organization, 2017)

Natural disasters only	Disease outbreaks/epidemics only	Both natural disasters and disease outbreaks/epidemics
Barbados	Guinea-Bissau	The Bahamas
Cuba	Guyana	Belize
Northern Micronesia	Jamaica	Cabo Verde
Vanuatu	Kiribati	Dominican Republic
	Mauritius	Maldives
	Samoa	Papua New Guinea
	São Tomé and Principe	South Pacific
	Seychelles	
	Solomon Islands	
	Suriname	
	Tonga	

Zoonoses such as leptospirosis have been identified as part of the SIDS climate-related health risks, and interventions have been proposed in the SIDS climate-resilient health systems as intervention strategies targeting such diseases (World Health Organization, 2018). The highest leptospirosis annual incidence in tropical island countries (Costa, Hagan, et al., 2015; Pappas et al., 2008) may reflect the ability of saprophytic and pathogenic leptospires to form biofilms and survive in such environments which present ideal conditions for their maintenance, such as relative humidity, seasonal rainfall, presence of a large abundance of maintenance hosts, etc. Leptospires infect humans most often in such islands due to many factors not least being tropical rainfall, the abundance of rodents, and risky human behaviours (e.g. walking bare feet, not using protective gloves when handling soil etc. (Bovet, Yersin, Merien, Davis, & Perolat, 1999) as well as a limited

number of alternative reservoir species that may enhance transmission (Derne, Fearnley, Lau, Paynter, & Weinstein, 2011b) through the so-called amplification effect (Schmidt & Ostfeld, 2001).

A recent comprehensive review of leptospirosis in the Pacific islands has shown that there is great heterogeneity between the knowledge in human and animal epidemiology (Guernier, Goarant, Benschop, & Lau, 2018). Some countries had no data (e.g. Nauru, Pitcairn islands, Tuvalu, Wake island) whilst others had continuous published data (e.g. Hawaii, French Polynesia, New Caledonia), indicating different resource capacity to survey, prevent and control leptospirosis (Guernier, Goarant, et al., 2018). Incidence data for human leptospirosis and animal investigation studies were seen in the association of domestic animals such as dogs and cats, as well as cattle being infected with *Leptospira* and thus participating in its transmission. A similar heterogeneity in leptospirosis incidence or data availability is seen in the Caribbean islands; however, the risk factors for transmission of leptospirosis included human, agricultural and environmental exposure, and were the same in both geographical groups of islands (Guernier, Goarant, et al., 2018; Vokaty et al., 2016). Authors reviewing human and animal leptospirosis data in both the Pacific and Caribbean proposed a One Health approach to further understand the exposure pathways and eventually improve prevention interventions. The projected increased burden of human leptospirosis in insular states was further seen by authors in the context of the risks presented by climate change, flooding, population growth, urbanisation, loss of biodiversity and agricultural intensification, which could play a role individually or synergistically (Guernier, Goarant, et al., 2018; Vokaty et al., 2016).

Leptospirosis in the South West Indian Ocean islands

The South West Indian Ocean islands comprise various islands of different sizes, geographic isolation and geological history. The islands of the South Western Indian Ocean (SWIO) span from the northernmost island of the Seychelles (Bird Island, 3°43′N) to the tip of Madagascar in the south (25°61′S), and from Rodrigues Island in the East (63°25’E), to Comoros archipelago (43°16’E) in the West of Madagascar. These tropical islands vary from the ancient massive granitic islands such as Madagascar or dispersed granitic inner islands of Seychelles, to oceanic islands that have emerged *de novo* from volcanic hotspots including Mascarenes (Mauritius, Rodrigues and La Réunion) and Comoros archipelagos as well as the isolated coral atolls found in the Seychelles archipelago (Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). The region is a recognised biodiversity hotspot including UNESCO World Heritage Listing

for six insular nature sites, and three cultural sites. These islands have been visited by a melting pot of seafaring Arabians and Africans expanding from the west, Indians from the north, Austronesians from the east, and finally Europeans commencing with Portuguese discovery around the early 16th century, reflecting a long history of human colonisation (Myers et al., 2000; Russell, Cole, Zuël, & Rocamora, 2016).

A number of mainly serological studies carried out prior to 2012 suggested that the epidemiological situation of human leptospirosis in the SWIO is contrasted in terms of human incidence and circulating serogroups (P. Bourhy et al., 2012; Gomard et al., 2014; Pagès et al., 2014; C. Yersin et al., 1998). Seychelles appears as the island state with highest human incidence whereas human disease is hardly documented in other islands such as Mauritius, the Union of the Comoros or Madagascar. A number of molecular investigations aiming at typing pathogenic *Leptospira* present in human acute cases and animal reservoirs have confirmed this epidemiological paradox and shown that although leptospirosis is of main concern in the region, different islands shelter distinct transmission chains, composed of either endemic or introduced *Leptospira* lineages, as best exemplified in the French Islands of Mayotte and La Réunion (Tortosa, Dellagi, & Mavingui, 2017). The diversity of *Leptospira* species in Mayotte is perhaps relatively similar to neighbouring Madagascar where it has been shown that the prevailing species/lineages are host-specific and related to the diverse endemic fauna mainly composed of *Tenrecs*, bats and endemic rodents (Dietrich et al., 2018; Lagadec et al., 2016; Tortosa et al., 2017). In Mayotte there are 4 pathogenic *Leptospira* species identified in humans: *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* group B found for the first time in this island and later reclassified as a new species and named *L. mayottensis* (Pascale Bourhy, Collet, Brisse, & Picardeau, 2014; Pascale Bourhy et al., 2010). This species can be considered as endemic to the Malagasy region as it is strictly associated to tenrecs, a highly diversified family of insectivorous mammals endemic to the big Island (Dietrich et al., 2018; Lagadec et al., 2016).

In **Reunion**, *Leptospira interrogans* is overwhelmingly dominant among human acute cases (Guernier et al, 2016), and is represented by two STs, namely ST02 and ST34, the former being identified in over 85% of fully genotyped clinical *Leptospira*. Sampling of more than a thousand animals including 750 rats (*Rattus rattus* and *Rattus norvegicus*) showed that these rodents harbour ST02 only. Additional sampling of wild (bats, mice) as well as domestic (cattle, pigs and dogs) animals showed that both *L. interrogans* ST02 and ST34 are found in dogs. Although bats are massively infected with *Leptospira borgpetersenii*, these bat-borne lineages

have never been found in human acute cases. By contrast, infected cattle and mice harbour *L. borgpetersenii* with significant genetic similarity to lineages found in a minority of human cases. A recent introduction of leptospires into La Réunion is substantiated by the fact that there are only two species and three genotypes of pathogenic *Leptospira* present in human cases, and that these are found in rats, dogs, mice and cattle, all introduced to the island (Guernier et al., 2016; Tortosa et al., 2017).

Human leptospirosis cases are not well described in **Madagascar**, and this probably results from underreporting and under diagnosis. After few anecdotal reports including a case of a traveller with travel history from Madagascar subsequently developing leptospirosis (Pagès, Kuli, Moiton, Goarant, & Jaffar-Bandjee, 2015), a seroprevalence survey was conducted in the community of Moramanga in Madagascar revealing 2.8% seropositivity (Ratsitorahina et al., 2015). The work (unpublished ref. http://www.pasteur.mg/projets/) of Vigan Womas *et al* at Institut Pasteur Madagascar has revealed a *Leptospira* seropositivity of 5.6% to 10.6%, for IgM and 20.2% to 29.5% for IgG amongst a cohort of urban garbage removers in Antananarivo the capital city of Madagascar. It is clear that *Leptospira* are abundantly present in animals, findings supported by various animal studies in rodents, bats and various insectivores that show infections with unique bacterial lineages displaying astonishing levels of host-specificity (Lagadec et al., 2012; Dietrich et al., 2014; Gomard et al., 2016), with *Leptospira* seroprevalence in *R. norvegicus* as high as 45% (Muriel Dietrich personal communication).

In **Comoros**, there is much less available data for human leptospirosis, however a retrospective examination of sera stored at the "Programme National de Lutte contre le Paludisme" (PNLP) in Comoros revealed the presence of *Leptospira* serogroups Mini/Sejroe/Hebdomadis complex, Pyrogenes, Grippotyphosa, and Pomona, whereas the serogroup Icterohaemorrhagiae was not found (Gomard et al., 2014). Although leptospirosis is not reported in the Union of the Comoros, this serological pattern is close to that reported on the neighbouring Mayotte (P. Bourhy et al., 2012), and suggests that leptospirosis is indeed a disease of medical concern in this country, with likely comparable *Leptospira* lineages.

The situation of leptospirosis in **Mauritius** is unclear as published data is sparse. Leptospirosis is suspected to be present in human populations, as was demonstrated by a French traveller with recent travel history in this island (Simon et al., 2012).

Leptospirosis in Seychelles

Seychelles geography, population and climate

The Seychelles archipelago is a group of 155 granitic and coralline island in the Indian Ocean spread over an area between 4 and 10 degrees south of the equator and lying between 480 km and 1,600 km from the east coast of Africa. The Inner Islands group constitutes 41 of the oldest mid-oceanic granite islands on earth while the rest of the 74 islands form five groups of coral atolls and reef islets that are called the Outer Islands group. This Indian Ocean republic occupies a land area of 455 km² and an Exclusive Economic Zone of 1.4 million km². The Seychelles mid-year 2017 population estimate is approximated at 95, 843 (National Bureau of Statistics, 2018).

Due to its geographical location and maritime exposure, the climate of Seychelles is of the warm, humid tropical type with strong maritime influences, although outside of the cyclone belt. The islands of the Seychelles that are most populated are located south of the Intertropical Convergence Zone (ITCZ), which is a belt of low pressure circling the earth near the equator where trade winds of the Northern and Southern hemisphere come together. The climate in these northernmost islands of Seychelles can be divided into two main seasons, the Northwest Monsoon and the Southeast Monsoon separated by two relatively short Inter-Monsoon periods, called Am-climate (Group A tropical monsoon climate) by the Köppen-Geiger classification (H. E. Beck et al., 2018). These islands do not experience a distinct dry season, although the southeast monsoon is associated with the longest dry spells compared to the northwest monsoon, which has higher rainfall. The southernmost islands of this archipelago, which are also sparsely populated, have the Am-climate of mostly dry SE monsoon and wet NW monsoon as described above, however additionally lie in the cyclone belt.

Epidemiology of leptospirosis in Seychelles

Whereas leptospirosis is considered a leading cause of morbidity and mortality amongst zoonoses worldwide (Costa, Hagan, et al., 2015), in Seychelles it is still a major cause of hospitalisations even after last known published studies in 1992, and 1998 (see Fig. 10. Below). The cases represented in the Fig. 10., are not only from published studies, but also from serological data (collated by the Statistics Unit of the Ministry of Health of Seychelles from Clinical Laboratory data) in the intervening years up to 2013 when molecular screening was initiated and data included.

Cases occur throughout the year and while the dynamics of human leptospirosis cases is known to be related to rainfall, this relationship is not so obvious in Seychelles (C. Yersin et al., 1998). Transmission dynamics of *Leptospira* in putative reservoir hosts remains unexamined in this island country. It has been shown that leptospires can exist in biofilms (Gomes et al., 2018; Ristow et al., 2008), which may explain its endemicity in these humid islands through infected soil and water sources in the environment, which remains not well studied worldwide (Barragan, Olivas, Keim, & Pearson, 2017; Thibeaux et al., 2017).

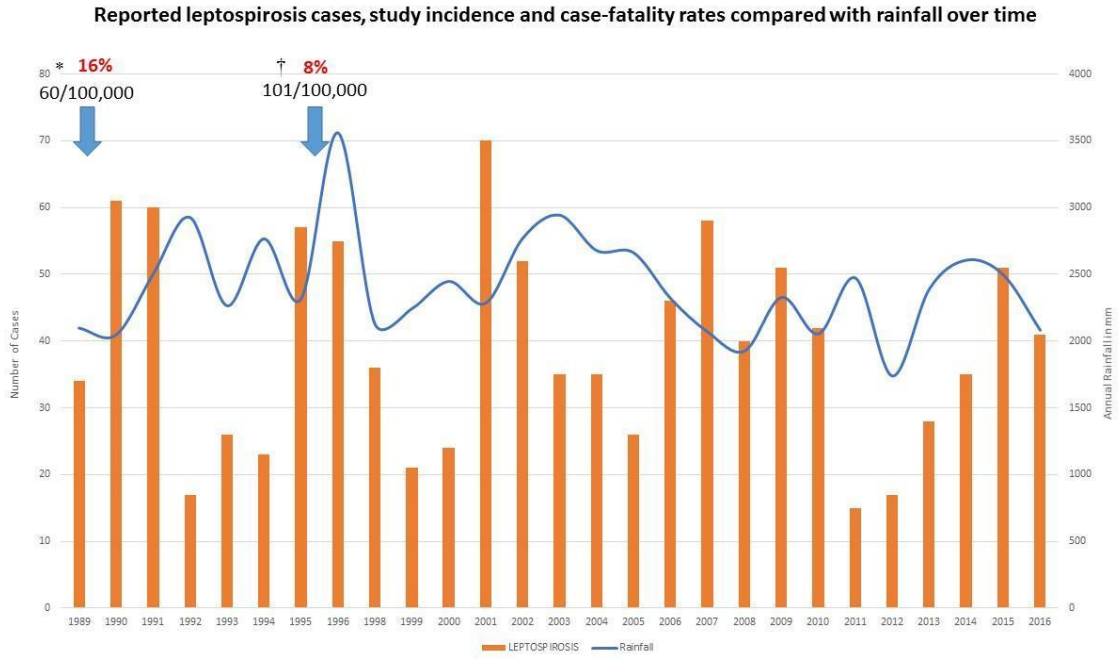

Fig. 10. Reported cases of leptospirosis from 1989 to 2016 in Seychelles, showing incidence and case-fatality rates from past studies as well as the annual rainfall trend. Source: Seychelles' Ministry of Health Statistics Unit and Seychelles Meteorological Station. (*Pinn, 1992 (Pinn, 1992); †Yersin *et al*., 1998. (C. Yersin et al., 1998))

Leptospirosis deaths have been fluctuating in Seychelles with peaks in 2007 and 2014 and lows in 2005, 2011, 2015 and 2017 when there was a marked decrease in deaths (Fig. 11), but nothing much can be read in that as the quantities are very small. However, for a small island developing state, the cases of deaths affect the population and are of national concern. Indeed, leptospirosis is mentioned amongst the core priority diseases of public health importance, which is required by International Health Regulations 2005, ratified by UN member countries.

Importantly, a reduction of 50% in case fatality by 2020 is mentioned as an indicator for quality care in the National Health Strategic Plan for 2016-2020 of the Ministry of Health of Seychelles.

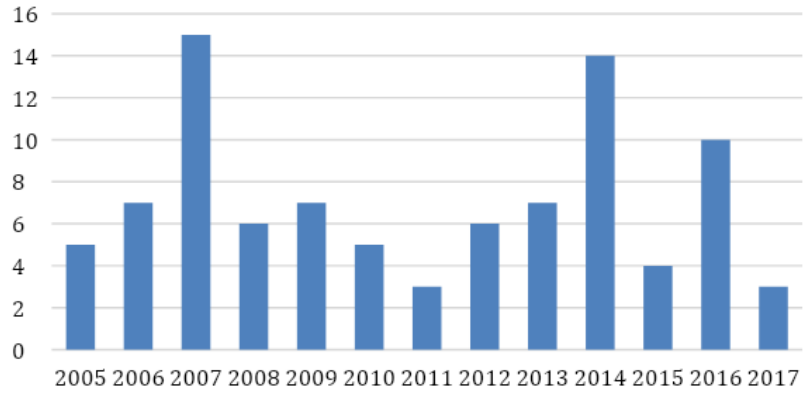

Fig. 11. Leptospirosis related mortality in all age groups from 2005 to 2017 in the Seychelles (Source: Disease Surveillance and Response Unit, Epidemiology and Statistics Section, Ministry of Health, Seychelles).

Case fatality rates are difficult to determine accurately as cases have been reported using different diagnostic methods in the past, namely serology based on IHA. This includes all the data on which the incidence rates for Pappas *et al*, is based (see Table 1.).

Box 1. Case definitions for leptospirosis

Case Definitions

Suspected case: Anybody reporting to a health centre (private or governmental) presenting with fever of ≥ 38ºC for more than three days with or without any of the following signs and symptoms; headaches, myalgia, haemorrhagic manifestations in the absence of any definite diagnosis.

***Probable* case**: Anybody meeting the suspected case definition criteria with a Positive ELISA IgM.

Confirmed case: Anybody meeting the suspected case definition criteria with a positive real-time PCR assay for pathogenic *Leptospira* spp. in blood and/or a positive MAT, a minimum four weeks after the onset of symptoms. A positive MAT is defined as one that displays an infective Serogroup with a four-fold seroconversion in paired sera, or acute sera with a Serogroup displaying a minimum titre of 1:400. The infective Serogroup in sera that had coagglutinatinating titres had the serogroup displaying two titre orders more than the rest as the definitive infecting serogroup.

Initiation of PCR tests started in 2013, and it has been used to provide confirmed leptospirosis cases. However, reports of cases still included serology until 2015 when case classification based on suspected, probable and confirmed was implemented (see Box 1. above).

Studies on Leptospirosis in Seychelles

Investigations on leptospirosis in Seychelles dated from Pinn in 1992 (based on studies in 1988-1990) showing that more males were affected with leptospirosis than females (a ratio of 10:1). Yersin *et al*. later (1998) reported the same trend in a study conducted in 1995-1996. These studies tally with the relative risks seen in males and females worldwide (Costa, Hagan, et al., 2015) (Fig. 12.).

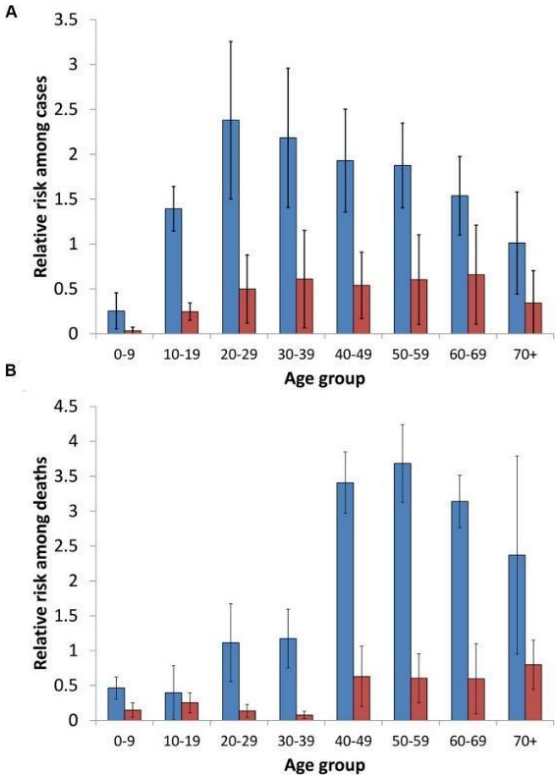

Fig. 12. Relative risks of leptospirosis amongst cases (A) and deaths (B), for age groups and sex.

Comprehensive studies on leptospirosis in humans were conducted in 1995-1996 (Bovet et al., 1999; C. Yersin et al., 1998; Claude Yersin et al., 2000), showing high morbidity and mortality due to leptospirosis in Seychelles with severe cases presenting with pulmonary haemorrhage being present in 20% of cases and associated with *Leptospira interrogans* serogroup Hurstbridge (ref. Fig. 13), which is still classified as non-pathogenic or intermediate. Pathogenic *L. interrogans* serogroup Icterohaemorrhagiae was the most prevalent amongst detected cases. PCR was used to confirm *Leptospira* species but bacterial DNA sequences were lacking.

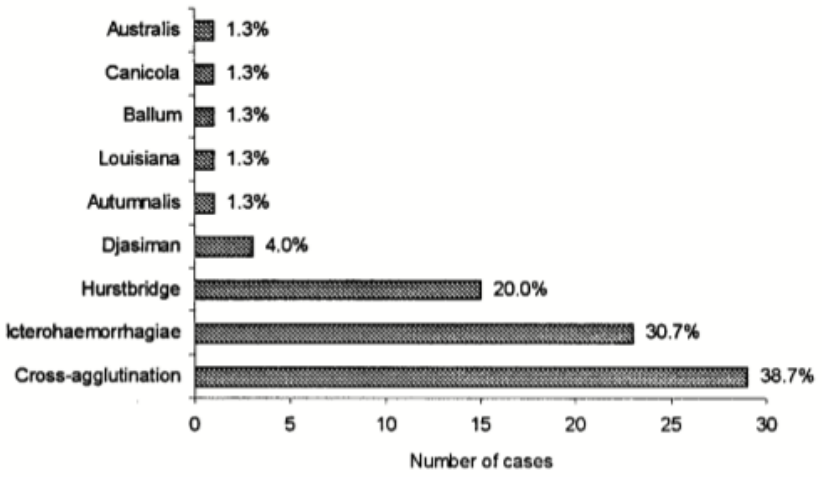

Fig. 13. Distribution of *Leptospira* serogroups amongst 75 cases from 1995-1996 study (Yersin *et al*., 1998).

The presence of cats around human cases and a mention of studies in rats and dogs was documented as a result of the studies in 1995-1996 (Bovet et al., 1999; C. Yersin et al., 1998). However, very little else was done to determine the actual animal reservoir of leptospirosis, which was presumed to be the notoriously abundant *Rattus* populations.

Rodent control

Historical efforts in controlling rodents in the Seychelles islands are from colonial periods. One of these such efforts, which was recorded by newspapers of the day, and re-printed, recently was implemented by the colonial government in August 1967. It involved a "tail tally", where islanders were asked to bring as evidence for a monetary reward the tails of rats that had been killed in an effort to control its population on the islands. A total of 25,590 tails were counted (Ernesta, 2019).

There are three main rodent species in Seychelles, namely *Rattus rattus*, *Rattus norvegicus* and *Mus musculus*. Rats have a destructive effect on biodiversity and hence their control has been of importance in Seychelles, which is rich in species biodiversity. Rat control efforts by conservation experts have helped in repopulating islands with species that are on the verge of extinction (Rocamora & Henriette, 2015b). Rodent control in Seychelles is multifaceted and involves several governmental ministries, including the Ministry of Environment, Ministry of Agriculture as well as the Ministry of Health. The involvement of rats as a vector of diseases such as leptospirosis has long been suspected and this has translated into programmes that are in place

to decrease the abundance of these pests. Various strategies are implemented to do this such as the use of rodenticides and/or rat-traps (Rocamora & Henriette, 2015b).

Statistics for the laying of these traps and utilisation of rodenticides is unfortunately not available from the health ministry, however the environment ministry has effectively implemented and maintained rat eradication and control of vulnerable islands harbouring at risk endemic species for conservation purposes (Rocamora & Henriette, 2015a). The conservation efforts at protecting natural ecosystems have recorded as of June 2015, 67 attempts at eradicating invasive animals, the most successful of these being eradication of black rats (*R. rattus*) followed by feral cats (Rocamora & Henriette, 2015a).

Situation at the beginning of the thesis

Leptospirosis is still a disease causing significant morbidity and mortality in many countries including the Seychelles (Fig. 10 and Fig. 11). However, in the Seychelles, there is a need to clarify the epidemiology of the disease in view of the continued high burden of the disease as shown by routine surveillance conducted by the Ministry of Health. The need to re-evaluate the status of leptospirosis in Seychelles through in-depth studies such as those conducted two decades ago is seen as a necessity to better understand the evolution of the disease in the country.

Incidence estimates based on serological data, which have last been done almost a decade ago, placed the country amongst the highest in the world (Pappas et al., 2008). With improved and now available molecular tools, there is a need to determine the molecular identity of *Leptospira* strains in circulation in Seychelles to be able to compare it with regional and global strains, as well as to identify putative reservoir animals that may harbour this pathogen. An identification of the reservoir hosts and a thorough understanding of transmission chains in Seychelles will allow fine-tuning control measures and better assisting evidence-based public health interventions to reduce the burden of this disease.

Deficiencies in sanitation and infrastructure has been associated with the risk of acquisition of leptospirosis (Hagan et al., 2016; Reis et al., 2008), however generally there are good housing and sanitation systems in the country. The importance of these factors remains to be independently examined, as with the effect of other abiotic factors on the evidently multidimensional disease of leptospirosis.

Globally there is an ever-increasing competition for space with wildlife as human activities (through industrialisation, urbanisation, tourism, fisheries, etc.) continuously encroach on more natural ecological sites, and this in spite of environmental protection efforts. This results in increased exposure of humans to diseases present in wildlife. It also exposes the accompanying domestic animals to wildlife from which they could acquire *Leptospira* infection, increasing the reservoir potential of domestic animals and strengthening the wild animal/domestic animal/human continuum. Given the general high prevalence of pathogenic *Leptospira* in animals together with a highly diverse regional wildlife, these components make leptospirosis a good model to determine the effects of human anthropogenisation on the environment and wildlife on disease maintenance and transmission from reservoir hosts to humans.

A thorough investigation of leptospirosis in a relatively closed environmental setting such as Seychelles will allow elucidating which are the prevalent bacterial lineages in this SIDS and identifying their associated animal reservoirs, which should intimate the transmission chains that are of clear medical concern.

Statement of the Problem

The actual status of leptospirosis in Seychelles is not well known as the last study was published almost 25 years ago. The current available data, which is the result of routine surveillance, is still based on serological data in spite of the introduction of PCR tests in 2013 (Integrated Disease Surveillance and Response guidelines, Ministry of Health, Seychelles). Additionally, **no genetic data is available** for the circulating *Leptospira* strains in Seychelles. There is also very little validated information available as to the reservoir(s) of leptospirosis in Seychelles and the effect of abiotic factors on leptospirosis transmission.

The burden of the disease has been consistently high throughout the years following the last detailed study almost twenty years ago. This burden remains high in spite of continuous national effort in implementing rodent control programmes to target the putative *Rattus* spp. reservoirs of the disease as described in multiple literature including the last study done locally, and has remained a major public health concern.

Research hypotheses of the thesis

Three research questions were set in this thesis:

Q1. How do the biotic and abiotic variables influence the transmission and maintenance of the disease? As rats are typically considered as a major reservoir, we modelled *Leptospira* infection in this animal using several biotic and abiotic variables such as land use, rainfall and altitude.

Q2. What are the specific *Leptospira* species/lineages involved in human leptospirosis in Seychelles and which are the putative reservoirs? This was achieved using a molecular epidemiology approach conducted through a One Health framework and consisting in comparing prevalence and genotypes of *Leptospira* circulating in wildlife and human acute cases.

Q3. What is the current incidence of leptospirosis in Seychelles and what are the epidemiological and behavioural variables that impact on the burden of the disease? We addressed the risk factors associated with human leptospirosis and compared data to those published 20-25 years ago in order to update our knowledge on behaviours, professional or recreational activities that protect or expose the Seychellois population to the disease.

To address these research questions, an acute fever surveillance programme (see Annex 1 for the study protocol) and animal sampling studies were put in place. Geospatial and climatic data were gathered to analyse abiotic factors influencing *Leptospira* positivity in humans and animals.

Animal sampling

Rats were trapped on Mahé island at 12 sampling sites representing a spread over seven regions (Victoria, Victoria periphery, North, Centre, East, West and South) and diverse habitats which were later simplified into two classes of urban and rural sites. Sampling was undertaken in two missions to represent the two seasons present in Seychelles, one during the Southeast monsoonal season in June-July 2013, and the other in the Northwest monsoonal season in February-March 2014. Sampling sites where rats were caught were georeferenced and habitat sites recorded. A range of 40 to 80 traps was laid 15 metres apart, trapped animals were euthanized, and samples including kidney, liver, lung, spleen and blood were collected. Grinded kidney tissue was immediately inoculated in culture medium. Morphological features (weight, head and body length, tail length, ear length, hind foot length) were recorded and used to differentiate *Rattus norvegicus* from *Rattus rattus*, whereas the latter was further confirmed through the sequencing of cytochrome b (*cytb*) gene in order to avoid miss-identification within the *R. rattus* complex. The sex and maturity of rats were also recorded.

Veterinarians euthanized cats and dogs discarded by owners at the Seychelles Veterinary Section and collected samples (urine and kidneys) were put in 70% ethanol and subsequently stored at – 80°C before shipping in liquid nitrogen.

Geospatial data

Remote sensing of satellite images to describe the land cover / land use of Mahé was collected to gather precise environmental information for analyses. SEAS-OI Station (http://www.seas-oi.org/) provided four high-resolution cloud-free Spot 5 images (© CNES - 2013, Distribution Astrium services / Spot images S.A., France, all rights reserved) at 2.5 meters spatial resolution in panchromatic mode and 10 meters in multispectral mode and acquired on December 6th 2012 and January 6th 2013. The raw images were pre-processed at level 1A with ENVI 5.1 software to have a set of images that were compatible with each other, i.e., correctly stackable (geometric and radiometric corrections).

An object-based image analysis (OBIA) with eCognition software (eCognition Developer 9.0.3, © 2014 Trimble Germany GmbH) was conducted involving a segmentation of the image pixels into objects and a classification of these objects according to intrinsic (reflectance, shape, texture), topologic (relations to neighbouring objects) and contextual properties (semantic relationships between objects) (Bordes et al., 2015; Révillion et al., 2015). Land cover / land use classes were chosen among the first level of the nomenclature used by the USGS and by adapting it to the tropical environment of Mahé (Anderson, Hardy, Roach, & Witmer, 1976). The classification was subsequently complemented with geographical vector data from the Seychelles Ministry of Habitat, Infrastructure and Land Transport to help differentiate between urban and peri-urban areas. A field observation campaign in January 2014 was organised to measure the quality of the land cover / land use classification and a confusion matrix was developed to verify whether the classes observed in the field were correctly identified on the satellite images.

The land cover / land use classification was used to compute landscape indices around the locations of each captured animal. These indices include the minimum distance between each animal and each land-use class. They also include the percentage area of each class in a buffer zone for which we chose different sizes (100, 500 and 1000 meters) in order to take into account potential distances travelled by animals. In each of these buffer zones, an index of landscape fragmentation (the total length of the contour of land use patches within each buffer, divided by the buffer area) was calculated. Ground elevation and slope was estimated by using the SRTM

(Shuttle Radar Topography Mission, http://srtm.usgs.gov/) digital elevation model (DEM) at 90 meters spatial resolution.

Climate data

Climatic data (monthly temperature and precipitation) was gathered from the Seychelles Meteorological Services, Mahé International Airport station and associated to the months of the human surveillance as well as those of the *Rattus* capture to see the impact on *Leptospira* positivity.

Human study

A prospective population-based survey was launched covering one-year period (1st December 2014 to 30th November 2015) on all acute febrile illnesses in the country. Enrolment of patients meeting an established case definition was referred to a leptospirosis clinic set up for the study, where trained interviewers gathered informed consent from participants, who were subsequently assessed for severity and were admitted to hospital according to severity. Patients below 13 years were not included in the study as this age category presents with a spectrum of acute febrile illnesses and additionally that age group does not suffer of a heavy burden of leptospirosis according to local surveillance data. The interviewer-administered questionnaire collected demographic and behavioural data on enrolled participants, as well as recorded results of clinical interventions. Biological samples were collected and submitted to a battery of tests, including those for leptospirosis diagnosis.

Chapter 1: Geospatial Analyses of *Leptospira*-infected *Rattus* spp. in Seychelles

Introduction

Anthropogenic activities on natural ecosystems have been shown to have multiplying effect in increasing exposure to emerging diseases such as zoonoses, which represent 60% of human infectious diseases (Lindahl & Grace, 2015; Taylor et al., 2001). Leptospirosis is a zoonotic disease that affects over a million people worldwide with mortality estimates up to 60,000 per annum (Costa, Hagan, et al., 2015). Identifying environmental factors that would indicate the presence of leptospires or the carriage of leptospires in animals could thus help targeting the settings where humans are most likely to contract leptospirosis. The anthropogenic drivers of leptospirosis infection are not well studied. Studies that model abiotic risk factors to *Leptospira* infection in reservoirs hosts as predictor of human infection are also limited. The modelling of *Leptospira* infection in rodents may serve as a good proxy to estimate the habitats, which may pose an increased risk of human transmission.

Remote sensing approaches which utilise satellite data in studies of the effect of habitats on disease prevalence have been used to help better understanding the impact of environmental factors in maintaining or influencing disease and have provided a more precise and objective measure of targeting mitigation efforts with the objective of improving health outcomes (L. R. Beck, Lobitz, & Wood, 2000; Zhang et al., 2013). Such approaches have never been done in Seychelles, and in view of the available satellite and rodent data, an analysis of this type was seen as an opportunity.

This chapter describes the abiotic risk predictors of *Leptospira* infection in *Rattus* species in **Article 1: Predicting the presence of leptospires in rodents from environmental indicators opening up opportunities for environmental monitoring of human leptospirosis.** The manuscript, which was submitted for peer-review in the Remote Sensing journal, is included below.

remote sensing

MDPI

Article

Predicting the presence of leptospires in rodents from environmental indicators opens up opportunities for environmental monitoring of human leptospirosis

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Abstract: Leptospirosis, an environmental infectious disease of bacterial origin, is the infectious disease with the highest associated mortality in Seychelles. In small island territories, the occurrence of the disease is spatially heterogeneous and a better understanding of the environmental factors that contribute to the presence of the bacteria would help implementing targeted control. The present study aimed at identifying the main environmental parameters correlated with animal reservoirs distribution and *Leptospira* infection in order to delineate habitats with highest prevalence. We used a previously published dataset produced from a large collection of rodents trapped during the dry and wet seasons in most habitats of Mahé, the main island of Seychelles. A land use / land cover analysis was realized in order to describe the various environments using SPOT 5 images by remote sensing (object-based image analysis). At each sampling site, landscape indices were calculated and combined with other geographical parameters together with rainfall records to be used in a multivariate statistical analysis. Several environmental factors were found to be associated with the carriage of leptospires in *Rattus rattus* and *Rattus norvegicus*, namely low elevations, fragmented landscapes, the proximity of urbanized areas, an increased distance from forests and, above all, increased precipitation in the three months preceding trapping. The analysis indicated that *Leptospira* renal carriage could be efficiently predicted using landscape fragmentation and rainfall only, with infection prevalence being positively correlated with habitat fragmentation and three months cumulative rainfall. This model may help decision makers in implementing policies affecting urban landscapes and/or in balancing conservation efforts when designing pest control strategies that should also aim at reducing human contact with *Leptospira*-laden rats while limiting their impact on the autochthonous fauna.

Keywords: Leptospirosis, rodents, *Rattus rattus*, *Rattus norvegicus*, spatial analysis, remote sensing, landscape metrics, satellite, ecology

1. Introduction

Leptospirosis is a bacterial disease caused by pathogenic spirochetes of the genus *Leptospira* [1,2]. Throughout the world, leptospirosis occurs mostly in tropical climates and in a variety of environments, affecting urban and rural populations [3] including on islands [4]. It has a major impact on human health with an estimation of over one million cases and about 60,000 deaths yearly worldwide [5,6]. Despite this considerable burden, leptospirosis remains a neglected disease as it affects the poorest populations, it is associated with flu-like unspecific symptoms and its diagnosis is challenging in low-income countries due to the usual unavailability of molecular diagnostic tools in these settings [6]. Over the past few years, research programs have multiplied to address the burden of the disease in human populations, identify main animal reservoirs and better understand its ecology [7,8]. However, many countries, especially in Africa, lack information on this disease [9].

Leptospirosis usually occurs after contact with a moist environment (water or soil) containing pathogenic leptospires [10]. These bacteria can survive from a few weeks to several months under favorable environmental conditions and hence infect a number of mammalian hosts [11,12] although non-mammal hosts have also been reported [13,14]. In reservoir hosts, leptospires colonize the renal tubules and are chronically shed and dispersed into the environment through urine [1]. Therefore, environmental conditions are determining factors of the transmission of these pathogens: they condition the survival time of bacteria in the environment and the presence of animal species able to act as reservoirs, which in turn contribute to the contamination of the environment. Identifying environmental factors allowing to predict the presence of leptospires in the environment and/or the carriage of leptospires in animal reservoirs can thus help identify the settings where humans are most likely to get infected.

Several studies have looked at the environmental patterns of leptospirosis epidemiology. Different risk factors have been highlighted depending on the geographic scales. At smaller geographic scales (*i.e.* over large areas), climate factors and habitat types are known to delineate species distribution as they mark the frontiers of their fundamental ecological niche [15,16]. These factors are, for example, temperature range and cumulative rainfall, which may be in turn related to altitude and land cover. Flooding has been shown as a major factor of bacterial dispersal and of increased human exposure [17]. In a bibliographic review, Mwachui *et al.* concluded that flooding and heavy rainfall were major drivers of leptospirosis incidence on islands and in Asia [8]. Also, in Cambodia, Ledien *et al.* showed that the detection of flooded areas helped to predict the risk of leptospirosis infection [18]. In New Caledonia, the El Niño Southern Oscillation (ENSO), which causes significant changes in rainfall, has been associated with outbreaks of leptospirosis and is therefore considered to contribute to the occurrence of leptospirosis [19]. Lastly, an association of leptospirosis to specific habitat types has been enlightened in some studies, as in southern Brazil (the state of Rio Grande do Sul), where the cases of leptospirosis in rural areas are restricted to specific ecoregions [20].

At a larger scale (*i.e.* over smaller areas), environmental characteristics, topography, meteorology, human presence and species interactions affect the presence and density of each species (as theorized for ecological niche modeling) [16]. Thus leptospirosis may present specific patterns related to each environmental setting [8]. In the Brazilian city of Salvador, an increased exposure to leptospirosis was associated with the location of households at lower elevations [21]. In Southeast Asia, an ecological sampling of rodents in various landscapes showed that their prevalence in different species of leptospires varies with their habitats (*i.e. L. borgpetersenii* is highly prevalent in non-floodable lands while *L. interrogans* and *L. borgpetersenii* co-exist with similar prevalence in rice fields and forests) [22]. Similarly, in northern Thailand, investigation of *Leptospira* spp. in rodents revealed a higher prevalence of infection in animals living in forested habitats than in

those living near villages where the prevalence of human leptospirosis is high (*i.e.* villages located in non-forested areas close to rivers) [23].

At such scales, these studies have demonstrated the need for and capacity of remote sensing techniques to analyze satellite imagery in order to construct environmental indicators allowing risk prediction. However, spatial and temporal resolutions of the data have an impact on the analyses. For instance, the detection of flooded areas is relatively difficult to assess by optical satellite imagery, since cloud cover is generally important during the rainy season. Also, flood maps are sometimes produced on an annual time scale, which is not appropriate to capture the local temporal impact of flooding on leptospirosis. In Thailand for instance, the comparison of flood maps with leptospirosis incidence at a national scale showed an inconsistent relationship [24]. These maps are hence an impressive piece of work based on hundreds of satellite images [25] but may not be suitable to catch the local temporal impact of flooding on leptospirosis.

Therefore, the question of whether environmental indicators obtained through the analysis of satellite images allow predicting the carriage of leptospires in reservoirs, or even the occurrence of human cases, remains open. Ledien *et al.* paved the way by showing the potential of flood-representative vegetation indices (especially the Modified Normalized Difference Vegetation Index – MNDWI, calculated from MODIS satellite images) in predicting leptospirosis seroconversion [18]. However, at the local scale, such predictions require finer data. To address this question, we looked at leptospirosis in a small area, the island of Mahé in Seychelles, where leptospirosis is a disease of major public health concern [14,26,27].

Mahé is the largest and most populated island (77,000 inhabitants) in the Seychelles Archipelago, located about 1,500 kilometers East of Africa, in the Indian Ocean. With its highest peak at 905 meters (Morne Seychellois), Mahé presents varied and hilly landscapes on a small territory. Several mammalian species have been introduced in Seychelles, all of which are likely to be reservoirs of leptospirosis. These are mostly introduced commensal rodents (*Rattus rattus*, *Rattus norvegicus* and *Mus musculus*) but also hares, rabbits, dogs, cats and tenrecs (*Tenrec ecaudatus*, a small mammal in the family Tenrecidae) [28]. Also, the endemic fruit bat of the inner islands of Seychelles, *Pteropus seychellensis*, is a potential reservoir with an unknown *Leptospira* species reported in one bat from a small sampling [14,29]. Rodents are the most abundant terrestrial mammals in Seychelles. They are found in all ecosystems, and can be abundant in towns and villages, agricultural lands and wooded hills. They are notoriously important actors in the maintenance, dispersion and transmission of leptospires to humans.

This study aimed at identifying environmental factors associated with murine rodents distribution and *Leptospira* infection. The objective was also to see the predictive potential of such indicators, which could be used for the environmental monitoring of leptospirosis and targeted control actions.

2. Materials and Methods

2.1. Sampling permit and ethics approval

Approval for the trapping and investigation of rats was received from the Seychelles Bureau of Standards (ref. A0157). The collection, handling, external examination and dissection of each animal followed the European Union legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and the reference rodent protocols [30–32]. The research protocol's ethical terms were defined under accreditation 03387 (FEDER POCT LeptOI 32913 project) and were approved by the CYROI Institutional Animal Care and Use Committee (Comité d'Ethique du CYROI n◎ 114, IACUC certified by the French Ministry of Higher Education and Research).

2.2. Animal sampling and Leptospira detection

The GPS coordinates and sampling schemes have been described elsewhere [14]. Briefly, rodents were captured on Mahé Island at 11 sampling sites (Casse Dent, Chemin Dame le Roi, Grand Bois/Mont Céphale, La Gogue, Fairview La Misère, La Réserve, Police Bay, Port Launay, Providence

Industrial Estate, Reclaimed land near the airport (Zone 21), Victoria) during the dry season (South-East tradewinds) in June and July 2013, and during the wet (North-Westerly tradewinds) monsoonal season in February and March 2014. A few animals were also captured from Beau Vallon out of this sampling protocol. Descriptive information was recorded in the field for each animal trapped, including a short description of the habitat and of the state of the trap. The captured animals were handled in a laboratory of the Ministry of Health to retrieve kidney, lung and spleen samples [14]. The infection status of rodents, determined through RT-qPCR or culture on the kidney samples [14] was used as a variable in the construction of the models. The trapping rate was calculated for a given species by dividing the number of animals caught by the total number of traps that caught a rat or remained opened (excluding those traps that caught other species).

2.3. *Landscape analysis*

In view of the relatively small size of Mahé Island and the diversity of rodent habitats, this study required precise environmental information. Such information did not exist in Seychelles and we consequently used remote sensing of satellite image to describe the land use / land cover of Mahé. SEAS-OI Station (http://www.seas-oi.org/) provided four high-resolution cloud-free Spot 5 images (© CNES - 2013, Distribution Astrium services / Spot images S.A., France, all rights reserved) at 2.5 meters spatial resolution in panchromatic mode and 10 meters in multispectral mode and acquired on December 6th 2012 and January 6th 2013. We preprocessed the raw images at level 1A with OrfeoToolBox 5.8.0 (OTB, https://www.orfeo-toolbox.org/) open source software to have a set of images that were compatible with each other, *i.e.*, correctly stackable (geometric and radiometric corrections).

We realized an object-based image analysis (OBIA) with eCognition software (eCognition Developer 9.0.3, © 2014 Trimble Germany GmbH) as already described [33,34]. The OBIA process involves a segmentation of the image pixels into objects and a classification of these objects according to intrinsic (reflectance, shape, texture), topologic (relations to neighbouring objects) and contextual properties (semantic relationships between objects). We chose the land use / land cover classes among the first level of the nomenclature used by the United States Geological Survey [35] further adapting it to the tropical environment of Mahé. The Seychelles Ministry of habitat, infrastructure and land transport provided complementary geographical vector data, which helped improving this classification, in particular to differentiate between urban and peri-urban areas. We organized a field observation campaign in January 2014 to measure the quality of the land use / land cover classification. We built a confusion matrix to verify whether the classes observed in the field were correctly identified on the satellite images [36].

We then used this land use / land cover classification to compute landscape indices around the locations of each captured animal [37]. These indices included the minimum distance between each animal and each land use class. They also included the percentage area of each class in buffer zones of different sizes (100, 500 and 1000 meters) in order to take into account potential distances travelled by animals. In each of these buffer zones, we calculated an index of landscape fragmentation, called edge density and obtained by dividing the total length of the contour of land use patches within each buffer by the buffer area. Lastly, we estimated the ground elevation and slope by using the SRTM (Shuttle Radar Topography Mission, http://srtm.usgs.gov/) digital elevation model (DEM) at a 90 meters spatial resolution.

2.4. *Meteorological data*

The Seychelles National Meteorological Services provided monthly rainfall data measured at 14 ground stations throughout Mahé Island. In order to estimate rainfall at each trapping site, we spatially interpolated these data using an inverse distance weighting method with ArcGIS software. We associated to each animal the average rainfall over 20 years (1993 - 2013) and the rainfall of the month of capture. In order to assess the impact of cumulative rainfall on leptospirosis, we calculated the rainfall for the month preceding the capture, as well as the cumulative rainfall for the two and three previous months.

196 *2.5. Cartography*

197 We used QGIS software for mapping the results. The Infectious Disease Surveillance Unit of the
198 Ministry of Health provided human leptospirosis data for the 22 districts of Mahé for the year 2015.
199 We obtained publicly available district population data from the National Bureau of Statistics of
200 Seychelles that were used to calculate and map the incidence of leptospirosis by district. We used a
201 district contour map provided by the Ministry of Habitat, Infrastructure and Land Transport of the
202 Republic of Seychelles.

203 *2.6. Statistical analyses*

204 We verified if the number of samples trapped at each site was sufficient to detect statistically
205 significant positivity by using a Poisson distribution, based on the average prevalence of the species.
206 We compared pairs of nominal variables using the Fisher's exact test with the assumption of
207 independence of these variables. We used Principal Component Analysis (PCA) to select the most
208 discriminant variables to be included in a multivariate analysis: we modeled the probability of
209 leptospirosis positivity as a function of environmental variables with a logistic regression (GLM with
210 logit function). We selected the best model with a backward elimination strategy based on the Akaike
211 Information Criterion (AIC). We also assessed the accuracies of models by estimating the receiver
212 operating characteristics (ROC) and the area under the curve (AUC) [38]. Statistical significance was
213 set at $P < 0.05$. All analyses were performed using the R 3.4.3 language and environment for statistical
214 computing [39].

215 **3. Results**

216 *3.1. Trapping success*

217 A total of 738 murine rodents were captured during the 1859 trap-nights of the study and
218 included in this analysis (Table 1, see also Biscornet *et al.* 2017 [14] for a comprehensive description of
219 the sample). They belong to two species: 687 *Rattus rattus* (RR) and 51 *Rattus norvegicus* (RN). No *Mus*
220 *musculus* were captured, probably because of the large size of the cage traps used.

221 **Table 1**: Rodents trapping results and *Leptospira* infection prevalence by sample site. The type of
222 environment is provided for each site: Urban (U), Peri-urban (P), Rural (R) and Natural (N).

Site	Trap-nights	Environment	*Leptospira* detection (RT-qPCR) Positive / Total sampled (% of positive)		
			Rattus rattus	*Rattus norvegicus*	All
Beau Vallon (out of protocol)	3	P	0/2 (0.0)	0/1 (0.0)	0/3 (0.0)
Casse Dent	149	N	2/65 (3.1)	0/2 (0.0)	2/67 (3.0)
Chemin Dame le Roi	179	R	2/75 (2.7)	-	2/75 (2.7)
Fairview La Misère	250	P	**8/69 (11.6)**	**8/15 (53.3)**	**16/84 (19.0)**
Grand Bois Mont Cephale	140	N	3/57 (5.3)	-	3/57 (5.3)
La Gogue	133	R	1/67 (1.5)	0/1 (0.0)	1/68 (1.5)
La Reserve	172	N	1/60 (1.7)	-	1/60 (1.7)
Police Bay	168	N	0/98 (0.0)	-	0/98 (0.0)
Port Launay	148	R	2/78 (2.6)	3/5 (60.0)	5/83 (6.0)
Providence Industrial Estate	33	U	0/6 (0.0)	2/2 100.0	2/8 (25.0)
Reclaimed Land (Zone 21)	142	P	**7/50 (14.0)**	**5/5 (100.0)**	**12/55 (21.8)**
Victoria	342	U	**4/60 (6.7)**	**9/20 (45.0)**	**13/80 (16.3)**
Total	1,859		**30/687 (4.4)**	**27/51 (52.9)**	**57/738 (7.7)**

223

We excluded "Beau Vallon" from the prevalence results, where there was only 3 trap-nights and 3 rats caught (2 RR, 1 RN). There was an average 169 trap-nights per site at the remaining 11 study sites. The trapping effort was lower at Providence Industrial Estate with only 33 trap-nights whereas the other 10 sites had at least 133 trap-nights. RR were trapped at all sites and averaged 43.3% with a maximum of 60.0% trapping rate at Police Bay. RN trapping was less successful (at 7 sites). The average trapping rate for RN was 3.8% and reached a maximum of 10.5% at Providence Industrial Estate and 10.3% at Fairview La Misère. There was no correlation between the percentages of trapping success of the two species per site. In the sampling sites where RN were caught, the trapping rates of RR were among the lowest. When adding the trapping rates of both species, the standard deviation decreases to 10.7% with an average of 47.1% (Figure 1).

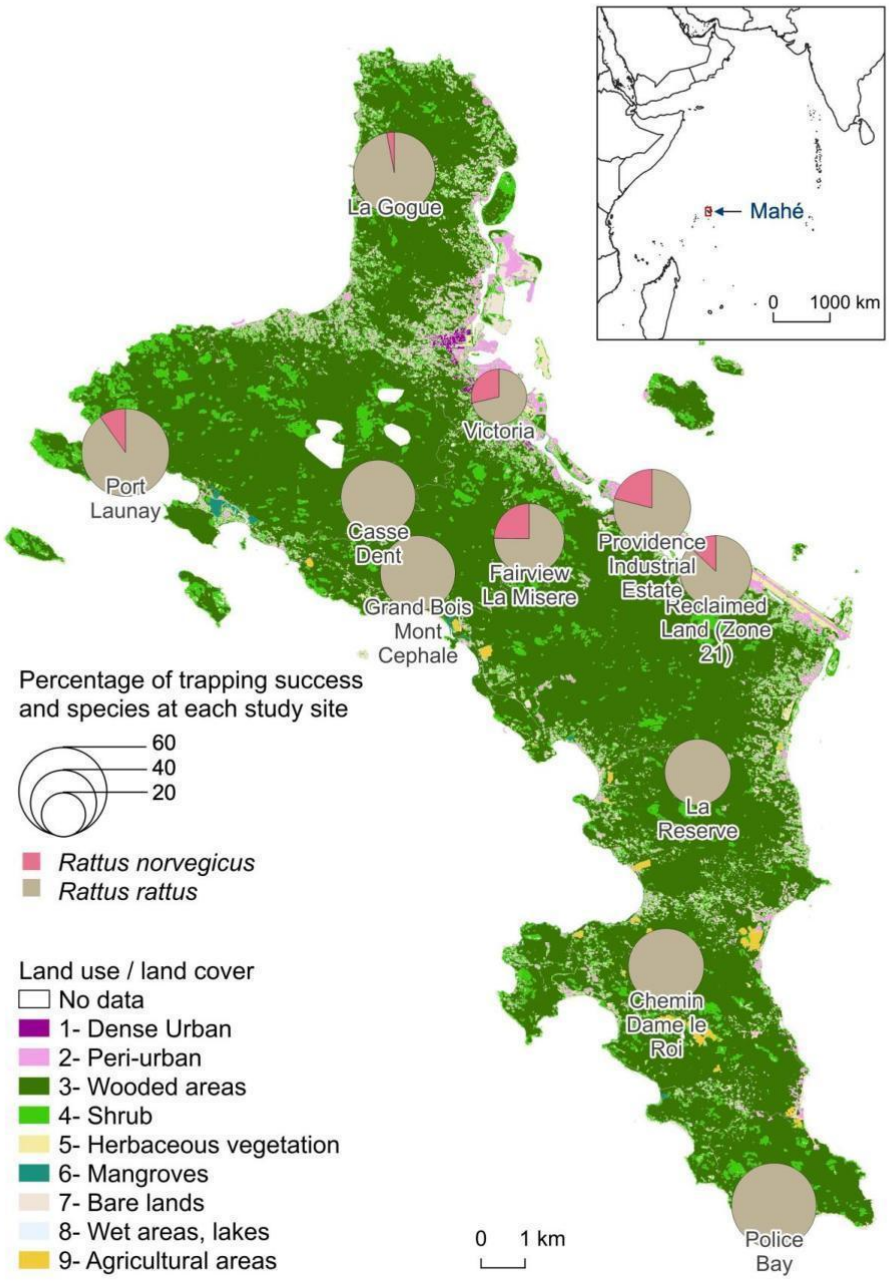


Figure 1: Percentage of trapping success of *Rattus norvegicus* and *Rattus rattus* in each study site

3.2. *Rat characteristics*

The sex ratio of males to females differed according to species although not significantly: 1.5 for RN (31/20) and 1.1 for RR (351/324). The adult to juvenile ratio was also higher for RN (16.0 *i.e.* 48

adults for 3 juveniles) than for RR (5.4 *i.e.* 580 adults for 107 juveniles), and very few RN juveniles were captured during sampling. Males were slightly larger in size and mass than females for both species but these differences in size were not significant between genders (average head and body length for RN males = 229.9 mm, RN females = 216.7, RR males = 182.8, RR females = 172.7). We also observed that the adult RN caught during the dry season were heavier (279.2 grams) than those caught during the wet season (218.2 grams), although not significantly. Adult RR had similar weight in both seasons (120.0 grams during the dry season and 122.5 grams during the wet season).

3.3. *Renal carriage of Leptospira spp.*

As previously reported [14], the overall prevalence of *Leptospira* infection was 7.7% (57/738) with a prevalence in RN (52.9%) significantly higher than in RR (4.4%) (Table 1). Maturity had no effect on infection in RR, positivity rate being similar in juveniles (4.7%) and in adults (4.3%). None of the 3 juvenile RN tested positive for leptospirosis, but their number is too small to test whether it is significant or not. In addition, there were no significant differences by gender for all animals tested and for both species. There was no significant difference in size between positive and negative animals, although positive adult RN had a larger body than negative animals (Figure 2).

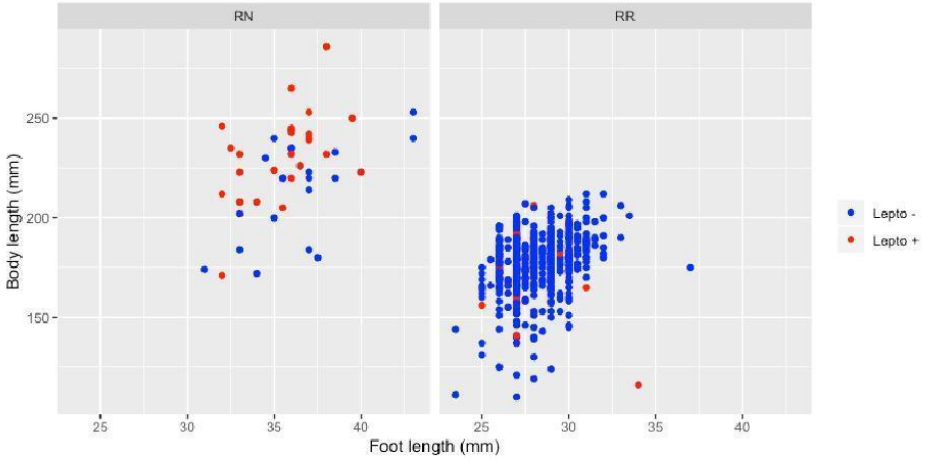


Figure 2: Relation between animal size (regarding body and foot length) and *Leptospira* infection, for *Rattus norvegicus* and *Rattus rattus*.

Leptospira-infected animals were found in 10 of the 11 sampled sites but with clearly distinct species distribution and infection prevalence (Table 1, Figure 3). At Police Bay, none of the 98 trapped rats (100% RR) were found positive for leptospire. Also, none of the three rats caught at Beau Vallon were positive. RN were found highly prevalent only at two sites, with similar infection prevalence: 45% at Victoria and 53% at Fairview La Misère (Central Mahé, about 500m high and 4-5 km South of the city of Victoria). RR were rarely captured on one site (Providence Industrial Estate) and were all non-infected. For the other 10 sites, a minimum of 50 RR was caught per site with an average prevalence of 4.4% and a standard deviation of 4.6%. The highest RR prevalence was reported from Reclaimed Land (Zone 21) (14%) and Fairview La Misère (11.6%).

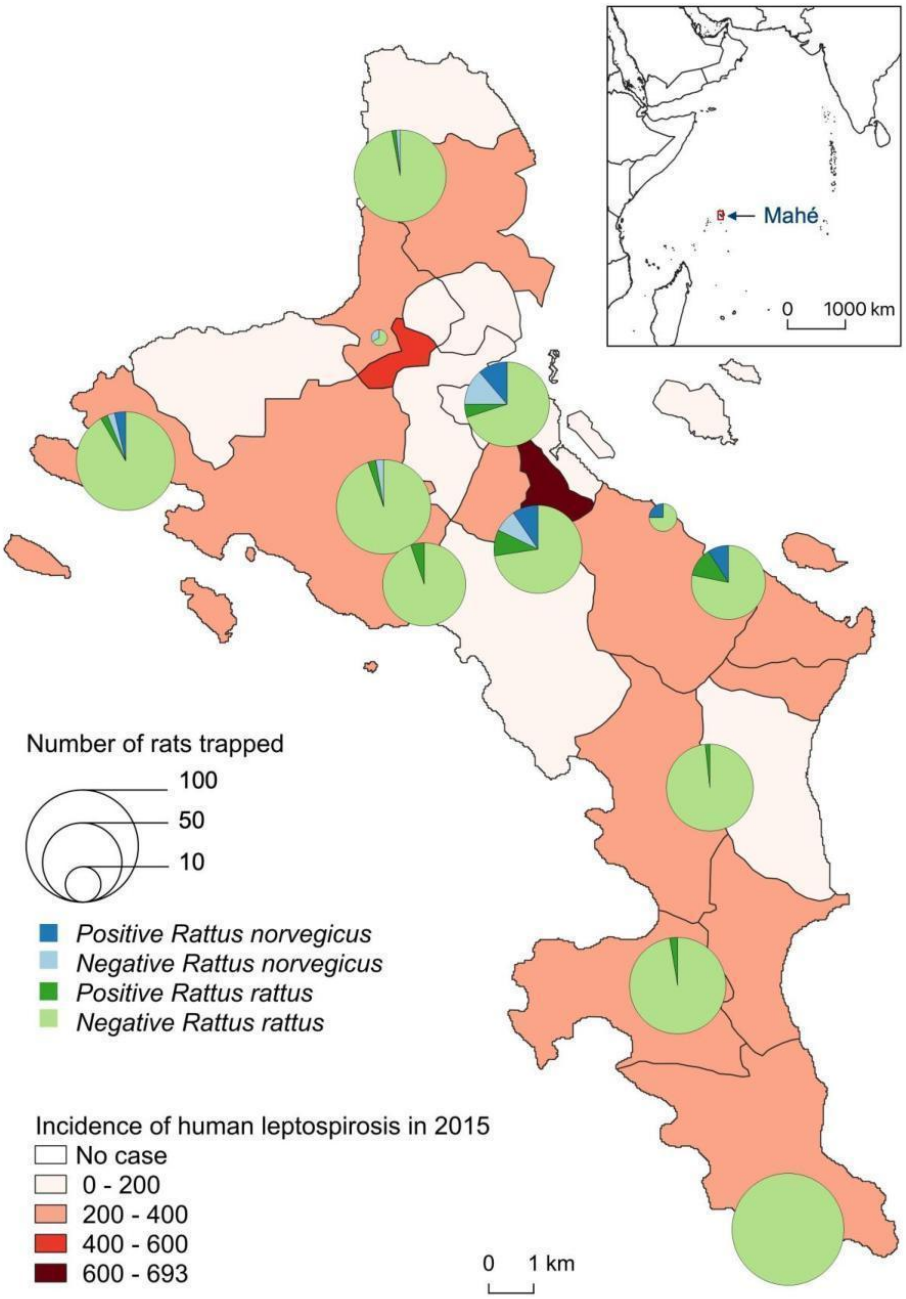


Figure 3: Number of *Leptospira*-infected (positive) and non-infected (negative) *Rattus* trapped, by species and site, compared to the incidence of human leptospirosis by district in 2015 (for 100,000 inhabitants).

3.4. Ecological patterns of rats distribution

The land use / land cover map obtained includes 9 classes (1- Dense Urban, 2- Peri-urban, 3- Wooded areas, 4- Shrub, 5- Herbaceous vegetation, 6- Mangroves, 7- Bare lands, 8- Wet areas, lakes, 9- Agricultural areas). The confusion matrix allowed us to calculate a Kappa index of 0.88, which is very satisfactory for future uses of this classification (Table 2). The least accurate class was bare soils due to confusion with the two urban classes that have similar spectral signatures. From this classification, we calculated 43 landscape indices to be used for statistical analyses. We also calculated five meteorological variables to complete the dataset.

Table 2: Confusion matrix of the land use / land cover classification.

Land use / land cover classes	1	2	3	4	5	6	7	8	9	Total	Author accuracy
1- Dense Urban	39				2		3			44	0.89
2- Peri-urban		56	3	2	1		4			66	0.85
3- Wooded areas			79			2				81	0.98
4- Shrub			4	28				1		33	0.85
5- Herbaceous vegetation		1	2	1	25		5			34	0.74
6- Mangroves			2	2		26				32	0.81
7- Bare lands				2		1	33	3		39	0.85
8- Wet areas, lakes						1		29		30	0.97
9- Agricultural areas		1			1				31	33	0.94
Total	39	58	90	35	29	30	45	33	31	390	
User accuracy	1	0.97	0.88	0.80	0.86	0.87	0.73	0.88	1		

[1] Total accuracy (Kappa) = 0.88.

Both rat species could be trapped over the entire range of altitudes with close averages (148.5 meters for RN and 154.4 meters for RR) (Figure 4A). Nevertheless, the majority of RN was caught at lower altitudes (with a median of 9 meters for RN and 123 meters for RR). Also, for both species, *Leptospira*-infected rats were found at a lower median elevation than non-infected ones. Of note, in Mahé Island, altitude is correlated with land use and proximity to urban areas, which are mostly located in coastal areas (*i.e.* low altitude). Infected rats of both species were observed at similar distances from dense urban areas (2513 meters for RN and 3548 meters for RR) while uninfected RN were observed at shorter distances (1400 m) and non-infected RR at greater distances with significant differences ($p<<0.001$) (Figure 4B). Non-infected RN were also significantly observed at greater distances from peri-urban areas than infected RN or RR ($p< 0.01$). Infected RN were found closer to water compared to non-infected RN, whereas there was limited difference between infected and non-infected RR. Distance to agricultural areas allowed species discrimination (RR being closer) but not the infection status of each species. Finally, the fragmentation index (edge density) also differentiated between infected and non-infected rats for both species. Amongst the three buffer sizes, the index calculated within a radius of 100 meters appeared as the most discriminating (Figure 4C). The median of the edge density (100m) of infected RR was almost twice that of non-infected RR and 1.5 times higher for infected RN compared to non-infected RN.

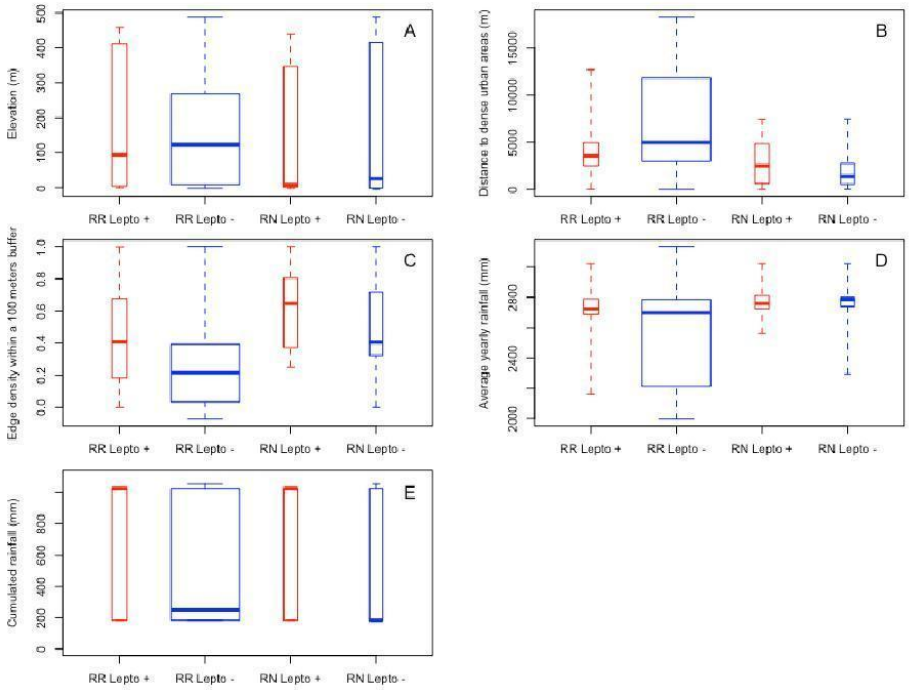


Figure 4: Boxplots of the most discriminant environmental variables, by species and *Leptospira* infection, measured from each sampling site: (A) Elevation, (B) Distances to dense urban areas, (C) Edge density within a 100 meters buffer, (D) Average yearly rainfall, (E) Cumulated rainfall during three months before capture.

Rain appeared as a factor influencing the distribution and infection rates of rats. First of all, rats sampled in the dryer locations (according to the 20-years average yearly rainfall) were mostly non-infected RR (Figure 4D). Also, when we considered the rains during the months preceding the capture, the cumulated rainfall of the last three months was the most discriminating variable (Figure 4E). Infected rats were sampled in places or during periods with more rainfall. Median values for infected rats were 4 (for RR) to over 5 times (for RN) higher than those of non-infected rats (1026 mm for positive RR and RN, 250 mm for negative RR and 183 for negative RN).

By projecting the variables on the first two axes (which account for 83.3% of the total inertia), a PCA showed that the distance to urban areas distinguishes non-infected RR from other rats. By contrast, the occurrence of infected RR and all RN (regardless of their infection status) was associated with higher rainfall and habitat fragmentation (Figure 5).

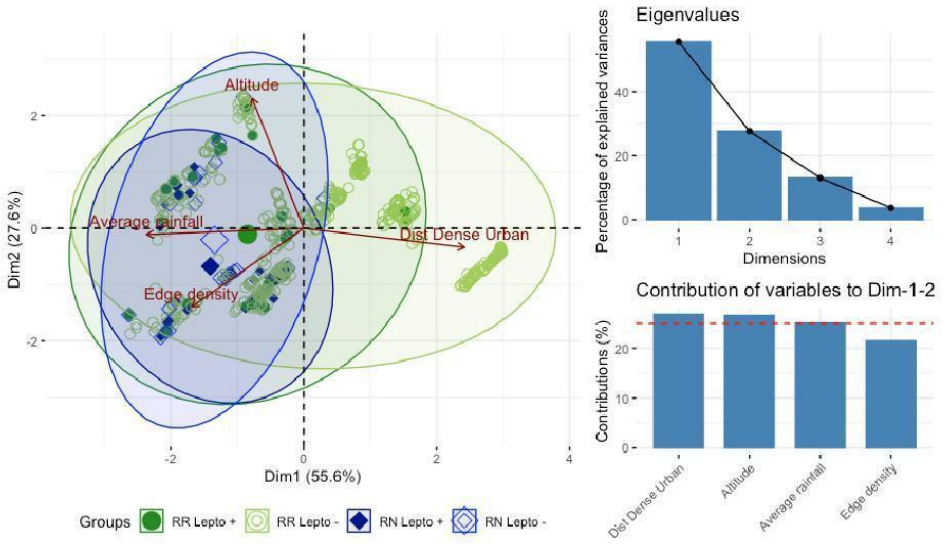


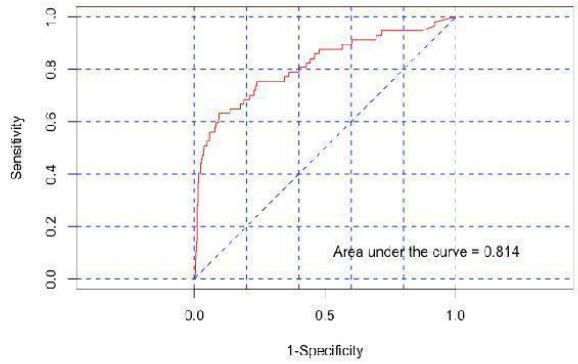
Figure 5: Principal Component Analysis showing the most discriminating variables.

The multivariate modeling with a logistic regression showed that the carriage of leptospires (infected *vs.* non-infected) could be predicted from environmental variables (Table 3). The best final GLM model GLM1 (AIC=305.77) identified the rat species, the average rainfall, the three months cumulated rainfall and the edge density (100m) as significant explicative variables. Removing the average rainfall variable did not change the explanatory power of the model (ΔAIC < 2). For this reason, we then kept the GLM2 model as our best simplest model (AIC=306.71). Its AUC of 0.814 confirmed a good performance (Figure 6). Considering only environmental variables and excluding the variable "Species", the best final GLM3 model (AIC=358.26) also included the average rainfall, the three months cumulated rainfall and the edge density (100m). Here, removing the average rainfall decreased its explanatory power. Its AUC of 0.775 was lower than GLM2 but still correct for a very simple model (Figure 7).

Table 3: Results of the best Generalized Linear Model (GLM) explaining the carriage of leptospires (infected *vs.* non-infected).

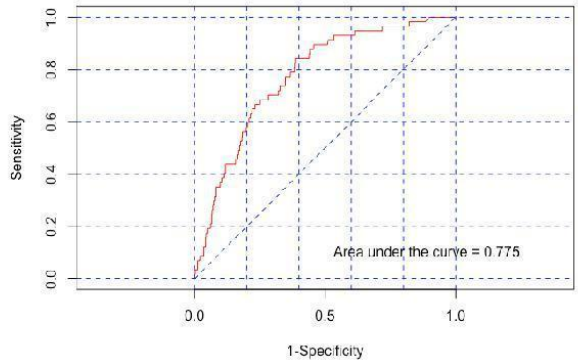
Model	Variables	Estimate	Std. Error	z value	P-value
GLM1 (Including variable Species) AIC = 305.77	(Intercept)	-4.966	2.185	- 2.273	0.023
	Species	-2.658	0.358	-7.436	1.04e-13
	20-years average rainfall	0.001	0.001	1.647	0.010
	3-months cumulated rainfall	0.001	0.000	2.228	0.026
	Edge density 100	1.556	0.596	2.610	0.009
GLM2 (Including variable Species) AIC = 306.71 ΔAIC (GLM2-GLM1)= 0.94	(Intercept)	-1.512	0.486	-3.110	0.002
	Species	-2.772	0.357	-7.759	8.58e-15
	3-months cumulated rainfall	0.001	0.000	2.180	0.029
	Edge density 100	2.048	0.546	3.751	0.000
GLM3 (Excluding variable Species) AIC = 358.26	(Intercept)	-8.700	1.974	4.407	- 1.05e-05
	20-years average rainfall	0.002	0.001	2.448	0.014

	3-months cumulated rainfall	0.001	0.000	2.488	0.013
	Edge density 100	2.139	0.529	4.045	5.23e-05
GLM4 (Excluding variable Species) AIC = 363.02 ΔAIC (GLM4-GLM3)= 0.94	(Intercept)	-4.125	0.364	11.345	- <2e-16
	3-months cumulated rainfall	0.001	0.000	2.475	0.013
	Edge density 100	2.893	0.474	6.100	1.06e-09



328

329 **Figure 6**: Sensitivity *vs.* specificity plot and area under the curve (AUC) for the GLM2 model
330 including the variable "species".



331

332 **Figure 7**: Sensitivity *vs.* specificity plot and area under the curve (AUC) for the GLM3 model
333 excluding the variable "species".

334 **4. Discussion**

335 *4.1. Rattus diversity and abundance*

336 Rodent trapping confirms the widespread presence on Mahé Island of the two most common
337 invasive commensal rodents (RR and RN). The high trapping rate of RR (average at 43.3% and highest
338 at 60%) reflects its relative higher abundance compared to RN, which was trapped less frequently

(average of 3.8% and highest of 10.5%) and mostly in or close to urban areas. Also, the coexistence of both rat species tends to modify the frequency of the two species due to competition, the presence of RN being correlated with less RR on a site. This phenomenon has been demonstrated in studies on various animals, including *Rattus* spp. [40,41].

Studies have shown that RN trapping rate varies greatly from place to place: recently in Salvador, Brazil, a study reported 13.1% of trapping rate [42], to be compared to 1.5% in Buenos Aires, Argentina [43]. The rat density seen on Mahé Island, Seychelles, could not be estimated as this study was not designed to answer that question, however high trapping rates (47.1% for both *Rattus* spp.) suggest a high abundance. This is generally expected on oceanic tropical islands due to the paucity of competitors and predators together with an abundance of suitable habitats and food sources for rats during much of the year [44]. Of note, a survey carried out on La Réunion Island by the same trapping team led to a trapping rate of 17.0% (808 RR and RN caught with 4,762 trap-nights) hence suggesting that rats abundance is particularly high in Seychelles, as previously reported by conservation-oriented research programs on various Seychelles islands such as the Aldabra Atoll, Mahé, Frégate and other granitic islands [44–46].

4.2. Leptospira-laden Rattus spp.

A higher *Leptospira* carriage in RN, compared to RR, is consistent with observations reported in many other countries. In Thailand, a meta-analysis of several surveys on murine rodents showed a prevalence of 20.8% (179/860) for RN compared to 5.8% (107/1858) for *Rattus tanezumi*, a *Rattus rattus* lineage [47]. Also, a meta-analysis of worldwide surveys on *Leptospira* carriage in RN reported an average prevalence of 20-25% with possible higher prevalence in specific countries [48], such as in a suburban area in Copenhagen, Denmark, where 53% of RN were found infected with *Leptospira* spp. [49] and in slums in Salvador, Brazil, where 88% of RN were infected [50]. Rarely, reverse trends have been shown: in the Los Rios region of Chile, *Leptospira* infection was 20.7% (51/246) in RR compared to 10.3% in RN, but the study was based on a small sample size (N=29) [51].

4.3. Rattus characteristics in relation to infection rates

Rats generally reach a larger size on tropical islands in view of the known island rule ecological hypothesis, which negatively correlates body size to co-occurring mammalian species: for instance, pacific islands have recorded gigantism in the invasive rodent *Rattus exulans* [52]. Although not significantly different, size differences between males and females were observed and may be related to the natural attribute of sexual dimorphism [42].

Captured RR were homogeneous with a sex ratio close to 1, a relatively large number of juveniles and a homogeneous weight according to the seasons. In contrast, for captured RN, males were caught in greater numbers than females, and very few young were caught. This reflects the differences in behavior between the two species, with RN females probably less mobile than RR females and a more conservative behavior for RN to protect their progeny. Indeed, RN are social animals that live in groups in their burrows [53]. They groom each other and females help each other to raise the juveniles [31].

Rural rats generally grow at a lower rate compared to urban rats. This has been postulated to be due to the relative abundance of nutritional sources compounded with less competition for it, in urban areas compared to rural areas, as well as the abundance of possible shelters afforded by urban landscapes [43,54,55]. This rapid reproductive capacity allows RNs to quickly colonize spaces. Knowing that they have high *Leptospira* prevalence, their population dynamics can create potential conditions for outbreaks or latency of leptospirosis.

4.4. Environmental factors in relation to rats distribution and infection rates

Mahé is a hilly island, with protected natural parks on its heights and the most populated areas on the coast. Trapping has revealed that rats are present on all sites, distributed throughout the island. However, spatial distribution differs between the two species. RR is the least selective species

and is found at all altitudes and in all environments, which corresponds to the known ecology of this commensal species [56]. Most RN were trapped at lower altitudes in urban settings. It should be noted that these urban areas include natural soil (an overlap also noted in the classification, "bare soils"), which is therefore the ideal environment for RN, as it tends to create its own shelter by digging burrows [57]. Rat movement has been shown to be restricted by food and shelter availability as well as physical features such as roads and waterways [58]. Associated with the fact that RN was relatively newly introduced [59], this may additionally explain the foci of RN in these urban landscapes.

The ecology of the two investigated *Rattus* species may at least in part drive the observed contrasted prevalence of infection, as RR is arboreal while RN nests in burrows, hence probably more exposed to environmental *Leptospira*. The different distributions of the two *Rattus* species, combined with a very high leptospires carrying rate in RN (12 times higher than RR), make the distribution of leptospires in rats logically similar to the heterogeneous distribution of RN. However, a few infected RR have been identified in the southern part of the island, at the heights of La Reserve (1.7% infected) and Chemin Dame le Roi (2.7% infected). But no infected rats were found in the extreme southern part of the island, at Police Bay (where no RN were found). Considering the average prevalence of infection of 4.4% in RR and the 98 samples tested at Police Bay, the probability of having at least one positive rat is 98.7% according to the Poisson distribution. We can therefore reasonably state that the RR population in the South of Mahé (and its environment) is free of leptospires.

The distribution of leptospires, as found in these rats, could correspond to a specific carrying capacity of the species and/or be related to the environmental factors that determine *Leptospira* survival during the environmental phase. For the first hypothesis, more genetic studies would be required, but we can still see that in environments where prevalence is higher, it is high for both species. The second hypothesis seems more plausible, with some environments being more favorable to the maintenance and transmission of leptospires. Our geospatial analyses have determined a selection of important factors strongly correlated with an increasing *Leptospira* positivity in *Rattus* spp., including lower elevation in relation to a specific distance range to dense urban areas and a higher distance to forested areas, a proximity to surface water for RN, a more fragmented landscape, and most of all higher rainfall during the three months before trapping. Some of these factors are also related to each other and, together, reflect anthropogenic disturbances, peri-urban areas, low elevation and near-water environments. The influence of habitat fragmentation on *Leptospira* positivity in both *Rattus* spp. can possibly be explained by the increased availability of food, water and shelter presented by such landscapes, in which density-dependent pathogen transmission could occur. Habitat fragmentation has been shown to promote an increase in host rodent presence and in turn on their parasites [60]. In Seychelles, fragmentation and urbanization seem to favour the presence of RN and *Leptospira*, which also leads to RR becoming more contaminated in these environments.

Rainfall appears to be a major factor influencing the distribution and infection rates of *Rattus* spp. The accumulation of water following rainfall may help transmission, as it brings *Leptospira*-infected environments closer to animal hosts. The runoff of water towards the coastal areas also accounts to the concentration of leptospires at lower elevations. The non-linear and lagged relationship between rainfall and human infection, as well as the role of floods, has been previously shown [61]. Our study is consistent with what is known of *Leptospira* infection of rats following rain [62]. However, the fact that it follows the same lagged relationship as in humans is interesting.

The multivariate modeling of leptospires carriage in *Rattus* spp. showed that leptospires carriage could be simply predicted using a few environmental variables (three months cumulated rainfall before capture and a landscape fragmentation index), without using molecular analyses. This prediction could be further improved if the reservoir species is known (RR *vs.* RN).

Finally, it is important to note that a previous investigation comparing *Leptospira* genotypes in both human acute cases and rodents in Seychelles has shown that rats can be considered the source of human infection in only a third of acute cases [14]. Our study confirms this previous result, as there is a poor overlap between the distribution of infected rats and human acute cases (Figure 3). As leptospirosis is still of great medical importance in this country, it is important to determine the environmental conditions that contribute to its survival and the animal species that act as carriers or

reservoirs in order to implement targeted control actions. Then, it would be interesting to complete this study at a micro-scale with chemical measurements of the soils in these environments to see more precisely the parameters that contribute to the survival of leptospires. Urban planning could be used to mitigate rat abundance and maximize the efficiency of rodent control (for example by limiting landscape fragmentation) with the aim of reducing humans contact with pathogens like *Leptospira* [63]. In certain areas (e.g. La Misère village), control strategies to limit the highly negative impact of rats on the autochthonous fauna and flora of Seychelles [45] should be designed to also contribute to this goal, and both advantages should be promoted to resident communities.

5. Conclusions

A geospatial analysis of infected and non-infected rodent reservoirs allowed us to identify favorable environments for their presence. In the Seychelles, we identified that the prevalence of leptospires in *Rattus* spp. can be predicted by landscape observation through remote sensing and rainfall measurement. This makes it possible to identify environments that are favorable to leptospires and that represent a risk for transmission to humans. This could help public health interventions strategies in the prevention and control of leptospirosis cases.

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625

Conclusion

This study showed that ***Leptospira*-infected *Rattus* were mainly located in areas of low elevations, fragmented landscapes, in proximity to urban areas, at an increased distance from forests and, particularly important, following recent precipitation**. A graphical abstract produced for publication (ref. Annex 2) summarises the remote sensing approach in the context of examining *Leptospira*-infected Rattus and the analyses of the impact of different habitats shown in the landscape covers.

A high trapping rate is indicative of the **large quantities of rats** present on Mahé island, which is expected for oceanic islands such as Mayotte for example, and which could explain the high human leptospirosis cases. Species of rats captured were *Rattus norvegicus* and *Rattus rattus* with the former being more infected with *Leptospira*. The study also predicts through multivariate statistical analysis that ***Leptospira* carriage in rats occurs when there is landscape fragmentation and recent rainfall.** However, contrary to what was expected, we show that there is a **poor overlap between the distribution of infected rodents and human acute case**s, which although there is not necessarily a causal link between presence of reservoirs and human cases as humans could have contracted the disease and travelled elsewhere, the inference is that there may be other alternative reservoirs of *Leptospira* present on Mahé (see chapter 2).

Results of the spatial modelling presented may **help decision makers implementing urban planning policies and/or in balancing conservation efforts when designing pest control strategies** that should also aim at reducing human contact with *Leptospira*-laden introduced rodents while limiting their negative impact on the autochthonous fauna.

Chapter 2: Molecular Epidemiology of Leptospirosis in Seychelles

Introduction

The insular islands of the SWIO display varied epidemiological features of incidence, mortality and diversity of leptospires. The Seychelles islands have consistently had high burden of this disease (Fig. 9 and Fig 10) and no formal investigation of the animal reservoirs have been undertaken. This second chapter aims at responding to these queries with the following objectives and strategies:

1) To determine the human incidence of leptospirosis in Seychelles amongst the cases of acute fevers of unknown origin,
2) To assess the clinical severity of incident leptospirosis in Seychelles,
3) To determine by molecular and serological methods the circulating *Leptospira* strains, and hence identify the main animal reservoir(s) of leptospirosis.

A prospective population-based study based was established as per study protocol (ref. Annex 1) to respond to the human leptospirosis objectives set forth. Two rat-trapping missions organised during the dry (SE) and wet (NW) monsoonal seasons were put in place to respond to the objectives set forth for the animal compartment. Sampling of domestic pets (cats and dogs) was carried out as well. Samples were submitted to serological and molecular screening, and all PCR positive samples were subsequently genotyped using MLST. The answers to these queries were the subject of **Article 2**: **Human leptospirosis in Seychelles: A prospective study confirms the heavy burden of the disease but suggests that rats are not the main reservoir.**

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RESEARCH ARTICLE

Human leptospirosis in Seychelles: A prospective study confirms the heavy burden of the disease but suggests that rats are not the main reservoir

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Abstract

Background

Leptospirosis is a bacterial zoonosis caused by pathogenic *Leptospira* for which rats are considered as the main reservoir. Disease incidence is higher in tropical countries, especially in insular ecosystems. Our objectives were to determine the current burden of leptospirosis in Seychelles, a country ranking first worldwide according to historical data, to establish epidemiological links between animal reservoirs and human disease, and to identify drivers of transmission.

Methods

A total of 223 patients with acute febrile symptoms of unknown origin were enrolled in a 12-months prospective study and tested for leptospirosis through real-time PCR, IgM ELISA and MAT. In addition, 739 rats trapped throughout the main island were investigated for *Leptospira* renal carriage. All molecularly confirmed positive samples were further genotyped.

Results

A total of 51 patients fulfilled the biological criteria of acute leptospirosis, corresponding to an annual incidence of 54.6 (95% CI 40.7–71.8) per 100,000 inhabitants. *Leptospira*

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carriage in *Rattus* spp. was overall low (7.7%) but dramatically higher in *Rattus norvegicus* (52.9%) than in *Rattus rattus* (4.4%). *Leptospira interrogans* was the only detected species in both humans and rats, and was represented by three distinct Sequence Types (STs). Two were novel STs identified in two thirds of acute human cases while noteworthily absent from rats.

Conclusions

This study shows that human leptospirosis still represents a heavy disease burden in Seychelles. Genotype data suggests that rats are actually not the main reservoir for human disease. We highlight a rather limited efficacy of preventive measures so far implemented in Seychelles. This could result from ineffective control measures of excreting animal populations, possibly due to a misidentification of the main contaminating reservoir(s). Altogether, presented data stimulate the exploration of alternative reservoir animal hosts.

Author summary

Leptospirosis is an emerging environmental infectious disease caused by corkscrew shaped bacteria called *Leptospira*. Humans usually get infected during recreational or work-related outdoor activities through contact with urine excreted by animal reservoirs. As a zoonotic disease, leptospirosis is a good example of the One Health concept for it links humans, animals and ecosystems in a web of pathogen maintenance and transmission. This zoonosis is highly prevalent in the tropics and especially in tropical islands. Seychelles archipelago has been reported as the country with highest human incidence worldwide, although figures are based on dated studies and/or poorly specific tests. The presented investigation aimed at providing an updated information on human leptospirosis burden in Seychelles and exploring the transmission chains in their environmental aspects. Presented data confirms that the disease still heavily impacts the country. Genotyping of pathogenic *Leptospira* in human acute cases reveals that three distinct Sequence Types (STs) are involved in the disease. However, rats typically considered as the main reservoir in Seychelles, harbor only one of these STs, found only in a minority of human cases. Hence, it appears that rats are likely not the main reservoir of leptospirosis in Seychelles, which has important consequences in terms of preventive measures to be implemented for a better control of human leptospirosis.

Introduction

Zoonoses are known to contribute to approximately 60% of human infectious diseases and represent 75% of emerging diseases [1]. Among them, leptospirosis is considered as one of the most prevalent bacterial zoonotic disease worldwide [2], as well as a (re)emerging disease [3]. It is considered by the WHO as being amongst the world's neglected tropical diseases with epidemic-prone potential causing significant public health impact [4,5]. Leptospirosis affects 1.03 million persons annually causing nearly 60,000 deaths [6]. This zoonosis is transmitted to humans and domestic animals through direct or indirect contact with infected urine excreted by reservoir/carrier hosts [7]. Leptospirosis prevalence is higher in tropical environments where conditions may be more conducive to *Leptospira* survival [8]. Prevalence is maximal in

tropical islands for unknown reasons, although reduced species diversity typical of insular ecosystems may boost pathogen transmission [9,10].

In the South West Indian Ocean islands (SWIO), human leptospirosis shows a contrasting epidemiology in terms of incidence, mortality and diversity of leptospires. In Reunion, a French administered island, the incidence of human leptospirosis is the lowest recorded in the region (8.2 cases per 100,000) and the disease is caused by two *Leptospira* species (*L. interrogans* and, to a much lesser extent, *L. borgpetersenii*), with Icterohaemorrhagiae serogroup being overwhelmingly dominant [11]. The higher incidence (74.5 cases per 100,000) in Mayotte [12], also administered by France and sharing a common health surveillance system with Reunion island, is compounded by a much higher diversity of bacterial species (*L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. mayottensis*) and serogroups [13,14]. Noteworthily in Mayotte, Icterohaemorrhagiae serogroup was not diagnosed through Microscopic Agglutination Test (MAT) since the implementation of active surveillance in 2008 [15]. In the Union of the Comoros, no human leptospirosis has been reported recently, but according to a recent study, the serological profile of *Leptospira* infecting humans is likely similar to that encountered in the neighboring Mayotte [16], the fourth island of the Comorian archipelago. In Madagascar, a considerable diversity of pathogenic *Leptospira* has been detected in bats and terrestrial small mammals [17–19], despite limited reports of human cases [20,21].

The Republic of Seychelles is an archipelago consisting of 155 granitic or coralline islands located between 4 and 10 degrees south of the equator and lying between 480 km and 1,600 km east of the African continent in the SWIO. The climate of Seychelles is of the warm, humid tropical type, and divided into two main seasons: the Northwest Monsoon, period of higher rainfall from December to March, and the Southeast Monsoon from May to October, separated by two relatively short Inter-Monsoonal periods [22]. The mid-year population estimate of Seychelles as at August 2015 is 93,419 [23], of which almost 90% live on Mahé island.

In reference studies on humans in Seychelles conducted up to 25 years ago [24–27], Seychelles has been reported as ranking first worldwide for leptospirosis incidence [8], and several serogroups have been identified by Microscopic Agglutination Test (MAT) [25,27]. However, in the absence of published molecular data, the *Leptospira* species of medical concern in Seychelles remains unknown. Rats have been considered as the main animal reservoir of *Leptospira* in Seychelles and have been the target of active population control; eradication of invasive rodents, including black rats (*Rattus rattus*) and Norway rats (*Rattus norvegicus*), have been a continuous activity of both the Public Health Authority and the Environment department in Seychelles [28]. However, the role of suspected animal reservoirs has never been properly investigated, and the only mention of *Leptospira* prevalence in rats is an anecdotal study where 24% of sampled rats (n = 25) were reported to be MAT seropositive for Icterohaemmorhagiae serogroup [25]. Hence, from a public health perspective, there is still a significant lack of information regarding a zoonosis considered as the most important infectious disease in the country, based on local surveillance data collected over the past three to four decades.

The context of insular countries, such as Seychelles, which inherently have limited geographical distribution and diversity of vectors/reservoirs, represents an opportune small-scale environmental setup for the investigation of disease ecology [29]. Such studies have a direct beneficial impact in providing concrete evidence-based data that may guide public health interventions implemented to control the transmission of zoonotic pathogens to humans. Hence, we carried out a comprehensive survey aiming at (i) determining the present incidence of human leptospirosis in Seychelles and compare it to the Yersin *et al.* (1998) study published almost twenty years ago, (ii) characterizing at the specific and infraspecific levels the *Leptospira* infecting humans and animal reservoirs in order to identify

transmission chains, and (iii) identifying biotic and abiotic variables having a major impact on the epidemiology of the disease in Seychelles.

Materials and methods

Ethical approval and sampling permits

The Health Research and Ethics Committee of the Ministry of Health of Seychelles approved the study protocol for humans (Research Proposal 1405). Signed consent forms were obtained from participants enrolled in the study before questionnaires were administered and samples taken. Written informed consent was sought from parents of minors included in the study. The Seychelles Bureau of Standards gave the approval for the trapping and investigation of rats (A0157). All animal procedures carried out on rats were performed in accordance with the European Union legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). The research protocol's ethical terms were defined under accreditation 03387 (FEDER POCT LeptOI 32913 research program) and were approved by the CYROI Institutional Animal Care and Use Committee (Comiteé d'Ethique du CYROI n° 114, IACUC certified by the French Ministry of Higher Education and Research). Veterinarians of the Veterinary Services of the Seychelles Agricultural Agency, Ministry of Agriculture and Fisheries, carried out sampling of dogs and cats.

Inclusion of human acute cases

A national leptospirosis surveillance program was launched in Seychelles in December 2014. The study was designed as a prospective population-based survey conducted for one year from the 1st December 2014 to the 30th November 2015 on all acute febrile cases of unknown origin in Seychelles. Physicians were requested to refer all cases more than 13 years of age with acute fever of unknown origin and meeting the case definition (Box 1) to the reference leptospirosis clinic established at the Seychelles Hospital. Patients below 13 years were not included, in view of the larger spectrum of non-specific acute fever cases affecting this age group and the low prevalence of leptospirosis among this young age class based on local surveillance data. Referred patients were enrolled in the study after providing informed consent, and were assessed for severity and admitted to hospital if required as per established criteria. Demographic data *i.e.* age, sex and residential address were collected on all enrolled patients, as well as the outcome of clinical intervention, and a questionnaire was administered. Biological samples were collected to conduct an array of standard laboratory tests: 3 to 5 ml of whole blood was collected in tubes with and without anti-coagulant.

Animal sampling

Rats were trapped throughout Mahé at 12 sampling sites representing a spread over seven regions (Victoria, Victoria periphery, North, Centre, East, West and South); habitats were defined by descriptions of the sampling sites (S1 Table). Two trapping missions were conducted, one during the Southeast monsoonal season in June-July 2013, and the other in the Northwest monsoonal season in February-March 2014. All information including GPS coordinates and habitat types are provided as supplementary material (S1 Table). Trapping was conducted following a standard protocol [30] using wire cage traps baited with roasted coconuts. At each sampling site, 40 to 80 traps were placed 15 meters apart in line. Trapped animals were collected the following morning and brought back to a laboratory facility of the Ministry of Health. Animals were euthanized by cervical dislocation, blood collected by cardiac puncture, and dissected organs (kidney, liver, lung, spleen) were stored immediately in liquid nitrogen.

Box 1. Case definitions and exclusion criteria

Case definitions

Suspected case: Anybody aged 13 years and above reporting to a health center (private or governmental) during the 12-month period presenting with fever of $\geq$ 38˚C for more than three days with or without any of the following signs and symptoms; headaches, myalgia, hemorrhagic manifestations in the absence of any definite diagnosis.

***Probable* case**: Anybody meeting the suspected case definition criteria with a Positive ELISA IgM as per the diagnostic criteria.

Confirmed case: Anybody meeting the suspected case definition criteria with a positive real-time PCR assay for pathogenic *Leptospira* spp. in blood and/or a positive MAT, a minimum four weeks after the onset of symptoms. A positive MAT was defined as one that displayed an infective Serogroup with a four-fold seroconversion in paired sera, or acute sera with a Serogroup displaying a minimum titer of 1:400. The infective Serogroup in sera that had coagglutinatinating titers had the serogroup displaying two titer orders more than the rest as the definitive infecting serogroup. For minors less than 18 years old, the accompanying guardian or parent was asked to give consent and to provide the relevant information.

Exclusion criteria

A person was excluded as a case if unwilling to participate in the interview and biological examinations and/or no samples were collected after enrolment.

Grinded fragments of fresh kidney tissue were immediately inoculated to culture medium (see below). *Rattus* species were identified using morphological characters [31] and animals identified as *R. rattus* were further sequenced at the cytochrome b (*cytb*) locus in order to avoid misdiagnosis within the *R. rattus* complex [32]. Dr. Jimmy Mélanie and Dr. Maria Tirant, respectively Principal Veterinary Officer and Veterinary Officer at the Seychelles Veterinary Section of the Seychelles Agricultural Agency in the Ministry of Environment and Agriculture, collected over a period of 4 months (December 2015 to March 2016) kidneys from healthy dogs and cats that were to be euthanized as part of the routine practice, *i.e.* from owners who wished to dispose of their unwanted animals. These kidney samples were stored in 70% ethanol at –80˚C until shipment using dry ice.

Nucleic acids preparation

Total nucleic acids were extracted from a pool of kidney, lung and spleen tissues collected from rats. Organs were dissected on ice, thin slices of approximately 20 mg of each tissue were pooled in 750 μL of DMEM (PAN-Biotech GmbH, Aidenbach, Germany) and grinded using a tissue lyser (QIAGEN, Les Ulis, France) and two 3 mm tungsten beads agitated at 25 Hz for 1 min. The resulting homogenate was centrifuged for 5 min at 10,000 rpm and 200 μL of the resulting supernatant was added to 200 μL of AVL buffer for subsequent extraction. In addition, 5 μL of bacteriophage MS2 (final concentration of 5μM) was added to one sample of every batch run and used as internal extraction control as previously described [33]. Extraction was performed on an EZ1 Advanced XL robot (QIAGEN, Les Ulis, France) using EZ1 virus

extraction kit following the manufacturer's instructions and using a final elution volume of 60 μL [18,19].

Nucleic acids were extracted from human serum samples either manually or using a QIAcube robot (QIAGEN, Les Ulis, France) as per manufacturer's instructions using QIAGEN Viral minikit. A reverse transcription step was performed on 10 μL of the eluted total nucleic acids from human and animal origin using a GoScript reverse transcriptase (RT) kit (Promega, Charbonnières-les-Bains, France), by adding 1.25 μL of random primer hexamers, incubating at 80˚C for 3 min and then holding at 4˚C. To this mix was then added 4 μL of Buffer 5X, 2 μL of $MgCl_2$ (25 mM), 1 μL of dNTPs (10 mM), 1 μL of RT (200 U/mL), 0.05 μL of RNAse inhibitor, and 0.7 μL of RNAse free H_2O. Reverse transcriptions were carried out using the following conditions: 25˚C for 5 min, 42˚C for 60 min, 70˚C for 15 min and then held at 17˚C.

As dog and cat kidneys were preserved in 70% ethanol, samples were first rehydrated overnight in osmosis water before carrying out DNA extraction using DNeasy Blood and Tissue extraction kit (QIAGEN) following manufacturer's instructions. Reverse transcriptions were also carried out on these samples following the conditions mentioned above. All produced cDNAs were stored at −80˚C until PCR detection.

PCR detection and genotyping through MLST

The screening of cDNA using a probe-based real-time PCR (Polymerase Chain Reaction) for pathogenic *Leptospira* spp. was done adapting Smythe's protocol to target the *rrs* (16S) gene [34]. Amplifications were performed in 25 μL containing 12.5 μL of Absolute Blue real-time PCR Low Rox Mix (Thermo Scientific, Waltham, MA, USA), 0.5 μL (10 μM) (initial stock concentrations shown) of each primer, 0.4 μL (10 μM) probe and 6.1 μL of nuclease free water. The PCR conditions were 95˚C for 15 min, followed by 45 cycles at 94˚C for 30 sec and 60˚C for 1 min. For human samples detection, a cut-off criterion was set at Cycle threshold (Ct) <35 for positivity following Ahmed *et al.* [35]. *Leptospira interrogans* DNA serially diluted and leading to a Ct of 30 was used as a positive control for RT and end-point PCRs. A minimum of three distinct negative (water) controls were performed for each RT-PCR run and one single negative (water) control for each end-point PCR. PCR detection was first performed in triplicates and samples producing at least two positive reactions were considered positive, while samples producing a single positive reaction were submitted to an additional triplicated PCR. Moreover, samples with amplification at only one or two out of six real-time PCR runs were not considered positive unless a *Leptospira* sequence was achieved. Additionally, samples that had 35<Ct<40 for more than two replicates out of six were considered positive. Animal samples were submitted to a single real-time PCR with a cut off criterion set at Ct<45. Genotyping of positive samples was carried out through Multi Locus Sequence Typing (MLST) scheme#3 (http://pubmlst.org [36]). This scheme was chosen instead of two other available schemes as a number of investigations on other islands of the SWIO region have been carried out using this same MLST scheme. The amplification of *adk*, *icd*A, *lipL*32, *lipL*41, *rrs*2 and *secY* genes was performed using generic primers [36]. In case of PCR failure, samples were submitted to an alternative PCR using degenerated primers [18] and/or to an alternative amplification of *rrs*2 gene using previously published LA/LB primers [37]. All amplicons were sequenced on both strands (GenoScreen, Lille, France) and sequences were edited using Geneious 9.1.3 [38]. Original sequence types (STs) were deposited on the pubMLST database. DNA sequences were deposited on GenBank and accession numbers are listed on S2 Table.

Leptospira culture from rat kidneys

Kidneys from freshly dissected rats were aseptically sectioned and finely minced with a blade before inoculating three distinct media: (i) Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid basal medium (Difco, Detroit, MI, USA) supplemented with Albumin Fatty Acid Supplement (AFAS, purchased at OIE and National Collaborating Centre for Reference and Research on Leptospirosis Academic Medical Center, Department of Medical Microbiology, Amsterdam) [39,40]; (ii) EMJH liquid basal medium supplemented with AFAS, rabbit serum and foetal calf serum (1% each); and (iii) semisolid Fletcher medium (Difco, Detroit, MI, USA) supplemented with rabbit serum (8%). All media were supplemented with 5-fluorouracil (5-FU) at a final concentration of 200 $\mu g.mL^{-1}$. Cultures were incubated at 28˚C, visually checked for the presence of *Leptospira* using a dark field microscope once a week for four months, and positive cultures were further sub-cultured in fresh EMJH liquid basal medium supplemented with AFAS but deprived of 5-FU. A detailed protocol is available at http://dx.doi.org/10.17504/protocols.io.ifccbiw.

Serological screening through ELISA and Microscopic Agglutination Test (MAT)

All acute human sera were screened through an in-house IgM ELISA test using 96-well Immulon 1B polystyrene plates coated with *Leptospira biflexa* serogroup Patoc antigen (already prepared at 11×10^8 leptospires/mL from cultures and stored at 4˚C). The antigen preparation was used at a dilution of 1:30 to test all 223 human sera on ETI-Max 3000 (DiaSorin, Saluggia, Italy) at the GHSR-CHU (Groupe Hospitalier Sud Réunion-Centre Hospitalier Universitaire) hospital of Saint Pierre in Reunion Island. Absorbances were read at 450/620 nm. MAT was based on a panel of twenty *Leptospira* strains (see S3 Table) allowing detecting most serogroups that have been previously reported in humans [13,14,25,41] and animals [42] in the SWIO islands. All patients enrolled in the prospective study were tested by MAT using the initial blood sample (acute phase) to measure the prevalence of antibodies to *Leptospira* in the cohort (reflecting either ongoing, recent or old infections). For 46 patients of the cohort, we could obtain a second blood sample at least four weeks after the onset of the first signs and symptoms (convalescent phase) and these 46 paired sera were tittered with MAT. Sera were tested at dilutions ranging from 1:50 to 1:3200. A MAT titer of more than or equal to 1:100 (cut off value of the test) indicated a seropositive sample and the reactive serogroup as the one allowing agglutination at two titer orders more than the other coagglutinins. MAT serology was diagnostic of acute leptospirosis only if the MAT titer was $\geq$ 1:400 on the acute phase sample and/or demonstrated a four-fold increase in titer (*i.e.* seroconversion) on paired sera. Since Icterohaemorrhagiae was the serogroup previously reported as most prevalent in human acute cases [25] and as Patoc cross-reacts with most serogroups, we carried out MAT in two steps. First, a screen at 1:100 using Patoc and Icterohaemorrhagiae serogroups allowed highlighting putative positive samples. Second, all the samples reactive against Patoc and/or Icterohaemorrhagiae were serially diluted, up to a titer of 1:3200 and submitted to MAT using the full panel listed in S3 Table.

Diagnostic criteria of human leptospirosis

Overall, a patient was considered positive for leptospirosis if one of the following conditions was fulfilled: (i) MAT titer of acute serum $\geq$1:400; (ii) evidence of seroconversion on paired sera attested by a fourfold increase of MAT titers; (iii) real-time PCR with Ct<35 for at least two replicates out of six; (iv) real-time PCR with 35<Ct<40 for at least 3 replicates out of six;

(v) real-time PCR with Ct<40 on one or two attempts and simultaneously positive for IgM by ELISA; (vi) real-time PCR with 35<Ct<40 and with at least a sequence achieved on one of MLST loci. Samples that were positive for IgM ELISA only were considered negative due to the possibility of rheumatoid factors giving a false positivity [43] and also for the well-known long-term persistence of anti-*Leptospira* IgM antibodies months and years after acute infection [44,45].

Mapping

Georeferencing and mapping of human cases and *Leptospira* positive *Rattus* spp. was carried out using QGIS v2.18.0 "Las Palmas", [46] freely available at http://www.qgis.org/en/site/.

Statistical analyses

We investigated the effects of different variables on the infection status of rats: host "Species" (*R. norvegicus vs R. rattus*) and "Maturity" (juvenile *vs* adult), "Seasons" (wet *vs* dry), as well as environmentally variables such as "UrbanOrRural" (representing the type of habitat, urban or rural), and "Region" (Victoria, Victoria periphery, North, Centre, East, West and South). Variable "UrbanOrRural" was determined in accordance to descriptions recorded during sampling at each site (refer S1 Table): "urban" habitats are built or heavily disturbed habitats while "rural" sites are residential, mixed-agricultural or natural habitats. Pairs of variables were compared using Fisher's exact test for count data. Generalized linear models including "Species", "Maturity", "Seasons", "UrbanOrRural", and "Region" as explicative variables were performed using a binomial distribution and logit link function (log-likelihood type 1 test). Analyses were conducted using "R" software [47].

Minimum-spanning tree

A minimum-spanning tree (MST) was built using goeBURST Full MST algorithm (PHYLOViZ 1.1, 2014), by concatenating six MLST gene markers (*adk*, *icd*A, *lipL*32, *lipL*41, *rrs*2 and *secY*; fused in that order) and comparing with previously published STs found in various hosts worldwide.

Results

Incidence of human leptospirosis in Seychelles

Overall, 225 patients out of 226 presenting with acute fever of unknown origin were enrolled in the study (one patient refused to participate). Two patients for whom no sample could be obtained were excluded, leaving a total of 223 patients effectively investigated for acute leptospirosis. There were 23 females and 198 males (gender information was missing for two patients) with a mean age of 33 years old (range 13 years– 60 years) and a median of 34 years old. A total of 51 patients (49 males and 2 females) were diagnosed with leptospirosis (see below) of whom 6 patients (5 males and 1 female) died of their disease, leading to a case fatality rate for leptospirosis of 11.8%.

There was a moderate agreement (44.5%) between MAT and real-time PCR results: out of 46-paired sera (representing 20.6% of all patients), 11 were MAT positive out of which six were also real-time PCR positive. Of the 35 paired samples that were MAT negative, 88.6% were also negative by real-time PCR. Agreement by Cohen's Kappa (Table 1) between confirmed positives as defined by the diagnostic criteria and the different performed tests were categorized as: good for PCR (87.8%) and MAT (81.4%), and moderate for IgM by ELISA

Table 1. PCR, MAT and IgM ELISA, agreement and test performance values. Number of positive and negative samples is defined as per the diagnostic criteria. Kappa (κ) / [Agreement Criteria]: ≤ 20 / [Poor]; 21–40 / [Fair]; 41–60 / [Moderate]; 61–80 / [Substantial] and 81–100 / [Good].

		Tests		
		PCR	MAT	IgM ELISA
Confirmed positive (n = 51)	Positive	30	19	18
	Negative	21	32	33
Confirmed negative (n = 172)	Positive	0	0	0
	Negative	172	172	172
Sensitivity (%)	Value	58.8	37.3	35.3
	95% CI	44.2–72.4	24.1–51.9	22.4–49.9
Specificity (%)	Value	100	100	100
	95% CI	97.9–100	97.9–100	97.9–100
Positive Predictive Value	Value	100	100	100
	95% CI	97.9–100	97.9–100	97.9–100
Negative Predictive Value	Value	89.1	84.3	83.9
	95% CI	85.5–91.9	81.3–86.9	81.0–86.5
Cohen's Kappa (%)	Value	87.8	81.4	45.7
	95% CI	79.7–93.5	72.4–88.5	35.7–56.0

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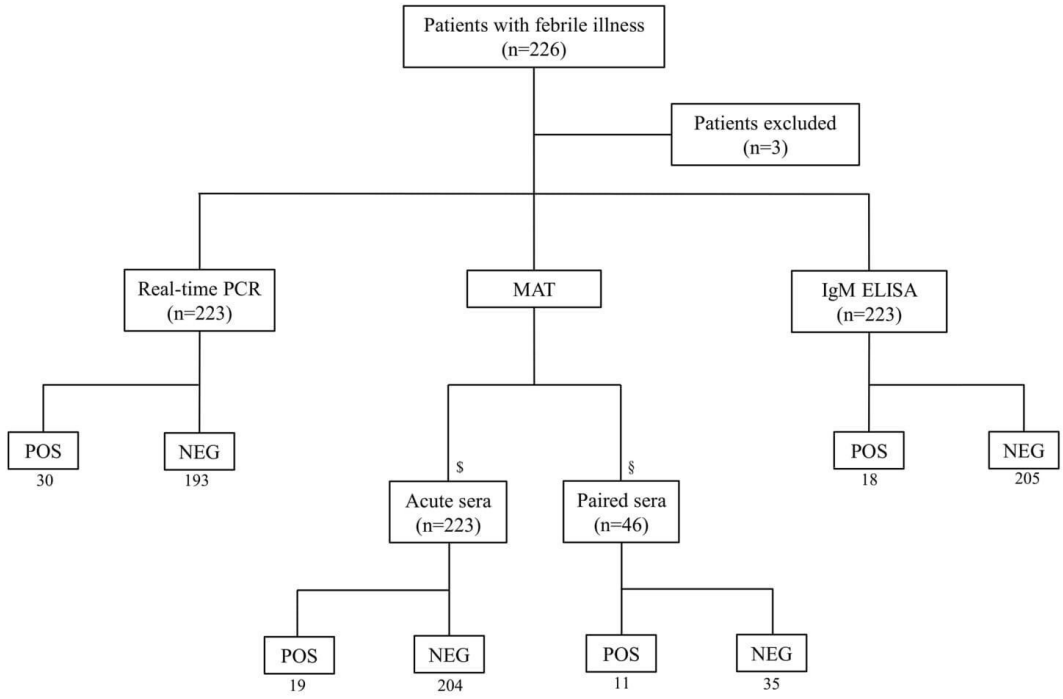
(45.7%). A flow chart of the tests conducted and the positive and negative results are shown in Fig 1.

Altogether, 51 patients fulfilled the diagnostic criteria of acute leptospirosis either by real-time PCR (n = 30) (as per criteria (iii) and (iv)) IgM ELISA with PCR (n = 9) (as per criteria (v)) and confirmed MAT (n = 19) (as per criteria (i) and (ii)), as well as the aforementioned sample for which a sequence was produced (diagnostic criteria (vi)). Considering the current population of Seychelles [23], incidence of human leptospirosis was evaluated to be 54.6 (95% CI 40.7–71.8) per 100,000. Temporal analysis of the prevalence of cases over the one-year period from December 2014 to December 2015 shows a decreasing trend from the January to March period, corresponding to the humid Northwest monsoon, towards the usually dry season, where fewer cases were reported (Fig 2). There is some seasonality depicted by a high number of cases after a period of high rainfall, although not as sharp as that reported in other insular ecosystems of the region (see Discussion).

In order to identify the most prevalent serogroups, we carried out MAT screening of all patients enrolled in the study using the blood sample collected at inclusion (acute phase serum samples). Forty-five acute phase sera out of 223 tested seropositive by MAT (*i.e.* titer > 100) at inclusion. Among the seropositives, the serogroup Icterohaemorrhagiae was dominant (n = 8), followed by Autumnalis (n = 5), Hurstbridge (n = 4), Australis (n = 4), Djasiman (n = 3) and Sejroe (n = 1). The serogroups Ballum and Canicola, previously reported in Seychelles [25], were not detected in our sample while the previously identified serogroup Louisiana was not included in our panel. We did have two sera that were Patoc positive but did not agglutinate with any of the 20 reference strains of our panel. As a significantly large number (n = 18) of sera in our sample set displayed cross-agglutination, we identified the infective serogroup as the one allowing agglutination at two titer orders more than the other coagglutinins. According to this criterion, the distribution of major cross-agglutinating serogroups, in decreasing order, were Icterohaemorrhagiae (n = 18), Autumnalis (n = 8) and Hurstbridge (n = 8). A single sample displayed equal cross-agglutination to Pomona and Hardjobovis serogroups (see S4 Table for tabulated MAT results).



$ MAT titers for acute sera positive when ≥ 1:400
§ MAT titers for paired sera positive when ≥ 1:400 and/or with four-fold seroconversion

Fig 1. Diagnostic flow chart of tests done, number of enrolled patients and diagnostic results.

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Genetic diversity of *Leptospira* infecting humans

MLST sequences were produced for 24 out of the 32 real-time PCR positive patients, distributed for the six gene loci as follows: *adk* (n = 21), *icd*A (n = 19), *lipL*32 (n = 22), *lipL*41 (n = 21), *rrs*2 (n = 22) and *secY* (n = 20). *Leptospira* sequences that were obtained from these 24 patients were all identified as *Leptospira interrogans*. Complete six-loci MLST was achieved for 18 patients leading to three different STs: one was identified in pubMLST database as ST02, whereas two STs were not previously reported/registered in the database and thus considered as novel. These two STs were submitted to the pubMLST database and consequently assigned as ST142 and ST143. Thus ST02 (n = 4), ST142 (n = 11) and ST143 (n = 3) represented 22.2%, 61.1% and 16.7% of positive human samples with full MLST, respectively. In order to use the whole sequence data, we arbitrarily assigned an ST to those samples for which only partial genotyping was achieved. For this, after establishing that all alleles or combination of alleles were compatible with ST02, ST142 or ST143, we included in the analysis those human samples for which the obtained sequences allowed unambiguous ST assignation. This

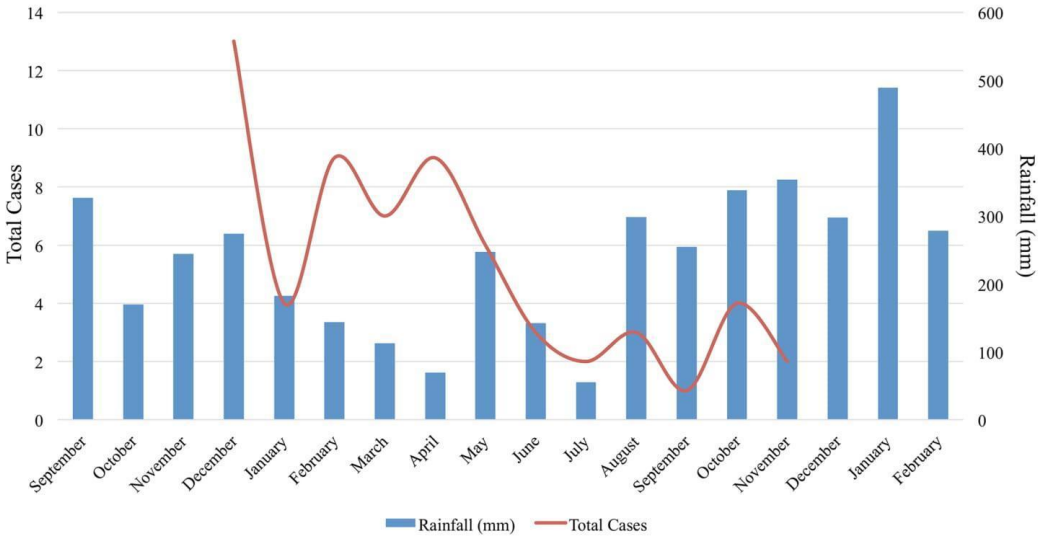

Fig 2. Leptospirosis total positive cases over a one-year period (1st December 2014 to 30th November 2015) in relation to rainfall data (mm) obtained from Seychelles airport and including three months before and after the study period.

https://doi.org/10.1371/journal.pntd.0005831.g002

allowed us to assign an ST to 21 out of the 24 fully or partially sequenced human samples. With this dataset, ST02 (n = 7), ST142 (n = 11) and ST143 (n = 3) were found in 33.3%, 52.4% and 14.3% of human samples, respectively.

Leptospira carriage and genetic diversity in rats

Altogether 739 rats were sampled and screened for *Leptospira* carriage, leading to an overall prevalence of 7.7%. Genotyping provided sequences for 34 out of the 57 positive animals (see S1 Table) distributed as follows: 24 sequences for *adk* gene, 18 sequences for *icd*A, 21 sequences for *lipL*32, 20 sequences for *lipL*41, 29 sequences for *rrs*2 and 27 sequences for *secY*. Additionally, 13 sequences were achieved for *rrs*2 gene using LA/LB primers [37] revealing sequences for two additional samples which were not successfully genotyped using MLST scheme#3 [36]. Full MLST was obtained from all 12 *Leptospira* positive cultures attempted from 74 rat fresh kidney tissues as well as from three uncultured tissue samples. Full genotyping of these 15 samples revealed the exclusive presence of *L. interrogans* ST02. Following the same procedure as that used for human samples analyses, we arbitrarily assigned an ST to those samples for which only partial genotyping was achieved. The allelic profiles of these remaining samples were all compatible with ST02, ST142 or ST143 but only nine allowed unambiguous ST assignation and were all indicative of ST02. A minimum-spanning tree presented in Fig 3 shows alleles differences between all three STs in fully genotyped human and rat samples.

Influence of biotic/abiotic variables on infection prevalence in rats

Overall, *Leptospira* prevalence was significantly higher (p-value < 0.0001) in *R. norvegicus* (52.9%, n = 51) than in *R. rattus* (4.4%, n = 688). Several biotic and abiotic variables affected

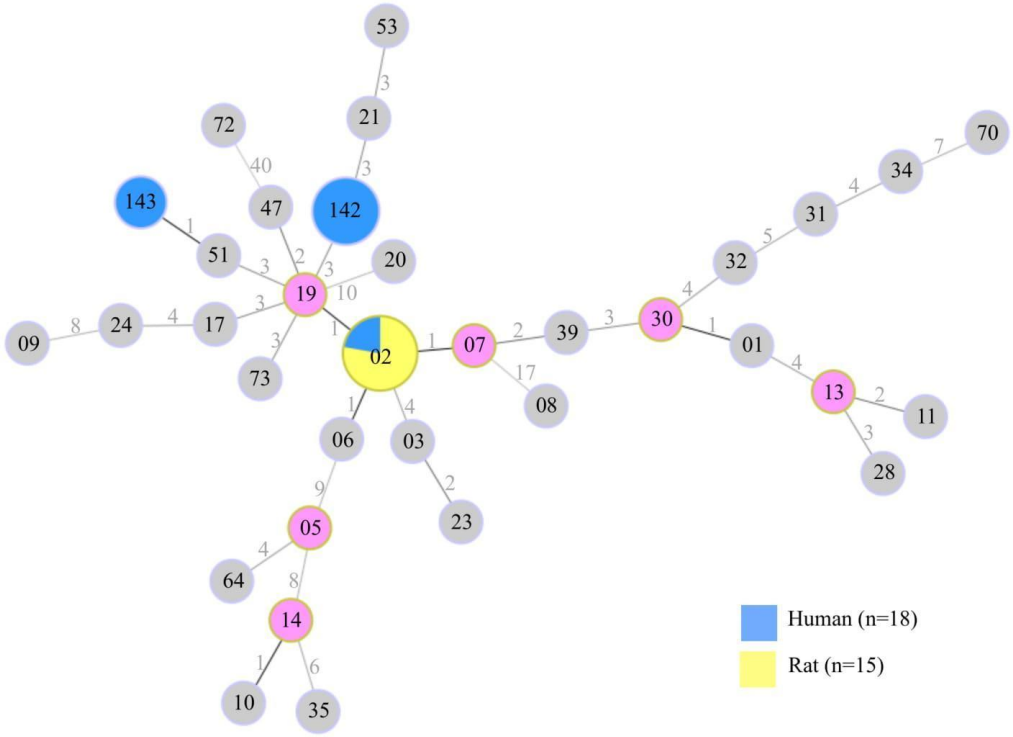

Fig 3. Minimum-spanning tree of *Leptospira interrogans* sequence types (STs) based on the MLST scheme #3 (http://www.pubmlst.org/leptospira/). Sequence Types from humans (in blue) and rats (in yellow) from Seychelles were included into a network constructed with previously published STs reported from various hosts worldwide and shown in grey circles. Group founders are shown in purple circles. The circle sizes of ST02, ST142 and ST143 reflect the relative abundance of each ST in the data set acquired from human and rat samples. The numbers indicated on branches represent the number of mutations between each ST.

https://doi.org/10.1371/journal.pntd.0005831.g003

the prevalence of *Leptospira* renal carriage in rats, we describe hereafter the influence of each analysed variable. Infection prevalence appeared significantly affected by the sampling season. Prevalence of *Leptospira* carriage was 5.4% (n = 464) during the dry season *vs*. 11.6% (n = 275) during the wet season (Fig 4) (p-value = 0.003). An analysis of urban versus rural habitats irrespective of rat species and season showed that there was also a significantly higher positivity rate in urban (18.7%; n = 230) than in rural (2.8%; n = 509) habitats (p-value < 0.0001; see Fig 4). When each rat species was analysed separately, the difference in infection prevalence was not significant for *R. norvegicus* (55.8% in urban *vs*. 37.5% in rural) but remained significant for *R. rattus* (10.2% in urban *vs*. 2.2% in rural, p-value < 0.0001). The higher prevalence in urban habitat was still significant when each season was analyzed independently: *Leptospira* carriage in rats was 13% (n = 138) in urban and 2.2% (n = 326) in rural habitats during the dry season (p-value <0.0001) while during the humid season, infection reached 27% (n = 92) and 3.8% (n = 183) in urban and rural habitats (p-value = 0.009), respectively.

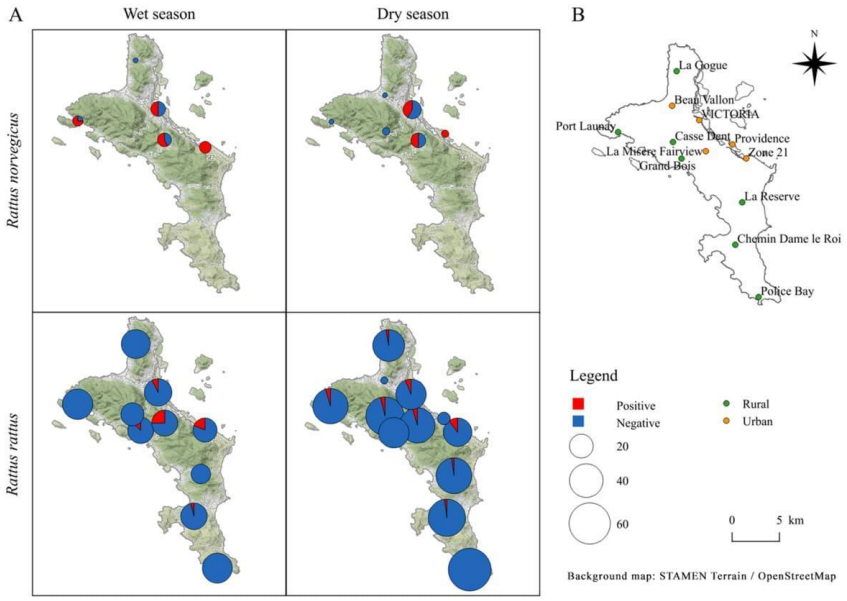

Fig 4. Distribution of sampled rats on Mahé Island, with infection status. A. The quadrants show the distribution of *Leptospira*-infected *Rattus norvegicus* and *R. rattus* plotted by Wet Season (Northwest monsoon, February-March 2014, n = 464) and Dry Season (Southeast monsoon, June-July 2013, n = 275). Circle sizes represent the relative number of rats captured at each site with a representation of the positives (in red) and negatives (in blue). **B**. Sampling sites are plotted with urban and rural habitats appearing in orange and green, respectively (see S1 Table for details including GPS coordinates). Maps were produced using QGIS, and the Mahé Island shape file obtained from OpenStreetMap (https://www.openstreetmap.org).

https://doi.org/10.1371/journal.pntd.0005831.g004

When infection prevalence was compared for each season independently, it appeared that *R. norvegicus* was still significantly more infected than *R. rattus* during both wet (64% *vs* 6.4%; p-value < 0.0001) and dry seasons (42.3% *vs* 3.2%; p-value < 0.0001). *Leptospira* carriage was also significantly higher in *R. norvegicus* than in *R. rattus* whether animals were trapped in urban (55.8% *vs* 10.2%; p-value < 0.0001) or rural habitats (37.5% *vs* 2.2%; p-value < 0.0001).

Overall, 4.5% (n = 5) of juvenile rats (n = 110) were carriers of *Leptospira*, representing 0.7% of the total rats sampled. Conversely 8.3% of adult rats (n = 629) were carriers of *Leptospira* representing 7% of total rats sampled. An analysis of maturity status of *Rattus* species in relation to *Leptospira* carriage showed that adult *R. norvegicus* were more infected (56.3%, n = 48) than adult *R. rattus* (4.3%, n = 581; p-value < 0.0001); whereas there was no significant difference detected amongst juveniles, possibly due to the very low number of caught *R. norvegicus* juveniles (n = 3). Both adult (p-value < 0.0001) and juvenile rats (p-value = 0.005) were more infected in the urban than in the rural habitats, adult rats being more infected in the wet (11.7%, n = 248) than in the dry (6.0%, n = 381) season (p-value = 0.017).

We tested the distribution of *Rattus* spp. in urban *vs*. rural habitats, showing that both rat species were unevenly distributed (see Fig 4), *R. norvegicus* colonizing preferentially urbanized habitats (84.3% of sampled rats, n = 51) while *R. rattus* was dominant in rural settings (72.8% of sampled rats, n = 688; p-value < 0.0001). Hence, the observed significant distribution of

infected rats may be indirectly related to the uneven spatial distribution of *Rattus* spp. We addressed this hypothesis by performing a generalized linear model (glm) including all variables (*i.e.* "Species", "Seasons", "UrbanOrRural", "Maturity" and "Region") acting alone or in interaction. Using this model, the effect of *Leptospira* carriage amongst rats showed a borderline effect of season (Wet > Dry; p-value < 0.1), whereas increasingly significant differences of *Leptospira* carriage were observed for habitat (urban > rural; p-value < 0.05) and *Rattus* species (*R. norvegicus* > *R. rattus*; p-value < 0.001). Performing a glm with "Species" and "Region" as explicative variables highlighted significant *Leptospira* infection in *R. norvegicus* ($p < 0.0001$) in the East region ($p < 0.005$) of Mahé. No other effect was highlighted for "Maturity" and "Region" variables. Further analyses of the interactions of variables "Species", "Seasons", "UrbanOrRural", "Region" and "Maturity" did not highlight any significant interaction between these variables when taken together. A stratified general linear model analysis based on host species (*i.e.* by *R. norvegicus* and *R. rattus* separately) against all the other variables did not reveal any significant interaction between variables either.

Leptospira prevalence and diversity in dogs and cats

Following the investigation of rats and humans, it appeared that rats were likely not the only reservoir involved in human leptospirosis, as two thirds of PCR confirmed human cases were infected with a *L. interrogans* ST that was virtually absent from our genotyped rats sample. In an attempt to explore other possible reservoirs, we collected kidney samples from 12 cats and 24 dogs. One cat (C13; Ct = 40) and one dog (D18; Ct = 39) were diagnosed as infected with *Leptospira* by real-time PCR. Only the dog sample allowed the production of sequences for *adk*, *lipL*32 and *lipL*41. Although no complete MLST could be achieved, the allelic profile obtained using the three sequenced *loci* was consistent with the novel ST142, representing the most common ST found in human leptospirosis cases.

Discussion

We report a human incidence of 54.6 (95% CI 40.7–71.8) per 100,000 inhabitants in Seychelles. This incidence is higher than that reported in Reunion island (8.2 cases per 100,000) and second in the region following Mayotte island, which displays a comparable high incidence (74.5 cases per 100,000) [12]. This incidence falls within the mean incidence of 41.1 (95% CI 29.5–55.7), calculated from a ten-year period (2005–2014) based on routine data collated by the Health Statistics Unit (Epidemiology and Statistics Section, Public Health Authority). However, the differences in incidence to previous studies in spite of the overlapping confidence intervals, from 101 cases per 100,000 previously reported in 1995–96 [25], to 54.6 cases per 100,000 in 2014–15 reported herein, requires some discussion. It must be emphasized that methods implemented in both studies are close but not identical. We carried out real-time PCR, IgM ELISA and MAT, while the previous study used end-point PCR and MAT only as the defined diagnostic criteria. The real-time PCR used herein is highly sensitive, likely because of the short stretch of amplified DNA. Considering our diagnostic criteria, we state that our screening was at least as sensitive as that implemented 20 years ago. However, the case definition used in this study was more restricted than the previous study and may have missed early cases presenting without fever or late stage cases presenting with jaundice. Lastly, for feasibility reasons, all human samples were first screened through MAT using Icterohaemorrhagiae and Patoc serovars only, which might have led to some false negative samples. Hence, the incidence reported herein may actually be underestimated.

Although lower than previously reported [25], the incidence of human leptospirosis in Seychelles remains high, indicating that Seychelles still has a heavy disease burden. The collected

demographic data reveals a strong bias towards males in our sample (90.5% males vs. 9.5% females). This bias is unexpected as all patients with acute febrile illnesses were included in the study, but is actually close to biases reported in two previously published leptospirosis studies in Seychelles (89% and 84% males reported in [24] and [25], respectively). Part of the explanation is that leptospirosis is actually an important portion of acute fever illnesses and is well known to be much higher in males than females [48], but such a high bias is still difficult to understand with our limited knowledge of acute febrile illnesses in Seychelles. We cannot rule out that although the protocol was designed to include all patients presenting with acute febrile symptoms of unknown origin, physicians who were informed of the general objectives of the project, *i.e.* an estimation of leptospirosis burden, may have spontaneously biased the recruitment towards putative leptospirosis patients, and hence towards males known to be significantly more affected than females by the disease. If so, reported figures may actually be under estimated. Interestingly, our data confirm the high case fatality rate of leptospirosis in Seychelles and the maintenance of the severity of the disease over the years: 11.8% in 2014–2015 versus 16% in 1988–1990 [24] to 8% in 1995–1996 [25]. Despite considerable improvements in the diagnosis over the last 20 to 30 years, the morbidity and severity of the disease does not seem to have decreased. The MAT data are overall in accordance with the major serogroups previously reported by Yersin *et al.* [25]. The high amount of cross-agglutinations that we report may be an indirect indicator of how much the Seychellois population is exposed to *Leptospira* with the presence of co-agglutinins possibly indicative of intense exposure and iterative reinfections.

Molecular investigation of rats and human acute cases brings in original data that enlightens the epidemiology of the disease in Seychelles. We report a very limited *Leptospira* diversity within rats and human cases. Indeed, *L. interrogans* was the single species found in both human acute cases and rats. When overlaying leptospiral diversity found in rats and human acute cases, it appears that ST02 is the only ST detected in rats while it is detected in only 22.2% (n = 18) of clinical samples with full MLST profile, and in 33.3% of clinical samples with full and partial MLST profile (n = 24). Noteworthily, two novel STs reported herein, namely ST142 and ST143, are dominant in human acute cases but were not detected in any of the fully or partially genotyped rats. Although it cannot be excluded that our sampling was not sufficient to capture the whole diversity of *Leptospira* maintained by rats, we can reasonably propose that other animal reservoir(s) or carrier(s) are actually involved in the epidemiology of human leptospirosis. Indeed, prevalence of *Leptospira* carriage reported herein in rats (7.7%, n = 739) is notably lower than that reported in other islands of the region such as Reunion (36.3%, n = 732) or Mayotte Islands (15.9%, n = 289) [49,50]. A recent report has suggested that domestic animals such as dogs may act as possible vectors in the transmission of *Leptospira* in the environment and consequently indirectly affecting humans [66]. Although serogroup Canicola commonly associated with dogs was not detected in our samples except in co-agglutination, this important issue needs to be addressed considering the abundance of stray dogs in the islands of Seychelles. Interestingly, the probable presence of ST142 in one dog (substantiated by sequences at three MLST loci), suggests that dogs may actually be shedders of this ST, found in over 50% of genotyped human samples. The situation in Seychelles can be compared to that occurring in Reunion Island where rats were found exclusively reservoirs of ST02 as well. In this French Island, two *Leptospira interrogans* lineages were found in humans, ST02 and ST34, and partial sequencing of *Leptospira* supports the presence of both lineages in dogs [49]. Although the present sampling setup was not designed to investigate the dogs' compartment, our results strongly call for a proper exploration of these as well as other domesticated animals or wild fauna.

Our study addresses the role of biotic and abiotic factors in the epidemiology of *Leptospira* carriage in rats, which can be overlaid on the dynamics of human leptospirosis. The data shows that there is a higher prevalence of *Leptospira* carriage in rats during the humid season than in the dry season, which has similarly been described in other insular tropical territories like Martinique and Reunion Islands [51,52]. This higher prevalence can be reasonably attributed to humid conditions prone to the maintenance of *L. interrogans* in the environment for long periods [53–56]. However, leptospirosis cases can be observed throughout the year, which can be best explained by an elevated and constant temperature all year round together with bouts of rainfall occurring even during the traditionally dry period (June-August). Altogether, it appears that the seasonality of leptospirosis in Seychelles is not as sharp as that reported in Mayotte and Reunion islands [11,57], likely because of a more equatorial rather than tropical climate in Seychelles and a more pronounced rainfall seasonality found in the two French overseas territories. However, rainfall might not explain all the variability observed, and long-term surveillance may help pinpoint other variables at play.

The importance of urbanization in predicting *Leptospira* carriage in rats is significant (Fig 4). In fact, 68% of *Leptospira*-positive rats were sampled in the urban environment during the dry season and this increased to 78% during the wet season. A general trend highlighted by our data is that habitat degradation is positively correlated with *Leptospira* carriage in rats. For instance, the only site with no infection detected in rats during both sampling seasons was at the southernmost extremity of Mahé at Police Bay, which is actually a natural habitat. Other natural environments such as La Réserve, Casse-dent, La Gogue and Grand Bois, sheltered infected rats but with lower prevalence as compared to those trapped in urbanized sites. Therefore, the traditional association of leptospirosis with occupational and environmental risk factors such as farming and agricultural zones, although still of importance in many countries, may not be so much an issue compared to the exposure of human populations to urban environments as shown in the case of Seychelles. It has to be noted that few residences are located in what we have classified as urban environments of Victoria, as these contain mainly office blocks, banks, port, factories, and commercial zones. People come to work or for leisure activities in these zones however mostly reside in other areas, hence human exposure in this zone may be low although the infection in the urban zones could play a role as a reservoir of the pathogen, which could diffuse to contiguous areas or to other animal species.

A glowing pattern highlighted by our data is that *R. norvegicus* appears dramatically more infected than *R. rattus*. When urban and rural environments were analyzed independently, *R. norvegicus* was again significantly more infected than its sister species. Hence, it appears that in Seychelles, *R. norvegicus* might be epidemiologically much more involved than *R. rattus* in the contamination of the environment with pathogenic *Leptospira*. However, the abundance of each *Rattus* species in each habitat should be comprehensively addressed in order to conclude on the weight of each respective species in the epidemiology of human disease. The distribution of *R. norvegicus* on Mahé must be overlaid upon ecological studies and observations done in Seychelles [28,58,59]. The distribution of *R. norvegicus* in the mainly urban areas on Mahé is consistent with studies showing its commensalism to humans [60,61]. However, such urban distribution contrasts with its past distribution (before the species was eradicated) on smaller islands of Seychelles (*i.e.* Frégate, D'Arros and Conception) where *R. rattus* was absent [62] and with reports from other countries where *R. norvegicus* is distributed in all habitats including rural areas [63].

Observer ecological records place the introduction of *R. norvegicus* in Seychelles relatively recently (*i.e.* within the last century), rats being first reported from D'Arros and Conception islands (later found to be occupied solely by *R. norvegicus*) in 1944 and 1965 respectively [28], whereas by comparison, *R. rattus* was first reported in Seychelles in 1773 and was probably

previously present on the islands [28,58]. The current distribution of both *Rattus* spp., and particularly the more restricted distribution of *R. norvegicus* on Mahé, probably results from the more recent colonization of the latter, its stronger human synantropism and preference for wetter habitats (which in turn also correlates with higher *Leptospira* carriage in rats), but the respective importance of these factors is still to be investigated.

In addition to the present investigation of animals, a recent study has notified the presence of a *L. kirschneri*-like species (sequenced on one gene only) in *Pteropus seychellensis* (Dietrich *et al*, submitted), a common frugivorous bat in Seychelles. The absence of this bacterial species in our human samples infers a lack of any role of these bats in the epidemiology of the disease. Again, the situation in Seychelles is quite similar to that recently reported on Reunion Island where an insectivorous endemic bat species, *Mormopterus francoismoutoui*, massively excretes *L. borgpetersenii* although this specific bat-borne lineage was not found in human cases or in rats [49]. Also, our study did not investigate a third and more discrete species of rodent present in Seychelles, the House mouse *Mus musculus*, a highly commensal species identified as a *Leptospira* carrier in other island countries. Lastly, studies around the world have revealed the existence of *Leptospira* carriers in animals other than mammals, including birds and reptiles [64], and even invertebrates [65], stimulating further exploration of alternative animal carriers in Seychelles.

Conclusion

From a biogeographic perspective, the present study not only completes previous studies carried out in Seychelles but also brings in original data showing that the South Western Indian Ocean islands actually shelter distinct epidemiological situations. Some oceanic islands such as Seychelles and Reunion Island [49] host a narrow diversity of cosmopolitan pathogenic *Leptospira* possibly of recent introduction, while other territories such as Mayotte [13,14] and Madagascar [17,18,19] are host to a much higher *Leptospira* diversity, including endemic lineages of medical importance [50]. Altogether, the presented data further confirms that insular ecosystems facilitate the exploration of infectious diseases, as these environmental settings are home to peculiar species assemblages that are in turn involved in unique transmission pathways.

This study has completed previously sparse information regarding human leptospirosis in Seychelles, which can still be considered as one of the countries with highest incidence worldwide. Presented results may guide the public health intervention strategies in the prevention of human leptospirosis and the control of animal reservoirs in Seychelles. The patterns of environmental exposure revealed herein support rat control efforts targeting the urban areas of Seychelles as they are expected to have a significant impact in reducing the risk of leptospirosis transmission and hence reducing the overall incidence in humans. However, genotyping of *Leptospira* from animals and human acute cases reveals that rats are potentially involved in less than a third of human infections. The insignificant change in mortality caused by leptospirosis together with a persistent high incidence of the disease in humans, highlights undetectable improvement in management of acute cases in humans worsened by a limited efficiency of preventive measures. This may result from insufficient rodent control measures or, as suggested by our study, from a misidentification of the main reservoir(s) still to be identified and controlled.

Supporting information

S1 Table. *Rattus* samples tested by *Leptospira* 16S real-time PCR and/or culture, including MLST typing data, habitat type, season, sexual maturity and GPS coordinates.
(XLSX)

S2 Table. GenBank accession numbers of sequences generated in this study that are representative of the *Leptospira interrogans* STs found.
(XLSX)

S3 Table. List of *Leptospira* spp. strains used for the MAT panel.
(DOCX)

S4 Table. Table of results for ELISA IgM, PCR consolidated (after two triplicate runs), MAT serogrouping (on acute and paired human sera) and sequence type of fully genotyped samples.
(XLSX)

S1 Checklist. STROBE checklist.
(DOC)

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Conclusion

The results from this molecular epidemiological study show that ***Leptospira interrogans*** **is the only species** present in human cases on the islands with a **low diversity indicative of a recent introduction** into these islands. The results also confirm the findings of previous studies of similar *Leptospira* serogroups with a higher prevalence of serogroup **Icterohaemorrhagiae**.

Our data also show for the first time **the presence of three distinct *Leptospira* STs**, two of which are unique to the Seychelles. These **novel genotypes contribute to the greater majority of human cases** and importantly, **are not found in rats**.

The latter finding means that the greater majority of human cases of leptospirosis are originating from yet to be determined alternative reservoir(s), which stimulates the exploration of these potential reservoirs. Our small sampling has shown **some association with dogs**, as one out of 24 sample dogs harboured one of the novel genotypes, which is importantly the most prevalent ST in human cases.

Finally, the study confirms the **high burden of leptospirosis incidence in Seychelles** and points to the misidentification of the reservoir of the disease as a possible reason for this in spite of the various public health intervention efforts of the Seychelles' health authorities. **Leptospirosis seasonality following the seasonal rains** was confirmed although not as marked as in the previous study conducted over 20 years ago, an effect which is postulated as resulting from climate change.

Chapter 3: Epidemiology of human leptospirosis in Seychelles.

Introduction

Environmental and behavioural factors are known to influence the risk of being exposed to *Leptospira* spp., however the relative importance of different exposures have not been well described in the SWIO. Similarly, descriptions on the clinical features associated with leptospirosis are limited. In Seychelles, only two previous studies looked at the clinical features or leptospirosis and the occupational and behavioural risk factors contributing to *Leptospira* infection (Bovet et al., 1999; Pinn, 1992; C. Yersin et al., 1998).

In the first study (Pinn, 1992), conducted over a two-year period from 1988-1990, leptospirosis was clinically diagnosed in 80 patients, leading to an incidence of 60 per 100,000 inhabitants although 58 were confirmed serologically and autopsy confirmed a further 7 cases. Pinn indicated that the annual incidence figure is most likely an underestimation due to the non-specific symptoms of leptospirosis. It was shown that a majority of males (89%) were affected with most cases (23.9%) coming from 20-29 age group and this was similar (22.5%) when considering both sexes. Regular alcohol consumption was associated with 75% of cases, and Pinn postulated that this might be associated with the consumption of traditional brews, which are produced in unhygienic environments with potential for contamination; however, this could not be confirmed. Biological features were most associated with raised (> 40U/L) alanine aminotransferase (87% of cases), raised (> 92U/L) CPK (76% of cases), raised (> 20µmol/L) bilirubin (75% of cases), raised (> 100µmol/L) creatinine and haematuria (69% of cases). Thrombocytopaenia (< 100×10^9/L) was in 50% of the 14 patients with pulmonary haemorrhage. The most common symptoms were fever (89%), myalgia (85%) and dark urine (53%) whereas the most common signs were liver tenderness (78%), fever (76%) and jaundice (76%). Pinn noted that myalgia was the most useful clinical symptom with some patients being severe enough as to prevent walking. Serum samples were obtained for 64 cases out of which only 12 had convalescent sera. ELISA IgM was positive for 58 (90.6%) of these available sera and out of 29 MAT positive reactions, 27 (23%) were Icterohaemorrhagiae and 2 (7%) were Autumnalis. Occupations most associated with leptospirosis were labourer (26.3%), being unemployed or retired (23.9%) and being a farmer or gardener (11.3%). Additionally, amongst positive cases in women, two thirds were housewives. Pinn noted that the diagnosis of leptospirosis in a jaundiced patient in the absence of malaria and yellow fever and rare cases of viral hepatitis would be simple.

In the second study conducted in 1995-1996 (Bovet et al., 1999; C. Yersin et al., 1998), Yersin et al. (1998) and Bovet et al. (1999), respectively described clinical features and risk factors associated with leptospirosis from a prospective population-based case-control study. A total of 75 cases of acute leptospirosis were detected corresponding to an annual incidence of 101 per 100,000 (95% CI: 79-126). The majority of cases were of males (84%) and the most affected age group was 20-29 years (32%). Eight serogroups were identified with Icterohaemorrhagiae (31%) and Hurstbridge (20%) being the most frequent, whereas influenza-like forms accounted for 37% of cases. Additionally, the most marked clinical features were jaundice (52%), acute renal failure (28%) and pulmonary haemorrhage (19%). Case fatality was at 8%. The prevalence of subclinical infection was high with 9% of healthy adult males having leptospiraemia (PCR positive) and 37% showing evidence of past leptospiral infection in the absence of any current or past history of infection, which the authors extrapolated to mean that a substantial proportion of the exposed population may have undiagnosed or subclinical infection. The concentration of cases was in adult males with manual outdoor occupations; however, other notable classes were housewives, students and workers with indoor activities.

Risk factors that were related to increased exposure to environment were forest activities, gardening, wet soil around the home, refuse not collected by public service, living in a house with corrugated iron and having a kitchen accessible to rats. Highlighted risky behaviours were washing clothes or bathing in the river, walking barefoot outside the home and conditions such as skin wounds. The relationship with rainfall was weak and explained by an undefined rainfall season. Acute leptospirosis was strongly associated with cats at home, however authors described this relationship as controversial in view of what was known at the time. Interestingly there was a weak association between rat density around the home to leptospirosis and the authors explained that this variable could have been attenuated in the study due to its imprecision, high rat density in the country and discrepancy between rat trails and what could be their actual habitats. A positive association was found with alcohol intake but was not explained further than to be correlated with outdoor occupations which may have confounded the association. Independent association was found for gardening, wet soil and skin wound which the authors declared to be consistent with the fact that leptospires survive in humid soils which may then cross abraded skin. Authors concluded that health education was of paramount importance in reducing the risk to leptospiral infection, and stressed the need to provide preventative protection equipment in risky activities, such as wearing footwear and gloves. Rodent control was acknowledged as a means of protection however

authors stressed that behaviour change was required for effective and sustainable prevention and control of leptospirosis in tropical areas like Seychelles where there needs to be a multifactorial integrated approach.

After 25 years from the first study conducted in the Seychelles, the need to clarify the prevailing clinical features of leptospirosis in Seychelles and how this can help promptly diagnose future cases is the subject of the following chapter. Prospective studies to determine the risk factors associated with leptospirosis are still rare (Felzemburgh et al., 2014), and the need to clarify and update the possible impact of environmental and behavioural risk factors associated with leptospirosis in Seychelles was the subject of **Article 3: An observational study of human leptospirosis in Seychelles.** The manuscript of the article was re-submitted after first review to the *American Journal of Tropical Medicine* and is included below.

Title: An observational study of human leptospirosis in Seychelles

***Running head**: Epidemiology of leptospirosis in Seychelles*

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23 **Abstract**

24 A one year population-based prospective study was launched in Seychelles, a country with one
25 of the highest human incidence of leptospirosis worldwide, in order to describe the
26 characteristic features of the epidemiology of the disease and highlight the most prominent risk
27 factors. Diagnosis was based on IgM ELISA, MAT and RT-PCR. A standardized questionnaire
28 was administered to 219 patients aged ≥ 13 years consulting for acute febrile illness.
29 Leptospirosis, which high incidence in Seychelles was confirmed by the study, was particularly
30 severe as the case fatality rate reached 11.2%. Leptospirosis was positively associated in
31 univariate analysis with socio-professional and clinical variables including gardening/farming,
32 oliguria, jaundice, conjunctivitis, history of HCV infection, anemia, thrombocytopenia and/or
33 biological renal failure. Epidemiological analyses of the questionnaires highlighted a link of
34 the disease with living in houses, the presence of animals around and in houses, gardening and
35 misuse of personal protective equipment. Multivariate analyses indicated that being a
36 farmer/landscaper and having cattle and cats around the home are the most significant drivers
37 of leptospirosis. Biological features most associated with leptospirosis were thrombocytopenia,
38 leukocytosis, high values for renal function tests and elevated total bilirubin. We report changes
39 in behavior and exposure compared to data collected on leptospirosis 25 years ago, with
40 indication that health care development has lowered case fatality. Continuous health education
41 campaigns are recommended as well as further studies to clarify the epidemiology of human
42 leptospirosis and especially the role of domestic animals. [238 words]
43

Introduction

Leptospirosis is an often neglected tropical infectious disease caused by spirochetes of the genus *Leptospira* [1–3], and is considered as a (re-) emerging zoonosis. Humans are infected when they come in direct or indirect contact with the urine of infected animals [4,5]. The disease affects over 1 million persons annually causing 59,000 deaths [6] and an estimated 2.9 million Disability-Adjusted Life Years (DALYs) lost per annum [7] reflecting its high socio-economic impact. Clinical diagnosis of leptospirosis is difficult as symptoms are non-specific leading to confusion with other infections such as dengue fever, influenza and hepatitis, hence contributing to underreporting. Moreover, 90% of cases are asymptomatic or mild [8]. Symptomatic disease in humans escalates from mild, self-limited febrile illness to severe forms displaying multisystemic complications leading to fulminant life-threatening illness [9]. Risk factors associated with leptospirosis include behavioral and environmental variables such as rainfall and temperature [10]. Rodents have traditionally been considered as the main reservoir of *Leptospira* spp., although several other animals (such as cattle, buffaloes, dogs and cats) can act as reservoirs [11–14].

Although reported worldwide, leptospirosis is most prevalent in tropical insular countries [15] where it is of major public health concern, including in the South Western Indian Ocean islands (SWIOI) such as Comoros, La Réunion, Mayotte and Seychelles (where incidence is amongst the highest worldwide) [16]. High disease incidence in such environments may be due to the warm and humid natural conditions that are conducive to the maintenance and transmission of *Leptospira* spp. In addition, the limited number of animal species typical of insular habitats [17] may facilitate transmission between competent reservoirs and hence contribute to increase leptospirosis incidence.

Environmental (particularly rainfall and flooding) and behavioral factors are recognized as risk factors for developing leptospirosis. However the seasonality of the disease in Seychelles has not seen to be as marked as in other locations such as Reunion Island [16,18], possibly because Seychelles lies closer to the equator.

Molecular investigations have stressed the low diversity of pathogenic *Leptospira* in

humans and rats within Seychelles [16] as both species are infected by *Leptospira interrogans*. Interestingly, despite the high incidence of disease in humans, the *Leptospira* carriage in the rats is low (7.7%). Most importantly, Multilocus sequence typing (MLST) has revealed that Sequence Types associated with human acute cases or with rat kidney carriage are different and indicate that most (68.7%) of clinical cases have likely not originated from rats [16]. Lastly, the highest infection rates in rats are found in non-residential urban areas. These characteristics highlight that rats are not the main reservoir of *Leptospira* infecting humans and that an alternative reservoir is yet to be determined in Seychelles [16].

In the present study, we evaluated the risk factors contributing to leptospirosis in Seychelles and describe the clinical features of the disease and their changing patterns compared to the data reported in previous studies conducted in the country some 25 years ago.

Materials and Methods

Ethics statement

The study protocol for humans was reviewed and approved by the Health Research and Ethics Committee of Seychelles (Research Proposal 1405). A written informed consent was provided by all adult patients enrolled on the study or by parents/guardians of minors. All samples were anonymized prior to laboratory testing.

Study sites and inclusion criteria

During December 2014 – November 2015, all patients aged ≥ 13 years with febrile illness (≥ 38°c) for more than 3 days were included in an observational study at all governmental health facilities (14 clinics, 3 cottage hospitals and 1 referral hospital) in Seychelles. Patients without fever the day of inclusion were included if an history of fever in the previous days was documented in their medical file e.g. before implementation of antipyretic treatment. The study coordinator center was the Seychelles Hospital on Mahé Island. Patients unable to give a good exposure history, to provide clinical information upon

admission, or refusing blood testing or to participate in the interview were excluded from the study.

Microbiological investigations and laboratory procedures

For each included patient, the following biological tests were performed: full blood count, liver function tests, renal function tests, blood culture if hospitalized, PCR for Chikungunya and Dengue viruses. Patients who reported a history of travel were also tested for malaria parasites by PCR. For each patient, only maximum values observed during the hospital stay were considered. The biological diagnosis of leptospirosis was done using real-time RT-PCR and serological screening through ELISA and the Microscopic Agglutination Test (MAT) following protocols that have been thoroughly described elsewhere [16].

Leptospirosis case definition

A confirmed case of leptospirosis was defined as a suspected case with a positive real-time RT-PCR assay for pathogenic *Leptospira spp*. in blood and/or a positive MAT, a minimum four weeks after the onset of symptoms. A positive MAT was defined as one that displayed an infective serogroup with a four-fold seroconversion in paired sera, or acute sera with a serogroup displaying a minimum titer of 1:400. The infective serogroup in sera that had co-agglutinating titers had the serogroup displaying two titer orders more than the rest as the definitive infecting serogroup.

Other fever etiologies

At the end of the study, all clinical records were reviewed to describe the etiology of acute fever in Seychelles. The diagnosis made by the practitioner based on clinical arguments was confronted to results of biological investigations. Clinical manifestations and biological disturbances were compared between patients with confirmed leptospirosis and patients with other diseases.

Clinical and epidemiological investigations

A questionnaire was administered to eligible outpatients or inpatients included in the study by trained medical personnel (doctors and nurses). This questionnaire included clinical, socio-demographic, current and past medical history, educational, professional, occupational,

126 environmental and behavioral variables. Association between leptospirosis and those variables
127 were analyzed in univariate and multivariate analyses. For alcohol consumption, heavy drinkers
128 have been defined, as regular drinkers having a calculated average alcohol intake of ≥100
129 ml alcohol a day[19].

130 **Statistical analysis**

131 Data from the administered questionnaire were recorded using EPIData 3.1® [20] and analyzed
132 with R® statistical package [21] using the chi-squared test or Fisher test for observed frequencies
133 and the t-test or Kruskal-Wallis test for quantitative data. Multivariate analysis was done using
134 a Logistic regression model including all variable with a level of significance < 0.20 ($p < 0.20$).
135 Data of the Population and Housing Census 2010 Report (from National Bureau of Statistics,
136 Seychelles) and data from the National Survey of Non-communicable Diseases in Seychelles
137 2013-2014 (from Ministry of Health, Seychelles) were used to compare the study sample to the
138 national data [22,23].

139

140 ## Results

141 **Case selection**

142 During the 12-months study period, 223 febrile patients on 226 eligible were included
143 in the study. Only 219 patients accepted to participate in the study (none of the four patients
144 refusing to participate was positive for *Leptospira*). The epidemiological part of the
145 administered questionnaire could be completed for 209 patients. When leptospirosis cases
146 dying before completion of the form were not included, the non-response rates between
147 leptospirosis cases and other causes of fever were not statistically different (6.2% *vs* 2.4%,
148 Fisher test, $p > 0.05$).

149

150 **Review of clinic registers**

151 Of the 219 patients presenting with acute fever, 197 were males and 22 were females. To
152 understand the reasons of this distorted sex ratio, the clinic registers of attendance of four clinics
153 were reviewed for the study period. These clinics are distributed throughout Mahé Island, which

154 hosts over 90% of the total population: one is located in the north, one in the south-east, one in
155 the west and one in center of the island. The four selected clinics were representative of other
156 clinics according to their level of attendance and location. Altogether, 75 cases of fever (50
157 men and 25 women) and 14 suspicions of leptospirosis (10 men and 4 women) were diagnosed
158 out of 29,391 consultations, representing an incidence of 0.25 fever per 1000 consultations and
159 an incidence of suspicion of 0.05 leptospirosis per 1000 consultations. In the clinics, males
160 accounted for 67% of consultations for fever during the study period. During our study period,
161 there was disproportionate representation of men among the consultants for fever in health
162 centers with a sex ratio of 2.
163
164 **Age and sex distribution**
165 The mean age of the study sample was 36 years with no significant difference between
166 genders (40 years for female and 35 years for male, $p > 0.05$, Kruskal-Wallis test). Leptospirosis
167 was diagnosed in 23.3% (51/219) of patients corresponding to an annual incidence of 54.6 (95%
168 CI 40.7-71.8) per 100,000 population with 96% (49/51) of cases occurring in men. There was
169 no difference in terms of age between leptospirosis cases and cases due to other causes of fever
170 (33 years [min 13; max 60] *vs.* 37 years [min 13; max 80], $p > 0.05$, Kruskal-Wallis test). The
171 distribution by sex, age group and leptospirosis infection of the 219 included cases of fever is
172 shown in Figure 1.
173 **Figure 1**: Distribution by sex, age group and leptospirosis infection of 219 included cases of
174 fever, Seychelles, December 2014 to November 2015.
175
176 **Travel history**
177 Out of the 219 included patients, 31 were not of Seychellois nationality. The positivity
178 rates for leptospirosis were not statistically different ($p > 0.05$, Fisher test) between Seychellois
179 and non-Seychellois patients (25% *vs.* 12.9%). Among the 31 foreigners, four were confirmed
180 leptospirosis cases: three had not recently travelled outside of Seychelles whereas the fourth

case occurred in a Malagasy 8 days after his arrival in Seychelles and was probably an imported case from Madagascar.

Travel outside of Seychelles was recorded in 213 out of 219 patients, with only 10 (4.7%) reporting to have travelled during the study period. Among them, two were further diagnosed as leptospirosis cases: the Malagasy patient returning from Madagascar and a Seychellois returning from Mauritius with a date of onset 16 days after his return. The positivity rates were not significantly different between travelers and those who had not been travelling (20% *vs.* 23.6%, $p > 0.05$, Fisher test).

Acute fever and leptospirosis incidence by district

Data by district including the number of included cases, the number of confirmed leptospirosis cases, the inclusion rate, leptospirosis incidence and percentage of leptospirosis cases are presented in Table 1. Acute fever cases were included from all inhabited districts except from La Digue. There was no significant difference in the inclusion rates by district except in La Digue. The incidence of leptospirosis by district was of 54.6 per 100 000 inhabitants (95% CI; 41 to 72) ranged from 0 in La Digue (95% CI; 0 to 90) to 136 per 100 000 inhabitants in St Louis (95% CI; 58 to 318).

Table 1: Number of included cases, number of leptospirosis cases, incidence per 1000 population of acute fever and leptospirosis cases by districts in Seychelles, from December 2014 to November 2015.

Occupational risks associated with leptospirosis

The main work activity was recorded for 213 participants (the distribution of leptospirosis cases by occupation is presented in table 2). Thirty percent of leptospirosis cases detected in our study were reported in landscapers and farmers though these work groups accounted for only 12% of our study sample. In these two work groups, almost half of patients consulting for acute fever were ultimately confirmed as leptospirosis cases. When compared to other activities, landscaping and farming were nearly 3 times more at risk to acquire

leptospirosis than other activities (RR = 2.8[1.7; 4.4]). When activities classically at higher risk of leptospirosis (landscaping, farming, builders, etc.) are pooled together, they were significantly more represented among the group of leptospirosis (61.7%) than in the group of patients with other causes of fever (43.8%): RR = 1.7[1.1; 2.9]. In multivariate analysis, working as a landscaper or farmer was found to be significantly associated with leptospirosis ($p < 0.0005$).

Table 2: Number, distribution of leptospirosis cases and positivity rates by occupation in Seychelles, from December 2014 to November 2015.

Hospitalization rate of leptospirosis cases

Out of the 219 patients with acute fever, 173 (79%) were hospitalized and 46 (21%) were treated as outpatients. The hospitalization rate and the mean duration of stay were higher for leptospirosis cases than for other causes of fever, respectively 90% *vs.* 75.6% (RR = 1.2 [1.0; 1.3]) and 3.6 days *vs.* 2.9 days ($p = 0.01$). The delay before hospitalization for leptospirosis cases was on average 3.6 days after the onset of symptoms versus 2.6 days for the other causes of fever ($p = 0.01$). In the same way, the delay before the first blood sampling was higher for hospitalized leptospirosis cases: 4.2 days compared to 3 days for the other causes of fever ($p = 0.01$).

Effect of other disease conditions

We screened for a possible influence of other diseases or habits on leptospirosis rate. There was no difference in clinical history between our sample and the general population of Seychelles except for high blood pressure prevalence and heavy drinking that were significantly lower in our study sample: 10.8% *vs.* 19.3% ($p = 0.01$) and 0.9% *vs.* 11% ($p = 0.0001$), respectively. There was no difference of prevalence between leptospirosis and other causes of fever for most of the listed medical antecedents: diabetes (5.1% of participants), high blood pressure (10.8% of participants), previous history of leptospirosis (2.4% of participants), hepatic diseases (4.2% of participants), renal diseases (0.9% of participants), HIV infection (0.9% of participants), alcoholism (0.9% of participants), intravenous drug use (IVDU; 3.2%

237 of participants) and cardiac diseases (1.4% of participants). Of note, the record of a previous
238 infection by HCV was significantly more frequent in leptospirosis cases (6.2% *vs.* 0.6%, RR =
239 10.2 [1.1; 95.7]).
240
241 **Clinical and biological features of leptospirosis**
242 Clinical and biological features by leptospirosis infection or other causes of fever are
243 presented in Table 3. In univariate analysis, only conjunctivitis, jaundice oliguria, myalgia-
244 arthralgia and fever were significantly associated with leptospirosis. In the same way, only
245 cough was clearly infrequent in leptospirosis cases.
246 **Table 3**: Clinical and biological features by leptospirosis infection or other causes of fever in
247 Seychelles, from December 2014 to November 2015.
248
249 Results of biological investigations were available only for 156 patients and were
250 missing for 27 inpatients (15%) and for 36 outpatients (78%). In univariate analysis, anemia,
251 severe anemia (< 11g/dl), thrombocytopenia, leukocytosis, neutrophilia, urea elevation,
252 creatinine elevation, biological renal failure, alkaline phosphatase elevation and bilirubin
253 elevation were significantly linked with leptospirosis infection. In multivariate analysis,
254 thrombocytopenia ($p < 0.0005$), leukocytosis ($p < 0.05$), high values for renal function tests (p
255 < 0.05) and elevated total bilirubin ($p < 0.005$) were still significantly associated with
256 leptospirosis infection.
257 **Severe and fatal leptospirosis cases**
258 Death occurred in 11.2% (6/51) of leptospirosis cases *vs.* 1.8% (3/165) in non-
259 leptospirosis cases (RR = 6.5 [1.7; 25.4]). Leptospirosis cases were classified in mild or severe
260 forms according to clinical and biological manifestations. Severe forms (21) represented 41.1%
261 of leptospirosis cases: five acute renal failure, 4 acute renal failure with hepatic failure, 4
262 pulmonary hemorrhage, 3 acute hepatic failure, 3 acute renal failure with pulmonary
263 hemorrhage, 1 endocarditis and 1 death at arrival.

There was no difference of prevalence between mild and severe leptospirosis forms for all of the listed medical backgrounds: diabetes, high blood pressure, leptospirosis, hepatic diseases, renal diseases, HIV infection, HCV infection, alcoholism, intravenous drug use and cardiac diseases. Among the 16 listed symptoms (fever, back pain, hematemesis, melena, hemoptysis, myalgia-arthralgia, hematuria, oliguria, abdominal pain, jaundice, headache, conjunctivitis, meningitis, cough, diarrhea and dyspnea), two were significantly more associated with severity: oliguria (40% *vs.* 13.3%; RR = 2.1[1.1; 3.9]) and jaundice (45% *vs.* 6.7%; RR = 2.9[1.6; 5.1]). The case fatality rate was not significantly different according to the clinical presentations (9.5% for severe forms *vs.* 6.7% for non-complicated forms). Some biological anomalies were significantly associated with severe forms: severe anemia (< 11) (70% *vs.* 14.8%; RR = 3.7[1.7; 7.9]), urea elevation (85% *vs.* 11.5%; RR = 7.4[2.5; 21.7]), creatinine elevation (70% *vs.* 18.5%; RR= 3.4[1.6; 7.3]), biological renal failure (85% *vs.* 40.7%; RR = 3.8[1.3; 11.3]) and bilirubin elevation (95% *vs.* 53.9%; RR = 7.5[1.1; 50.3]). The prevalence of thrombocytopenia was not significantly different between simple and severe forms of leptospirosis (80.8% *vs.* 95%) but the severity of the thrombocytopenia was significantly higher for severe forms (mean number of platelets 53,000 *vs.* 99,500, p = 0.003, Kruskal-Wallis test). There was no difference of prevalence between recovered and deceased leptospirosis cases for all listed medical histories: diabetes, high blood pressure, leptospirosis, hepatic diseases, renal diseases, HIV infection, HCV infection, alcoholism, intravenous drug use and cardiac diseases. Among the 16 listed symptoms (see above), only abdominal pain was significantly associated with fatal outcomes: 60% *vs.* 15%; RR = 6[1.1; 31.2]). The case fatality rate was not significantly higher in pulmonary leptospirosis than in other forms (33% *vs.* 11%). Severe anemia was more frequent in fatal issue with an average hemoglobin level reaching 8.4 *vs.* 11.4 in survivors. The thrombocytopenia was in average more profound in fatal issues (49,000 platelets *vs.* 96,000 in survivors) but this difference was not statistically significant.

290 **Accuracy of clinical diagnoses**

291 Clinicians established for 121 patients a tentative diagnosis of leptospirosis on clinical
292 grounds. The accuracy of this diagnosis was challenged by results of the biological tests
293 (available for 113 patients). On the one hand, 38% of patients clinically considered as
294 leptospirosis cases (including mild and severe forms) were not confirmed by the specific tests.
295 On the other hand, 8.7% of confirmed leptospirosis were diagnosed by clinicians as
296 endocarditis, cholecystitis, cellulitis, acute gastroenteritis, viral fever or heroin overdose. The
297 positive predictive value (PPV) of the diagnosis of leptospirosis in the clinical file was only of
298 62% and the negative predictive value of other diagnostic (NPV) was of 90.8%. Among the 65
299 patients for whom the cause of acute fever was not leptospirosis, infectious diseases accounted
300 for 90,8% (URTI, LRTI, gastroenteritis, cellulitis, hepatitis, pyelonephritis, dengue and
301 malaria) while non-infectious diseases represented 9,2% (pancreatitis, urinary lithiasis,
302 polyarthritis, bowel obstruction, gastrointestinal bleeding, malignant hyperthermia due to
303 exercise).
304
305 **Knowledge on leptospirosis**

306 Level of knowledge of the sample is presented in Table 4 by leptospirosis status. There
307 was no difference between groups either in the level of knowledge of the participants or on the
308 mode of contamination of leptospirosis as well as on protective measures. However, the level
309 of knowledge on leptospirosis was very low in all patients. Similarly, preventive measures were
310 overall poorly known.
311 **Table 4.** Level of knowledge on leptospirosis of participants according to their leptospirosis
312 status in Seychelles, from December 2014 to November 2015.
313
314 Housing conditions, environmental factors and behaviors by leptospirosis status are
315 shown in Table 5. Leptospirosis cases were more frequently living in houses than in apartments
316 (89% *vs.* 69%, RR = 2.9[1.2; 6.9]). There was no difference in the frequency of garbage
317 collection, the type of sewage system, the type of soiled water disposal system, the type of

318 waste disposal, the type of houses (type of floor, type of ground, type of materials used for
319 walls and roofs), the type of water used for drink or cooking (treated water for 87%) nor the
320 type of water used for bathing (treated water for 84%) between leptospirosis and non-
321 leptospirosis cases. The use of PPE during risky activities was very low in our sample both in
322 leptospirosis and non-leptospirosis cases. Among patients using PPE (gloves and boots), those
323 using boots only were more frequent in the leptospirosis group (65% *vs.* 41%; RR = 2.2[1.3;
324 5.5]) but we didn't success in identifying a link between PPE use or misuse during risky
325 activities in the four weeks preceding the onset of symptoms. In univariate analysis, presence
326 of animals in the vicinity (especially cattle, cats, poultry, dogs but not rats) and presence of pets
327 at home were significantly more frequent around leptospirosis cases. Importantly, with the
328 exception of regular gardening, none of the behaviors classically described as associated with
329 leptospirosis risk in Seychelles or in other places was retrieved in our study. Lastly, multivariate
330 analysis highlighted the presence of cattle and cats around homes as significantly associated
331 with leptospirosis cases ($p < 0.0005$).
332 **Table 5.** Housing conditions, environmental factors and at-risk behaviors by leptospirosis
333 status in Seychelles, from December 2014 to November 2015.
334

335 **Discussion**

336 Leptospirosis data used in this study combined RT-PCR, IgM and MAT screening [16].
337 The turnaround time before molecular test results were available was less than 5 days for 75%
338 of non-leptospirosis cases and over 7 days for only 7 suspected-cases. The probability for false
339 negatives was hence low and controlled by the additional diagnosis of IgM and MAT, which
340 are sensitive in the second phase of the disease and hence complementary to molecular
341 screening. Therefore, case classification bias in this study, if existing, is probably low. Most
342 cases were autochthonous, hence the epidemiological patterns highlighted herein are mostly
343 relevant for describing the situation prevailing in Seychelles. As the inclusion rate was the same
344 in all health districts except La Digue, we can consider that the protocol of inclusion was
345 homogenously applied in all districts.

There was an overrepresentation of males (96%) in our study sample despite the generic criteria of inclusion (acute fever) which was rather due to a sex bias in the attendance of health facilities, than to a bias in patient inclusion. It is well known that access to care is sometimes more difficult for women in low to middle income countries, including admission in ICU or hospitalization [24,25]. The reasons of this difference are still not clearly understood and include difference in clinical presentations or decision-making. This bias is actually quite similar to that reported in 1992 (89%) [26] and in the 1995-96 (84%) [27] studies in which half and two thirds of cases, respectively, were diagnosed among males aged less than 40 years.

Leptospirosis on Seychelles is a severe disease leading to fatal outcomes mainly due to unusual and severe acute clinical manifestations such as pulmonary hemorrhage [28,29]. However, the distribution of leptospirosis cases by age group and by sex in Seychelles is very similar to that of Reunion Island. Another similarity is the high Case Fatality Rate (CFR) in both islands (more than 11% in Seychelles, 3 to 5% in Reunion Island). Interestingly, the epidemiological situation on Seychelles and Reunion Island is quite distinct from that reported on Mayotte, a SWIO island part of the Comoros archipelago, in terms of sex ratio, age group and severity of the disease. Indeed, on Mayotte, females represent a third of cases [30,31] and CFR is significantly lower (0.9%). In Seychelles and Reunion Island, *L. interrogans* is responsible of the vast majority of human cases while in Mayotte, *L. interrogans* is involved in a minority of severe cases [32,33]. These contrasting features highlight the large differences that exist in the epidemiology of leptospirosis in the region, possibly resulting from the distinct virulence of *Leptospira* lineages/species prevailing on each island as recently substantiated through experimental infection [34].

Besides, the leptospirosis CFR has decreased in Seychelles in the last decades (16% in 1992) similar to Reunion Island in the 70's [16,26,35]. This decrease likely results from improvements in case management such as the development of ICU and the generalization of modern resuscitation techniques as dialysis and mechanical ventilation including extracorporeal membrane oxygenation [35] The CFR in Seychelles is still higher than that reported in Futuna (0.5%), Fiji or Philippines (7%), similar to that reported in Mexico (12.8%)

374 but lower than in other areas of the world (19% in Taiwan) [30,31,36–40]. Pulmonary hemorrhage is
375 considered as a predominant cause of death due to leptospirosis in Seychelles and Reunion
376 Island. In our study, seven inpatients (13.5%) developed a pulmonary form, two of which died,
377 but the difference in CFR was not statistically significant when compared to other clinical
378 forms. Pulmonary forms, male sex, delayed treatment, thrombocytopenia, oliguria and
379 hemoptysis are associated with fatal cases in different studies worldwide [38,39,41]. In our study,
380 there was no difference in the delay before treatment between recovered and deceased
381 leptospirosis cases. However, severe anemia and the presence of abdominal pain upon
382 admission was significantly associated with death. We also found that thrombocytopenia was
383 lower in fatal cases than in recovering patients (49,000 in average *versus* 96,000) but this
384 difference was not statistically significant. Lastly, in our study we were unable to find a higher
385 mortality in severe forms when compared to uncomplicated forms, which is probably due to
386 missing information for patients who died on admission.
387 Clinical (oliguria, jaundice) or biological (severe anemia, severe thrombocytopenia,
388 high bilirubin, renal failure) factors are associated with severe forms as previously reported in
389 different studies [42–47]. We were not able to assess links with hypotension or coagulation
390 abnormalities as this information was missing in the medical files. The delay in treatment is
391 often associated with severe forms [39,45,47] while delays before treatment was very short in our
392 study probably due to the small size of the country and access to free health facilities in most
393 parts of the islands. Final clinical diagnosis was available only for 113 cases. The PPV of the
394 clinical diagnosis of leptospirosis was low. It appears that more than 8% of leptospirosis cases
395 were misdiagnosed, therefore without biological confirmation the burden of leptospirosis
396 would have been overestimated by 30%. Diagnosis was available for only 61 other causes of
397 fever. More than 90% of those fevers were due to infectious diseases with respiratory infections,
398 e.g. URTI (Upper Respiratory Tract Infection), LRTI (Lower Respiratory Tract Infection) and
399 tonsillitis, accounting for more than 50% of other causes of fever. This explains why cough at
400 first examination was significantly associated with other causes of fever rather than
401 leptospirosis.

Several domestic behaviors have been associated to leptospirosis risk worldwide. A recent meta-analysis has shown that footwear use decreases significantly the exposure to leptospirosis (OR = 0.59 [0.37; 0.94]) [48]. Walking barefoot outside house has been often associated with leptospirosis contamination worldwide [49–52] including in the SWIOI [30,32,53]. In our study, walking barefoot was not significantly linked to leptospirosis contrary to figures reported in a previous study in Seychelles [53]. This is probably due to a decrease of this practice in the general population (22% in our sample, 26% in leptospirosis cases and 20% in non-leptospirosis cases) compared to the precedent study (39% in leptospirosis cases and 17% in controls).

Presence of rats around houses or during outdoor activities is a known risk factor for leptospirosis in many temperate or tropical countries like Hawaii and the SWIOI [30,31,54]. Although rats were predominantly found around houses of leptospirosis cases in 1993 (40% *vs.* 25%) in Seychelles, the presence of rats reported during our study was not different around leptospirosis and non-leptospirosis cases. This situation suggests that rodents are not the main source of human contamination in Seychelles, as shown by a recent investigation [16]. In our study, animals (variable including rats, cattle, poultry, cats, dogs and rats) were present around 86% of leptospirosis cases compared to 65% around non-leptospirosis cases (RR = 2.5[1.1; 5.7]) but a significant difference was found only for poultry, cats, cattle and dogs. Multivariate analysis of animal presence showed significant association of cattle ($p < 0.0001$) and cats ($p < 0.05$) around positive leptospirosis cases. Cattle are considered in many countries (Asia, east Africa, Indian subcontinent, Oceania and Europa) as an important source of environmental contamination by *Leptospira* [40,53–56]. Similarly, concerns about the role of pets in human leptospirosis are currently growing [57]. In our study, cats were reported around all leptospirosis cases (RR = 1.2 [1.1; 1.4]). Serological evidence of cats infection have been published in countries like Chile [58] and cats can shed leptospires in their urines during acute clinical infection and may even act as chronic shedders [59–61]. Furthermore, their involvement in the epidemiological cycle in rural areas is suspected [62]. Dogs, and mainly stray dogs, have been also suspected or are involved in the epidemiology of human leptospirosis worldwide including

in the SWIO [33,49,57,63]. In 1995-96, dogs were equally present around leptospirosis cases and controls but in 2014-15, dogs were found significantly more frequently (RR = 1.5[1.4; 1.8]) around leptospirosis cases (100%) than around non-leptospirosis cases (63%). Importantly, one dog in Seychelles was recently reported as shedder of *L. interrogans*, which genotype is found in the majority of human cases but was noteworthily absent from hundreds screened rats [16]. Lastly, poultry was significantly (RR = 10.3[3.3; 31.9]) more present around leptospirosis cases than around non-leptospirosis cases. The presence of poultry or poultry breeding is often considered as due to exposure to rats dropping around poultry, as backyard poultry farming is a marker of rural environment, which is in turn associated with leptospirosis contamination. Few studies are available about poultry leptospirosis but a seroprevalence study conducted in Grenada and Trinidad showed that 11% of poultry harbored antibodies against *Leptospira spp.* suggesting that chicken are exposed to leptospires [64]. Furthermore, laboratory infections of chicken embryos showed that chicks that hatched from infected embryos developed a clinically recognizable leptospirosis and that leptospires can readily be observed in the circulating blood [65]. Therefore, the possible role of cattle, poultry, cats and dogs in human leptospirosis in Seychelles has to be investigated.

We did not find a significant difference in the proportion of subjects that had been working in the forest during the four last weeks before inclusion between leptospirosis cases and non-leptospirosis cases (35.6% vs 25.6%), contrary to the analyses conducted in the 1995-1996 study in Seychelles or in others part of the world [31,32,66–68]. Gardening was reported as an activity at risk in Seychelles as in other parts of the world [30–32,53,56]. The absence of link with professional gardening or gardening in the last four weeks, might result from to a lack of power of our study. Another explanation is that it may be easier to identify a link with a regular rather than with an accidental exposure. It is probably why we found a clear link between professions at high-risk to leptospirosis that have a regular exposure and leptospirosis. Those people accounted for 61.7% of leptospirosis cases in our study and have about twice as much risk to contract leptospirosis (RR = 1.7[1.1; 2.9]). People practicing landscaping and/or farming as their main work activity accounted for about one quarter of the total of leptospirosis cases and

more than half (53.8%) were confirmed leptospirosis cases. Compared to other main work activities, landscapers or farmers were almost three times more at risk of leptospirosis (RR= 2.8[1.7; 4.4]). This finding was also confirmed by multivariate analysis, where working as a landscaper or farmer was found to be significantly associated with leptospirosis cases ($p< 0.0005$).

In conclusion, our study confirms the heavy burden of the disease reported 20 years ago in Seychelles, but highlights some striking differences in several epidemiological parameters that result at least in part from improvements in health care and in behavior changes. The development of health care has lowered case fatality of leptospirosis despite a high disease incidence in the country. However, we report a rather low level of knowledge on leptospirosis urging the need for implementing continuous information campaigns about this disease. Lastly, data pinpoints cattle, poultry and pets as risk factors of the disease. Since a recent study has shown that rats are probably not the main reservoir, complementary studies aiming at testing cattle and pets as additional reservoirs are paramount. These will allow the development of a comprehensive picture of the overall human and animal epidemiology which is required to further refine preventive measures in order to mitigate the burden of this devastating disease.

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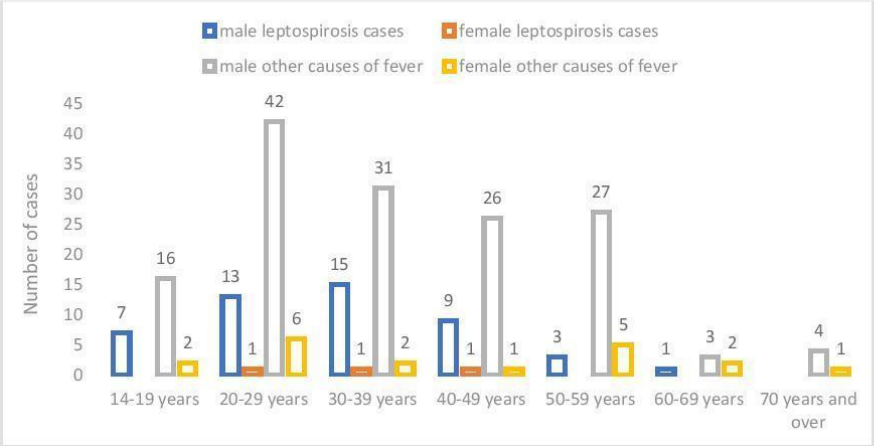
753 Figure 1: Distribution by age by sex and by leptospirosis infection of the 219 fever cases included,

754 Seychelles 2014-2015



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757 **Table 1**: Number of included cases, number of leptospirosis cases, incidence per 1000 population of
758 acute fever and leptospirosis cases by districts in Seychelles, from December 2014 to November 2015.
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District	Number of included cases	Number of confirmed leptospirosis cases	District population	inclusion rate per 1000 inhabitants	leptospirosis incidence per 1000 inhabitants	Percentage of confirmed leptospirosis cases
Anse aux Pins	11	5	3673	2.99	1.36	45.5%
Anse Boileau	12	5	4183	2.87	1.20	41.7%
Anse Etoile	20	3	5018	3.99	0.60	15.0%
Au cap	6	2	3743	1.60	0.53	33.3%
Anse royale	13	4	3818	3.40	1.05	30.8%
Baie Lazare	13	3	3227	4.03	0.93	23.1%
Baie st Anne (praslin)	3	0	3626	0.83	0.00	0.0%
Beau vallon	12	4	4142	2.90	0.97	33.3%
Bel air	1	0	3015	0.33	0.00	0.0%
Bel Ombre	3	0	4163	0.72	0.00	0.0%
Cascade	11	4	4088	2.69	0.98	36.4%
Glacis	5	0	4157	1.20	0.00	0.0%
Grand Anse (Mahé)	7	3	2842	2.46	1.06	42.9%
Grand Anse (Praslin)	4	3	4056	0.99	0.74	75.0%
La digue	0	0	3506	0.00	0.00	0.0%
English River	7	2	4252	1.65	0.47	28.6%
Mont Buxton	5	0	3173	1.58	0.00	0.0%
Mont Fleuri	6	0	3966	1.51	0.00	0.0%
Plaisance	14	2	3690	3.79	0.54	14.3%
Pointe La rue	11	2	3245	3.39	0.62	18.2%
Port Glaud	7	3	2378	2.94	1.26	42.9%
St Louis	18	2	3436	5.24	0.58	11.1%
Takamaka	7	0	2580	2.71	0.00	0.0%
Les Mamelles	13	3	2537	5.12	1.18	23.1%
Roche Caiman	9	1	2893	3.11	0.35	11.1%
Total	223	51	93419	2.39	0.55	22.9%

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765 **Table 2.** Number, distribution of leptospirosis cases and positivity rates by occupation in Seychelles,

766 from December 2014 to November 2015.

Occupation (N, %)	Number of Leptospirosis cases and positivity rates by occupation	Distribution of 47 leptospirosis cases by occupation in percentage
Farmer or lanscaper (26 ; 12%)	14 (54%)	30%
Builders (32 ; 15%)	3 (9%)	6%
Mechanic (8 ; 4%)	1 (12%)	2%
Student (19 ; 9%)	5 (26%)	11%
Retired (7 ; 3%)	0 (0%)	0%
Unemployed 15 ;7%)	3 (20%)	6%
other occupations at risk (29 ; 14%)	9 (31%)	19%
Not at risk other occupations (77, 36%)	12 (15%)	26%

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769 **Table 3**: Clinical and biological features by leptospirosis infection or other causes of fever in Seychelles,

770 from December 2014 to November 2015.

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	Leptospirosis cases	Other causes of fever	P value	Relative Risk
	Clinical features			
Fever confirmed at enrolment	38 (75%)	97 (57%)	p = 0.02	1.4 [1.1-4.6]
Myalarthralgia	37 (66%)	76 (45%)	p = 0.009	1.4 [1.1-1.8]
Oliguria	12 (24%)	13 (9%)	p = 0.007	2.6 [1.3-5.3]
Jaundice	11 (22%)	13 (8%)	p = 0,006	2.7 [1.3-5.8]
Conjunctivitis	9 (18%)	10 (6%)	p = 0.01	2.9 [1.3-6.8]
Back pain	17 (34%)	36 (21%)	NS	
Haematemesis	3 (6%)	4 (2%)	NS	
Melena	0 (0%)	1 (0,6%)	NS	
Haemoptysis	3 (6%)	9 (5%)	NS	
Haematuria	3 (6%)	6 (4%)	NS	
Abdominal Pain	10 (20%)	38 (23%)	NS	
Headache	21 (42%)	89 (53%)	NS	
Meningitis	2 (4%)	6 (4%)	NS	
Diarrhea	3 (6%)	9 (5%)	NS	
Dyspnea	1 (2%)	2 (1%)	NS	
Cough	2 (4%)	24 (14%)	p = 0.03	0.3 [0.06-0.9]
	Biological anomalies			
Anemia	48 (96%)	108 (64%)	P < 10-7	1.5 [1.3-1.7]
Severe anemia (Hb <11g/dl)	19 (38%)	24 (14%)	p = 0.0009	2.6 [1.5-4.7]
Thrombocytopenia	44 (87%)	63 (38%)	P < 10-7	2.3 [1.7-3.0]
Leukocytosis	34 (66%)	66 (39%)	p = 0.002	1.7 [1.2-2.3]
Neutrophilia	44 (87%)	106 (63%)	p = 0.004	1.4 [1.1-1.6]
High urea	22 (43%)	10 (6%)	P < 10-7	7.3 [3.1-17.0]
High creatinine	21 (40%)	22 (13%)	p=0.0001	3.3 [1.7-5.5]
Biological renal failure	30 (59%)	52 (31%)	P=0.001	1.8 [1.3-2.7]
High alkaline phosphatase	21 (40%)	17 (10%)	P= 0.01	4 [1.7-9.,5]
High total bilirubin	36 (72%)	53 (31%)	P < 10-4	2.3 [1.5-3.4]

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775 **Table 4:** Level of knowledge on leptospirosis of participants according to their leptospirosis status in

776 Seychelles, from December 2014 to November 2015.

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	Leptospirosis cases	Other causes of fever	P value	Relative Risk
	Global knowledges on leptospirosis			
Has never heard about leptospirosis	12 (27%)	52 (31%)	NS	
Know that leptospirosis is a deadly disease	41 (92%)	111 (68%)	P=0.03	4.3[1.3-13.5]
know that leptospirosis is a curable disease	24 (54%)	97 (59%)	NS	
	Knowledge on leptospirosis transmission			
No knowledge on leptospirosis transmission	13 (29%)	56 (34%)	NS	
Know that walking barefoot is a risk factor for leptospirosis	7 (15%)	31 (19%)	NS	
Know that contact with garbage is a risk factor for leptospirosis	1 (2%)	8 (5%)	NS	
Know that rats are involved in leptospirosis transmission	25 (57%)	80 (49%)	NS	
Know that contamination can occured in fresh water	8 (18%)	29 (18%)	NS	
	Knowledge on leptospirosis prevention			
No knowledge on leptospirosis prevention	7 (16%)	56 (34%)	P=0.02	0.43[0.2-0.9]
Avoiding unprotected contatct with garbage	1 (2%)	7 (4%)	NS	
Avoiding unprotected contact with freshwater	2 (4%)	13 (8%)	NS	
Avoiding garbage accumulation	2(4%)	11 (7%)	NS	
Using Personal Protective Equipment	20 (44%)	65 (40%)	NS	
Wearing shoes outside	13 (28,9%)	48 (29,3%)	NS	

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780 **Table 5:** Housing conditions, environmental factors and at risk behaviors by leptospirosis status in

781 Seychelles, from December 2014 to November 2015.

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	Leptospirosis cases	Other causes of fever	P value	Relative Risk
	Housing			
Living in a house	40 (87%)	113 (69%)	p = 0.008	2.9 [1.2-6.9]
Wood house with corrugated sheet	5 (11%)	26 (16%)	NS	
Separate kitchen	4 (9%)	25 (15%)	NS	
Connection to municipal sewerage	1 (2%)	18 (11%)	NS	
Closed bins available	12 (26%)	35 (22%)	NS	
Weekly garbage collection only	5 (11%)	21 (13%)	NS	
Using untreated water	7 (17%)	12 (8%)	NS	
	Environmental factors			
Rats in the vicinicity	24 (55%)	79 (48%)	NS	
Animals in the vicinicity	30 (86%)	109 (67%)	p = 0.01	2.5 [1.1-5.7]
Dogs in the vicinicity	45 (100%)	103 (63%)	p < 10-5	1.5 [1.4-1.8]
Cats in the vicinicity	45 (100%)	52 (32%)	P <10-7	1.2 [1.1-1.48]
Poultry in the vicinicity	30 (67%)	20 (12%)	P <10-6	10.3 [3.3-31.9]
Cattle in the vicinicity	30 (67%)	1 (0,7%)	P <10-7	42.3 [13.2-134.9]
Direct interactions with animals	20 (44%)	66 (40%)	NS	
Pets at home	17 (38%)	37 (23%)	p = 0.04	1.7 [1.0-2.9]
Animals bites	1 (2%)	5 (3%)	NS	
River in the vicinicity	16 (35%)	52 (32%)	NS	
Previous leptospirosis case at home	4 (9%)	8 (5%)	NS	
Previous leptospirosis case in the vicinicity	4 (9%)	7 (4%)	NS	
	Behaviors			
Walking barefoot at home	23 (52%)	93 (57%)	NS	
Walking barefoot outside	11 (25%)	36 (22%)	NS	
Swimming in the sea	9 (21%)	28 (17%)	NS	
Swimming in fresh water	5 (11%)	17 (10%)	NS	
Swimming in swimming pools	1 (2%)	1 (0,7%)	NS	
Using river water for bathing	9 (21%)	17 (10%)	NS	
Washing clothes in river	4 (9%)	11 (7%)	NS	
Hikking in swamps	2 (5%)	4 (3%)	NS	
Hikking in forest	6 (14%)	15 (10%)	NS	
Not always washing fruits or vegetables	8 (18%)	44 (27%)	NS	
Regular alcohol consumption	10 (22%)	36 (22%)	NS	
Regular gardening	22 (50%)	48 (30%)	p = 0.01	1.9 [1.1-3.2]
Working outside the previous four weeks	16 (35%)	42 (26%)	NS	
Construction work in the previous four weeks	5 (11%)	36 (22%)	NS	
Presence of wounds	18 (40%)	59 (36%)	NS	
Using boots without gloves as protective equipment	29 (65%)	66 (40%)	p = 0.004	2.2 [1.3-5.5]

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Conclusion

The results of this epidemiological investigation has shown through univariate analyses that leptospirosis was positively associated with clinical variables including **gardening/farming, oliguria, jaundice, conjunctivitis, history of HCV infection, anaemia, thrombocytopaenia and/or biological renal failure**. The epidemiological variables associated with cases were if patients were **living in houses, the presence of animals around and in houses, gardening and misuse of personal protective equipment**.

Multivariate analyses showed that **being a farmer/landscaper and having cattle and cats around the home** were the most significant drivers of leptospirosis. Clinical features most associated with leptospirosis were **thrombocytopenia, leukocytosis, high values for renal function tests and elevated total bilirubin**.

The study showed **changes in behaviour and exposure as compared to 25 years ago** when the previous studies were conducted and reported that the **lowered case fatality** may be due to improved clinical case management. This study leads to recommendations for con**tinuous health education campaigns** to continuously maintain awareness of the disease amongst the population as well as **further studies to clarify the epidemiology of human leptospirosis and especially the role of domestic animals**.

Overall Discussion and Perspectives

Any efforts to describe the epidemiology of zoonotic pathogens should consider their presence in reservoir hosts, which maintain and transmit disease, their survival as free organisms in the environment, the factors influencing transmission to humans, and finally the distribution and effects of the disease in humans. Because of a high prevalence in the environment and an extreme diversity, *Leptospira* spp. represent an excellent model of zoonotic pathogen to examine the interactions between reservoir host(s), the environment and humans. Within the frame of this thesis, we explored the epidemiology of leptospirosis in Seychelles through geographic modelling, molecular epidemiology and human epidemiology approaches. Produced data allowed us to **determine the biotic and abiotic factors that influence and predict *Leptospira* spp. transmission and infection in *Rattus* species through a geospatial modelling approach**, the results of which are described in **Chapter 1**. We **updated the incidence of leptospirosis in these islands and identified the specific involved *Leptospira* lineages as well as their putative reservoirs**. The results of this investigation are presented in **Chapter 2**. We finally used questionnaire data to **explore the epidemiological and behavioural variables that impact the burden of the disease as well as describe the main clinical manifestations**, and the results of this are presented in **Chapter 3**. Summary of all the main findings of the studies done in Seychelles is shown in Fig. 14.

Previous to this study, reports of leptospirosis epidemiology in Seychelles was dated and mostly non-existent in terms of exploration of the putative reservoirs and the effect of biotic and abiotic factors in affecting disease morbidity and infection prevalence in animals (C. Yersin et al., 1998; Bovet et al., 1999; Claude Yersin et al., 2000). An understanding of leptospirosis epidemiology in Seychelles was of particular public health priority in view of the continued burden of the disease in this island state and in view of the fact that the last reports placed it as having the highest incidence of the disease worldwide (Pappas et al., 2008).

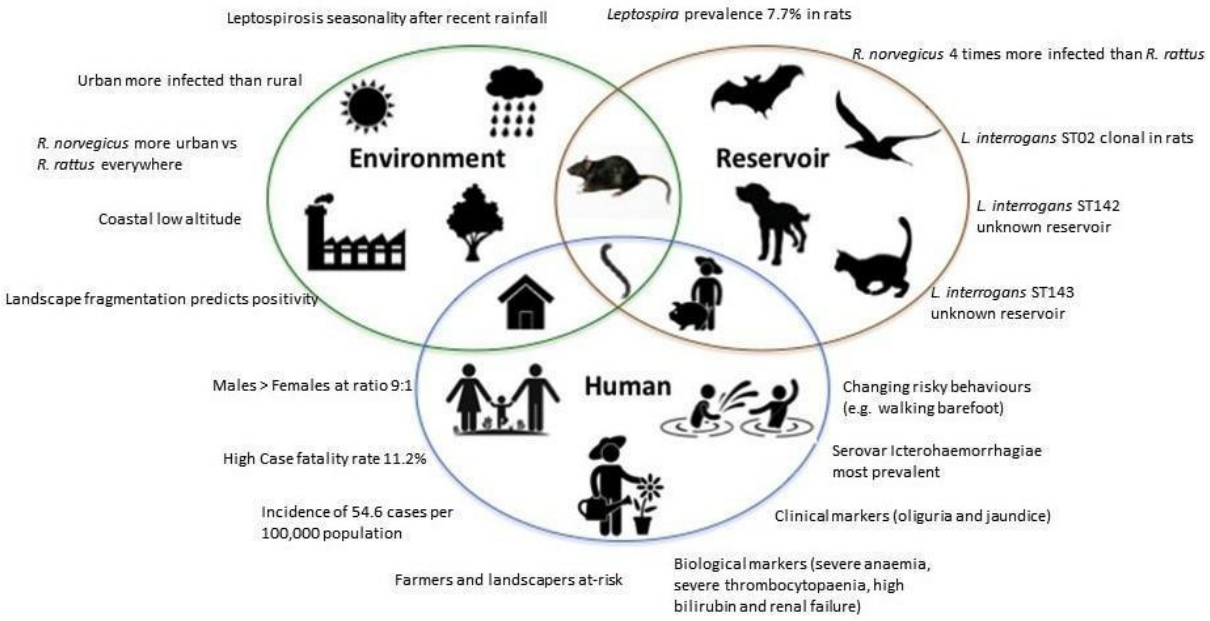

Fig. 14. Summary of salient features of leptospirosis in Seychelles produced in the frame of this thesis.

Geospatial Analyses of *Leptospira*-infected *Rattus* spp. in Seychelles

A precise geospatial analyses of infection prevalence was undertaken using *Rattus* as a model of *Leptospira* exposure on Mahe, the largest island of the Seychelles archipelago, where the majority (>90%) of the population resides. Rat diversity and trapping success was estimated in our study although this was not the main focus of the sampling. We show a trapping rate averaging 43.3% and peaking at 60%, which is indicative of a relatively high abundance of *Rattus norvegicus* and *Rattus rattus*. Although the used sampling protocol did not intend to measure the absolute abundance of rats in Seychelles, these numbers can be compared with trapping success recorded using the same protocols and human resources on La Réunion and Mayotte. For La Reunion, trapping rates are 14.4% (2013) and 10.8% (2017) compared to 51.4% (2012), 57.7% (2014) and 28.3% (2017) for Mayotte. These data were gathered from LeptOI project in 2012 and 2014 and on Typhus project in 2017 (source; Gildas Le Minter, IRD). Comparing these figures to Seychelles, it can be seen that Mayotte displays similar high rapping rates that are commonplace in oceanic islands, which harbour low competitors and predators coupled with the abundance of habitats and food resources, whereas trapping success in La Reunion suggests a lower abundance perhaps related to its slightly varied habitat and possibly focussed effort in rodent control. The relative high

abundance of rats in Seychelles is also consistent with the reports from conservation-focussed programs (Harper & Bunbury, 2015; Rocamora & Henriette, 2015b).

Rattus norvegicus was found to be significantly more infected than *R. rattus*, a pattern that has been also reported in other tropical countries like Brazil (Costa, Wunder, et al., 2015; Panti-May et al., 2016) and Thailand (Herbreteau, Bordes, Jittapalapong, Supputamongkol, & Morand, 2012). This may be related to the ecology of these mammal species, with *R. rattus* being mainly arboreal while *R. norvegicus* breeds in burrows and is hence probably more exposed to infections of environmental origin. As Norway rats are also more associated with urban landscapes, their role in human leptospirosis is probably more prominent (Harper & Bunbury, 2015).

For the first time, we show the impact of various environmental factors in relation to rodent distribution and *Leptospira* infection rates on Mahé island, Seychelles. Sampling data show that *R. rattus* is widespread on the island as opposed to *R. norvegicus*, which is mostly found in lower altitudes and urbanized habitats. The differences in the distribution of *Leptospira*-laden *Rattus* spp. may be related specifically to (i) the carrying capacity and/or (ii) the distribution of each *Rattus* species. Both non-exclusive hypotheses may be the subject of future studies, the former being genetic in nature while the latter looks more specifically to the ecology of *Rattus* spp. Indeed, the distribution of *R. norvegicus* appears contrasted among different islands of SWIO. *Rattus norvegicus* is mostly distributed in coastal urbanized areas in Madagascar but recent surveys suggest a colonization of the colder highlands in the last decades (JM Duplantier, personal communication). In La Réunion, trapping along two altitudinal transects crossing the eastern humid and western dry portions of the island have shown that *R. norvegicus* is restricted to urbanized coastal areas and to elevated (>1000m) forested areas in the West portion of the island (unpublished data from LeptOI project). In Seychelles, it is thought that *R. norvegicus* has colonized Mahé through Victoria harbour within the last 50 years (Cheke, 2010). Hence, the current distribution may be dynamic and *R. norvegicus* may expand its range towards more elevated and natural habitats in the next future, which in turn could have epidemiological consequences. Factors from our study that are most associated with *Leptospira* presence in *Rattus* spp. are lower altitudes and proximity to urban areas, elevated distance from forested areas, close proximity to surface water for *R. norvegicus*, habitat fragmentation and rainfall within the last 3 months.

Although the majority of *Leptospira*-infected rats are the Norway rats, there are some black rats infected at altitudes located at Chemin Dame Leroi (elevation approx. 170m) and La Reserve (elevation approx. 280m), which are natural and semi-natural sites. Strikingly, the southernmost part of the island contains a dearth of any *Leptospira*-carrying rats. This may result from an environment non-conducive to *Leptospira* maintenance (e.g. soil chemistry and its importance in maintaining leptospires survival) or from the absence of *R. norvegicus*, which is 12 times, more infected than *R. rattus* and most probably a foci of infection of *R. rattus* and other species.

Molecular epidemiology of leptospirosis in Seychelles

As a result of a prospective epidemiological study that we were able to put in place (ref. study protocol in Annex 1), we were able to confirm that Seychelles shares an epidemiological profile similar to Reunion where most clinical cases result from infection with *Leptospira interrogans* (Biscornet et al., 2017; Guernier et al., 2016). In fact, Reunion Island also shares the same *Leptospira interrogans* strain sequence type ST02 in rats, indicative of a recent introduction of this *Leptospira* spp. in these islands compared to other regions of the world. In Mayotte, for example, a portion of human cases is associated with *L. mayottensis*, a recently identified *Leptospira* spp. found first in Mayotte but subsequently found to originate from Madagascar and present uniquely in *Tenrecs* (P. Bourhy et al., 2012; Pascale Bourhy et al., 2014; Dietrich et al., 2014; Lagadec et al., 2016; Dietrich et al., 2018). In Seychelles however, the finding of two novel genotypes (ST142 and ST143) and the absence of these bacterial lineages in rats stimulates interest in elucidating their reservoir(s), especially as these STs represent 2/3 of human cases included in the frame of the present study. Interestingly, a small sampling was able to show an association with dogs where one out of 24 sampled dogs harboured ST142, which is note worthily the most prevalent ST in human cases. Interestingly, on Reunion Island, two distinct STs, namely ST02 and ST34, were found in human cases. As in Seychelles, all infected rats were shedding ST02 only, while dogs were found shedding both ST02 and ST34. Dogs are predominant in Seychellois households and frequently as strays. They have been shown to be involved in leptospirosis epidemiology in other parts of the world as well (Gay, Soupé-Gilbert, & Goarant, 2014; Guernier et al., 2016; Miotto et al., 2018; Zaidi et al., 2018), and hence deserve further exploration in the Indian Ocean islands.

More *Leptospira* positive patients were from semi-rural and rural areas and there was a strong association with agricultural activities as shown in epidemiological studies (see below). It was difficult to determine where the patients were infected and even the STs did not show any

apparent structuration in geographic distribution (Biscornet et al., 2017). This finding is consistent with the molecular epidemiological profile determined in the frame of the study, which identified two novel sequence types (ST142 and ST143) of which the reservoir(s) is/are not rats and which represent the majority strain of *Leptospira interrogans* found in human patients. This is further supported by the finding that only a 7.7% molecular prevalence of *Leptospira* in rats from Seychelles whereas a review of country-specific study data showed that some countries have more than 60% infection prevalence of *Leptospira* in rats (Boey, Shiokawa, & Rajeev, 2019).

The finding that rats are not the major reservoir for human leptospirosis in Seychelles is major for two main reasons. Firstly, rats have been historically associated with leptospirosis and considered as the main reservoir of human leptospirosis (Haake & Levett, 2015a). Leptospirosis is actually termed "rat disease" in France. However, recent studies have begun shifting dogmatic stances on this subject to enable the consideration of other animal species as being major contributors for the transmission of pathogenic leptospires (Jimenez-Coello et al., 2010; Desvars, Naze, Benneveau, Cardinale, & Michault, 2013; Gay et al., 2014; Guernier et al., 2016). In a recent study carried out in Tanzania where rats were sampled in zones of high incidence of human leptospirosis, no rats (zero out of 320 rats) were found to carry leptospires whereas in contrast positivity was found in cattle, sheep and goats suggesting that ruminants play a more important role (Allan et al., 2018). Secondly, this finding brings to the fore the importance of other putative reservoirs whereas the focus of public health educational information provided has always been targeted mainly on the importance of controlling rats with the supposed consequent effect of reducing cases of leptospirosis. The decades of rodent control efforts have obviously not significantly influenced human leptospirosis epidemiology, which further highlights the presence of alternative reservoir(s) for pathogenic leptospires in Seychelles.

Epidemiology of leptospirosis in Seychelles

The one-year population-based prospective study that was put in place involved the gathering of epidemiological information through the use of a standardised questionnaire and recording results of clinical diagnostic markers of leptospirosis in humans, with the objective of revealing the characteristic features of the disease and describing the risk factors that may be involved. We argue that case classification bias is low in the study due to the combined use of RT-PCR, IgM ELISA and MAT screening. We also determined that the epidemiological patterns that have been identified are likely descriptive of the situation in Seychelles due to a limited number of imported cases of leptospirosis.

A sex distribution bias was confirmed in leptospirosis epidemiology in Seychelles with a significant over-representation of males, as reported in previous studies in Seychelles in 1992 (89%) (Pinn, 1992) and 1995-1996 (84%) (C. Yersin et al., 1998) at an average ratio of 9:1, although the difference in 2014-2015 (96%) was even more markedly biased towards males. The higher risk of developing leptospirosis experienced by adult males has been shown in numerous studies worldwide and summarised in a recent review article by Costa, *et al* (Costa, Hagan, et al., 2015). Although, exposure-related bias may be an important driver for this gender difference (Haake & Levett, 2015a), the effect of gender bias in acquisition of infectious diseases has been expounded recently as related to differences in sex hormone and immune effectors (Guerra-Silveira & Abad-Franch, 2013; Skufca & Arima, 2012). This hypothesis may also be coherent with a low incidence in children, who are typically exposed in tropical islands but rarely diagnosed with leptospirosis.

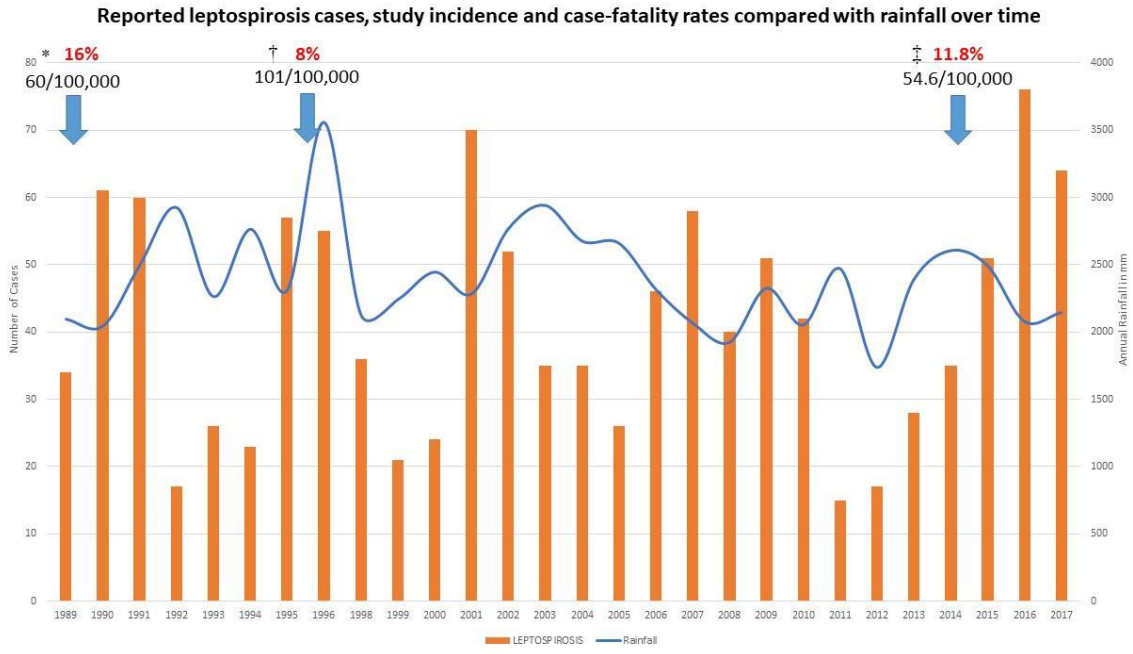

Fig. 15. Updated chart of reported cases of leptospirosis from 1989 to 2017 in Seychelles, showing incidence and case-fatality rates from past studies as well as the annual rainfall trend. Source: Seychelles' Ministry of Health Statistics Unit and Seychelles Meteorological Station. (*Pinn, 1992 (Pinn, 1992); †Yersin *et al*., 1998. (C. Yersin et al., 1998) and ‡Biscornet et al, 2017 ((Biscornet et al., 2017)).

The apparent 2 to 3 year cyclical peaks and troughs in number of leptospirosis cases is yet to be explained. A similar cyclical pattern of *Leptospira* shedding was seen in bat maternity although the cyclical peaks were annual and there was no connection to human disease (Dietrich et al., 2015), therefore the implication for Seychelles context is not known. A small sampling of bats (*n*=50) did not reveal any pathogenic *Leptospira* spp. (Biscornet et al., 2017). An in depth sampling of the yet unexplored other potential *Leptospira*-infected animals is yet to be done and could shed light on the reason for the peaks and troughs, which seem to be independent of years with heavy rainfall.

We report a case fatality rate of 11.8% for leptospirosis in Seychelles (see Fig. 15 for comparison of previous studies), which in the region is closest to Reunion island at 3-5% CFR, and considerably higher than on the island of Mayotte where CFR is at 0.9%. These differences tally with the findings that the *L. interrogans* strains identified in the Seychelles and Reunion with the highest CFR are likely newly introduced and so likely exhibit the highest virulence attributes in causing severe disease, as compared to the endemic leptospires (*L. mayottensis* and bat-borne *L. borgpetersenii*), which are mainly found in endemic tenrecs and rodents of Mayotte and Madagascar and may be less virulent (Tortosa et al., 2017). Indeed in a recent article, this hypothesis was confirmed in experimental infections with leptospires isolated from wildlife from the islands of the SWIO and showing that endemic tenrec-borne and bat-borne leptospires are less virulent than the likely introduced rat-borne *L. interrogans* genotyped as ST02 (Cordonin et al., 2019).

The CFR due to leptospirosis was shown to have decreased in the last decades between studies conducted from 1992 (Pinn, 1992) when it was 16% to 11.8% in the last study conducted in 2014-2015. In the study conducted in 1995-1996 (Claude Yersin et al., 2000), pulmonary haemorrhage was seen as the main cause of death amongst leptospirosis cases, where all fatalities (n= 6) that occurred presented as such dying shortly after symptom onset. In our study, although there were similarly 6 fatalities, however, only a third presented in pulmonary form at autopsy. We suggest that the reduction in CFR and changes seen in the main cause of death may be due to the improvements in the clinical management of severe leptospirosis during the intervening years, as well as implementation of dialysis and mechanical ventilation.

We also show that as with studies done elsewhere, clinical (oliguria and jaundice) and biological (severe anaemia, severe thrombocytopaenia, high bilirubin and renal failure) markers

are associated with severity and could thus serve as indicators to guide physicians. A recent article demonstrated how the implementation of a simple score based on markers of severity can help to predict acute and severe leptospirosis (Fish-Low et al., 2019; Smith et al., 2019). A similar score implemented in Seychelles where leptospirosis is endemic and where severe forms are frequent could be lifesaving.

We report in our study changes in risky behaviours compared to previous studies conducted 20 years ago. Walking barefoot which was found to be a significant risk factor for leptospirosis in previous studies for example, was not found to be significantly linked with cases of leptospirosis, which we argue points to changes in such practices.

Most interestingly, we found that the presence of rats around houses of leptospirosis cases has not changed between previous studies and ours, which we argue further suggests that rats are not the main source of transmission of leptospirosis in Seychelles (Biscornet et al., 2017). In our study we also highlight the importance of other potential animal reservoirs of leptospirosis, as leptospirosis cases was found to be associated with cattle, cats, dogs and poultry. Whereas previous studies did not show any difference in the presence of dogs around leptospirosis cases compared to non-leptospirosis cases, our study showed that dogs were present around 100% of leptospirosis cases compared to 63% for non-leptospirosis cases. This further highlights the probable involvement of dogs as a shedder of pathogenic leptospires as a genotype representing one of the novel and main infecting strain was found in a dog (Biscornet et al., 2017).

The professions with the highest risk of being a leptospirosis case was landscaping and farming, with three times more risk in these two professions. Integrating these factors in the aforementioned scoring index could assist in early detection and treatment of potential leptospirosis cases (Tubiana et al., 2013; Goarant, 2016; Smith et al., 2019).

Perspectives

Although the investigations carried out in the frame of the present thesis have enlightened the epidemiology of the disease, results have also led to new research questions, some of which need to be addressed in order to fully understand what is actually going on in Seychelles. Obviously, one of the most salient remaining questions is related to the identification of reservoir(s) alternative to rats. As the majority of human cases are associated with two new STs, identifying reservoirs involved in their maintenance and shedding would undoubtedly help in prevention. A sampling to include the House mouse (*Mus musculus*), which was not trapped in our

study, could be relevant as highlighted by a recent investigation implemented in Puerto Rico and revealing this species as the most *Leptospira*-infected rodent in cattle farms (Benavidez et al., 2019). A large exploration of dogs, including stray dogs and pets, will allow answering whether dogs are indeed major reservoir of the disease. If so, obligatory vaccination of pets and proactive control of stray dogs would have a tremendous positive impact on disease incidence. There have been discussions already with the Chief Veterinary Officer of the National Biosecurity Agency, Dr. Jimmy Melanie, about conducting animal sampling and this is expected to start as soon as the study protocol is complete which is currently in draft phase. Sampling of the urine and/or kidneys as well as blood of animals has been proposed. We also propose to include yet untrained staff in this future study, e.g. newly recruited veterinarians and lab staff who will be interested in the study.

As far as reservoirs are concerned, we can establish protocols to allow testing whether the increased infection prevalence in Norway rats results from the intrinsic capacity of this species to support chronic infection, and/or from the distribution of this species which occupies low elevated fragmented areas. Indeed, the stark difference in *Leptospira* prevalence between Norway and black rats leads to exploring this feature to understand the underlying reasons for the differences.

A growing number of studies explores *Leptospira* infection in environment samples such as mud or rivers (Sato et al., 2019; Thibeaux et al., 2018), and environmental DNA may be highly relevant as a proxy to determine the levels of infection of specific habitats. Actually, data presented herein shows that infection prevalence in rats might not be such a relevant proxy of *Leptospira* exposure of the environment. Hence, a direct measure of the infections in the environment may bring in complementary information. Currently the SPHL is in process of establishing an MoU with the Seychelles Agricultural Agency (SAA) and the Soil and Plant Diagnostic Laboratory (SPDL) to be able to collaborate on these areas. They would already be in possession of interesting data on soil chemical composition and characteristics, which could be analysed together with past and future data on leptospires presence in the environment or animals or human leptospirosis cases. There could well be important abiotic factors accounting for the survival of *Leptospira* spp. in the environment and so explain the epidemiology of the disease seen within and between these islands.

The human epidemiology data presented in chapter 3 highlighted farmers and landscapers as an at-risk group. A seroprevalence study in this group could further clarify their actual exposure, and eventually propose measures to specifically lower the burden of the disease in this group. This

could also be the subject of collaboration to be formalised in the MoU currently being drafted between the SPHL of the Public Health Authority and the SAA.

In view of the prevailing high CFR seem in Seychelles, a retrospective analysis of case files to examine the specific details of fatal cases would also be a very interesting prospect. The possibility of there being other pathologies that worsen the leptospirosis cases could also be explored by such a retrospective study. In previous studies, the association of pulmonary haemorrhage to disease severity has highlighted (Yersin et al., 2000), however this has not been of particular significance in recently conducted studies.

Lastly, 77.1% of the included patients were actually diagnosed as negative for leptospirosis. This sample is precious and will be used to identify other pathogens responsible for acute fevers in Seychelles. Such study will be carried out using high throughput serological screenings, and will allow identifying the pathogens of highest medical importance in the country. This is especially important as following the study, the epidemiological profile of acute fevers in Seychelles had changed dramatically with the most massive waves of dengue infection seen in these islands to date (Lustig, Wolf, Halutz, & Schwartz, 2017), which in turn further complicates the diagnosis of leptospirosis. The presence of other rat-borne diseases such as murine typhus (caused by *Rickettsia typhus*) is yet to be explored, a fact that becomes important in view of the abundant rats present on the islands and as murine typhus has been recently reported in regional islands like Reunion (Balleydier et al., 2015) and Madagascar (Rakotonanahary et al., 2017). Causes of what has been termed as “rat bite fever”, which in literature mentions *Streptobacillus moniliformis* and *Spirillum minus* as causative agents, could be explored as well amongst the large percentage of patients that were diagnosed negative as well as in future prospective studies or surveillance examining acute fever syndromes. Altogether, presented data show how investigations carried out under the One Health framework are relevant from basic and public health perspectives. In Seychelles, such studies are facilitated by the insular nature of the country, which typically shelters a limited number of animal (and potentially reservoir) species and facilitate the identification of human imported cases (Tortosa et al., 2012). In addition, Seychelles shelter a limited number of health infrastructures and the reduced size of the human population virtually gives access to most if not all human cases.

Annex 1: Leptospirosis Study Protocol

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Assessment of the current burden and the epidemiology of acute Leptospirosis amongst fevers of unknown origin in Seychelles.

Principal investigator

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Réseau SEGA-COI
CRVOI-La Réunion

DSRU

Table of Contents

List of Acronyms

CI: Confidence Interval

CIRE OI: Cellule de l'InVs en région Océan Indien

CRP: C- Reactive Protein

CRVOI: Centre du Recherche et de Veille de l'Océan Indien

CXR: Chest X Ray

DSRU: Disease Surveillance & Response Unit

ECG: Electrocardiogram

ELISA: Enzyme Linked Immunosorbent Assay

EMGH : Ellinghausser, Mc Cullough, Johnson and Harris medium

ESR: Erythrocyte Sedimentation Rate

ID: Identification

IDSR: Integrated Disease Surveillance & Response

IgM: Immunoglobulin M

IHA: Indirect Haemagluttination Assay

MAT: Micro Agglutination Test

OR: Odds Ratio

RT PCR: Real Time Polymerase Chain Reaction

SEGA COI: Suivi et de Gestion des Alertes de la Commission de l'Océan Indien

WHO: World Health Organization

Introduction

The World Health Organization (WHO) has recognized Leptospirosis as a re-emerging communicable disease, which poses a major public health threat worldwide. It is marked by increasing incidence with half a million human cases annually, and a case fatality of 25% in severe forms (1). It is the most widespread zoonotic disease caused by a pathogenic spirochete of the genus *Leptospira* and is most common in temperate and tropical regions (2). The disease is considered neglected in many places and its real burden in Seychelles and globally is largely unknown. Therefore the study is expected to give a better understanding about the current epidemiological situation compared to results from the previous study done nearly 20 years ago, and to provide recommendations on the way forward (3).

Background Information

History of Leptospirosis

Leptospirosis (also known as Weil's syndrome, Canicola Fever, Canefield Fever, Nanukayami Fever, 7-day fever, Rat Catcher's Yellows, Fort Bragg fever, Black Jaundice, and Pretibial Fever) is the most widespread and prevalent bacterial zoonotic (affecting humans as well as animals) disease in the world caused by a pathogenic spirochete of the genus *Leptospira*(2).
It is a little more than 100 years since Weil; Professor of Medicine at Heidelberg (1886) first described this disease caused by *Leptospira interrogans, serovar icterohaemorrhagiae* or *copenhageni* (4). *Leptospira* bacteria seen at that time were not cultured and were named *Spirochaeta interrogans* by Stimson as early as 1907, in silver stained preparations of liver from a patient believed to have died of yellow fever. Its contagious nature and microbial origin were proved independently, first in Japan by Inada et al. *(Spirochaeta icterohaemorrhagiae)* in 1915, and soon after in Germany *(Spirochaeta icterogenes)* by Uhlenhuth and Fromme. Both cultivated isolates were described as pathogenic *Leptospira.* Later in 1914, a saprophytic leptospira, *Spirochaeta biflexa* was found in fresh water. Noguchi then proposed the name *'Leptospira'* in 1918, following detailed microscopical and cultural observations (5). In the 15 years or so, from discovery until the 1930s, many of the important serovars prevalent throughout the world, and their host sources were discovered. During the 1920s to 1950s, the milder forms of leptospirosis, the numerous related distinct serotypes and occupational relationships were elucidated in Japan, Indonesia and Germany. Electron microscopy revealed much of the detail of the structure during the 1960s and 1970s and Yanagawa and Faine (1966) showed that *Leptospira* were analogous to other bacteria in structure and that characteristic antigens were associated with structural elements (5).

Epidemiology

WHO and Hartskeerl et al., have recognized that leptospirosis poses a major public health threat, as a re-emerging communicable disease to the developing and the developed countries. It is a growing public and veterinary health problem, which requires active surveillance to understand changes in the demographics and patterns of infection (1). While it is generally accepted as a globally re-emerging disease, the true spread and increase of leptospirosis cases remain unknown. Pathogenic *Leptospira* survive longer in a warm and humid environment,

and the disease is thus most common in the late summer and early fall in temperate regions, and during rainy seasons in tropical regions (2).

Other factors facilitating the spread of infections in the temperate climate zone are socio-economical, such as the intensive migration of people or changes in the economic status of individuals or some communities, the consequence of which can result in poor hygiene status and transmission of infections (6). Epidemics have also often been related to heavy rainfall and flooding periods and the Indian state of Kerala has witnessed post-monsoon epidemics of leptospirosis in recent years (7).

Most mammalian species are natural carriers of pathogenic *Leptospira* which include feral, semi-domestic, farm and pet animals as important infection sources, whereby they live in the kidneys and genital tracts of their natural hosts. The risk of acquiring leptospirosis is through direct contact with infected animals or, much more commonly, through indirect contact with water or soil, contaminated urine of infected rodents or animals. The most likely to be infected are persons engaged in agriculture, sewage works, forestry, butchery, rice and sugar-cane field workers, veterinarians, slaughterhouse/abattoir workers, pet traders, rodent catchers, dairy workers and military personnel; it is also considered to be an occupational disease (8). However, individuals working directly with animals (farmers, cowherds, veterinarians, abattoir workers, etc.) can also acquire the infection during milking or after animal bites, contact with aborted foetuses, parts of placenta and infected carcasses (in slaughtering houses).

In addition, an increase of recreational exposure incidences has also been observed recently in Seychelles and in certain countries with temperate climates (6); risk associated with exposures occurring in various types of recreational activities, such as swimming, triathlons and water sports (kayaking, rafting, and fishing) in rivers or lakes where water contaminated by leptospires can be a source of infection for triathletes and other water sports enthusiasts. Cases have been reported in the USA, Germany, Austria, Ireland (7) and our neighbouring island, La Reunion [CIRE OI, 2013]. Apart from the danger connected with rodents, which are considered as the main vectors of leptospires, occurrence of the disease in dogs (stray and domestic) known as Stuttgart disease and cats can also generate a higher risk of infection for humans. For a long time, dogs were recognized as a reservoir of leptospires and a potential source of infection and results of recent investigations show that ticks are also potential vectors of leptospires (6).

Leptospires enter directly into the bloodstream or lymphatic system via skin abrasions and cuts, through the mucous membranes of the eyes, the nasopharyngeal mucosa by inhalation and mouth by consumption of contaminated items (9), genital tracts of domestic animals or through an invasion of the placenta from the mother to the foetus at any stage of pregnancy in mammals. Person-to-person transmission is extremely rare since man is a dead-end host for leptospiral dissemination although a recent report has shown that people can maintain leptospires in certain ecosystems (8).

In humans, infection with *Leptospira* spp. varies from being sub-clinical (asymptomatic), to a mild to a highly acute disease, depending on the infecting serovar, age, health and immunological competence .Typically a biphasic course of leptospirosis is often observed, the manifestation of signs is preceded by an incubation period of usually 5 to 14 days but ranges from 2 to 30 days and the illness can last from a few days to 3 weeks or more. Leptospirosis may present with a wide variety of clinical manifestations ranging from mild-flu like illness to a sometimes serious and fatal disease (10). The first phase of the disease, lasting up to 7 days, is connected with a period of leptospiraemia (the presence of leptospires in the blood). Unspecific signs, like abrupt fever, chills, headache, diarrhoea, nausea and vomiting, myalgia are observed during this phase. More rarely, conjunctivitis, maculopapular skin rash and sporadically icterus can be noted. After 5–7 days, the signs can retreat and disappear. In some cases, the patient can recover (sometimes without medication) or the disease can regress to a subclinical form, but usually, after 1–3 days of apparent remission, the second phase of the disease begins; the anicteric form. The anicteric form is a consequence of leptospires transfer from the blood vessels to the organs; it is milder and diagnosed more often in approximately 90% of cases.
The signs of meningitis and encephalitis (headache, neck stiffness) are most often observed in this form. In some patients, uveitis may also develop which can appear some weeks or even years after the onset of the disease (6).

The icteric form of the disease is a consequence of serious hepatic, renal or pulmonary disorders caused by leptospires located in these organs, whereby the patient will present with jaundice, haematuria, albuminuria, oliguria and anuria. Case fatality ratios are usually low but may reach 25% in patients with severe illness (10). Infections in pregnant women caused by leptospires, can result in various foetal disorders and often death. However, administration of appropriate antimicrobial agents can lead to the birth of healthy infants if detected early (6).

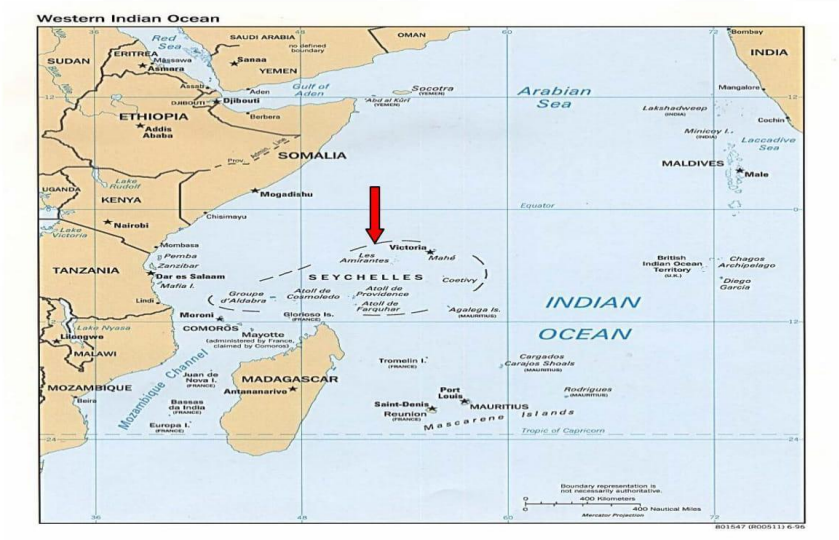
Leptospires induce a serovar-specific immune response consisting of a cellular and humoral mediated immunity against the infecting serovar, but does not necessarily protect against infection with other serovars. Sero-conversion may occur after 2 - 10 days after onset of disease, depending on the individual's immunological competence and infecting serovar.

IgM class antibodies usually appear earlier than IgG class antibodies, and remain for months or years at a low detectable titre, however IgG antibodies may not be detected at all, or for only a short period, or persist for several years. The duration of detectable antibodies in humans after natural infection with *Leptospira* vary substantially with sero-positive persons becoming sero-negative between 6 and 60 months after infection. Leptospirosis can mimic many other infectious diseases, namely influenza, hepatitis, dengue, chikungunya and it can also be misdiagnosed as Hantavirus infections or other viral haemorrhagic fevers, yellow fever, malaria, brucellosis, borreliosis, typhoid fever or other enteric diseases, and pneumonia (3), together with a range of abortifacient diseases in animals.

Leptospira characterisation has most often been carried out using serological tools, mainly through the Microscopical Agglutination Test together with monoclonal antibodies. These tests allow the discrimination of nearly 300 pathogenic *Leptospira* serovars . In general, each serovar is adapted to a certain mammalian host; rodents, insectivores, dogs, pigs and cattle comprise the best-known ones. Molecular methods are increasingly used to characterize pathogenic *Leptospira*, and to allow molecular epidemiological investigations at regional or global levels (11).

Regional context

The western Indian Ocean counts 21 inhabited groups of islands belonging to 12 countries. The terrestrial surface areas of the western Indian Ocean islands are between < 1km² of the Bassas of India and 587 041 km² of Madagascar. Most islands of this region have a tropical climate with two main seasons, a hot and rainy season known as the austral summer and a dry season known as the austral winter. However, in Seychelles, the climate is subequatorial with more than 80% of humidity all year round with distinct monsoons (12).



Leptospirosis constitutes a major public health problem in the southwestern Indian Ocean, particularly in La Reunion, Mayotte, Mauritius and the Seychelles with incidence rates shown to be amongst the highest in the world (12). Data available about human and animal leptospirosis are diverse; human leptospirosis has been extensively studied and reported in Reunion Island, Mayotte and the Seychelles. Serologic evidence has recently been found in the Comoros and in Madagascar. It has been described as endemic and non-endemic in the mammals of the western Indian Ocean islands in wild fauna as well as in pets, cattle and in commensal rodents other than rats (12, 13). It has been known in La Reunion since 1980 as a major infectious disease in cattle and in 2003, a study did confirm that it was the major cause of abortion in cattle (13).

The epidemiological role of bats in the transmission of *Leptospira* to humans attracts more and more scientific interest. In Madagascar, antibodies to *Leptospira* could not be evidenced in the fruit bat *Pteropus rufus*, but recently pathogenic *Leptospira* spp. were found in bats, in Madagascar and the Comoros (12), serological evidence has also been reported in lemurs from Mayotte but at a low seropositivity rate (13).

Local context

The Republic of Seychelles with a population of 89,949 inhabitants is a small island state spanning an archipelago of 116 granitic and coralline tropical islands, the majority of which are small and uninhabited. The landmass is approximately 459 km², but the islands are spread over an Exclusive Economic Zone of 1.4 million km². It lies between approximately 4ºS and 10ºS and 46ºE and 54ºE of the Equator, some 480 km and 1,600 km from the coast of Africa, in the Western Indian Ocean, to the northeast of the island of Madagascar and 1,600 km to East of Kenya. The neighboring islands, developing countries and territories are Zanzibar to the West, Mauritius and Reunion to the South, Comoros and Mayotte to the Southwest. It is composed of 25 districts, of which 90 % of the population lives on Mahé, 9 % on Praslin and La Digue. The main ethnic groups are those of African, French, Indian and Chinese origin.

The climate of Seychelles is tropical and mostly equable; average rainfall is 1,500–2,200 mm per year, the temperature ranges from 24 ° C to 32 ° C and the humidity is high, but its debilitating effect is usually enhanced by the prevailing winds. The monsoon of the Southeast from late May to September brings cooler weather and a more agitated sea, and the Northwest monsoon from December to April brings a hotter and humid weather that often reaches 80% or more and the sea is generally calm.
Medical care within a national health system is delivered free of charge and is easily accessible through community clinics (governmental and private), small cottage hospitals, and a main referral hospital.

Leptospirosis is considered endemic, particularly frequent and severe in Seychelles and has presented the highest incidence in the western Indian Ocean area and one of the highest incidences in the world. Between 1988 and 1990, the annual incidence was 60 cases per 100 000 inhabitants and in 1995-1996, following a population based study, the incidence was estimated to be 101 per 100,000 population (3). About ⅓ of the cases were mild forms; ⅔ had a more severe presentation with jaundice (without liver failure) and/or acute renal failure and/or pulmonary hemorrhage and autopsies showed that diffuse bilateral pulmonary hemorrhage was the main cause of death (14). Males were more predominantly affected representing 84% of the confirmed cases and it was found to be less frequent in children less than 15 years of age and in adults of more than 75 years of age. Traditionally the highest

incidence and the severity amongst males are related through the different occupational and recreational exposures. In Seychelles, it was found that the most affected were those who had high exposures to the environment either professionally (e.g farmers and environment workers) or recreationally (gardeners). Icterohaemorrhagiae and Hurstbridge were the most predominant circulating serogroups and five new serogroups: Ballum, Canicola, Djasiman, Hurstbridge and Lousiana were also identified and of which none had a predominance of severe clinical presentations (3).

However, it is possible that the disease susceptibility and clinical outcomes could be different according to sex as it has been reported in several studies. It has been proven that the excess of leptospirosis cases occur in males due to greater occupational and recreational exposures which puts them at risk of being in contact with *Leptospira* infected agents. (15). It has been suggested that children less than 5 years of age have limited contact with infected soil and water and that children less than 10 years of age show less severe reaction to leptospiral infection (10).

The absence of cases among the elderly can probably also be explained by limited contact with infected soil and water and because they are more likely to have developed immunity in a highly endemic setting.

Justification for the study

Fevers of unknown origin represent a public health problem in Seychelles with over 600 cases reported in 2013; leptospirosis is considered to be a main cause. In 2013, 584 suspected cases of leptospirosis out of the over 600 cases of fever of unknown origin was reported but only 28 (26 males/2 females) were actually confirmed through IHA diagnosis.It is important to note that current diagnosis only uses IHA testing, which notoriously lacks both specificity and sensitivity compared to other diagnostic tests. In addition, IHA will not be positive for acute cases of less than 6 days. From 2009 to 2013, 158 IHA confirmed cases of leptospirosis were reported. More cases were reported in 2009(51 cases) and 2010(47 cases) followed by a decline in 2011(14 cases) but have gradually increased from 18 cases in 2012 to 28 cases in 2013 (16).

To date, it is difficult to explain if the decline in 2011 was related to a reduction in the number of cases or to difficulties related to laboratory diagnostic issues.

An increase of 65% in confirmed cases in 2013 compared to 2012 suggests that the real burden of leptospirosis is underestimated. A total of 5 confirmed leptospirosis related deaths, all males were reported in 2013 age ranging from 30 to 51 years old, case fatality rate of 18% out of the total confirmed cases and a case detection rate of 4.8 per 100 tests (16). This case fatality rate is high compared to Reunion (4%), difference which could be explained by the delay in diagnosis, management and treatment but also due to the different pathogenicity of the strains circulating in the islands. To date, although testing facilities are now being made available, there is yet no substantial molecular information available on the *Leptospira* species actually circulating in Seychelles. In summary, the incidence of confirmed cases seems to decrease which contrast with the number of suspected cases that is increasing. Leptospirosis in Seychelles is underreported for many reasons but mainly due to difficulties in distinguishing clinical signs from those of other endemic diseases and a lack of appropriate diagnostic laboratory services.

The real burden of leptospirosis is thus currently unknown and questions that are being asked are, whether the decrease is related to changes in behaviour (*e.g.* walking bare foot) or other causes of unknown fever or whether leptospirosis cases are currently being misdiagnosed.
This study will allow us to clarify the burden of leptospirosis in Seychelles. Furthermore, the results of the study when compared with the previous population based study which was conducted in 1995-1996, will assist towards a better understanding of the current epidemiological determinants of human leptospirosis in Seychelles. Occupational factors such as outdoor activities, presence of cuts at body parts, environmental factors such as contact with rodents are some of a few that will be investigated. Hence, it will act as a critical step for designing interventions, to prioritize resources and plan public health interventions aiming at the control and stopping the transmission of the infection source. Importantly, those feverish cases diagnosed negative for leptospiral infection will be further investigated for the presence of arboviruses (West Nile virus, Chikungunya, Dengue, etc,) in order to provide the baseline for future fever surveillance activities.

Objectives

Main objectives:

1- To determine the current burden of acute human leptospirosis in Seychelles and to identify risk factors associated with the disease.
2- To describe the aetiology of acute fever of unknown origin in Seychelles.

Specific objectives:

The specific objectives of the study are:

1- To measure the proportion of acute fevers of unknown origin that is due to leptospirosis in Seychelles.
2- To identify possible environmental and behavioural risk factors associated with leptospirosis infection in Seychelles.
3- To determine by molecular and serological methods, the circulating *Leptospira* strains in the Seychelles.
4- To assess the clinical severity of incident leptospirosis in Seychelles.
5- To determine the proportion of arboviral infection amongst acute fevers of unknown origin in Seychelles.

Methodology

Study area and study population

The study will cover the whole territory of Seychelles, which consists of 116 islands situated in the Indian Ocean and a population of 89,949 inhabitants. The study population will be all males and females aged 13 years and above and residing in Seychelles.

1. ***Proportion of Leptospirosis cases amongst acute febrile illnesses in Seychelles.***

A prospective population based study will be conducted from August 2014 to July 2015 on all acute fever cases of unknown origin in Seychelles. All physicians from governmental and private institutions in the country will be repeatedly informed about the study. They will be requested to refer all cases with acute fever of unknown origin to the reference center (a specific center to be set up for the course of the study) at the Seychelles Hospital whereby the severity will be assessed and admission conducted as per admission criteria.

Data Collection

A clinical case questionnaire will be administered and an array of standard laboratory tests will be conducted on all acute fever cases of unknown origin for confirmation of leptospirosis *(refer to Annex 1&3 and diagnostic scheme below).*

Whole blood and serum for PCR, culture and serology along with other blood tests, urine for PCR will be obtained from consenting patients meeting the acute fever of unknown origin definition criteria. For serological and PCR tests, blood and urine will be collected as soon as the patient is enrolled in the study upon informed consent (on admission for most patients, within the first 24 hours). Samples requiring tests such as the Rapid diagnostic test (ELISA for IgM: Lepto Check Western Blot kit), IHA and RT-PCR will be sent to the Public Health and Clinical Laboratory situated at the Seychelles Hospital.

For other blood tests, maximum values observed during the hospital stay will be considered. All fatalities will undergo post-mortem examinations. Fevers of unknown origin testing negative for leptospirosis will be further investigated for other possibly prevalent aetiologies

(e.g. Dengue, Chikungunya, Sindbis virus). At the end of the study, all clinical forms will be reviewed to describe the aetiology of acute fever of unknown origin in Seychelles.

Daily rainfall measurements from collection sites will be obtained from the National Meteorological Services in order to verify and determine any correlation of cases to that of rainfall for the preceding weeks.

2. *Determinants of leptospirosis in Seychelles.*

Risk factors:

A matched case-control study will be conducted to determine environmental and behavioural factors of leptospirosis. The study will be conducted from August 2014 to July 2015 on all probable (IHA and or IgM positive) and confirmed (PCR positive) cases of leptospirosis seen at the Seychelles Hospital. Controls will be randomly recruited from the National Census Database, as described below.

Data Collection

An interviewer administered questionnaire which will include clinical, socio-demographic, present and past medical history, educational, professional, occupational, environmental and behavioral variables will be administered by trained medical personnel (trained doctors and nurses) to characterize the clinical illness and exposure history to eligible cases and to eligible controls upon consent. *(Refer to Annex 1&3).*

3. *Description of Leptospira species*

A *Leptospira* culture will be conducted on all PCR positive cases. For this, an aliquot of fresh heparinized serum will be used to inoculate fresh EMJH medium following a previously described procedure (17).

Data Collection

For all confirmed or probable cases, a MAT will be conducted on the initial blood sample and a second blood sample will be taken 4 weeks after the onset of the first signs and symptoms. This will allow the description of the serologic species and the comparison with historical data. Samples for MAT will be sent to CRVOI, La Reunion.

For all confirmed cases, DNA will be sequenced to allow the description of the molecular species responsible of human leptospirosis in Seychelles in comparison to the species identified in *Rattus spp*. and in Bats.

Leptospirosis Case definitions

Suspected case: Anybody aged 13 years and above reporting to a health center (private or governmental) during the 12 month period presenting with fever of ≥ 38º C for more than 3 days with or without any of the following signs and symptoms; headaches, myalgia, hemorrhagic manifestations in the absence of any definite diagnosis.

Probable case: Anybody meeting the suspected case definition criteria with a Positive IgM or with a positive IHA assay for *Leptospira* in blood.

Confirmed case: Anybody meeting the suspected case definition criteria with a positive RT-PCR assay for *Leptospira* in urine or blood and/or a positive MAT 4 week after the onset of symptoms.

For minors less than 18 years old, the accompanying guardian or parent will be asked to give consent and to provide the relevant information.

Exclusion criteria

A person will be excluded as a case if he/she is:

- Unwilling to participate in the interview and biological examinations

Control definition:

A control will be a male or female aged 13 years and above and residing in Seychelles, selected from the National Census Database.

Inclusion criteria

The person who has been randomly selected as per control definition and who has not been ill from an infectious disease for the past 8 weeks and with a negative IgM for *Leptospira*.

For each eligible case, 2 eligible controls will be randomly selected using random tables within the 10 days that follow and matched to the case according to the same sex and age and this will be repeated in cases of drop outs. Pair matching of sex and age for controls has been reported in various studies as variables well known to strongly correlate to leptospirosis and such will minimize the confounding effect.

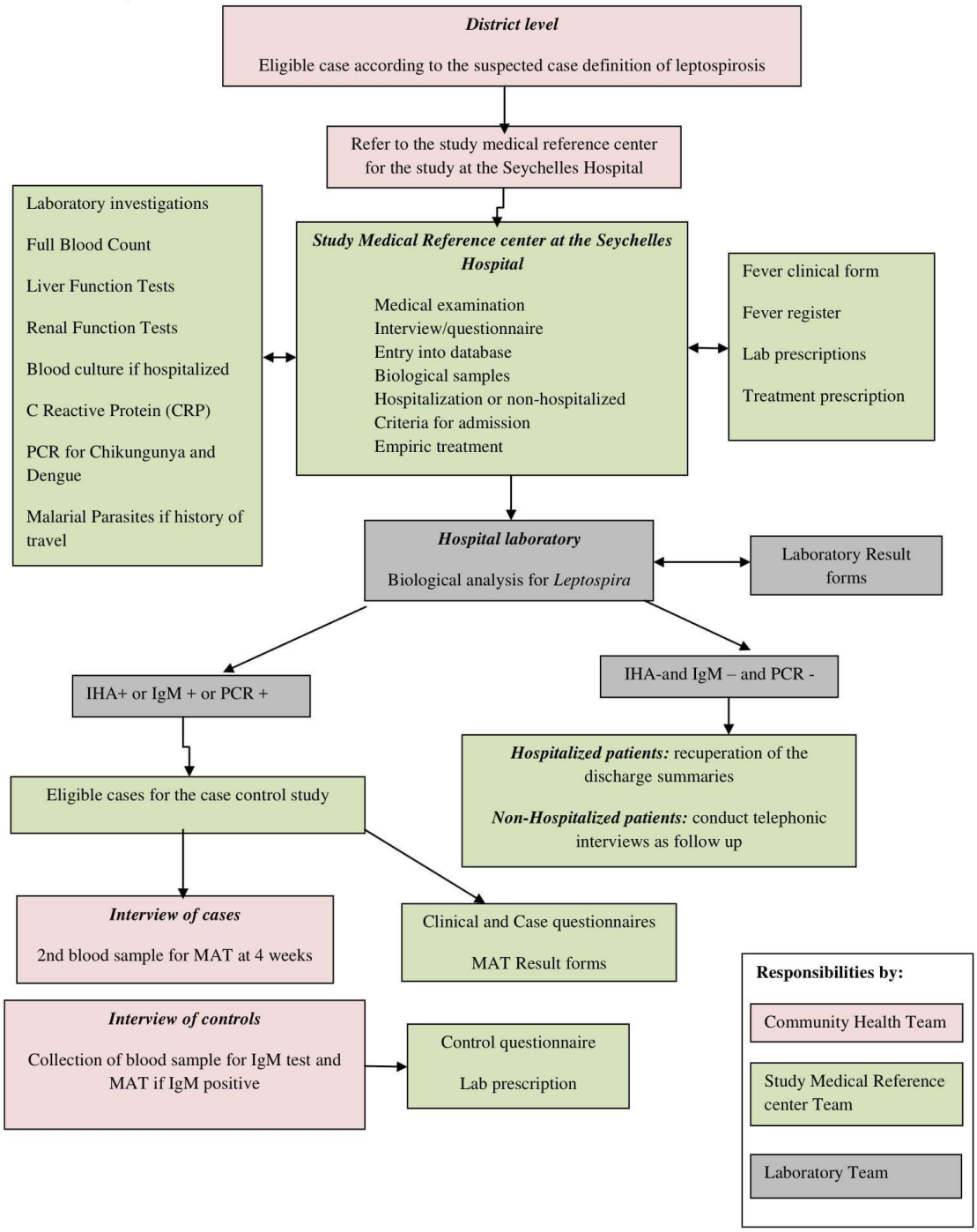
Age matching will be done within the range of plus or minus five years. However, if the first control does not meet the inclusion criteria, the second and a third one will be selected accordingly.

Exclusion criteria

However controls will be excluded if:

- the person has been ill from an infectious disease for the past 8 weeks and with a positive IgM for *Leptospira*
- the person is unwilling to participate in the interview and biological examinations

Study Flow Chart



Roles and responsibilities

Community Health Team
(nurses, doctors and public health officers)

- Identification of cases of fever of unknown origin concordant with the suspected case definition of leptospirosis
- Refer all patients with fever of unknown origin to the study medical reference center
- Perform blood sampling for 2nd MAT at 4 weeks for all leptospirosis confirmed cases
- Perform interviews for leptospirosis cases and controls
- Perform blood sample collection for controls at home

Study Medical Reference center team
(nurses and doctors)

Perform the patients' examinations, prescribe the biological examinations, prescribe the treatment and decide if hospitalization or not as per clinical guidelines, collect the written consent of the patient, complete the clinical and case investigation forms and collect the discharge summaries for hospitalized patients upon discharge from hospital and formally enroll eligible patients in the study.

Laboratory team

Perform the biological analyses for all fever of unknown origin cases and for all leptospirosis cases except for MAT; perform IgM detection for control; assure the conservation of biological samples in goods conditions for the initial MAT sample and the second sample 4 weeks after. MAT analyses will be done in La Reunion.

Study Team

Train the nurses, doctors and public health officers, supervise the district teams and the lab team; collect the leptospirosis biological test results, inform and include the leptospirosis cases in the case control study, select randomly two matched controls per case, inform the district level of the environmental investigations and interviews that they have to conduct for both cases and control. Check the completeness of data at each stage and at the end of the patient course.

Leptospirosis Diagnostic criteria

Upon arrival to the reference center, a rapid Ig M (LeptoCheck WB) will be conducted and molecular detections of leptospira DNA (RT-PCR)(18, 19) will be carried out from blood and/or urine. Routine leptospirosis IHA tests will also be done on all samples.

Acute fever specimen laboratory process

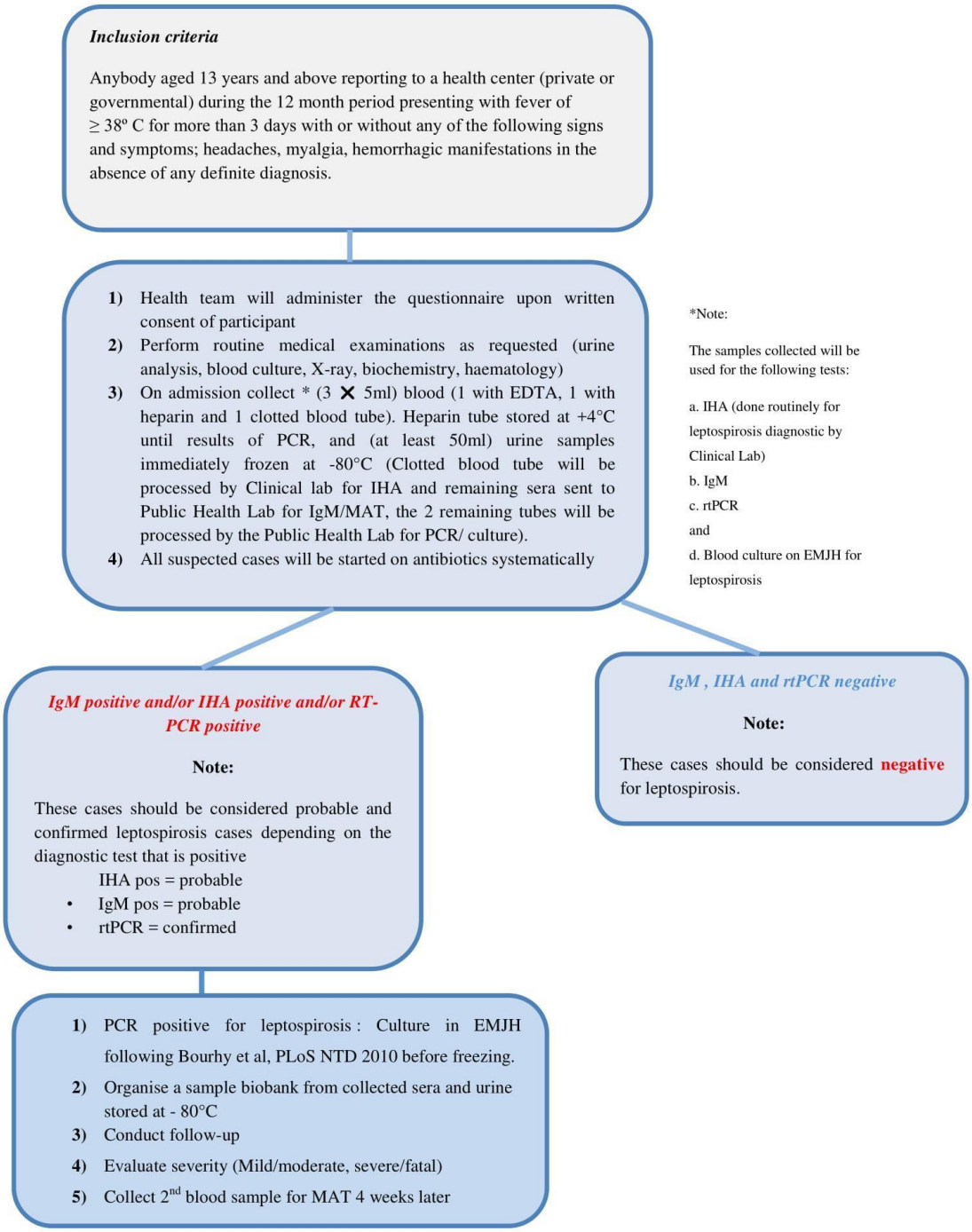
EDTA blood tubes will be stored at +4°C until *Leptospira* PCR. Total nucleic acids will be prepared using QIACube robot, immediately stored at -80°C with 10µl aliquots used for Reverse Transcription. A RT PCR will be performed as previously described (12) with cDNAs together with IHA and IgM with all sera.

- Leptospirosis positive specimens (either PCR, IHA or IgM): PCR positive sera will be used for culture of *Leptospira* as previously described (17) and remaining PCR positive sera together with PCR negative sera will then be frozen at -80°C.

- Leptospirosis negative specimens (sera, total nucleic acids, cDNAs and urine) will be stored at -80°C for subsequent investigations.

(Refer to Annex 5)

Diagnostic Scheme



Sample size calculation

The sample size calculations are based on the matched case-control study and the specific objective of knowing the determinants of leptospirosis in Seychelles.

The sample size for a matched case control study of 2 controls per case with a power of 80% and a two sided alpha risk of 0.05% (95% CI). An OR of 2.5 with a 20% exposure in controls suggests an estimated sample size of 165 participants (55 probable or confirmed cases and 110 controls).

Ethical considerations

Each potential participant (cases and controls) will be first provided with a detailed explanation of the purpose of the study, commitment and potential benefits involved in participating in the study and will be given an assurance of confidentiality (coding for laboratory specimens) followed by an informed and written consent from the prospective participant and the parent/guardian in the less than 18 years old, *(refer to Annex 6)*. Each prospective participant and parent/guardian will be given an opportunity to ask questions prior to recruitment and during the study and will be provided contact details of the principal and co-investigator should he /she wishes to contact during the study. Each potential participant will be free to decline participation and/or refuse to answer any specific questions.

Timeline / Planning

The study protocol will be submitted to the Medical Ethics Committee of Seychelles on the 30th May 2014 and once given the final approval; the study will proceed as planned from August 2014 to August 2015.
(Refer to Annex 8).

Data Management

Data collectors/interviewers and training

A maximum number of nurses and doctors from the wards and community health centers will be trained as interviewers and public health officers and laboratory technicians will provide the relevant data and results.

A training week will be organized in June 2014 and will have the following general objectives:

- to give an overview of the current global and local leptospirosis situation
- be made familiar with the study to be conducted, procedures, design and questionnaire
- develop specific and general qualities as data collectors/interviewers in communication skills and professional ethics
- pre-test the questionnaire

The objective of the training is for the participants to acquire a comprehensive knowledge of the entire study process.

Data collection instruments

An interviewer-administered questionnaire in both Creole and/or English will be used to collect clinical, socio-demographic, present and past medical history, educational, professional, occupational, environmental and behavioural variables data of the individual. The questionnaire will be administered by trained medical personnel (trained doctors and nurses) as designed to characterize the clinical illness and exposure history to eligible cases seen or admitted to Seychelles Hospital and to eligible controls at home upon consent. Variables included in the study questionnaire were identified through extensive literature review and formal discussion with epidemiologists, clinicians and laboratory technologists involved in leptospirosis disease prevention, control and management.

A unique identification number will be given to each case and control for laboratory investigations. A specific colour coded laboratory form with all biological investigations required will be used throughout the study period of which a completed copy with results will be attached to the completed questionnaire prior to being sent to the DSRU *(refer to Annex 9)*.

Pre-testing of questionnaire

Pre-testing of questionnaire will be done during the training and during the study pilot phase prior to the main study in order to:

- determine the time length of the interview
- improve on the wording of the questions to ensure understandability
- eliminate any unnecessary questions and potentially add new questions
- test question sequence
- correct and improve translation
- check adequacy of questionnaire instructions
- identify interviewers coding difficulties and reporting

Quality control assurance measures

The quality assurance measures will be directed at controlling bias, the interview technique, and the preparation for the study, the conduct of the study and finally the plausibility of the database. Constant and close supervision of data collection by the principal investigator and co-investigator will ensure the quality of data. The principal investigator and co-investigator will visit each ward 2-3 times a week for direct supervision and spot-checking.

After each interview, the interviewer will go over the questionnaire and check for consistency, accuracy and completeness of data. Completed questionnaires alongside the signed consent and laboratory results attached will be sent to the DSRU in a sealed envelope. Once at the DSRU, the questionnaire will be viewed for any possible inconsistent and inaccurate data prior to double data entry into SPSS and analysis by STATA.

Analysis Plan

Data processing and analysis

Upon interview, all variables and open-ended questions will be coded manually on a paper-based questionnaire by the interviewer. The final data will be then double entered by the data entry personnel using SPSS and analyzed by using STATA 12.1.

Any missing values will be kept but not replaced. Statistics will be computed for all variables for both cases and controls. Categorical variables will be summarized using percentages and compared using the Chi-square test or Fisher's exact test. Continuous variables will be summarized using means and standard deviation (SD) and compared using Student's t-test or the Mann-Whitney test as appropriate.

Univariate Analysis will be done to measure the association of the different exposures and leptospirosis. Crude matched odds ratio will be calculated to assess the association between each exposure and leptospirosis. The statistical significance of the association between the exposures and leptospirosis will be assessed using the p-value and the 95% confidence interval.

Conditional Multivariate Analysis will be done to identify the associations between the outcome and different exposures and to identify confounding factors.

Conditional logistic regression will be used to identify factors associated with leptospirosis and to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between exposure variables and leptospirosis cases. Variables with p values ≤ 0.20, variables that are well documented in the literature, confounders and effect modifiers will be introduced in the multivariate logistic regression model. A stepwise backward approach will be used to remove non-significant variables, only variables with an OR > 2 even if not significant and p values < 0.5 will be retained in the final model.

The main results of the study will then be compared to that of the previous study, which was conducted in Seychelles in 1995-1996.

Confounding factors

Confounding occurs when an observed association is due to three factors: the exposure, the outcome of interest, and a third factor which is independently associated with both the outcome of interest and the exposure. Socio- demographic and environmental factors in the study can be potential confounding factors such as people who does gardening or walk barefoot are positive risks to acquiring leptospirosis; hence this is why the study will be restricted to only leptospirosis confirmed cases. Pair matching of sex and age for controls will minimize the confounding effect.

Effect modifiers

Effect modification occurs when the effect of a factor is different for different groups and such giving different odds ratio such as males being more predominantly affected by leptospirosis or people engaged in outdoor or gardening activities are most at risk**.**

Privacy, Data Storage & Confidentiality

All data collected, questionnaires and results will be held in the strictest confidence at all levels. The principal investigator and co-investigator will be the only ones to have access to all the datasets and paper based questionnaires. Electronic data sets will be stored in a secure, password protected computer and paper based questionnaires will be locked at the DSRU.

Dissemination and reporting of results

Results of the study will be published in early 2016 and disseminated to relevant stakeholders and collaborators and to participants.

Expected outcomes of the study

The present situation of leptospirosis in Seychelles remains a major public health concern. With the results of the study, it is expected to assess the real burden of leptospirosis in Seychelles, to improve the diagnostic capacity in Seychelles. It will enable us to better understand the epidemiology of this infectious disease and constitute a critical step for designing interventions and consequently diminishing the risk of leptospirosis transmission in Seychelles.

Competing interests

The authors declare that they have no competing interests.

Funding and sponsors

The study will be funded by the Ministry of Health of Seychelles and co-funded by the Réseau SEGA-COI and CRVOI- La Reunion.

Laboratory diagnosis will be provided by the Ministry of Health of Seychelles and CRVOI-La Reunion.

Authors' contributions

Principal responsibility for the study design, conduct, analysis, interpretation and manuscript preparation was and will be assumed by Mrs. Jeanine Faure and Dr Jastin Bibi of the DSRU, from the Ministry of Health of Seychelles.

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Annexes

Annex 1: Acute Fever Clinical Questionnaire (English version)

Annex 2: Acute Fever Clinical Questionnaire (Creole version)

Annex 3: Leptospirosis Questionnaire (English version)

Annex 4: Leptospirosis Questionnaire (Creole version)

Annex 5: Leptospirosis Specimen Process

Annex 6: Consent Form (English version)

Annex 7: Consent Form (Creole version)

Annex 8: Timeline and Planning

Annex 9: Laboratory Results Form

Annex 1

Acute Fever Clinical Questionnaire

1. Unique identification number: Case No /Sex /2014 /2015
2. Date of investigation: ___/___/___
3. Investigator: .....
4. Respondent: Patient ☐ Concubine ☐ Relative ☐ Others……

Socio-Demographic Data

5. First name: …… 6. Surname: ……
7. Gender: Male Female
8. Date of birth: ___/___/___ 9. Age: ----
10. NIN: ___/___/___/___/___ or Passport No:

11. Marital status: Single Married Concubine Widowed Separated
12. Origin: Seychellois Expatriate If yes, specify from which country…….
13. Telephone number: (mobile) ---------- (home) ----------

14. Profession: .....
15. Education level: Primary Secondary Post-secondary University
    Never been to school others ……..

16. Address: …… 17. District: ……

18. GPS X: ---------- 19. GPS Y: ----------

Clinical Data

20. Hospitalization: No Yes If yes, Ward: ……

21. Date of hospitalization: ___/___/___ 22. Date of discharge: ___/___/___

23. Past Medical History

Diabetes:	No	Yes	Don't know
Hypertension:	No	Yes	Don't know
Cardiac Disease:	No	Yes	Don't know
Leptospirosis :	No	Yes	Don't know
Liver problems:	No	Yes	Don't know
Kidney problems:	No	Yes	Don't know

Others: ……

24. Date of onset of first symptoms: ___/___/___

25. Clinical Manifestations: (symptoms preceding admission 2 weeks)

Fever:	No	Yes	Don't know
Low backache:	No	Yes	Don't know
Haematemesis:	No	Yes	Don't know
Melaena:	No	Yes	Don't know
Haemoptysis:	No	Yes	Don't know
Myalgia and/or arthralgia:	No	Yes	Don't know
Haematuria:	No	Yes	Don't know
Oliguria:	No	Yes	Don't know
Abdominal pain:	No	Yes	Don't know
Jaundice:	No	Yes	Don't know
Headaches:	No	Yes	Don't know
Conjunctival suffusion:	No	Yes	Don't know
Meningism:	No	Yes	Don't know

Others: ……

26. Have you visited any medical doctor during the past 2 weeks? No Yes if No, skip → 31

27. Were you given any treatment: No Yes

28. Antibiotic treatment: No Yes

29. If yes, specify the name of the antibiotic: ……

30. Date started antibiotics: ___/___/___ 31. Duration of treatment: ……

31. Have you travelled to any foreign countries during the past month? No Yes
if No, skip → 34

32. If yes, which country …….. 33. Date of arrival: ___/___/___

Comments: ……...

Biological Data

Attention: Please attach a copy of the lab results form to the investigation form

34. Patient's unique ID: ___/___/___

35. Date of blood collection: ___/___/___ 36. Date of urine collection: ___/___/___

37. *Refer to Annex 4 to insert results of laboratory tests.*

Blood Group: _____

Urine: protein. (stick):_____ blood (stick):_____ nitrate (stick):_____

Stools: parasites:_____ Occult Blood_____

PCR (blood) Positive Negative Ongoing not done

PCR (urine) Positive Negative Ongoing not done

MAT Positive Negative Ongoing not done

ELISA Positive Negative Ongoing not done

1st serology (IHA TEST KIT) date: ___/___/___

Result:_____

2nd serology(IHA TEST KIT) date: ___/___/___

Result:_____

1st serology lepto (overseas): date: ___/___/___

Result: _____

2nd serology lepto (overseas): date: ___/___/___

Result: _____

1st serology Hepatitis A date: ___/___/___

Result:_____

2nd serology Hepatitis A date: ___/___/___

Result:_____

1st serology Hepatitis B date: ___/___/___

Result:_____

2nd serology Hepatitis B date: ___/___/___

Result:_____

1st serology Hepatitis C date: ___/___/___

Result:_____

2nd serology Hepatitis C date: ___/___/___

Result:_____

ECG

(description):_____

CXR

(description):_____

38. **Antibiotic treatment**

Penicillin: dosage: _____ Date started: ___/___/___ Duration in days:_____

Tetracycline : dosage:_____ Date started: ___/___/___ Duration in days:_____

Erythromycin : dosage:_____ Date started: ___/___/___ Duration in days:_____

Other drugs:_____

39. **Follow-up**

7-15 days after discharge: comments:

_____

28-45 days after discharge: comments:

_____

41. Evolution: RECOVERY SEQUELAES DEATH

42. If death: Date of death ___/___/__

43. Post mortem results:..…….....

Other comments:

……..…

Annex 2

Kestyonner klinik pou lafyev

Kod idantifyan inik: Ward /Nimero ka /Sex /2014 /2015

Dat interview: ___/___/___ Anketer :.....

Repondan: Pasyan Galan Fanmiy Lezot……

Detay sosyo-demografik

Non: …… Sinyatir: ……..

Sex: Zonm Fanm

Dat nesans: ___/___/___ Laz: ----

NIN: ___/___/___/___/___ oubyen Nimero passpor:

Pe viv: Tousel Marye Menaz Separe Vev

Lorizin: Seselwa Etranze Si wi, spesifye ki pei …….

Nimero telefonn: (mobile) ---------- (lakour) ----------

Profesyon: .....

Nivo ledikasyon: Primer Sekonder Post sekonder Liniversite

Zanmen al lekol Lezot …….

Ladres: …… Distrikt: ……

GPS X: ---------- GPS Y: ----------

Detay Klinik

Reste lopital: Non Wi Si wi, ki ward ……..

Dat ki oun antre lopital: ___/___/___ Dat ki oun sorti lopital: ___/___/___

Eski ou annan/oubyen deza ganny:

Dyabet : Non Wi Pa konnen

Tansyon : Non Wi Pa konnen

Malad leker : Non Wi Pa konnen

Leptospiroz : Non Wi Pa konnen

Problenm lefwa : Non Wi Pa konnen

Problenm ronyon : Non Wi Pa konnen

Lezot …….

Dat premye zour oun komans malad: ___/___/___

Problenm ki oun gannyen: (avan 2 semenn pase)

Lafyev : Non Wi Pa konnen

Leren fermal: Non Wi Pa konnen

Vomi disan: Non Wi Pa konnen

Al salel ek disan: Non Wi Pa konnen

Kras disan : Non Wi Pa konnen

Zwen ek/oubyen misk fermal : Non Wi Pa konnen

Pis disan : Non Wi Pa konnen

Pis pti ginn : Non Wi Pa konnen

Vant fermal : Non Wi Pa konnen

Lazonis : Non Wi Pa konnen

Latet fermal : Non Wi Pa konnen

Likou red : Non Wi Pa konnen

Conjunctival suffision : Non Wi Pa konnen

Lezot……

Eski oun war en dokter sa 2 semenn pase? Non Wi

Eski ou ti ganny okenn latizann: Non Wi

Antibyotik: Non Wi

Si wi, lekel sa antibyotik: ……

Eski oun voyaz dan okenn pei sa dernyen mwan? Non Wi

Si wi, ki pei…….. Dat ki ou ti retournen: ___/___/___

Komanter: …….

Detay biolozik

Atansyon: Silvouple atas en kopi bann rezilta biolozik avek sa form

Nimero idantifyan inik sa dimoun: ----/-----/-----

Dat ki oun kolekte disan: ___/___/___ Dat ki oun kolekte lirin: ___/___/___

Refer kot Annex 4 pou fer antre rezilta.

Blood Group: _____

Urine: protein.(stick):_____ blood(stick):_____ nitrate (stick):_____

Stools: parasites:_____ Occult Blood_____

PCR (blood) Positive Negative Ongoing not done

PCR (urine) Positive Negative Ongoing not done

MAT Positive Negative Ongoing not done

ELISA Positive Negative Ongoing not done

1st serology (IHA TEST KIT) date: ___/___/___

Result:_____

2nd serology(IHA TEST KIT) date: ___/___/___

Result:_____

1st serology lepto (overseas): date: ___/___/___

Result:_____

2nd serology lepto (overseas): date: ___/___/___

Result:_____

1st serology Hepatitis A date: ___/___/___

Result:_____

2nd serology Hepatitis A date: ___/___/___

Result:_____

1st serology Hepatitis B date: ___/___/___

Result:_____

2nd serology Hepatitis B date: ___/___/___

Result:_____

1st serology Hepatitis C date: ___/___/___

Result:_____

2nd serology Hepatitis C date: ___/___/___

Result:_____

ECG

(description):_____

CXR

(description):_____

Tretman antibyotik

Penicillin: Doz: _____ Konbyen zour :_____

Tetracycline : Doz:_____ Konbyen zour:_____

Erythromycin : Doz:_____ Konbyen zour:_____

Lot latizann:_____

Swivi

7-15 zour apre desarz:

komanter:_____

28-45 zour apre desarz:

komanter:_____

Evolisyon : BYEN ☐ SEQUELAES ☐ LANMOR ☐

Si lanmor: Ki dat ___/___/___

Rezilta letopsi: …….....

Lezot komanter:_____

Annex 3

Leptospirosis Questionnaire

Environment Data

Unique identification number: Case No /Sex /2014 /2015

44. Housing facility: House Flat Elderly home Homeless

45. Number of rooms excluding toilet and kitchen:

46. Separated kitchen: No Yes

47. Number of people living in the same house for the past 4 weeks:

Adults Children

48. Type of habitat: Concrete Wood Corrugated iron sheets Straw Others

49. Type of floor inside: Earth Concrete Wood Tiles

50. Type of ground outside house: Earth Concrete Grass

51. Type of sewerage system: Septic tank Gutters River Others ……

52. Type of waste disposal: Closed bins (home) Open bins Public bins

Trench River Others …

53. Water supply: Treated River Well Others ……..

54. Presence of rats around: No Yes if No, skip → 55

If yes, is it more during: Daytime At night

55. Presence of animals/pets:

Dogs Home Garden Close by

Cats Home Garden Close by

Poultry Home Garden Close by

Cattle Home Garden Close by

Others: …….

56. Occasional flooding: No Yes

Activity Data

57. Walking in the house: bare-foot with slippers with shoes don't know
58. Significant other (spouse) walking at home: bare-foot with slippers with shoes don't know
59. Walking outside the house: bare-foot with slippers with shoes don't know
60. Significant other walking outside of the house: bare-foot with slippers with shoes don't know
61. Where did you swim in the past 4 weeks? Sea River Lakes
62. Stream nearby: No Yes
63. Washing clothes in stream: No Yes
64. Bathe in stream: No Yes
65. Fishing in streams: No Yes
66. Do you do gardening? No Yes

If yes, is it professional leisure/home garden

67. Do you use untreated water for washing yourself, No Yes
68. For washing your utensils? No Yes
69. Have you had any cuts/ abraisons on hands, feet or other body parts in the last 4 weeks?

No Yes if yes, Foot or Leg Hand or Arm others…….

72. Alcohol consumption: Regularly Occasionally Non-drinker
73. Do you consume? Beer Spirits Baka Kalu
74. Baka: home made outside home produced by ……. number of bottles/week:
75. Kalu: self-collected bought produced by ……. number of bottles/week:
76. Lapire: home made outside home produced by…….. number of bottles/week:
77. Have you been working in the fields during the last 4 weeks? No Yes
78. In forests/woods during last 4 weeks? No Yes
79. Have you been working in your garden during the last 4 weeks: No Yes
80. Working in a building during the last 4 weeks: No Yes
81. Have you had any rat's bite? No Yes
82. Other animal's bite? No Yes

if yes, which one.....

83. Do you know of somebody having leptospirosis in your household? No Yes
84. In your neighbourhood? No Yes

if yes, at what distance is the house(s)……...

Annex 4

Kestyonner leptospiroz

Nimero idantik inik: Ward /Nimero ka /Sex /2014 /2015

Detay Lanvironman

Fasilite reste: Lakaz Flat Lakaz bann dimoun aze Napa landrwa reste

Kantite lasanm apard kabinen ek lakwizin: Lakwizin separe: Non Wi

Konbyen dimoun ki reste dan sa lakaz sa dernyen 4 semenn: Adilt Zanfan

Kalite lakaz: Blok Dibwa Tol Lapay lezot ……

Ater dan lakaz : Later Beton Dibwa Tiles

Kalite zarden: Later Beton Zerb

Kalite desarz : Pi perdi Rigol Larivyer lezot ……

Kote ou zet salte: Bin ferme (dan lakour) Bin ouver Bin piblik Transe Larivyer lezot …

Ki kalite delo ou servi: Trete Larivyer Pwi lezot ……..

Eski oun war lera dan zalantour: Non Wi

Si wi, eski I plis: Dan lazournen Aswar

Eski I annan delo monte tanzantan: Non Wi

Detay aktivite ek lwazir

Eski ou mars dan lakaz: Nipye Dan savat Dan soulye Pa konnen

Okenn lezot dimoun (partner) I mars andan : Nipye Dan savat Dan soulye Pa konnen

Mars deor: Nipye Dan savat Dan soulye Pa konnen

Okenn lezot dimoun I mars: Nipye Dan savat Dan soulye Pa konnen

Kote oun naze pandan sa dernyen 15 zour? Delo sale Larivyer Lak

Eski I annan en larivyer ki pre? Non Wi

Eski ou lav lenz dan larivyer: Non Wi

Eski ou benny dan larivyer: Non Wi

Eski ou lapes dan larivyer: Non Wi

Eski ou plante? Non Wi

Si wi, eski I: profesyonnel en pastan/zarden kot lakour

Eski ou servi delo ki pa trete pou benyen ? Non Wi

Eski ou servi delo ki pa trete pou lav zafer? Non Wi

Eski oun ganny okenn koupe/krose lo ou lanmen/lipye ou okenn lezot parti ou lekor pandan sa dernyen 15 zour? Non Wi

Si wi, lipye/lazanm lanmen/lebra Lezot…….

Eski ou servi lalkol? Regilyerman Okazyonelman Pa bwar

Eski ou bwar? Labyer Spirits Baka Kalu

Baka: fer dan lakour andeor lakour prodwir par ….Kantite boutey par semenn:

Kalu: fer dan lakour aste prodwir par … Kantite boutey par semenn:

Lapire: fer dan lakour andeor lakour prodwir par … Kantite boutey par semenn:

Eski oun travay dan bwa oubyen dan lapay sa dernyen 4 semenn? Non Wi

Eski oun travay dan zarden sa dernyen 4 semenn? Non Wi

Eski oun travay dan en batiman sa dernyen 4 semenn? Non Wi

Eski oun ganny morde avek lera? Non Wi

Okenn lezot zannimo? Non Wi

si wi, lekel.....

Eski ou konn okenn dimoun kinn ganny leptospiroz dan ou lakour? Non Wi

dan ou lantouraz ? Non Wi

si wi, ki zistans lakaz I ete……..

Annex 5

Leptospirosis Specimen Process

Diagnostic test	Specimens	When to collect	How to prepare, store and ship	Results
Positive serology: 1. A seroconversion demonstrated by serologic test using Indirect Haemagglutination Assay (IHA) (acute and convalescent phases tested) (by Focus Diagnostics). 2. An IgM positive (by LeptoCheck WB) immunochromatographic test Positive PCR Samples that are positive for PCR (tested following either (18) or (19), and will be used for cultures as previously described (17), whereas samples that are positive by serology will have the acute and convalescent sample tested by MAT	-Whole Blood -Serum -Urine	Collect from a suspected case of leptospirosis who meets the case definition and who has consented to be enrolled on the study. Collect 3 blood specimens per patient in acute phase (i.e. < 5days post-onset of symptoms). Specimens should be 5ml in volume (1 in EDTA tube, 1 in heparin tube and the other in normal blood clot bottle) Collect 2 blood specimens per patient in convalescent phase (i.e. 4 weeks post-onset of symptoms or 3 weeks after the 1st specimen). Both blood specimens should be 5ml in volume (1 in EDTA tube and the other in normal blood clot bottle) Collect up to 50ml of urine in a clean sterile container (provided by the medical practitioner) on recruitment on the study and freeze at -80°C immediately	Collect a minimum of 15 ml of venous blood distributed as 5 ml portions in an EDTA bottle for PCR, a heparinised blood bottle for culture and in a blood clot bottle for IHA,IgM and MAT. Urine samples will be immediately frozen at -80°C upon arrival In the event of delays store blood samples at 4 °C. It is important to note that Leptospira cultures require fresh sera samples (17). Sera will thus be stored at 4°C until PCR results. Culture procedures thus require/: -a PCR within 24-48h -a culture of PCR+ sera -the subsequent freezing of remaining sera and PCR negative samples at -80°C. For outlying islands ship sample using appropriate shipping package to prevent breaking or leaking and maintain cold storage during shipment.	Serological results will be available routinely after maximum a week for IHA and IgM tests. PCR results will also be available on a weekly basis MAT results will be available at the end of the study

Annex 6

EXPLANATION AND CONSENT FORM FOR THE STUDY OF THE EPIDEMIOLOGY OF ACUTE LEPTOSPIROSIS AMONGST FEVERS OF UNKNOWN ORIGIN IN SEYCHELLES

The Ministry of Health of Seychelles will be conducting a study on Leptospirosis and other causes of fever of unknown origin in the Seychelles over a year period from August 2014 to August 2015. I will now explain to you about the study process before you decide to participate. You will be free to decline your participation in the study.

Objectives of the study

As fever of unknown origin including leptospirosis are of major public health importance to Seychelles, the main objective of the study is to determine the respective burden and severity and to describe possible environmental and behavioral associated risk factors.

What will happen to me if I take part?

You will first be asked to give a written consent to take part in the study. Consent means you understand all parts of the study that has been explained to you and you can make a decision to participate. Once you have given the consent:

- The doctor or nurse will have an interview with you that might last about 20 minutes. Questions to be asked will be that of your personal details and activities of daily living.
- After the interview, you will be asked to give some urine, some blood will be taken from your arm for various blood tests including leptospirosis, hepatitis A, B and C ect.. Some tests will be done locally and some will be sent overseas for specific tests.
- If you are tested positive for leptospirosis, a second questionnaire will be administered at home and few other blood tests will be repeated 4 weeks after you had fallen sick.
- To participate in the study you must be ready to do the interview and give samples; if you do not wish to do so you will not be eligible to participate.

Will there be any risks if I take part in the study?

There are very few possible risks such as:

- it may hurt a little bit to give your blood and this may cause bruising
- some questions we will ask you can be personal and you may feel uncomfortable.

The staffs are trained to keep your information private and to make it safe for you to answer these questions.

What will be the benefits if I take part in the study?

If you take part in the study you will be screened for other infections other than leptospirosis. You will receive your results of the tests conducted and treatment will be given to you accordingly and follow up visits will be conducted upon your discharge from hospital.

What will happen to the results of the study?

The results of the study will be written in a report by the Ministry of Health and may be published in a journal. The results will help in better planning in designing effective and sustained prevention control programs to control the spread of leptospirosis in Seychelles.

If you have any problems or further questions regarding the study, do not hesitate to call:

Mrs Jeanine Faure or Dr Jastin Bibi; Telephone 4388012 or 4388082, Ministry of Health

I …….. hereby consent to participate in the leptospirosis study which is being conducted in the Seychelles of which has been clearly explained to me.

……..	……..
Signature or thumb stamp of the patient	**Signature of the interviewer**
Date: ___/___/___	**Date:** ___/___/___

Annex 7

LESPLIKASYON EK FORM KONSANTMAN POU LETID LO SITIASYON EPIDEMIOLOZIK LEPTOSPIROZ PARMI BANN KAS LAFYEV ENDETERMINEN SESEL

Minister Lasante pe al fer en letid lo leptospiroz e bann lezot lakoz lafvey endeterminen pandan enn an depi out 2014 ziska lafen out 2015.Mon pou eksplik ou bann diferan letap sa letid avan ki ou deside si ou pou partisipe. Ou lib pou refize partisipe.

Lobzektif sa letid

Etan ki leptospiroz e lafyev endeterminen I en problem mazer pou lasante piblik isi Sesel, sa letid pou ede determin lanpler e gravite sa maladi ek bann fakter konportmantal e anvironmantal ki kapab asosye avek.

Ki pou arive avek mwan si mon partisip dan sa letid ?

Premyerman ou pou bezwen donn ou konsantman an ekri, ki savedir oun konpran tou bann leksplikasyon ki noun donn ou e ou pare pou partisipe.
De ki oun donn ou konsantman :

- Ners oubyen dokter pou fer en interview avek ou pou apepre 20 minit.
  Kestyon pou enpe lo detay personel ek bann aktivite toulezour.
- Apre interview ou pou ganny demande pou donn enpe lirin e apre tir enpe disan kot ou lebra. Pou annan tes ki pou ganny fer lokalman e apre enternasyonalman.
- Si ou ganny teste positiv pou leptospiroz, en dezyenm kestyonner pou ganny administre e lezot tes disan pou osi ganny repete 4 semenn apre ki oun tonm malad.
- Pou partisip dan sa letid, ou pou bezwen pare pou donn en interview e apre fer tes lirin ek disan , sinon si ou pa pare ou pa pou kapab partisipe.

Eski I annan okenn risk si mon partisip dan sa letid?

I annan enn de risk tel ki:

- I kapab fer mal enpe ler ou pe tir disan e ou lebra i kapab vinn ble osi
- Bann kestyon ki pou ganny demande I kapab personel e enpe anbarasan pou reponn

Bann travayer in ganny formen pou kit ou bann lenformasyon konfidansyel.

Ki bann benefis si mon partisip dan sa letid?

Si ou partisip dan sa letid, ou pou kapab fer lezot tes apard leptospiroz.

Ou pou ganny ou bann rezilta, tretman e swivi ler ou sorti lopital dapre ou problenm.

Ki pou arive avek rezilta sa letid ?

En rapor pou ganny ekri par minister lasante e i kapab osi ganny pibliye dan bann zournal enternasyonal.

Bann rezilta pou ede travay lo bann progranm prevansyon pli spesifik pou pli byen kapab kontrol sa lepidemi.

Si ou annan okenn kestyon ou problenm konsernan sa letid, pa ezite pou kontakte :

Madanm Jeanine Faure oubyen Dokter Jastin Bibi; Telephone 4388012 or 4388082,

Minister Lasante, Sesel.

Mwan ……mon donn mon kosantman pour mwan partisip dan sa letid lo leptospiroz ki pe ganny fer Sesel e kinn ganny byen eksplike.

…….	…….
Sinyatir partisipan	**Sinyatir anketer**
Dat: ___/___/___	**Dat: ___/___/___**

Annex 8

Timeline and Planning

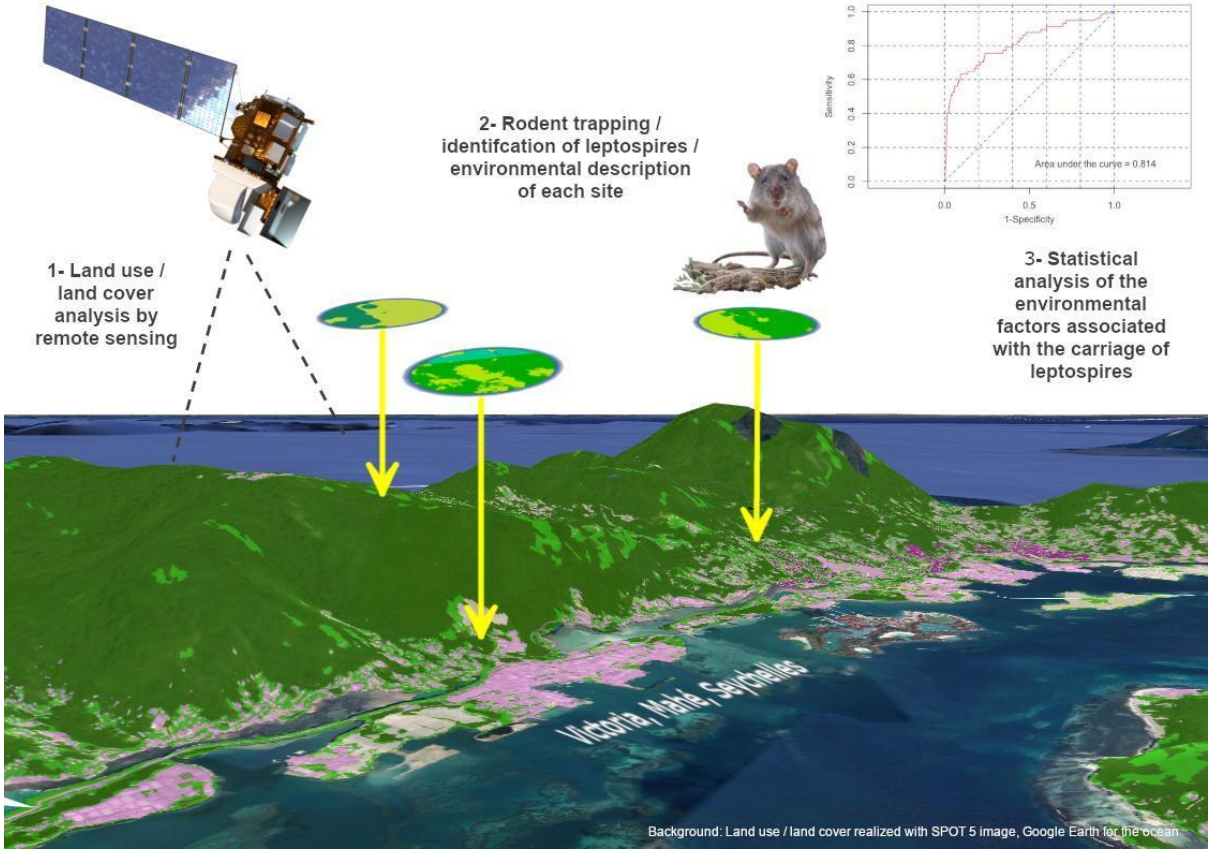
ACTIVITIES	DATES
Literature review	1st February 2014
Drafting of protocol	1st March to April 2014
Sharing of first draft	25th April 2014
Meeting with working committee	28th -30th April 2014
Sharing of second draft	2nd to 7th May 2014
Finalization of protocol	14th May 2014
Send by e-mail to Ethics committee	16th May 2014
Oral presentation to Ethics committee	29th May 2014
Amendments requested by Ethics committee	June 2014
Final validation by Ethics committee	June 2014
Training of study team	16 th 25th June 2014
Pre-testing of questionnaires	1st - 25th July 2014
Start study	1st August 2014
End study	31st July 2015
Data entry and analysis	1st August - 31st October 2015
Writing of study report	1st November – 15th December 2015
Validation of study report	21st December 2015
Dissemination of report	5th January 2015

Annex 9

Laboratory result form

	DATE	DATE	DATE	DATE	DATE	DATE	DATE	DATE	DATE
HB									
HT									
PLT									
ESR									
PT									
LEU									
NEU									
LYM									
EOS									
BASO									
NA+									
K+									
UREA									
CREAT									
ALK PHOS									
CK									
TOT BIL									
CONJ BIL									
UNCONJ BIL									
GOT									
GPT									
GT									

Annex 2: Graphical Abstract of Geospatial Analyses



Graphical abstract of article submitted to *Remote Sensing* based on geospatial analyses of *Leptospira* spp. in rats on Mahé island. © Herbreteau

Annex 3: Lettre D'Engagement de Non-Plagiat

UNIVERSITÉ DE LA RÉUNION

POLE RECHERCHE
Ecoles Doctorales

LETTRE D'ENGAGEMENT DE NON-PLAGIAT

Je, soussigné(e) ..... Leon BISCORNET .....,

en ma qualité de doctorant(e) de l'Université de La Réunion, déclare être conscient(e) que le plagiat est un acte délictueux passible de sanctions disciplinaires. Aussi, dans le respect de la propriété intellectuelle et du droit d'auteur, je m'engage à systématiquement citer mes sources, quelle qu'en soit la forme (textes, images, audiovisuel, internet), dans le cadre de la rédaction de ma thèse et de toute autre production scientifique, sachant que l'établissement est susceptible de soumettre le texte de ma thèse à un logiciel anti-plagiat.

Fait à ..... VICTORIA ....., le (date) ..... 17/09/2020 .....

Signature :

Extrait du Règlement intérieur de l'Université de La Réunion
(validé par le Conseil d'Administration en date du 11 décembre 2014)

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Les délits de contrefaçon, de plagiat et d'usage de faux peuvent donner lieu à une sanction disciplinaire indépendante de la mise en œuvre de poursuites pénales.

References

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