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GLOBAL WATER PATHOGEN PROJECT

**PART THREE. SPECIFIC EXCRETED PATHOGENS: ENVIRONMENTAL AND
EPIDEMIOLOGY ASPECTS**

LEPTOSPIRA AND LEPTOSPIROSIS

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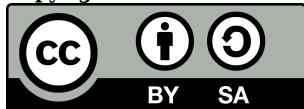
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Summary

Leptospirosis is an important and often ignored disease affecting more than one million people a year worldwide, from which 50,000 die. No serovar is known to be adapted to humans, so humans acquire the disease directly from animals (contact with urine or tissues) or indirectly (contact with urine contaminated water). The three main environments showing transmission risks are water-based, rodent-borne and livestock/pet-borne environments. Leptospirosis occurs worldwide but it is more frequently found in humid tropical climates, especially in regions with poor sanitary infrastructure, deficient drainage, and as a result large numbers of rats. The contact with natural bodies of water (wet farming) increases the risk of infection. Outbreaks tend to occur during rainy seasons and it has been associated with flooding. The disease has a broad geographical distribution due to the large spectrum of hosts including domestic and wild mammals. Leptospirosis affects risk groups that are exposed to animal reservoirs or urine contaminated environments and people in contact with natural bodies of water. Leptospirosis is regarded as the most widespread zoonosis.

Leptospirosis is caused by spirochetes of the genus *Leptospira* which comprises 20 species that are phylogenetically separated into 3 clusters: 9 pathogenic, 6 saprophytic, and 5 intermediate. The etiologic agents of this disease are pathogenic and intermediate species of *Leptospira* which comprise more than 260 serovars. Pathogenic and intermediate clusters have been reported to cause infection and in this text we will refer to them as parasitic *Leptospira*. The organism is a thin spirochete of 0.1 to 0.2 μm in diameter and 6 to 12 μm in length, so that it can be filtered through 0.45 μm filters. It is very motile showing translational motility, travelling approximately 20 μm in 2 to 3 seconds in ordinary media.

Leptospirosis is considered a neglected disease, and the spirochete causing this disease, *Leptospira* is difficult to recover in culture and only recently molecular genetics tools have been developed suitable for the study of this spirochete. These factors have contributed to a lag in the understanding of its virulence mechanisms, its immunogenic properties and finally a lag in the development of vaccines that may induce strong and durable protection.

To date, different methods for inactivation of leptospire for use as vaccines have been applied, these include formalin, phenol, ethanol, heat, freeze and thaw and radiation; and after 100 years the only licensed vaccines are whole leptospira inactivated bacterins. These have been used to immunize animals, mainly dogs, cattle and pigs. because of problems with components of the culture media and reactogenicity, such bacterins have not been widely accepted for use in humans, however human bacterin vaccines have been used successfully in several regions, including China, Japan, Cuba, and Europe. No live vaccines are currently licensed.

Studies of parasitic *Leptospira* in the environment are

very limited mainly because it is difficult to isolate these spirochetes from surface waters or soil without getting contamination with saprophytic *Leptospira*. However, genomic studies suggest that parasitic *Leptospira* evolved from an environmental (free living) *Leptospira* found in water or mud, similarly to the contemporary members of the saprophytic cluster (such as *L. biflexa*). There seems to be some evolutionary variation within parasitic leptospire; some species (such as *L. interrogans*) have retained genes associated to environmental survival whereas other parasitic leptospire (such as *L. borgpetersenii*) seem to have lost these genes. Leptospiral survival in water is bolstered by viscous material such as agar (and possibly biofilm). Some strains of parasitic *Leptospira* are able to produce biofilms when incubated in fresh water even under the absence of nutrients, furthermore *L. interrogans* (Pathogenic cluster) has been found in nature associated to biofilms formed by environmental bacteria. Some parasitic leptospira are able to produce biofilm when incubated in distilled water (low nutrient conditions) and seemed to detect nutrients such as fatty acids causing the dispersion (release) of leptospiral cells from the biofilm. It has been observed that leptospiral biofilm has a five to sixfold increase in antibiotic resistance in all the strains used. It is tempting to speculate that biofilms may protect pathogenic *Leptospira* against other toxic compounds in the environment. The role of biofilm in the transmission of *Leptospira* to susceptible hosts are still unclear.

Soil may also play a role in environmental survival; some researchers have shown that *Leptospira* survived in the wet soil on dry days and appeared in the surface water on rainy days, suggesting that soil could be a reservoir for leptospire in the environment. Even though leptospire are considered as fragile spirochetes, studies have shown that they can survive and even maintain their virulence despite unfavorable conditions such as cold and nutrient-poor acidic waters after up to 20 months.

There is no standard protocols to culture parasitic species of *Leptospira* from bodies of fresh water or soil. There are only few reports describing isolation of parasitic *Leptospira* from environmental samples and the reports show little success; the main difficulty in the isolation of these species is the overgrowth of environmental bacteria or the fast-growing saprophytic *Leptospira*. Molecular techniques are a lot more promising at detecting parasitic *Leptospira* in the environment. Many PCR protocols have been developed using mainly genes that are present only in pathogenic *Leptospira* such as the hemolysis-associated protein-1 gene, *lipL32*, or pathogenic specific sequences of genes such as *flaB*, *secY*, and 16S ribosomal gene. The main limitation of many of these analyses is that they fail to detect parasitic *Leptospira* of the intermediate cluster which are commonly found in water. Detection of genomic DNA sequences do not guaranty the viability of bacteria in water or other samples. RNA methods are under development to detect viable bacteria.

1.0 Epidemiology of the Disease and Pathogen(s)

Leptospirosis is a deadly bacterial infection caused by pathogenic members of the genus *Leptospira*. Human leptospirosis has long been recognized as an environmental disease, with documented reports dating at least a century ago (Noguchi, 1918). Today, it is clearly acknowledged that most human infections originate from environmental exposure to water and soils contaminated by the urine excreted by infected animals. Human exposure to contaminated water mostly occurs through bathing or swimming in natural freshwater bodies, but isolated contamination of water supplies has also been involved in outbreaks of leptospirosis (reviewed in Levett, 2001).

Leptospirosis is very challenging to diagnose clinically. Educating clinicians about considering leptospirosis when patients present with acute febrile syndromes, particularly when there has been recent exposure to freshwater, could greatly improve diagnosis. Although leptospires are considered to be very fragile bacteria, being highly susceptible to a number of physical and chemical agents (Faine et al., 1999), they present an astonishing capacity to survive in freshwater ecosystems for weeks to months. However, little is known about the fate of pathogenic leptospires in water, despite the importance of such knowledge towards preventing this potentially fatal bacterial infection in humans.

There are three main reasons for limited knowledge about the fate of leptospires in water. First, the genus *Leptospira* comprises both virulent species, which are the etiological agents of human and animal leptospirosis, and saprophytic species, which are non-virulent normal inhabitants of freshwater ecosystems. Second, the taxonomy of the genus has long been based on serology, which distinguished almost 300 serovars grouped into more than 23 serogroups, but only two species (*Leptospira interrogans sensu lato*, which grouped all virulent species, and *Leptospira biflexa sensu lato* for all saprophytic species until the 1980s). The two previous species grouping of the 1980s have since been declared invalid, based on more recent characterization using genetic molecular taxonomy, which to date has discriminated at least 35 species (Brenner et al., 1999; Ramadass et al., 1992; Yasuda et al., 1987; Thibeaux et al., 2018b; Thibeaux et al., 2018a). In addition, another group of *Leptospira* (named “intermediates”) has also been described (Schmid et al., 1986; Perolat et al., 1998), which currently incorporates 11 species with at least some displaying some degree of virulence. Early scientific reports did not always describe whether the leptospires studied had some virulence potential. The older literature therefore needs to be interpreted with caution to consider these taxonomical differences. Third, and finally, yet as importantly, pathogenic strains of *Leptospira* are slow-growing, and can easily become overgrown by saprophytic species and other microorganisms, even with selective culture medium. Still, the body of literature published over the last century is proving useful in building some knowledge about the occurrence, persistence and survival of leptospires in water. Putting this knowledge together can help improve prevention of this significant, possibly

reemerging, environmental disease.

1.1 Global Burden of Disease

1.1.1 Global distribution

One of the first descriptions of the disease now known as leptospirosis was reported by Larrey in 1812, during a blockade of Napoleon’s troops in Cairo; and more than a century ago Adolph Weil described a severe form of the disease, since known as Weil’s disease. Leptospirosis is an important and often ignored disease affecting more than one million people per year worldwide, with 58,900 deaths (Costa et al., 2015; Ko et al., 2009). Leptospirosis occurs worldwide, but it is more frequently found in humid tropical climates, especially in regions with poor sanitary infrastructure, deficient drainage, and as a result, large numbers of a key animal reservoir: rats. While inappropriate management of human excreta may support rodent densities and thereby be a contributing factor, human infections are often associated with exposures to natural bodies of water (wet farming). Outbreaks tend to occur during rainy seasons and it has been associated with flooding (Della Rossa et al., 2016; Ganoza et al., 2006; Trevejo et al., 1998; Concepcion-Acevedo et al., 2018; Reardon, 2017). The disease has a broad geographical distribution due to the large spectrum of hosts, including domestic and wild mammals, including rats and mice, which harbor the bacterium in their kidneys and release it through their urine (Carmona Gasca et al., 2013; Dietrich et al., 2015; Ko et al., 2009). Various animal hosts are further discussed in Section 1.3.2. Leptospirosis affects risk groups that are exposed to animal reservoirs or urine-contaminated environments, such as veterinarians, abattoir workers, sewage workers, military personnel, people in contact with natural bodies of water, and increasingly including individuals partaking in water sports and recreation (Agampodi et al., 2014; Katz et al., 2002; Ko et al., 2009; Lau et al., 2010; Morgan et al., 2002; Sejvar et al., 2003). Leptospirosis is regarded as the most widespread zoonotic disease (Evangelista and Coburn, 2010) and is reemerging in regions once thought to be relatively free of the pathogen, such as northern Sweden (Strand et al., 2015) (see Table 1 below).

1.1.2 Symptomatology (morbidity and case-fatality ratios)

The symptoms of leptospirosis vary from asymptomatic to a severe hemorrhagic and/or multi-organ disease. The severity of the disease depends on the *Leptospira* species (Alston, 1935) and the ability of the strain to cause infection. The disease can be divided in two stages; in the first stage (septicemic phase), leptospires multiply in the host’s blood and other tissues and cause fever, chills, headache, anorexia, myalgia (mainly in calves) and nausea or fulminant cases, and in the immune phase conjunctival suffusion and jaundice. Headache, photophobia, nausea, retro-orbital pain may indicate the presence of *Leptospira* in the cerebrospinal fluid (Picardeau, 2013). Severe leptospirosis (Weil’s disease or severe pulmonary hemorrhage syndrome) can develop after the septicemic phase. Weil’s disease is characterized by hemorrhage,

jaundice, and renal failure (Bharti et al., 2003), while severe pulmonary hemorrhage is linked to massive intra-alveolar bleeding. Mortality due to these 2 syndromes may range from 5-15% and renal failure may affect 16-40% of cases. Pulmonary pathology may occur in 20-70% of the cases and symptoms may range from cough, dyspnea, hemoptysis, to respiratory distress syndrome (Adler and de la Peña-Moctezuma, 2010). Death can occur from kidney failure, pulmonary hemorrhage, or other serious multi-organ system dysfunction.

1.2 Taxonomic Classification of the Agent(s)

Leptospirosis is caused by spirochetes of the genus *Leptospira*, which currently comprises 35 species that are phylogenetically separated into three clusters: 13 pathogenic, 11 saprophytic, and 11 intermediate (Adler and de la Peña-Moctezuma, 2010; Thibeaux et al., 2018b; Thibeaux et al., 2018a). The etiologic agents of this disease are pathogenic and intermediate species of *Leptospira*, which comprise more than 260 serovars (Adler, 2015a). Pathogenic and intermediate clusters have been reported to cause infection and, in this Chapter, we will refer to them as “virulent” *Leptospira*. The organism is a thin spirochete of 0.1 to 0.2 μm in diameter and 6 to 12 μm in length, so it may even pass through 0.22 μm filters. Hence 0.22 μm pre-filtration is often used to decontaminate *Leptospira* cultures from other bacteria (Evangelista and Coburn, 2010). It is a highly motile bacterium showing translational motility, travelling approximately 20 μm in 2 to 3 seconds in ordinary media. It has been demonstrated that such a motility is due to its coiled periplasmic flagellum that rotates at the leading end, generating a helical wave that propels the cell by rolling in a direction opposite to that of the flagellar gyration (Goldstein and Charon, 1988, Picardeau et al., 2001).

1.3 Transmission

1.3.1 Routes of transmission

Leptospira, as a fragile spirochete, is not resistant to desiccation, so aqueous environments are necessary for survival and transmission among mammals sharing a common environment (Adler, 2015a). Transmission of *Leptospira* to susceptible hosts usually occurs through contact with urine or water sources contaminated with animal urine (Ko et al., 2009). Sick animals usually shed virulent leptospires during the immune phase and convalescence of the disease (asymptomatic carriers). Rodents, on the other hand, usually are resistant to disease, but can remain infected and act as continuous reservoirs, shedding infecting leptospires through urine to environment for life (Thiermann, 1981). Most mammals can carry leptospires with no signs of disease or only mild clinical manifestations. Such subclinical infections are caused mainly (but not exclusively) by host-adapted serovars. The three main environments indicating human transmission risks are water-based, rodent-borne and livestock/pet-borne environments.

1.3.2 Human, animal, and environmental reservoirs

Leptospirosis is primarily an animal disease affecting wild and domestic mammals. Acute leptospirosis in animals can present with the same clinical patterns as in humans, mostly in young animals. After the septicemic phase, once the circulating antibody titers have risen, pathogenic serovars of *Leptospira* usually accumulate in kidneys, inside the renal tubules; so that infected animals shed potentially infecting leptospires to the environment through their urine. It causes important losses to livestock operations (especially cattle and swine), mainly because of abortion and infertility. In dogs the disease shows at least three different pathologic syndromes: icteric syndrome, uremic syndrome and hemorrhagic syndrome (Adler and de la Peña-Moctezuma, 2010). Seroprevalence in cattle may be as high as 84% and in dogs 35% (de Freitas et al., 2010, Ellis et al., 1986b, Oliveira Lavinsky et al., 2012, Schuller et al., 2015). Lower incidences have been found in other domestic animals among them sheep acting mainly as asymptomatic reservoirs (Cerri et al., 1996, Ellis et al., 1994, Lilenbaum et al., 2009); in horses the disease usually has a chronic course resulting in recurrent uveitis (ERU) being the main cause of equine blindness (Halliwell et al., 1985, Malalana et al., 2015, Wollanke et al., 2001). Cats are usually resistant to infection unless a concurrent immunosuppressive disease might be affecting them, such as feline leukemia virus or feline AIDS (Azocar-Aedo et al., 2014, Hartmann et al., 2013). Outbreaks of leptospirosis have also been described in wild animals such as sea lions in California (Avalos-Télez et al., 2016, Colagross-Schouten et al., 2002, Prager et al., 2013). Other wild species such as rhinoceroses and wolves may suffer of acute leptospirosis, but probably only when kept in captivity (Neiffer et al., 2001). Rodents play an important role in transmission and are considered the principal reservoirs that maintain leptospires present in environment and the main source of infection to susceptible hosts such as pigs, dogs, zoo animals and humans (Athanzio et al., 2008, Costa et al., 2014, Haake and Levett, 2015). De Faria et al. (2008) reported that up to 80.3% of Norway rats (*Rattus norvegicus*), a species that spread almost all over the globe, were culture positive and 68.1% were shown positive by antibody titers of at least 1:100 in a microscopic agglutination test (MAT). Other species of wild mammals have been detected as leptospiral carriers (Andreoli et al., 2014, Duncan et al., 2012, Pinna et al., 2012, Scialfa et al., 2013). In France, seropositivity rates were found to range from 31% in farmed American mink, 74% in wild European mink and up to 89% in stone martens (Moinet et al., 2010). In the United Kingdom, seropositivity rates were 6.2% in water voles (Gelling et al., 2015). Bats from different continents have also been shown as carriers of different *Leptospira* species, with a variable excretion rates (from 6 to 45%) (Dietrich et al., 2015). Most infected animals recover and become asymptomatic carriers for long periods of time, shedding leptospires through their urine (Della Rossa et al., 2016, Ko et al., 2009, Villanueva et al., 2014). Some serovars are adapted to a specific animal species, serovar Canicola to dogs (Morrison and Wright, 1976), serovar Hardjo to cattle (Ellis et al., 1986b), serovars Pomona and Bratislava to pigs (Ellis et al., 1986a; Stalheim,

1968), serovar Pomona to horses (Halliwell et al., 1985, Verma et al., 2013a), serovar Ballum to mice (Carmona Gasca et al., 2013; da Silva et al., 2010; Brem et al., 1995), serovar Icterohaemorrhagiae to rats (Thiermann, 1981; Adler and de la Peña-Moctezuma, 2010). Except in specific endemic settings (Ganoza et al., 2010), chronic renal carriage in humans is very rare and does not significantly contribute to the epidemiology of leptospirosis. Human to human transmission is a very rare event and humans mostly acquire the disease directly from animals (i.e., contact with urine or tissues) or more frequently indirectly (i.e., contact with urine-contaminated water) (Koizumi et al., 2009, Ko et al., 2009).

1.3.3 Incubation period

Leptospirosis has a variable incubation period depending on virulence of the infecting strain, infecting dose and the patient susceptibility (Levett, 2001). Entrance of pathogenic *Leptospira* to susceptible hosts occurs through mucous membranes or skin abrasions (Adler and de la Peña-Moctezuma, 2010; Levett, 2001). Leptospire reach the bloodstream and replicate in the absence of specific antibodies (leptospiremia), disseminating to many tissues (Coutinho et al., 2014, Ko et al., 2009). This phase is called septicemic phase and typically is characterized by fever, myalgia, arthralgia, erythema and cephalgia (Alston, 1935) and lasting from three to ten days. After this phase, serum antibodies become elevated and leptospire correspondingly disappear from the bloodstream (Ko et al., 2009; Levett, 2001). Studies in animals, particularly hamsters, have revealed that most tissues are invaded, especially kidneys, where leptospire allocate into the renal tubules away from the circulating specific antibodies (Coutinho et al., 2014). Leptospire are subsequently shed to the environment via urine (leptospiuria). This second phase of the disease, in which leptospire are shed, is called the immune phase and can last for long periods of time, even for life in reservoir animals (Ko et al., 2009). Many of the severe symptoms of this phase of the disease may be due to immunopathology (Fraga et al., 2011).

1.3.4 Period of communicability

Although very rare cases of inter-human transmission have been suspected and reported, leptospirosis is not a contagious disease. Therefore, this section discusses communicability from the animal reservoir and human susceptibility. Three main factors intervene to define the period during which sick animals, asymptomatic carriers or reservoirs may shed infecting leptospire to the environment (Adler and de la Peña-Moctezuma, 2010; Holt et al., 2006).

1. Serovar virulence. Virulence obviously and directly relates to the infective dose. It has been shown that *Leptospira* serovars vary in their level of pathogenicity. There are differences in virulence (level of pathogenicity), even among isolates of the same serovar. Some extremely virulent serovars have an experimental LD₅₀ of four or less bacteria (CFU) per animal in the hamster model (Sánchez,

2008; Watanabe et al., 2014). In any case, the infectious dose is crucial to the severity of the disease. Even though some species-adapted serovars may cause mild or no obvious signs of disease, they may still be shed to the environment through urine.

2. Immune status of the host. Animals that have never been in contact with a virulent *Leptospira* serovar, young or immunosuppressed individuals are more prone to acquire a severe clinical infection, relative to adults or immune competent individuals that develop a mild disease. Severe clinical infection may end in death of sick animals. Chronic carriers, in contrast, may shed infectious leptospire for long periods of time, even for life. Of note, leptospirosis immunity is both short lasting and serogroup-specific. People in hyperendemic areas may get leptospirosis several times, either from strains belonging to different serogroups or after post-infection immunity has declined.
3. The serovar - animal species relationship. It is well known that species-adapted serovars develop mild or subclinical infections in the corresponding animal species, frequently leading to chronic carriage in their animal host and chronic urinary shedding (Levett, 2001). In contrast, serovars non-adapted to the infected species usually produce severe or even fatal infections, but result in short-term excretion.

Depending on these three factors, an animal may, on the one hand, become severely sick and even die from the leptospiral infection, so that the period of shedding might be short or even non-existent. On the other hand, if the infection is not so severe as to kill the patient/animal, the relapse and recovery can lead to a state of chronicity and animals then become asymptomatic carriers for long periods of time and even for life (Adler, 2015a). Numbers shed in urine are rarely reported, but are usually high, e.g. Gillespie and Ryno reported a direct microscopic count of up to 108 leptospire per milliliter shed in cow's urine (Gillespie and Ryno, 1963).

1.3.5 Population susceptibility

As mentioned above, leptospirosis affects more than one million people a year worldwide, and almost 60,000 fatal cases per year (Costa et al., 2015; Ko et al., 2009). Farmers and suburban slum inhabitants are at the highest risk of infection (Costa et al., 2014), however there is one report suggesting that natural bodies of water in urban settings may pose higher risks of severe leptospirosis than rural ones (Ganoza et al., 2006). There have been reports of human leptospirosis outbreaks associated with urine contamination of water sources presenting a fatality rate of 8.6% (3 out of 35) (Cacciapuoti et al., 1987). Most outbreaks however are related to flooding following heavy exceptional or seasonal rainfall (Wynwood et al., 2014; Smith et al., 2013; Easton, 1999; Gaynor et al., 2007; Weinberger et al., 2014). However, there is at least one report where the main risk factor was the opposite, subnormal rainfall that may have contributed to the risk of exposure in military personnel exposed to contaminated

water sources (Corwin et al., 1990). The environmental exposure is detailed in section 2. Of note, males are infected more frequently than females, possibly reflecting both an occupational nature of the transmission and sex-

related susceptibility (Costa et al., 2014; Haake and Levett, 2015; Gomes et al., 2018). Global incidence rates are indicated in Table 1.

Table 1. Incidence of diagnosed disease for virulent *Leptospira*

Geographical Area of Study	Gender	Incidence per 100,000 Inhabitants ^a	Year of Data Collection
World-wide	Both	1.03E+06 cases /year	1970 to 2008
Africa	Female	8.6	1970 to 2008
Africa	Male	25.5	1970 to 2008
Americas	Female	6.6	1970 to 2008
Americas	Male	23.3	1970 to 2008
Asia and Oceania	Female	10.9	1970 to 2008
Asia and Oceania	Male	37.5	1970 to 2008
Europe	Female	2.3	1970 to 2008
Europe	Male	7.8	1970 to 2008

Source: Costa et al., 2015

^aper 100,000 inhabitants unless otherwise noted but, no data available for prevalence or seasonality

1.4 Population and Individual Control Measures

1.4.1 Vaccines

After the first isolation of *Leptospira* in Japan in 1914, Japanese researchers showed two years later that a phenol-inactivated culture of a virulent *Leptospira* elicited immunity against infection in Guinea pigs (Ido et al., 1916; Adler, 2015b). To date, different methods for inactivation of leptospires for use as vaccines have been applied, these include formalin, phenol, ethanol, heat, freeze and thaw and radiation. After 100 years, the only licensed vaccines are whole *Leptospira* inactivated bacterins (Adler, 2015b). As leptospirosis is largely a zoonotic disease, it is important to consider animal immunization as part of a control strategy - hence the detailed discussion in this section on vaccines. Current practice should consider immunization of local animals, such as dogs, cattle and pigs.

Leptospirosis is considered a neglected disease, and the spirochete causing this disease, *Leptospira*, is very difficult to recover in culture. Only recently have molecular genetics tools been developed that are suitable for the study of this spirochete (Adler, 2015a; Evangelista and Coburn, 2010; Ko et al., 2009). These factors have contributed to a lag in the understanding of its virulence mechanisms, its immunogenic properties and finally a lag in the development of vaccines that may induce robust and lasting protection (Adler, 2015b; Evangelista and Coburn, 2010).

1.4.1.1 Global coverage and future prospects

To date, available *Leptospira* vaccines only provide a

short lasting and serogroup-specific immunity. Problems with components of the culture media and reactogenicity have also been suspected, although not demonstrated (Benbrik et al., 2001). Therefore, such bacterins have not been widely used in humans. However human bacterin vaccines have been used successfully in several regions, including China, Japan, Cuba, and Europe (Verma et al., 2013b; Zamora et al., 2005), notably to prevent occupational leptospirosis in sewage-workers (De Serres et al., 1995; Nardone et al., 2004). Importantly, animal vaccination might still prove useful in reducing leptospirosis burden in humans, by decreasing the environmental contamination. In this regard, dog vaccination is routinely performed in a number of countries, aiming at protecting both the dog and its owner (Gay et al., 2014). Livestock vaccination is widely implemented in New Zealand and correlated with a significant decrease in human cases (Thornley et al., 2002). Further details on vaccines are presented in Appendix A.

1.4.2 Hygiene measures

Currently, implementing rodent control through improved sanitation and food residuals management seems the most effective measures to limit leptospirosis. Farmers and other professionals in occupations at risk should use protective gear (apron, gloves, boots and protective mask), e.g. in abattoirs or when using high pressure water (for cleaning) to avoid infection by aerosolized animal urine. Additionally, animal vaccination may reduce leptospiral shedding (Subharat et al., 2012). For workers exposed to municipal wastewaters (e.g. sewage workers), vaccination, even in developed regions would seem appropriate for risk

management (De Serres et al., 1995), however vaccines for humans are available only in a few countries.

2.0 Environmental Occurrence and Persistence

Studies of virulent *Leptospira* in the environment are very limited, mainly because it is difficult to isolate these spirochetes from surface waters or soil without contamination with saprophytic *Leptospira*. However, there is a growing interest in understanding the lifestyle and survival capabilities of virulent *Leptospira* in the environment. In addition, genomic studies suggest that virulent *Leptospira* evolved from an environmental (free-living) *Leptospira* present in water or mud, similarly to the contemporary members of the saprophytic cluster (such as *L. biflexa*) (Saito et al., 2013; Thibeaux et al., 2018b; Thibeaux et al., 2018a). There seems to be some evolutionary variation within virulent leptospires; some species (such as *L. interrogans*) have retained genes associated to environmental survival whereas other virulent members (such as *L. borgpetersenii*) seem to have lost these genes. These findings have been corroborated by in vitro experiments showing that *L. interrogans* survives better in distilled water than *L. borgpetersenii* (Bulach et al., 2006, Picardeau et al., 2008), which may imply that *L. borgpetersenii* is not transmitted by water but rather relies on direct host-to-host transmission (Bulach et al., 2006).

Leptospiral survival in water is bolstered by viscous material, such as agar, which possibly translates to biofilm in natural environments. Some strains of virulent *Leptospira* are able to produce biofilms when incubated in fresh water, even in the absence of nutrients (Barragan et

al., 2011; Ristow et al., 2008). Furthermore *L. interrogans* (pathogenic cluster) has been found in nature associated with biofilms formed by environmental bacteria (Vinod Kumar et al., 2015a). Some virulent *Leptospira* are able to produce biofilm when incubated in distilled water (low nutrient conditions) and appear sense the presence of nutrients, such as fatty acids, which triggers release of leptospiral cells from the biofilm (Barragan et al., 2011). It has been observed that biofilm-borne leptospiral cells display a five- to six-fold increase in antibiotic tolerance (Vinod Kumar et al., 2016a). It has also been speculated that biofilms protect pathogenic *Leptospira* against other toxic compounds and stressors in the environment. Taken together, *Leptospira* survival in the environment seems to be favored by biofilm formation, which could contribute to disease transmission among risk groups such as sewage and agricultural workers (Della Rossa et al., 2016; Ganoza et al., 2006; Koizumi et al., 2009; Trueba et al., 2004).

Soil may also play a role in environmental survival. Some researchers have shown that *Leptospira* survived in the wet soil on dry days and appeared in surface water on rainy days, suggesting that soil could be a reservoir for leptospires in the environment (Saito et al., 2013; Thibeaux et al., 2017). Even though leptospires are considered fragile spirochetes, studies have shown that they can survive and even maintain their virulence for up to 20 months despite unfavorable conditions, such as cold and nutrient-poor acidic waters (Andre-Fontaine et al., 2015). However, there is limited knowledge about possible differences in the nutritional requirements of saprophytic and virulent species and about ability of virulent leptospires to multiply in surface water or soils. General data on the occurrence of leptospires in the environment is presented in Tables 2-5.

Table 2. Pathogenic *Leptospira* occurrence in sewage and wastewaters

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	Reference
Brazil (Salvador, Pau da Lima)	2011 to 2012 (July to January)	Sewage Water	36% (121/335)	<i>LipL32</i> qPCR on 40 mL water sample	Casanovas-Massana et al., 2018
Colombia (Cordoba)	2009 to 2011	Waste water (from pig stable)	5.56% (1/18)	Culture enrichment 0.5 mL with and without filtration through 0.45µm filter	Calderon et al., 2014 ;
UK (London)	1977	Sewage (cattle market and farms)	100% (2/2)	Culture of 20 mL serogroup Tarassovi identified	Coghlan and Kmety, 1987
UK (Oxford)	1980 to 1981	Sewage and sewage sludges	15.9% (30/189)	Culture ^a 20ml reduced to 0.2ml Via centrifugation/filtration tarrassovi serotype identified	Jones et al., 1981

^aLiquid and solid EMJH supplemented with 5-Fluoro-Uracil (100 µg/ml), rabbit serum 1%, Amphotericin B (50µg/ml), Sulphathiazole (50 mg/L), neomycin sulphate (5 mg/L), actidione (0.5 mg/L) and incubated at 29°C. Identification by serological typing.

Table 3. Pathogenic *Leptospira* occurrence in water samples

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	Reference
Brazil (Pelotas)	NR ^a	Stagnant Water (abandoned swimming pool with dead possums and rats)	100% (1/1)	Culture (Few Drops)	Forster et al., 2013
Brazil (Rio de Janeiro, Petropolis)	2009 (May to July)	Community water supplies, small lakes, and surface water	0.1% (1/100)	Multiplex PCR on 125 mL <i>lipL</i> -32 and 16srRNA both genes amplified in pathogenic species	Vital-Brazil et al., 2010
Brazil (Salvador, Pau da Lima)	2011 to 2012 (July to January)	Standing Water	34% (82/250)	<i>lipL</i> 32 qPCR on 40 mL water sample	Casanovas-Massana et al., 2018
Chile (Los Rios)	2010 to 2012 (November to April)	Puddles	19.3% (36/306)	<i>lipL</i> 32-PCR on 50mL to 1.0L water	Muñoz-Zanzi et al., 2014
Chile (Los Rios)	2010 to 2012 (November to April)	Water in open containers	45.5% (10/22)	<i>lipL</i> 32-PCR on 50mL to 1.0L water	Muñoz-Zanzi et al., 2014
Chile (Los Rios)	2010 to 2012 (November to April)	Water Canals/Rivers	3.9% (4/103)	<i>lipL</i> 32-PCR on 50mL to 1.0L water	Muñoz-Zanzi et al., 2014
Chile (Los Rios)	2010 to 2012 (October to April)	Puddles	27.3% (84/208)	PCR and sequencing on 50 mL to 1L water	Mason et al., 2016
Chile (Los Rios)	2010 to 2012 (October to April)	Water in open containers	14.4% (28/194)	PCR and sequencing on 50 mL to 1L water	Mason et al., 2016
Chile (Los Rios)	2010 to 2012 (October to April)	Water Canals/Rivers	9.7% (13/134)	PCR and sequencing on 50 mL to 1L water	Mason et al., 2016
Colombia (Cordoba)	2009 to 2011	Well Water	0% (0/18)	Culture enrichment 0.5 mL with and without filtration through 0.45µm filter	Calderon et al., 2014
Colombia (Cordoba)	NA ^b	Untreated water samples	0% (0/13)	15 mL water samples collected on farms Centrifuged 14,000 rpm / 5 min. Pellet washed by PBS. PCR and sequencing.	Ensunchó-Hoyos et al., 2017

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	Reference
France (Lyon)	2008 (Spring)	Water	6.38% (3/47)	qPCR on 50 mL of water 103 to 104 genome-equivalents/mL	Vein et al., 2012
India (Nicobar and Andaman Islands)	2012	Urban Water Samples	9.7% (11/113)	PCR on 20 mL water sample, Centrifuged 8,000 g for 10 min. DNA extracted from pellet	Lall et al., 2016
India (Nicobar and Andaman Islands)	2012	Rural Water Samples	6.7% (9/133)	PCR on 20 mL water sample, Centrifuged 8,000 g for 10 min. DNA extracted from pellet	Lall et al., 2016
India (Nicobar and Andaman Islands)	2012	Paddy Fields	15.9% (18/113)	PCR on 20 mL water sample, Centrifuged 8,000 g for 10 min. DNA extracted from pellet	Lall et al., 2016
Island of St. Kitts	NA	Water (Environmental water: ponds, puddles, water dams, mountain spring and stream)	18.18% (8/44)	qPCR on 300 mL environmental water sample (20.6 to 56 genomes per 300 mL)	Rawlins et al., 2014
Italy	2005	Water (From toilet and tap)	50% (2/4)	Environmental source tracking after fatal case	Luchini et al., 2008
Malaysia	2012 to 2013 (December to November)	Water	0% (0/72)	40 mL of water filtered through 0.2µm, filtrate centrifuged 4,000g/27°C/20 min before culture.	Azali et al., 2016
Malaysia (Lubuk, Yu, Pahang)	2011	Stagnant Water	100% (8/8)	PCR on 100 mL of water	Sapian et al., 2012
Malaysia (Kelantan)	2016 (June to September)	Water	0% (0/21)	100 mL sample, cultured 1 mL filtered through 0.22µm with 5-Fluoro-Uracil	Mohd Ali et al., 2018
Malaysia (Kelantan and Terengganu)	2009 (April)	Water after culture pre-enrichment	6.78% (12/177)	PCR after enrichment in culture, 5-10 drops used	Ridzlan et al., 2010
Malaysia (Sarawak)	2014 to 2015 (April to February)	Water (Drain effluent, river, lake, puddle)	1.96% (6/324)	PCR on 50 mL water sample	Pui et al., 2017b
Malaysia (Terengganu)	2012 (February and March)	Water	7.5% (3/40)	Culture (EMJH media) and PCR 2.5 ml of 0.22µm filtrated water or pellet of 985 ml	Ismail et al., 2014
New Caledonia (North province regions)	2016 (March to June)	Water	0% (0/10)	PCR/Viability-PCR on 10 mL of water Linked the identity of environmental <i>Leptospira</i> to human infecting strain	Thibeaux et al., 2017

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	Reference
Russia (lake Nero, Yaroslav region)	1974 (June to September)	marsh and bank of lake (moisture: 69.5 to 70.3%, pH 7.4 to 7.8)	1.1% (7/630)	1 g sample suspended into 5 ml Inoculation to hamsters Isolated 4 serogroups grippityphosa and 1 serogroup hebdomadis	Karaseva et al., 1977
Thailand (Bangkok)	2011 (November)	River Water	0% (0/2)	1.5-6 L qPCR Bacteria collected on 0.2 µm filter membrane	Chaturongkasumrit et al., 2013
Thailand (Bangkok)	2011 (November)	Flood Water	8.33% (1/12)	1.5 to 6 L qPCR Bacteria collected on 0.2 µm filter membrane (Single positive from urban residential area, sample was turbid with pH 8.05, Vibrio cholerae detected)	Chaturongkasumrit et al., 2013
Thailand (Bangkok province, Don Muang district and Nakhon Pathom province)	2011 (November and December)	Floodwater	0.9% (1/110)	PCR and Culture on 100 mL of flood water (50 mL for PCR, 50 mL for culture)	Thaipadungpanit et al., 2013
Thailand (Khon Kaen and Nakhon Ratchasima Province)	2005 to 2006 (Nov to Jan)	Water	23% (23/100)	Duplex PCR	Tansuphasiri et al., 2006
Thailand (Nan Province)	2013 to 2016	Water	21.4% (3/14)	Rrs nested PCR and MLST performed on 50 mL of water. Underground water source used in animal husbandry, drinking/cleaning pens, and/or rice fields	Kurilung et al., 2017
USA (IL)	1998 (July)	Water from coastal and non-coastal areas Lake Springfield (Triathlon)	3.7% (1/27)	PCR	Morgan et al., 2002

^aNR: Not Reported; ^bNA: Not Available

Table 4. Pathogenic *Leptospira* occurrence in soil, soil and water, and misc.

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	REF
India (Archipelago of Andaman and Nicobar)	NR ^a	Waterlogged paddy field soil	60% (6/10)	PCR on five sterile glass rods (buried up to 3" deep in different waterlogged paddy field soils and allowed to rest for 48 h)	Vinod Kumar et al., 2015b
India (Nicobar and Andaman Islands)	2014 (July to August)	Paddy field Water and Leaves	24% (34/142)	PCR on sterile glass slides incubated for 48 h in different sources of water. Paddy leaves were collected and cut to 1 cm in length at air-liquid interphase	Vinod Kumar et al., 2016a
Japan (Hokkaido and Okinawa)	2014 to 2016 (July to April)	Soil	9.1% (12/132)	20 g of soil, culture with STAFF and 16S rDNA for identification	Masuzawa et al., 2018
Malaysia	2012 to 2013 (December to November)	Soil	1.39% (1/72)	No sample volume for soil soil resuspended with sterile water, supernatant filtered through 0.2µm before culture.	Azali et al., 2016
Malaysia (Kelantan)	2016 (June to September)	Soil	33.3% (7/21)	20 g soil sample, cultured 1 mL washings filtered through 0.22µm with 5-Fluoro-Uracil	Mohd Ali et al., 2018
Malaysia (20 miles around Kuala Lumpur)	1961 to 1962	Water and Soil	2.9% (397/13,848)	1 to 5 ml of water 0.5 ml washing of topsoil Inoculation to hamsters/guinea pigs serogroup Pomona isolated ^b	Baker and Baker, 1970
Malaysia (Lubuk, Yu, Pahang)	2011	Surface Soil	50% (2/4)	PCR of 100 g of soil	Sapian et al., 2012
Malaysia (Lubuk, Yu, Pahang)	2011	Deep Soil (30 cm)	12% (3/25)	PCR of 100 g of soil	Sapian et al., 2012
Malaysia (Sarawak)	2014 to 2015 (April to February)	Surroundings of housing areas, landfills, open field, and lake soil	11.6% (34/292)	PCR on 20 g	Pui et al., 2017a

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	REF
New Caledonia (North province regions)	2016 (March to June)	Soil	56.69% (30/52)	PCR/Viability-PCR on 250 mg soil Pathogenic leptospire were alive and present in soils several weeks after the infecting event	Thibeaux et al., 2017
New Caledonia (North province regions)	2016 (March to June)	Vegetal Float debris	12.2% (2/11)	PCR/Viability-PCR on 250 mg vegetal debris	Thibeaux et al., 2017
Philippines (Leyte Province)	2014 (January)	Wet Soil	48% (11/23)	PCR and culture enrichment on 10 g of wet soil (16S rRNA gene sequence similar to <i>L. kmetyi</i>)	Saito et al., 2014
Poland (Lublin province)	2010 to 2013	Water and Soil	0.94% (2/212)	Traditional and Nested PCR targeting <i>lipL-32</i> (5L of water and soils were collected in 50mL falcon tube)	Wojcik-Fatla et al., 2014
Taiwan (Yunlin, Chiayi, Tainan)	2010 (May-December)	Soil	30.6% (33/108)	Nested PCR on 100 mg soil sample Investigation following two flood hazards due to typhoons Morakot and Fanapi	Fuh et al., 2011
Thailand (Western Part)	2016 (November)	Water and Soil	35.3% (6/17)	Cultured in liquid and solid EMJH supplemented with 100µg/ml of 5-Fluoro-Uracil followed by a rrs nested PCR. Water: 0.5 mL filtered through 0.22 µm from 30 mL, cultured at 28°C Soil: 50 g, washings filtered through 0.22µm cultured at 28°C	Chaiwattananarungruengpaisan et al., 2018
UK (London)	1935	Slime from London sewers	6.7 (2/30)	Inoculation to guinea pigs	Alston, 1935
USA (FL, Tampa)	2017 (December)	Water Soil	0.0% (0/15) 0.0% (0/11)	500 µl to 30 ml of water cultivated in EMJH medium with 5-fluorouracil	Stern et al., 2010
USA (IA)	1964 (August to September)	Big Creek water Sampled with soil	30% (1/3)	Inoculation to guinea pigs 5ml Intraperitoneal injection	Diesch and McCulloch, 1966

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	REF
USA (CA, Long Valley, Little Hot Creek Spring)	2015 (June)	Dendrolitic cone	100% (1/1)	16S metagenomic sequencing (Illumina MiSeq platform) V4 region of the 16S rRNA gene (positions 519 to 802, <i>Escherichia coli</i> numbering). Dendrolitic cones ranged in height from 0.5-2.0 cm	Bradley et al., 2017

^aNR: Not Reported; ^bIsolated from blood cultures of moribund animals

Table 5. Pathogenic *Leptospira* occurrence in drinking water samples

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	References
Chile (Los Rios)	2010 to 2012 (November to April)	Animal drinking water ^a	14.5% (12/83)	<i>lipL32</i> -PCR on 50mL to 1.0L water	Muñoz-Zanzi et al., 2014
Chile (Los Rios)	2010 to 2012 (November to April)	Human drinking sources	19.1% (9/47)	<i>lipL32</i> -PCR on 50mL to 1.0L water	Muñoz-Zanzi et al., 2014
Chile (Los Rios)	2010 to 2012 (October to April)	Animal drinking water ^a	16.5% (14/85)	PCR and sequencing on 50 mL to 1L water	Mason et al., 2016
Chile (Los Rios)	2010 to 2012 (October to April)	Human drinking sources	14.7% (14/95)	PCR and sequencing on 50 mL to 1L water	Mason et al., 2016
China (Nanjing, Bejhekou drinking water plant)	NA ^b	Drinking Water	0.45% of total metagenomic reads	Metagenomics (454 pyrosequencing) <i>L. interrogans</i> still in tap water after chlorination	Huang et al., 2014
Colombia (Cordoba)	2009 to 2011	Animal drinking water ^a	5.56% (1/18)	Culture enrichment 0.5 mL with and without filtration through 0.45µm filter	Calderon et al., 2014

Area	Year	Sample Description	Occurrence Percent Positive (Sample Numbers)	Detection Method	References
Colombia (Cordoba)	NA	Drinking Water	0% (0/13)	15 mL water samples collected on farms Centrifuged 14,000 rpm / 5 min. Pellet washed by PBS. PCR and sequencing.	Ensuncho-Hoyos et al., 2017
Thailand (Bangkok)	2011 (November)	Tap Water	0% (0/11)	1.5-6 L qPCR Bacteria collected on 0.2 µm filter membrane	Chaturongkasumrit et al., 2013
Thailand (Bangkok)	2011 (November)	Filtered Tap Water	0% (0/5)	1.5-6 L qPCR Bacteria collected on 0.2 µm filter membrane	Chaturongkasumrit et al., 2013

^aNon-potable drinking water for animals; ^bNA: Not Available

2.1 Detection Methods

Leptospire might be detected by either culture and isolation or using molecular techniques like (quantitative) PCR. *Leptospira* spp. are fastidious slow-growing organisms with very specific nutritional requirements. However, as for most bacterial species, initial work used classical, culture-based isolation procedures. Although several different culture media have been developed and used for *Leptospira*, most studies have used the oleic acid - albumin culture medium Ellinghausen-McCullough-Johnson-Harris (EMJH) or the rabbit serum-enriched Korthof's enrichments; as liquid or semi-solid media for at least initial culture, thus preventing quantification. Since the mid 1960s, the control of contaminants has been achieved by including 5-Fluoro-Uracil into the culture medium (Johnson and Rogers, 1964). More recently, a combination of selective antimicrobial agents as proposed by Chakraborty et al. (2011) has increasingly been used (sulfamethoxazole, 40 µg/mL; trimethoprim, 20 µg/mL; amphotericin B, 5 µg/mL; fosfomycin, 400 µg/mL; and 5-fluorouracil, 100 µg/mL). Whether these culture media and selective agents favor the growth of all *Leptospira* species and strains is still unknown, and the isolation process most likely results in the loss of *Leptospira* diversity. In addition, although viable but non-culturable (VBNC) *Leptospira* have not been reported, the ability of different strains to grow in vitro in culture media is still very poorly established.

As with any culture-based isolation technique, fast-growing saprophytic species are most frequently isolated and may jeopardize the detection and isolation of slower-growing virulent leptospire. Historically, to gain insight into pathogenic *Leptospira* from waters and soils, direct inoculation of environmental samples into susceptible animal models have been used. Pathogenic leptospire that

induce infection and disease in such test animals and cultures from their blood or target organs allows for the recovery of pure cultures. Examples are provided in the following section 2.2, pathogenic leptospire were isolated from recreational waters in the USA after inoculation into guinea pigs (Diesch and McCulloch, 1966).

A number of pathogenic leptospire were similarly recovered from soils and waters in Malaysia after inoculation of hamsters (Baker and Baker, 1970). This technique used many susceptible animals (hamsters or guinea pigs), and is currently unacceptable from an ethical perspective. Of note, this technique does not allow a quantitative assessment, since the pathogenic leptospire are amplified or lost in the test animals by immune clearance, depending both on their own virulence and their initial concentration in the sample studied (minimal infective dose). Animals might also die from another infection that may mask a virulent leptospire. To summarize, this technique has an excellent specificity if *Leptospira* isolation is successful, but may suffer from a low sensitivity presumably leading to a number of false negative results. Consequently, the proportion of positive samples observed with this animal-enrichment technique are expected to underestimate the true occurrence.

Once the leptospire are isolated, there is no easy and specific phenotypic technique for their identification, even at the species level. This further complicated the early studies of *Leptospira* in the environment, but modern molecular methods are now reducing this problem. The International Leptospirosis Society regularly produces a position statement on the speciation of Leptospiral isolates, which is publicly available (<https://leptosociety.org/resources>). The most recent recommendation is to use Whole Genome Sequencing

techniques and genomic comparisons for speciation.

2.1.1 Culture from environmental samples

There is no standard protocol for culturing virulent species of *Leptospira* from bodies of fresh water or soil. Furthermore, there are only a few reports describing the isolation of virulent *Leptospira* from environmental samples; as discussed above, the main difficulty in the isolation of these species is the overgrowth of environmental bacteria or the fast-growing saprophytic *Leptospira* (Barragan et al., 2011; Benacer et al., 2013; Saito et al., 2013). Culture-based protocols include the use EMJH (Ellinghausen and McCullough modified by Johnson and Harris) culture medium containing cocktails of antibacterial and anti-fungal compounds such as sulfamethoxazole, trimethoprim, amphotericin B, fosfomycin, and 5-fluorouracil (Benacer et al., 2013; Saito et al., 2015; Saito et al., 2013). Alternatively bacterial contamination in water samples can be reduced by utilizing 0.22 µm filtrate before enriching in EMJH medium, then isolating colonies on solid medium (Saito et al., 2015; Wuthiekanun et al., 2013).

2.1.2 Molecular based methods: amplification of leptospiral DNA from environmental samples

Due to difficulties in culturing *Leptospira* from environmental samples, numerous PCR-based techniques have been developed that mainly target genes only present in pathogenic *Leptospira*, such as *lipL32* (Muñoz-Zanzi et al., 2014; Vinod Kumar et al., 2016b; Aviat et al., 2010) or pathogenic specific sequences of genes, such as *flaB* (Villanueva et al., 2014), *secY* (Perez and Goarant, 2010; Guernier et al., 2018) and the 16S rRNA gene (Ganoza et al., 2006). Because of sequence polymorphisms, PCR targeting *lipL32* or *secY* fail to detect virulent *Leptospira* of the intermediate cluster (e.g. *L. fainei*, *L. broomii*, *L. inadai*, *L. wolffii*, *L. licerasiae*), which are commonly found in water (Ganoza et al., 2006, Thaipadungpanit et al., 2013).

A second problem with PCR methods used to date is that detection of genomic DNA sequences provides no indication of the viability of bacteria in water or other matrices. In addition to the possible use of PMA or EMA to reduce cell membrane compromised or free DNA detections (Thibeaux et al., 2017; Soupé-Gilbert et al., 2017), targeting pre-rRNA appears to be an alternative with possibly greater precision in detecting viable bacteria (Cangelosi, 2009; Gedalanga and Olson, 2009). While yet to be reported for *Leptospira*, targeting pre-rRNA before and after media stimulation and assaying by qRT-PCR to identify differences in the pre-rRNA copies present should correspond to viable *Leptospira* (Cangelosi, 2018).

Most recently, detailed procedures for the molecular detection of pathogenic leptospires have been published, which will probably trigger further studies on leptospires in the environment (Beigel and Verma, 2017; Riediger et al., 2016; Thibeaux et al., 2018a). There has also been increased use of high throughput sequencing technologies. For example, 16S rRNA amplicon sequencing yielded evidence of the presence of *Leptospira* in various water

sources (Huang et al., 2014; Zhang et al., 2017; Escobedo-Hinojosa and Pardo-Lopez, 2017). Studies using such so-called next generation DNA sequencing approaches will likely provide improved information on *Leptospira* ecology in the coming years.

2.2 Data on Occurrence

2.2.1 Excreta in the environment

Pathogenic *Leptospira* live and multiply in the proximal kidney tubules of reservoir animals, which are frequently asymptomatic, and are then shed through the urine. Thus, they reach soils and waters in the environment, which are the sources of most human infection. However, very little is known on the actual numbers of leptospires that are shed by reservoir animals. A detailed analysis of the kinetics of excretion in mice shows that excretion increases with age, reaching 3.0×10^7 /ml of urine and suggesting that older rodents excrete larger amounts of leptospires (Soupé-Gilbert et al., 2017). A meta-analysis indicated that reservoir rats excrete the greatest concentration of leptospires (5.7×10^6 per mL of urine), but large mammals produce the greatest quantities (up to 10^9 leptospires per day) because of the larger volume of urine (Barragan et al., 2017). This meta-analysis suggested that humans could shed approximately 10^6 leptospires per day. However, except in very specific situations, humans usually do not become chronic carriers and only excrete leptospires for a couple of days to weeks (Levett, 2001). Therefore, human excreta are usually not considered to play an epidemiological role in transmission of leptospirosis.

2.2.2 Sewage

Leptospirosis has been historically associated with sewage and is still an occupational hazard in sewer-workers. Pathogenic leptospires were isolated from sewage or sewage slime very early (Alston, 1935; Coghlan and Kmety, 1987; Jones et al., 1981). Using molecular techniques, pathogenic leptospires were evidenced from sewage in the Peruvian Amazon (Ganoza et al., 2006) and from a wastewater sample in Colombia (Ensuncho-Hoyos et al., 2017). In Brazilian slums, open sewers are considered as a major source of human leptospirosis, yet may be more a function of rat urine presence than human sewage. A recent study in an urban slum in Salvador, Brazil, demonstrated the presence of pathogenic leptospires in more than one third of sewage water samples, with a concentration that range 2.0×10^3 to 1.7×10^6 /100 mL. The highest concentration were reported during the rainy season and at sampling sites nearest the bottom of the valley and thus most vulnerable to flood waters (Casanovas-Massana et al., 2018).

2.2.3 Manure

Due to the huge quantities of leptospires potentially excreted in the urine by large mammals, manure can be a major source of contamination. Direct detection in or isolation of pathogenic leptospires from manure is a major

challenge, because of the enormous bacterial burden and the difficulties of selectively growing leptospires, as well as the major concern of PCR inhibitors preventing a relevant use of molecular detection. Cattle manure was shown to be a source of pathogenic leptospires in field conditions (Gillespie and Ryno, 1963). A study that modeled the shedding of leptospires in cattle manure found they were resilient in an oxidation ditch designed for nutrient removal, persisting for upwards of two months (Diesch, 1971). More recently, a change in the management of livestock manure in Korea was associated with a significant decrease in leptospirosis incidence (Ryu et al., 2017).

2.2.4 Surface waters

As described above (see section 2.1), studies using only culture-based methods mostly detected saprophytic leptospires, which are normal inhabitants of water and soils and frequently overgrow virulent leptospires in culture. Using inoculation to susceptible animals (guinea pigs or hamsters), pathogenic leptospires have been isolated from both temperate and tropical surface waters (Diesch and McCulloch, 1966; Crawford Jr et al., 1969; Baker and Baker, 1970; Alexander et al., 1975; Jackson et al., 1993). Molecular detection has been used since the late 1990s and notably allowed some DNA from pathogenic *Leptospira* to be detected from lake water associated with a recreational outbreak of leptospirosis after a triathlon in Illinois, USA (Morgan et al., 2002). Later molecular methods detected pathogenic leptospires from 23% of various surface water sources in Thailand (Tansuphasiri et al., 2006). Of note, classical PCR cannot provide direct quantitative information about the leptospires, but both studies cited above suggest low concentrations.

The use of quantitative real time PCR (qPCR) does enable direct quantitative estimates of concentrations of leptospires in water sources. Concentrations in the range one to seventeen thousand leptospires per mL (including both pathogenic and intermediate strains) were measured in various surface water sources in Peru, notably showing both higher frequency and higher concentrations in urban (especially in a market area) than in rural area (Ganoza et al., 2006). In Hawaiian streams, *Leptospira* were detected in 87 of 88 water samples (98.8%), with concentrations from 5 to 10^4 genomes / 100mL (approx. 1 to 2.0×10^3 leptospires / 100 mL), with a strong correlation with turbidity. Further sequencing of the PCR product evidenced only species from the intermediate cluster (Viau et al., 2011). In a temperate environment near Lyon, France, pathogenic *Leptospira* were detected in 3 of 47 (6.38%) water samples collected from ponds with concentrations in the range 10^5 to 10^6 / 100 mL (Vein et al., 2012). In the temperate region of Los Rios in South-Central Chile, pathogenic leptospires evidenced by PCR were more common in standing waters (19.3% of puddles and 45.5% of open containers) than in running waters (3.9% in canals or rivers). The overall detection rate was 77 out of 570 (13.5%) water samples, revealing the ubiquity of pathogenic *Leptospira* in surface waters in this temperate region (Muñoz-Zanzi et al., 2014). Particular attention should be paid to floodwaters, which

are known to be responsible for numerous leptospirosis infections and outbreaks in humans. Although floodwaters have rarely been studied (Thaipadungpanit et al., 2013; Chaturongkasumrit et al., 2013), they should be considered as periods of increased leptospirosis risk, as shown by the high numbers of leptospirosis outbreaks triggered by floods (Smith et al., 2013; Park et al., 2006; Matono et al., 2015; Gaynor et al., 2007; Easton, 1999) (Table 2-5).

2.2.5 Groundwater

Few studies have investigated leptospires in ground water. Recent 16S rRNA gene amplicon studies confirm a greater abundance of *Leptospira* in surface than in underground waters (Delafont et al., 2016). Pathogenic *Leptospira* were reported from underground water sources in Thailand, but the study did not provide hydrologic details about the water sources investigated (Kurilung et al., 2017).

2.2.6 Drinking water

Leptospira have been detected in drinking water samples in several studies. Saprophytic leptospires are normal inhabitants of freshwater, which do not involve any sanitary risk. However, there has also been evidence of pathogenic leptospires from drinking water for animals (Calderon et al., 2014; Muñoz-Zanzi et al., 2014; Mason et al., 2016; Kurilung et al., 2017), but also for humans (Muñoz-Zanzi et al., 2014; Mason et al., 2016). Very interestingly, a shotgun metagenomics study retrieved a *Leptospira* spp. draft genome from chlorinated drinking water, suggesting an ability to resist some degree of chlorination (Zhang et al., 2017). Similarly, a 16S rRNA gene amplicon study found *Leptospira* spp. sequence reads in a drinking water network, both right after chlorination (initial concentration of 0.6-0.8 mg/L free chlorine) and in final tapwater (0.1 mg/L residual chlorine); in this study, a higher relative abundance of *Leptospira* spp. sequence reads in tap water compared to initial water further supports the hypothesis of resistance to chlorination (Huang et al., 2014). Whether this resistance is related to *Leptospira* ability to form biofilms (discussed above in section 2) remains to be determined.

2.2.7 Seawater

Leptospires are thought to survive poorly in seawater. However, there is limited evidence of some degree of saltwater tolerance by some *Leptospira* strains. One study reported the isolation of a virulent leptospire that was apparently tolerant to seawater (Grune Löffler et al., 2015). Another study, using 16S rRNA gene amplicon, reported *Leptospira* in marine waters and soils in the Gulf of Mexico (Escobedo-Hinojosa and Pardo-Lopez, 2017). On the other hand, leptospirosis cases observed in marine mammals could be due to coastal contamination by virulent leptospires arising from terrestrial runoff and contamination of coastal marine environments (Bogomolni et al., 2008; Cameron et al., 2008; Prager et al., 2013).

2.2.8 Sludge

Leptospire have been reported from sewage and sewage sludge since the 1930s (Alston, 1935; Jones et al., 1981; Coghlan and Kmety, 1987), yet there has been little interest generally in such reports because human sewage does not appear to be a significant source of human disease.

2.2.9 Soil

Noguchi (1918) appears to be one of the first to report soils as a possible environment where *Leptospira* survive. However, because of the technical challenges of studying delicate slow-growing organisms in soils, this environmental compartment has been poorly studied. Using animal inoculation, pathogenic *Leptospira* were recovered from Malaysian soil washings in the early 1960s. The authors noted a higher isolation frequency from soil washings than from waters (Baker and Baker, 1970). Quite similarly, a study in Minnesota, which failed to isolate virulent strains, noted that isolation of leptospire was more frequent from soils than from adjacent waters (Henry and Johnson, 1978). Similarly in Hawaii, leptospire were isolated from 7 of 13 water samples, but from all 16 soil samples examined (Wilson and Fujioka, 1995). In New Caledonia where leptospirosis is endemic, a source tracking study used viability-PCR and detected viable pathogenic leptospire in river soils weeks after the contamination events (Thibeaux et al., 2017). A combination of selective agents was proven to be very effective for the culture and isolation of *Leptospira* from environmental samples (Chakraborty et al., 2011). Using this selection techniques, a great biodiversity of leptospire were isolated from tropical soils in New Caledonia, supporting the hypothesis that soils may be the original habitat of the genus *Leptospira* and a possible environmental reservoir of pathogenic strains (Thibeaux et al., 2018b; Thibeaux et al., 2018a). This hypothesis is also supported by other findings, notably the positive correlation between *Leptospira* concentration and turbidity (Viau and Boehm, 2011) or the role of floods in triggering leptospirosis outbreaks, as presented above.

2.2.10 Irrigation water and on crops

Irrigation waters are mostly surface waters, though groundwater is sometimes used. Most of the risks associated with irrigation waters should be evaluated by knowledge acquired from the water source considered and described in the corresponding section. Very few studies have considered *Leptospira* irrigation water.

There is both direct and indirect evidence of a high risk of leptospirosis in humans that rely on irrigated crops. In Korea, pathogenic leptospire were isolated from the water of a rice paddy field identified as a source of a leptospirosis outbreak (Kim, 1987), providing direct evidence. Indirect evidence relies on the epidemiological association of human leptospirosis with activity in rice paddy fields (Kim, 1987; Ryu and Liu, 1966; Ivanova et al., 2012), but also other irrigated crops, such as irrigated taro fields (Massenet et

al., 2015).

Crops are not considered to represent a direct risk for leptospirosis, but improper storage can lead to rodent population growth, constituting an indirect risk of leptospirosis via increased excretion of urine.

2.2.11 Fish and shellfish

Leptospira isolates have historically been recovered from a number of cold-blooded animals, notably amphibians and reptiles (Andrews et al., 1965; Minette, 1983). More recently, molecular detection suggested *Leptospira* carriage in the kidneys of some reptiles (Jobbins and Alexander, 2015). Positive serology was sometimes reported from freshwater fishes (Mgode et al., 2015), but this finding is considered anecdotal and does not mean that the seropositive fishes had the ability to chronically carry and shed live virulent leptospire. Current scientific consensus is that cold-blooded vertebrates and invertebrates are not involved as reservoirs of human-pathogenic leptospire, and they have not been reported as possible vehicles of human contaminations. On the other hand, freshwater fish farming might pose an occupational risk for leptospirosis, through extensive and prolonged contact with freshwater, as well as through the possible presence of rodents at feed stock facilities.

2.2.12 Air

Leptospirosis is not an airborne infection and is only exceptionally acquired through the respiratory system. Aerosols from infectious sources (contaminated water sources, mammal urines, urine-humid beds of rodents in animal facilities) can occasionally induce leptospirosis through the mucous membranes of the respiratory tract (WHO, 1999; Levett, 2001). Leptospire were isolated from an air-conditioning plant in New Zealand however, the strains isolated did not demonstrate virulence in susceptible animal models, suggesting that they were not pathogenic leptospire (Ris and Hamel, 1979).

2.3 Persistence

Environmental survival and persistence of virulent *Leptospira* was recognized very early on as key to leptospirosis epidemiology (Noguchi, 1918). Virulent leptospire are thought to multiply in animal hosts and only survive in the environment. However, because of the technical difficulties involved in culturing and identifying *Leptospira* in complex environmental samples, limited information on environmental *Leptospira* survival has been published. Most studies have used microcosms in laboratory experiments to study the survival of pathogenic leptospire under different physico-chemical conditions. In general, limited quantitative data was identified for *Leptospira* persistence or survival in excreta or environmental matrices. Nonetheless, factors likely to increase persistence include coaggregation with different bacteria within biofilms (Vinod Kumar et al., 2015b). Hence, pathogenic *Leptospira* may persist for months in surface waters (Barragan et al., 2011).

One century ago, Noguchi (1918) initially demonstrated both the survival and virulence of pathogenic leptospires for up to one week in drinking water. Later in a more complex experimental design, the influence of temperature and pH were studied, showing a longer survival at neutral to slightly alkaline pH and an optimal temperature around 25-27°C, with temperatures above 60°C killing leptospires in a few seconds (Chang et al., 1948). This same study showed survival of 100 days with the addition of 1% horse serum in sterile tap water. Based on inoculation studies using susceptible animal hosts, the survival and infectivity was found to be maintained in soils for more than 40 days (Smith and Self, 1955). Such evidence, as well that from other as other studies, has built the case that leptospires can survive and remain infective for weeks to months in a favorable environment. However, it must also be noted that bacterial survival is not only environment-dependent, but also strain-dependent and different pathogenic *Leptospira* strains have shown different survival capabilities under the same environmental conditions (Smith and Turner, 1961).

The longest survival reported in nutrient-deprived conditions was reported from an experiment in a mineral bottled water held at 30°C, where a *Leptospira interrogans* serogroup Icterohaemorrhagiae survived and even induced a lethal disease in gerbils after 593 days (Andre-Fontaine et al., 2015).

In natural environments, the isolation of an identical strain five months apart in the same rain puddle suggested its ability to survive in wet soil between rain events (Saito et al., 2013). Similarly, using viability-PCR and genotyping, the *Leptospira interrogans* strain involved in human infections was shown to persist in soils from river edges and sediments where human infections had occurred 9 weeks earlier (Thibeaux et al., 2017). Although repeat contamination of natural areas is possible, there is strong evidence that pathogenic *Leptospira* not only survive, but also remain infective for weeks to months in wet soils and freshwater ecosystems.

Table 6 shows early studies done on persistence more recent data are not available.

Table 6. Persistence of *Leptospira* in different matrices

Area (Year)	Starting Concentrations (Species)	Matrix/ Conditions Temperature (°C)	Maximum Survival in Days	Reference
New York (1918)	Unknown (<i>L. interrogans</i> serogroup Icterohaemorrhagiae)	East river water	<1	Noguchi, 1918
New York (1918)	Unknown (<i>L. interrogans</i> serogroup Icterohaemorrhagiae)	sewage water	<1	Noguchi, 1918
New York (1918)	Unknown (<i>L. interrogans</i> serogroup Icterohaemorrhagiae)	stagnant water	<1	Noguchi, 1918
New York (1918)	Unknown (<i>L. interrogans</i> serogroup Icterohaemorrhagiae)	horse stool emulsion	<1	Noguchi, 1918
New York (1918)	Unknown (<i>L. interrogans</i> serogroup Icterohaemorrhagiae)	sewer filtrate	<1	Noguchi, 1918
New York (1918)	(Pathogenic <i>Leptospira</i> strain Flanders)	non sterile distilled water with few large motile bacilli	3	Noguchi, 1918
New York (1918)	(Pathogenic <i>Leptospira</i> strain Flanders)	drinking water	7	Noguchi, 1918
North Queensland, Brisbane, Australia (1950s)	20 mL of a 7 day culture into soil Pathogenic <i>Leptospira</i> (strain Australis A)	Soil	43 days in soil 3 days in water	Smith and Self, 1955
North Queensland, Brisbane, Australia (1950s)	20 mL of a 7 day culture into soil Pathogenic <i>Leptospira</i> (strain Australis A)	Rain water from soil	15 days in soil 4 days in water	Smith and Self, 1955

3.0 Reductions by Sanitation Management

Very limited data exists on the specific removal efficiencies of leptospires via sanitation management and traditional wastewater treatment processes. Nonetheless, general disinfection methods and physical removal techniques would be considered necessary for the control of Leptospirosis. Adequate surrogates for *Leptospira* removal in engineered systems have not been identified, but traditional fecal indicators (i.e., *Escherichia coli* and Enterococci) have recently been used to assess environmental risk during a leptospirosis outbreak in Israel (Dadon et al., 2018). Sports events in apparently pristine surface waters have been frequently linked to leptospirosis outbreaks (Sejvar et al., 2003), suggesting that coliforms and *E. coli* counts may not be a good indicator of leptospiral risk (Morgan et al., 2002). Therefore, whether they may be reasonable substitutes for evaluating removal techniques remains to be determined. *Campylobacter* spp. could also serve as a surrogate due to it also being a Gram-negative bacterium with a similarly thin and elongated physiology. *Leptospira* is generally considered to be an environmentally-associated pathogen where human infection is strongly linked to flooding events or outside contamination of surface waters from animal urine. Outbreaks of leptospirosis generally do not occur in regions with proper sanitation practices and may be a reason for the lack of data in engineered systems. What published data is available on *Leptospira* in sanitation and related systems is summarized in the following subsections.

Overall, effective rodent control and urine management from various domesticated animals are important aspects in managing leptospires associated with sanitation systems.

Recommendations for a person in a developing region to manage *Leptospira* include:

1. Use chlorine tablets to treat drinking water. If no chlorine is available, heat the water past 50°C for at least one minute.
2. Do not bathe in, or use recreationally, surface waters after flooding events or with known sources of fecal contamination. This is especially regarding individuals with open cuts or sores.
3. Compost manure/digestate if you plan on applying it to land or otherwise (see below section 3.1).
4. Prevent livestock excrement from surfaces waters, outbreaks are traced to animal feces and urine in frequently used waters.
5. Control pests like rats, mice, and other rodents by improving food storage and garbage disposal conditions.

3.1 Wastewater Treatment

3.1.1 On-site waterless sanitation

The reduction of *Leptospira* spp. being introduced into the environment, originating from either human or animal, would help in reducing the frequency of human contact with pathogenic serotypes. Over a third of the world does

not have access to adequate sanitation facilities (WHO, 2006), and open defecation is still a common practice in some of these regions. Because of the direct link between flooding events and outbreaks of Leptospirosis, there is further emphasis made on the physical containment of unabated excreta from entering the environment. During the rainy season, these uncontained wastes allow runoff into surface waters where they collect and allow for transmission to humans downstream. The implementation of basic sanitation infrastructures can vastly aid in the overall reduction and prevention of waterborne pathogenic *Leptospira* transmission.

3.1.1.1 Pit Latrines, vault toilets, dry toilets

The application of basic types of toilets or pit latrines to create physical separation between waste streams and humans would be considered best practice for hygiene and sanitation management of leptospires. The pathogen content of urine is much less than in feces (Höglund et al., 2002), suggesting the prioritization of solids partitioning of human waste if at all else.

3.1.1.2 Composting

Composting is considered an effective, passive, waterless approach to reducing the overall biological activity and concentration of pathogenic bacteria in highly concentrated wastes and has been found to be effective in reducing pathogenic *Leptospira*. The main factors influencing the inactivation of *Leptospira* spp. in sludge and manure composting are the temperature and pH characteristics of the compost. Parker and Walker (2011) simulated the pH and temperature profiles traditionally occurring during composting, evaluating their effect on the survivability of the pathogenic leptospires. Via logistic regression, they concluded that a temperature profile above 45°C for at least 4 hours has the potential to kill leptospires. Thermophilic temperatures >50°C for composting would then have the potential to kill leptospires in a few seconds, given that the entirety of the waste is subjected to comparable temperatures (Chang et al., 1948; Parker and Walker, 2011). Leptospires have a slight favorability towards alkaline pH profiles (Smith and Turner, 1961), but in regards to the heterogeneity of compost matrices, the pH dependency of *Leptospira* survivability seems to dissolve (Parker and Walker, 2011). Composting would be considered a viable and effective solution for the management of leptospires in manure.

3.1.2 Oxidation ditch

Diesch (1971) performed a study simulating an oxidation ditch being fed cattle manure from infected animals at a 1:10 scale laboratory model as well as field sampling. Leptospires were identified via dark-field microscopy in the aerated portion of the ditch for >60 days during summer temperature profiles as compared to 5 days in the anaerobic portion of the sludge. This suggests that available dissolved oxygen is critical to the survival of leptospires in comparable matrices. The study ended before a final contact time could be established for the complete

removal of leptospire from the aerated portion.

3.1.3 Waste stabilization ponds

Lined or unlined waste ponds are used worldwide to passively treat wastewater streams. Depending on the climate of the region, the effluents of these stabilization ponds can vary greatly, with a preference for warmer weather that allows for higher kinetic removal rates (Hickey et al., 1989). Although specific reductions in *Leptospira* concentrations in waste stabilization ponds have not been documented, it can be inferred that the level of dissolved oxygen in these ponds would dictate the efficacy of *Leptospira* reduction. Being that this a passive technique (no mechanical mixing or aeration), the dissolved oxygen content would be minimized in the lower portions of the stratified pond where a reduction of active leptospire would be anticipated.

3.1.4 Wetlands

Wetlands or “constructed wetlands” are artificial environments used to treat either municipal or industrial wastewaters. These systems are designed for the passive reduction of nutrient loadings on the environment by mechanisms already present in a natural ecosystem (nitrification, denitrification, phosphorus removal). These systems are not designed nor intended for pathogen removal and would not be considered a viable alternative. The persistence of leptospire in wet and aerated soils and sediments is well documented.

3.1.5 Aerated lagoons

No reports of *Leptospira* spp. reductions by aerated lagoons were identified.

3.1.6 Wastewater treatment facilities

3.1.6.1 Primary/preliminary treatment

No reports of *Leptospira* spp. reductions by primary/preliminary treatment were identified.

3.1.6.2 Activated sludge

Morphological spirochetes have been found in the activated sludge of treatment facilities in Amsterdam, but specific structural markers of the *Leptospira* genus were not found (Deinema, 1976). Concentrated levels of spirochetes were seen in the interior of aggregated flocs

and as motile cells on the floc surfaces. Being that *Leptospira* is an aerobic and chemoheterotrophic organism, it is inferred that the activated sludge portion of a treatment facility engenders their proliferation.

3.1.6.3 Membrane bioreactors/trickling filters

No reports of *Leptospira* spp. reductions by membrane bioreactors or trickling filters were identified.

3.1.6.4 Anaerobic digestion and biogas

The absence of oxygen, even in otherwise favorable growth conditions, will inhibit the survivability of leptospire in manure and sludge matrices (Diesch, 1971). It can then be inferred that anaerobic digestion of sludges would be a favorable environment for the eradication of infective leptospire. A study done by McGarry and Stainforth (1978) investigated the effect of biogases on the survivability of *Leptospira icterohaemorrhagica*, finding that the spirochetes died within 31 hours after introduction of biogas as compared to 300 hours in the control.

3.2 Disinfection as a Tertiary (or Post Primary) Treatment and Drinking Water

3.2.1 Chlorine disinfection

As a tertiary treatment method for wastewater, chlorine appears to be the most relied upon treatment for the eradication of pathogens, including *Leptospira*, and is the recommended approach by the EPA (USEPA, 1999). Depending on the chlorine demand of the secondary effluent matrix, the recommended dosage range for tertiary treatment is 5-30 mg/L. These values are corroborated by a 1948 study where all leptospire were killed within one minute at a neutral pH and chlorine residual of 3.5 mg/L, or within three minutes at a residual of 1 mg/L (Chang et al., 1948). Unfortunately, specific Ct (concentration x time) values could not be identified.

3.2.2 UV disinfection and natural processes

Leptospire are also considered highly susceptible to UV light and *Leptospira* cultures were killed by direct exposure to sunlight for 1-2 hours in distilled water (Faine et al., 1999). In a study, exposure to UV light (10 J/m²) resulted in inactivation of most pathogenic *Leptospira* strains in contrast to non-pathogenic leptospire which were more resistant to the same UV radiation dose; this study also showed that *Leptospira* is more sensitive to UV radiation than other bacteria such as *E. coli* (Stamm and Charon, 1988).

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