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Investigation of leptospirosis in febrile patients from Manabí communities (Ecuador)

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DEDICATORIA

A mi familia, por motivarme y darme su apoyo incondicional en la realización de mis sueños.

Y a todas esas personas que buscan aprender un poco más acerca del mundo que les rodea y, que aportan con un grano de arena todos los días, para el progreso de la humanidad.

RESUMEN

El objetivo de este estudio fue determinar si la fiebre está asociada a la presencia de ADN de *Leptospira* detectado en suero humano con PCR (Polymerase Chain Reaction). Se extrajo ADN de 576 muestras de suero humano (513 febriles y 63 no febriles) obtenidos entre Febrero del 2014 y Julio del 2015 en la parroquia semi-urbana de Calderón y el cantón Santa Ana en Portoviejo. Para este análisis se utilizó PCR en tiempo real (PCR-RT) seguido de PCR convencional, para amplificar el gen de la subunidad 16S ribosomal (*rrs*) de *Leptospira* sp. Finalmente los amplicones de 331 pares de bases (pb) obtenidos, fueron secuenciados. Se detectó secuencias del gen *rrs* en 2 de 513 (0.5%) de pacientes febriles y 0 de 63 (0.0%) de las muestras de personas no febriles.

Palabras clave: *Leptospira*, fiebre, leptospirosis, zoonosis, PCR

ABSTRACT

The aim of this study was determine whether fever is associated with the presence of leptospiral DNA in human sera detected with Polymerase Chain Reaction (PCR). DNA was extracted from 576 samples of human serum (513 febrile and 63 non-febrile) obtained between February 2014 to July 2015 from semi-urban parishes Calderón and Santa Ana in Portoviejo city. DNA was analyzed first with real time PCR (PCR-RT), followed by conventional PCR to amplify the 16S ribosomal RNA (*rrs*) subunit from *Leptospira* sp. Finally, the 331 base pairs (bp) amplicons obtained were sent to be sequenced. The *rrs* sequences were detected in 2 out of 513 (0.5%) febrile patients and 0 of 63 (0.0%) from non-febrile patients.

Keywords: *Leptospira*, fever, leptospirosis, zoonosis, PCR

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INTRODUCTION

Leptospira sp. (family *Leptospiraceae)* is a highly motile and spiral shaped spirochete (Adler, 2015; Bharti et al., 2003) that has a protoplasmic tubular cell body, coiled helically around two periplasmic flagella, which are responsible for its distinctive corkscrew motility and hooks at both cell ends (Adler, 2015). In order to move, *Leptospira* cells contract and expand producing their clockwise rotation and translating movement (Adler, 2015; Bharti et al., 2003). Furthermore, its distinctive spiral shape is due to its peptidoglycan layer and cytoskeletal proteins (Adler, 2015; Slamti, de Pedro, Guichet, & Picardeau, 2011).

Leptospira species acquire their major energy and carbon sources through beta-oxidation of long chain (>C15) fatty acids usually obtained from the growth medium or from fatty acids located at solid-liquid interfaces in natural environments (Adler, 2015; Henneberry & Cox, 1970). Other main nutritional requirements are ammonium salts, vitamins including vitamin $B_{12,}$ thiamine, biotin (for some strains), phosphate, calcium, magnesium and iron (Stalheim & Wilson, 1964).

Leptospira sp. grows in aerobic or microaerophilic locations, displays cytochrome c, oxidase and catalase activities. Saprophytes and pathogenic leptospires have an optimal growth range *in vitro* of 28-30°C. Whereas saprophytes exhibit growth at low temperatures (11-13°C) pathogenic leptospires grow at 37°C. The optimal pH range for growth is 7.2-7.6 (Adler, 2015; Slamti et al., 2011).

Leptospira spp. are classified in the base of their phenotypic and genotypic

characteristics which in most cases don't coincide (Adler, 2015). The phenotypic classification is defined by resistance to 8-azaguanine and serology, distinct by agglutination after cross-absorption of rabbit antisera to leptospiral antigens (Adler, 2015). Resistance to 8-azaguanine is a characteristic of free living leptospires (*Leptospira biflexa* sensu lato) while parasitic leptospires (*Leptospira interrogans* sensu lato) are sensitive (P N Levett, 2001). Based on serology the parasitic leptospires are divided in over 200 serovars (assembled into 24 serogroups), whereas de saprophytic ones have around 60 serovars (P N Levett, 2001).

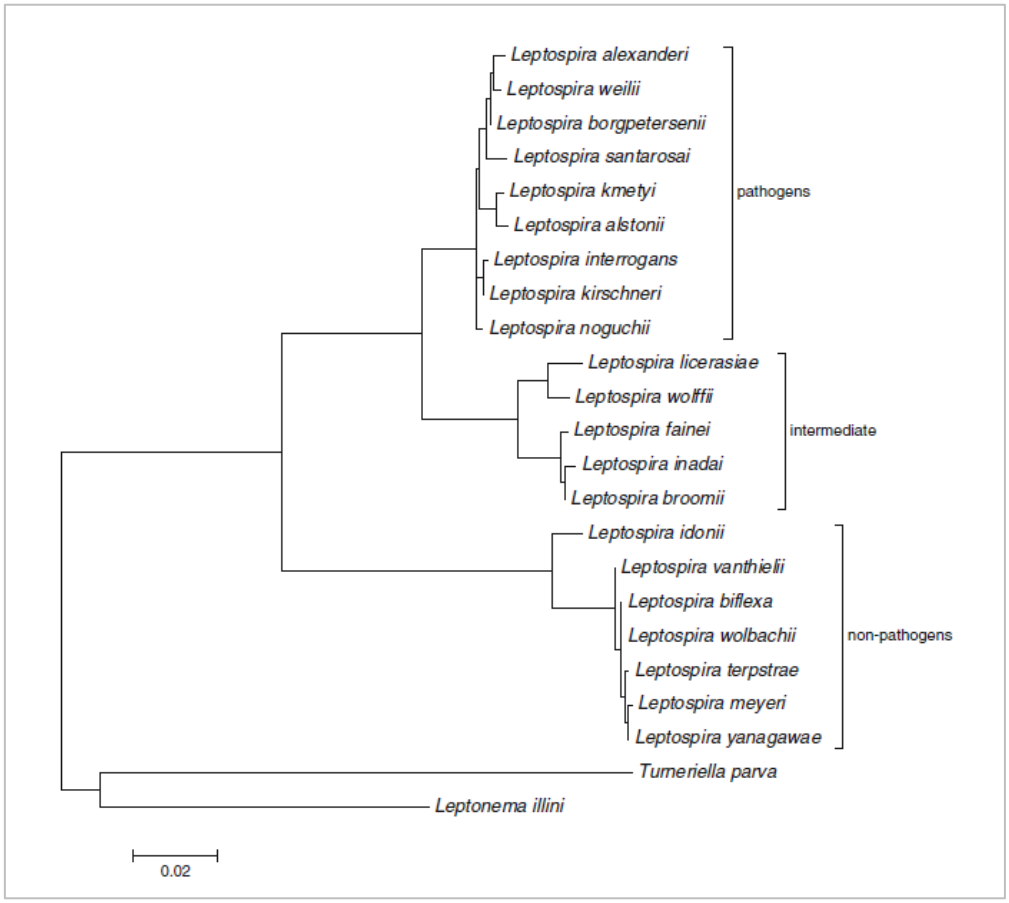

Figure 1.1: "Molecular phylogenetic analysis of Leptospiraceae 16S ribosomal RNA (*rrs*) gene sequences by maximum likelihood method, based on the Tamura-Nei model, using MEGA5" (Adler, 2015)

The second classification is defined by the phylogenetic analysis of leptospiral genes and separates *Leptospira* into more than 21 species which cluster into three groups: pathogens, non-pathogens, and an intermediate group, and as it is displayed in Figure 1.1 (Adler, 2015). In 2014, a new pathogenic *Leptospira* was isolated from patients in Mayotte (Indian Ocean), a new species called *Leptospira mayottensis* (Pascale Bourhy, Collet, Brisse, & Picardeau, 2014). The phenotypic and genotypic classifications do not correspond so it is important to carry out both characterizations (Adler, 2015; Bharti et al., 2003).

Genetic characteristics

Leptospira spp. are characterized by a G+C content of approximately 35-42 mol% with a genome extent ranging between 3.9 to 4.6 Mbp (Baril, Herrmann, Richaud, & Margarita, 1992; Pascale Bourhy, Louvel, Girons, & Picardeau, 2005). The genome is big compared to other spirochetes genomes which may be related to its aptitude to survive in different environments (animal tissues, fresh water and soil) (Barragan et al., 2011; Bharti et al., 2003). The genome of *Leptospira* contains two circular chromosomes *cI* and *cII*; *cI* has 3.6 Mb in size, gene density of 75-92% and encodes most of the protein and housekeeping genes and there are between one and two genes for each rRNA (16S

, 23S, and 5S), disseminated in this chromosome that differentiates *Leptospira* from other bacterial genomes (Baril et al., 1992; Mathieu Picardeau et al., 2008). The chromosome *cII* has 278-350 Kb in size and carries other indispensable genes such as *fltB* (glutamate synthase), *asd* (aspartate semialdehyde dehydrogenase), and *metF* (methylene tetrahydrofolate reductase) which form part of important metabolic pathways (Adler, 2015; Mathieu Picardeau et al., 2008; Zuerner, Herrmann, & Saint

Girons, 1993). *Leptospira biflexa* has a third chromosome-like called chromid p74 (74 kb) and it has plasmid or phage replication machinery (Harrison, Lower, Kim, & Young, 2010).

There is limited information about pathogenic and intermediate *Leptospira* phages and related genomic islands (Adler, 2015). Even though there is no evidence about phages infecting pathogenic *Leptospira*, it was demonstrated that a genomic island can excised from the *L. interrogans* chromosome and form a circular intermediate (P. Bourhy et al., 2007). On the other hand, saprophytes have a fully sequenced and detected phage called LE1 similar to the group A1 in the *Myoviridae* family and, it can replicate as a circular replicon in *L. biflexa* (Pascale Bourhy, Frangeul, et al., 2005).

The genome of *L. interrogans* serovar Lai, and other *L. interrogans* strains have approximately 3,718 coding sequences (CDS). Mutations such as single nucleotide polymorphism (SNP), insertions, and deletions, were identified in 101 genes (2.7% of the entire genome content) (Zhong et al., 2011). Gene redundancy is more frequent in *L. interrogans* in comparison to the non-pathogenic *Leptospira*. The presence of repeated sequences makes them predisposed to recombination, and could be the origin of *Leptospira* diversity (Adler, 2015). In addition, the genome of *L. borgpetersenii* serovar Hardjo is smaller (2,800 CDS), which may be the reason for its inability to survive outside de host (Bulach et al., 2006).

Numerous insertion sequences (IS) have been recognized in *Leptospira* (Zuerner & Huang, 2002). The copy number of these IS elements is different among serovars and between the isolates of a serovar (Adler, 2015). For example, in the genome of

L. borgpetersenii, there are much more IS elements than other species like *L. interrogans* or *L. biflexa* (Bulach et al., 2006). It seems possible then, that the reduction of *L. borgpetersenii* serovar Hardjo genome might be the consequence of genomic deletions or relocations of IS elements (Adler, 2015).

Comparison between proteins of the genomes of leptospires revealed that they have a common backbone of 1,547 proteins (Ricaldi et al., 2012). The non-pathogenic *L. biflexa* has more genes encoding environmental sensing and metabolic proteins in contrast to pathogenic species, possibly because *L. biflexa* can live in diverse environments (Adler, 2015). When the genomes of the pathogens *L. interrogans* and *L. borgpetersenii* and the non-pathogen *L. biflexa* are compared, there are 893 genes related only to pathogenic species (Adler, 2015). Also, 78% of 655 unique proteins with no defined purpose of *L. interrogans*, probably function as exclusive mechanisms of pathogenicity (Adler, 2015). Finally, the intermediate *Leptospira* species, represented by *L. licerasiae* has more proteins and genes linked to pathogens than saprophytes, suggesting that they are more closely related to pathogens (Ricaldi et al., 2012).

Leptospirosis

Leptospirosis is a prevalent and potentially fatal zoonosis caused by *Leptospira* sp. (Reis et al., 2008). This infection ranges in severity from an asymptomatic illness to a violent life-threatening malady (Ashford et al., 2000). Because of its variability, leptospirosis is qualified as a polymorphic human disease (F Merien, Baranton, & Perolat, 1995). In Ecuador, this infection behaves like an endemic disease with sporadic epidemic outbreaks (Espín Jaramillo, 2015; Reis et al., 2008).

Epidemiology.

The occurrence of leptospirosis depends on three factors (Figure 1.2): the environment, the host and the type of leptospiral strain (Adler, 2015). Environmental aspects consist of sanitation, housing, precipitations and possibly physical, chemical and biological changes in fresh water (Adler, 2015; Barragan et al., 2011). Host´s susceptibility depends on age, genetic factors, skin integrity, and whether protective clothing was worn. The means in which the host and the bacteria interact determine the route, exposure and dose of the pathogen (Adler, 2015). There is abundant evidence that different strains or species of pathogenic and intermediate *Leptospira* differ in their ability to cause disease, probably because of differences in virulence factors, motility and ability to survive the immune attack (Adler, 2015; Chiriboga et al., 2015; Ricaldi et al., 2012). The nature of reservoir hosts determines the types of pathogens present in a particular location (Bharti et al., 2003).

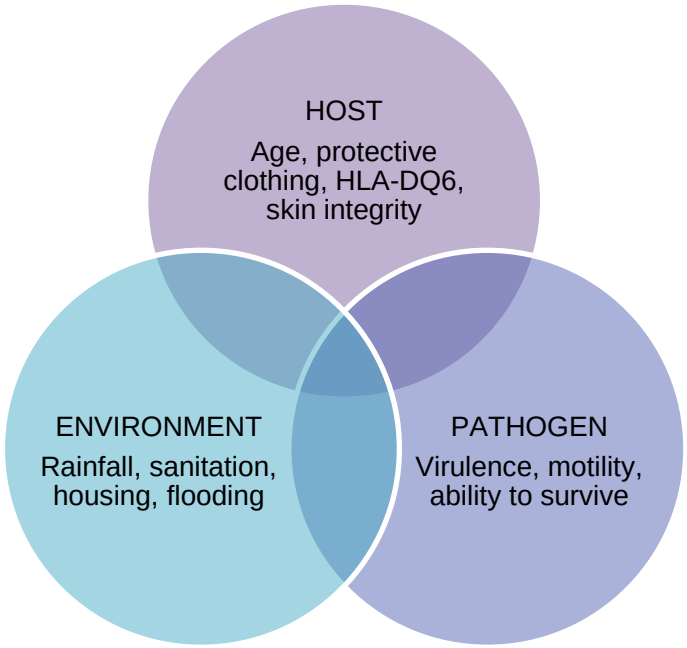

Figure 1.2: Factors contributing to leptospirosis (Adler, 2015)

Leptospires are ubiquitous in the environment, because they are abundant in the kidneys of many wild and domestic reservoir mammals. The leptospiral life cycle comprises shedding in the urine from the animal reservoir, persistence in the environment, new host infection and kidney colonization (Adler, 2015; Bharti et al., 2003). When leptospires reach the renal tubular lumen in the kidney, they invade the brush border of the proximal renal tubular epithelium, from which urinary shedding can continue for long periods of time without affecting its host (Adler, 2015). Depending on the animal host and the leptospiral strain the infection could cause a wide range of clinical outcomes from asymptomatic to hemorrhagic diseases (Rusbridge, Caldow, Crawshaw, & Gunn, 1986).

Even though rats are treated as significant reservoirs, large herbivores are supplementary noteworthy sources of infection (Adler, 2015). A study in the leptospirosis endemic area of Portoviejo (Manabí-Ecuador) (La Hora, 2010; Palma, 2011) suggested cattle and pigs may be more important carriers of leptospirosis than rats during certain periods of time (Sosa Moreno, 2015).

Leptospirosis is mainly a zoonosis (Bulach et al., 2006), nevertheless, human shedding of *Leptospira* sp. does happen through the infection, disseminating the bacteria in the environment also (Adler, 2015).

Cuts, scratches and mucous membranes are the *Leptospira* gateways for entry. Infection may occur upon direct contact with an infected animals or contact with urine contaminated soil or water (Steneroden, Hill, & Salman, 2011). Veterinarians, farm workers, slaughterhouse workers, hunters, animal shelter workers, sewer

maintenance workers, military personnel and similar others are individuals exposed to the disease (Bharti et al., 2003). The extent of the risk hangs on the local prevalence of *Leptospira*, the degree and frequency of exposure (Adler, 2015).

Transmission in Portoviejo.

The most important economic activities of the province of Manabí are agriculture and stockbreeding (El Diario manabita de libre pensamiento, 2012). According to the last population and housing census, 27.63% work in farming activities, and most of this people live in rural areas (El Diario manabita de libre pensamiento, 2012; INEC, 2010). These occupations implicate that people will be exposed to soil or water contaminated with urine of rodents, humans or other animals with *Leptospira* (Adler, 2015; Bovet, Yersin, Merien, Davis, & Perolat, 1999).

In the parishes of Calderón and Santa Ana, direct contact with animals is a common occurrence due to; farmers and slaughterhouse workers do not always use protective gear to avoid contact with blood, urine, and other fluids. Indirect contact with *Leptospira* by contaminated water or soil is much more common because, livestock in Santa Ana y Calderón approach the rivers to drink and usually contaminate them with urine; while, people also use the same rivers as a source of water and for occupational/recreational activities (Adler, 2015; Barragan et al., 2011).

Finally, the rapid growth of poor and disorganized neighborhoods in Portoviejo Ecuador, render deficient sanitary infrastructure, drainage systems, and garbage management (Benacer et al., 2016); heavy rains and flooding and the presence of animal reservoirs (rats, dogs and livestock) increase the risk of exposure to

leptospires in these communities (Adler, 2015; Morikawa et al., 2015; OCHA, 2006).

Diagnosis.

The range of symptoms in leptospirosis is wide, making clinical diagnosis very difficult (Tilahun, Reta, & Simenew, 2013). It is even more challenging in endemic and tropical areas, such as Ecuador, where illnesses with similar symptoms like dengue fever, influenza, malaria, chikungunya fever or zika fever are present as well (Centers for Disease Control and Prevention, 2014; Stoddard, Gee, Wilkins, McCaustland, & Hoffmaster, 2009). The success of the diagnosis depends on the type of sample and the phase of the infection; different tissues (or fuids) contain leptospires at different moments. (See Figure 1.3) (Adler, 2015). The methods for diagnosis in laboratory are serology, culture and PCR.

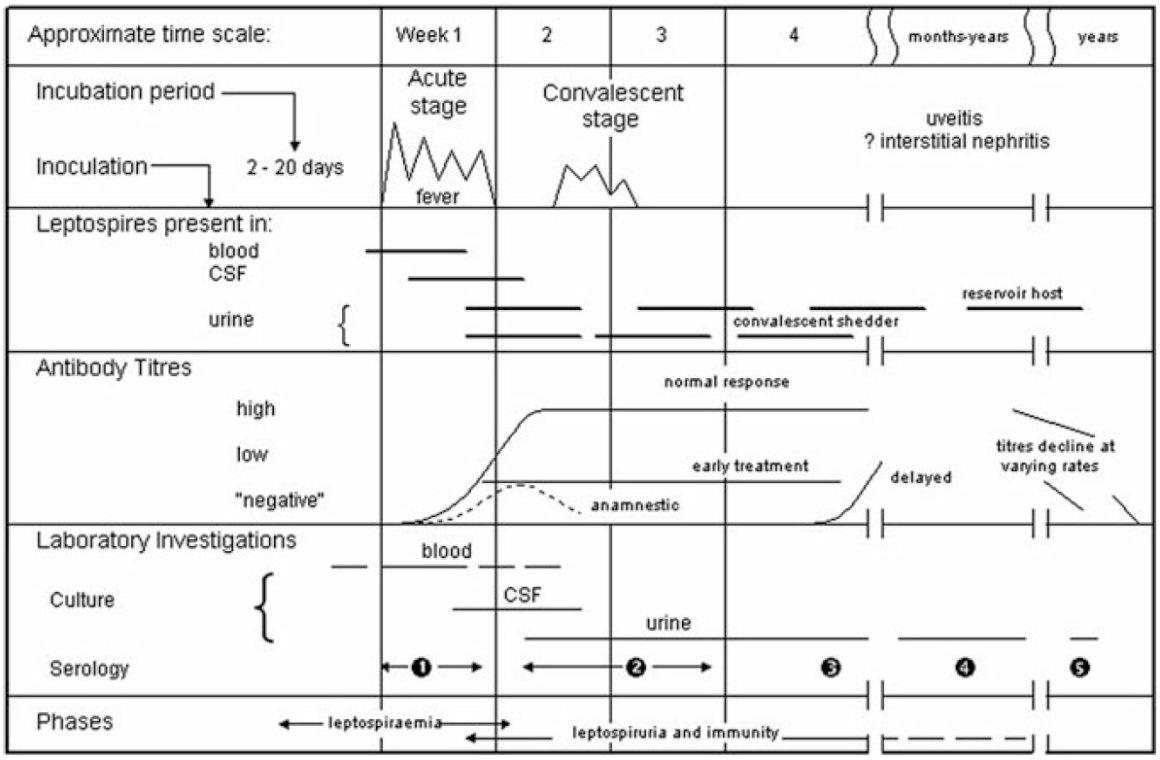

Figure 1.3: Biphasic nature of Leptospirosis and relevant investigations at different stages of the disease (Adler, 2015)

Serological detection.

Serological methods are divided in two, serogroup specific such as the Microscopic Agglutination Test (MAT) and genus specific like the Enzyme-Linked Inmmuno Sorbent Assay (ELISA) (Adler, 2015). The MAT is the international reference standard in serological methods to detect leptospirosis (Vanasco et al., 2016). This test requires two samples of human sera to react with live antigen suspensions of different *Leptospira* serovars, in order to detect rising titers between the samples (Adler, 2015). After incubation, the serum/antigen compounds are examined by dark field microscopy for agglutination and the endpoint is the highest dilution of serum in which 50% of agglutination occurs (Adler, 2015). The advantage of MAT is that is serogroup specific; nevertheless cross reactions between serogroups occur particularly in the acute phase where it detects both IgG and IgM antibodies as it is shown in Figure 1.4 (Adler, 2015; Paul N Levett, 2003; Vanasco et al., 2016). Other disadvantages of MAT are the detection of leptospirosis in the late acute phase when antibiotic treatment is less effective and patients with the fulminant variety of the disease may die before sero-conversion (Adler, 2015; Vanasco et al., 2016). Also, microagglutination is a complicated test because live cultures must be maintained for all the serovars required to use them as antigens and the person who interprets it must be highly experienced (Adler, 2015; Vanasco et al., 2016). It is recommended to use this test only to determine the common serogroups of a population because, it does not detect the infecting serovar (Adler, 2015; Paul N Levett, 2003).

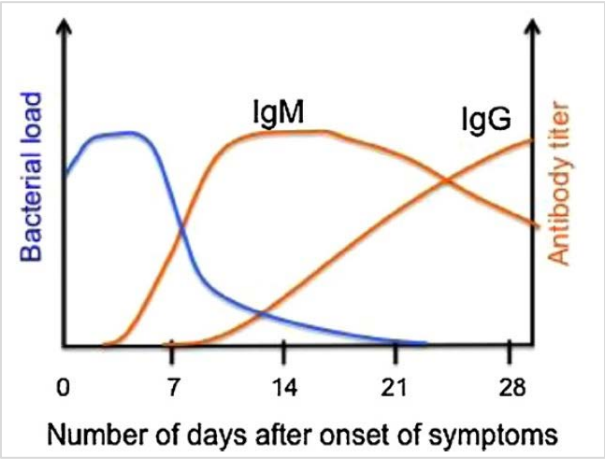

Figure 1.4: Kinetics of Leptospirosis in blood (M. Picardeau, 2013)

The ELISA test has been developed to detect IgM and IgG antibodies of leptospirosis present in human sera (Adler, 2015). ELISA commercial kits, which detect *Leptospira* antibodies, are prepared with cells from *L. biflexa* and *L. interrogans.* ELISA can detect any leptospiral serovar (Adler, 2015; Winslow, Merry, Pirc, & Devine, 1997). The advantage of ELISA is its sensibility and that it can be used in the first week of illness where the IgM antibodies can be detected (Adler, 2015; Vanasco et al., 2016). ELISA is a more accurate test than MAT and it is characterized by its reproducibility providing objective results when it is used (Vanasco et al., 2016). Even though it is an accurate test, in endemic regions the presence of previous antigens of past infection cause cross reactions because *Leptospira* IgM can last for months an even years (Cumberland, Everard, & Levett, 1999; Silva et al., 1995) giving false positive results, that is why is indispensable to test two samples to detect rising titers in an active infection (sero-convertion) (Adler, 2015). Furthermore, ELISA presents a poor performance detection in leptospirosis in endemic areas because a high percentage of the population seems to be seropositive but they are not in a current infection (Desakorn et al., 2012). And finally, ELISA also gives false positive results in the presence of other diseases (Bajani et al., 2003; Desakorn et al., 2012; Winslow et al.,

1997). Nonetheless, serology is still considered the most sensitive method to detects cases of leptospirosis (Adler, 2015).

***Cultivation*.**

Leptospira sp. can be cultivated from blood, peritoneal dialysate and cerebrospinal fluid if they are obtained in the acute stage of the illness (Adler, 2015; Vanasco et al., 2016). Urine should be cultured on the second week of the disease in the convalescent stage because leptospires invade the brush border of the renal tubular epithelium after the leptospiremia phase (see Figure 1.4) (Adler, 2015). The first three fluids must be inoculated in a minimal quantity (three drops) in specialized media for the bacteria and in the case of urine it has to be neutralized first and diluted before inoculation (Adler, 2015). Cultures are incubated in 28°-30°C and checked every week in dark field microscopy for approximately two months (Adler, 2015). Advantages are that the presence of the bacteria is proof of infection. Although, to make a clinic diagnosis this technic has more disadvantages because it is a tedious process that takes too much time that the patient does not have. Also, if the analyst is not careful in the weekly process of examination, the cultures could get contaminated with bacteria and fungi (Adler, 2015). Finally, it is probable that, even if the patient is diagnosed with the illness, *Leptospira* will not developed in the culture (Adler, 2015; Vanasco et al., 2016).

Molecular detection.

To detect *Leptospira* sp. DNA serum, whole blood, urine, aqueous humor, cerebrospinal fluid, and a few organs are used (Adler, 2015). but the best sample to detect leptospiral DNA during febrile episodes may be serum (Agampodi, Matthias,

Moreno, & Vinetz, 2012). However, once immune phase (convalescent stage) of the disease starts; urine may be the most suitable sample (see Figure 1.3). Furthermore, due to the small amount of *Leptospira* present in serum samples, very sensitive diagnostics test are required like PCR-RT (Pascale Bourhy, Bremont, Zinini, Giry, & Picardeau, 2011), and in order to have a better amount of DNA in the samples a second round of PCR amplification should be performed (Chiriboga Miño, 2014; Chiriboga et al., 2015; Sosa Moreno, 2015) so there would be an observable result in the detection of *Leptospira* DNA in electrophoresis and sequencing.

There have been defined many PCR assays which target different genes, divided into two groups: housekeeping genes (*rrs*, *gyrB* or *secY*), and genes associated with pathogenicity (*lipL32*, *lig*, or *lfb1*) (Ahmed, & P. Grobusch, 2012). Most of the PCR-RT tests are designed to identify genes from pathogenic *Leptospira* such as, *lfb1* (Pascale Bourhy et al., 2011), *gyrB* (Slack, Symonds, Dohnt, & Smythe, 2006), *lipL32* (Pascale Bourhy et al., 2011)*, secY* (Ahmed, & P. Grobusch, 2012; Pascale Bourhy et al., 2011) and *ligA*/B (Palaniappan et al., 2005), nevertheless, there are few PCR techniques that have been validated for clinical use and they also use 16S rRNA (*rrs)* and *lip32* to detect the bacteria (Thaipadunpanit et al., 2011; Villumsen et al., 2012). The PCR-RT detection threshold is 10 to 100 leptospires/mL in blood or urine (Pascale Bourhy et al., 2011; Smythe et al., 2002; Stoddard et al., 2009).

The only gene validated to detect *Leptospira* species in general is *rrs* but, unless is used with a TaqMan probe (Smythe et al., 2002) or in a nested PCR previously to PCR-RT (Fabrice Merien et al., 2005), the results may give a high percentage of false positives because some primers detect other bacteria genus from clinical

samples that may be considered as contamination (Chiriboga Miño, 2014; Sosa Moreno, 2015; Villumsen et al., 2012). For this reason, in preview studies the amplicons of most of the genes, in particular *rrs*, are sequenced because it is indispensable to differentiate the *Leptospira* species of anomalous amplification products (Chiriboga et al., 2015).

These PCR procedures could be used to detect leptospiral DNA in blood samples in the acute phase of the disease (Pascale Bourhy et al., 2011), because the bacteria are found in the bloodstream of the patient and could be quicker than other type of laboratory techniques (Agampodi et al., 2012) although, this technique is very expensive and requires equipment, experienced technicians and a laboratory to make it so it is not possible to use it in the most affected areas (Thaipadunpanit et al., 2011). Other disadvantages are that PCR does not identify the species' serovar yet, and the detection of only pathogenic *Leptospira* genes is not identifying the intermediate group which is now the cause of mild infection as some studies had probe (Chiriboga et al., 2015; Schmid et al., 1986; Zakeri et al., 2010).

Artifacts in polymerase chain reactions

Polymerase chain reactions (PCR) have become in molecular biology an indispensable tool to detect DNA from a variety of samples. This technique is very efficient and sensitive, however when it analyzes a heterologous sample, some factors give large bias to the results, causing the detection of artifacts as false positives (Schloss, Gevers, & Westcott, 2011). There are two types of artifacts produced in PCR, called heteroduplexes and chimera (Boers, Hays, & Jansen, 2015;

Kalle, Gulevich, & Rensing, 2013; Kanagawa, 2003; Stevens, Jackson, & Olson, 2013).

First, there are three forms of heteroduplexes which are formed in the stage of annealing: a homoduplex between complementary strands, a heteroduplex caused by the cross-hybridization of heterologous sequences and duplex between primers and templates. Heteroduplexes cause several problems, especially in sequencing where the strand that is being sequenced is either the correct one or the heterologous sequence and the results will be full of noise, giving the appearance of having two types of DNA. *In vivo*, there are repair enzymes that detect the parent strand and eliminate the bad paired sequence, but in PCR, the enzymes choose independently either strand as a template for resynthesize of the complimentary base (Schloss et al., 2011). These heteroduplexes can be identified in the migration of the DNA products in and electrophoresis like an extra band with less weight. These heteroduplexes are formed in significant amounts in the later PCR cycles when the concentration of PCR products in high enough to compete with the primers for annealing (Kanagawa, 2003).

Second, there are two kinds of mechanisms to form chimeras: annealing of an incompletely extended primer and template switching during DNA synthesis. In the first case, an incompletely extended primer can act as a primer in the subsequent PCR cycles. If the incomplete fragment anneals to a different template having a partially homologous sequence, a chimeric product will result from the assay. In the second case, during primer extension, the extending strand switches templates form the original to one which annealed to the original downstream of the priming site and

a chimeric molecule is produced. In both cases, the formation of chimeras occurs in the later cycles of PCR, when the concentration of the incompletely extended primers is high enough to compete with the original primer for annealing (Kanagawa, 2003).

Therefore, to avoid the formation of any type of artifacts or bias the PCR cycles have to be limited to a maximum of 35 cycles, and use specific fluorescent probes (Taqman®) or specific fluorescent primers (SybrGreen®), to monitor the PCR products in real time, when the DNA quantity is low (Kanagawa, 2003) which is the case in *Leptospira* DNA in serum.

Persistent DNA in samples after spirochetes infection

Molecular detection techniques, that use primers such as *rrs*, give an excellent help to diagnosis of a disease caused by fastidious microorganisms, which are difficult to be isolated and cultivated (Branger, Casalta, Habib, Collard, & Raoult, 2003). Nevertheless, false positives can occur particularly in *rrs* primers that detect not only the intended bacteria but also environmental contaminants or amplicon carryover (Branger et al., 2003; Kalle et al., 2013; Thoreson et al., 1999). Another drawback is that these methods do not differentiate between living or dead organisms, and even bacteria killed by ultraviolet light, heat, acid, hydrogen peroxide, drying, starvation, antibiotics and autoclaved cells have given positive results for DNA detection with PCR (Branger et al., 2003; F Merien et al., 1995; Sheridan, Masters, Shallcross, & MacKey, 1998) making difficult to scientist to interpret some obtained results. The half-life of DNA varies depending on the environment which it persist, and there is evidence that DNA maintains for months and even years in some tissues (Branger et al., 2003; Pícha, Moravcová, Va, & Hercogová, 2013).

The persistence of bacterial DNA is currently a matter of intense controversy (Ellis et al., 2014; Pícha et al., 2013; Salo, Jaatinen, Söderström, Viljanen, & Hytönen, 2015; Weis, Yang, Seiler, & Silver, 1997). Few studies have probed that after antibiotic treatment, *Borrelia burgdorferi'* DNA (an spirochete like *Leptospira* sp.), can persist in certain tissues like ligaments, which are not reachable by antibiotics or the immune system, making the infection chronic or relapsing (Häupl et al., 1993). Moreover, another study has found patients positive for spirochete DNA, after 8 to 10 months of treatment with antibiotics, in synovial membrane, but they did not correlate this to the presence of the bacteria but with the possibility of a still active infection because patients were still presenting symptoms (Priem et al., 1998). This has led to the proposal of the "Amber hypothesis" which explains that spirochetes travel through the bloodstream to joint structures like synovial membrane, joint capsule, tendons, ligaments cartilage o menisci where bacterial cells get caught by a collagenous matrix and from there their microbial material is released in a undefined period of time, making the infection seem chronic or relapsing (Wormser, Nadelman, & Schwartz, 2012), but proof of living spirochetes in tissues has not been found (Pícha et al., 2013).

Leptospira and *Leptospira* DNA could present similar behavior. In a study, after giving treatment with antibiotics to patients with leptospirosis, serum samples were taken in the acute and convalescent period to be examined by conventional PCR and dot-blot (F Merien, Amouriaux, Perolat, Baranton, & Girons, 1992; F Merien et al., 1995). Most of the patients cleared the bacteria from their system but it was possible for them to find DNA in a minor percentage until day 56 of infection presenting a

similarity to *B. burgdorferi* (F Merien et al., 1995). Due to this effect, DNA from Leptospires, like other spirochetes, could persist in tissues for long periods of time. There was a case about a man that proved to be positive for *Streptococcus pneumoniae* DNA in heart tissue after seven years of its original treatment (Branger et al., 2003). This DNA was not present in an unreachable tissue like it was mention before, but in a heart valve. The patient was not febrile, and his white blood cell counts were normal and all blood cultures were negative, but DNA was still there. If other DNA bacteria like *S. pneumonia* can persist after long periods of time in human tissue, maybe *Leptospira* DNA could persist too. This becomes a probable consequence in prevalent communities for leptospirosis, such as Portoviejo, because people are exposed to the pathogen constantly and they may have developed “herd immunity” causing asymptomatic infections (Ashford et al., 2000; Johnson et al., 2004) and therefore constant presence of *Leptospira* DNA in blood.

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SCIENTIFIC PAPER

Leptospiral DNA in febrile patients from semi-rural communities in Manabí-Ecuador

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Key Words: *Leptospira*, fever, leptospirosis, zoonosis, PCR

Introduction

Leptospirosis is a neglected potentially fatal zoonosis, caused by intermediate [1-3] or pathogenic [4,5] species of *Leptospira* [2,6,7]. This infection produces diverse clinical signs and symptoms that range from a subclinical to a potentially fatal hemorrhagic disease [4,8]. The real incidence of the disease is unknown because it is underreported [4,9]. The World Health Organization (WHO) estimates that over one million severe human cases per year affect people around the world, and the tropics are the most affected regions, particularly during rainy seasons [2,4,10,11]. Leptospirosis has a worldwide distribution because its transmission occurs in countryside and cities as well [4,12]. Peridomestic rodents are considered the

reservoirs of the bacteria, but large herbivores are an additional significant source of diverse *Leptospira* species contaminating with their urine, rivers and soil [4,5].

Ecuador is the 18th country with the highest incidence of leptospirosis (11.6 cases per million habitants) [13]. Rural communities like Abdón Calderón and Santa Ana in Portoviejo city (Manabí province) are regions of high incidence of leptospirosis [9,13] since their most important economic activity is agriculture and most of the population lives in slums near rivers, where they are in constant contact with the bacteria [5,14]. The cycle of transmission of this disease in Ecuador is still unknown [15,16]. However, there have been outbreaks and reported cases in Portoviejo, diverging from year to year, with no specific pattern [2,9].

The detection of leptospirosis is difficult, particularly in tropical areas [5] such as Ecuador, where infectious diseases with similar symptoms are present as well [9,17]. Nonetheless, the most accurate and quick diagnosis of leptospirosis, in early stages of the disease, is accomplished by molecular detection of leptospiral DNA of the microorganism or its components in body fluids or tissues [5,18,19]. In this study, leptospiral DNA was amplified by real time PCR (PCR-RT) targeting the 16s rRNA gene (*rrs*), from serum samples [2,6]. As a contribution to elucidate the behavior of the pathogen in Ecuador, the aim in this study was to determine whether fever is associated with the presence of leptospiral DNA in serum.

Materials and methods

Study region

The study was conducted in 2 communities in the Manabí province, Ecuador: communities of Abdón Calderón (population ~14,164 inhabitants), and Santa Ana (population ~47,385 inhabitants) [20].

Study design

Between February 2014 and July 2015, a total of 576 (513 febrile and 63 non-febrile) serum samples were collected by field staff in health posts from Santa Ana and Calderón. The study communities were visited 22 times. During each visit, serum samples were collected for every febrile patient that had fever in a 24-hour period or more, plus one or more of the following symptoms: chills, retroocular pain, muscle or articular pain; we excluded patients with intestinal or upper respiratory disease symptoms., who attended the health post. We also used serum samples from non-febrile patients from the same communities whose blood was obtained, by personnel at the same medical post, to study conditions other than infectious diseases.

Leptospiral culture

Three drops from 53 blood samples from random febrile patients were cultured in semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium [5]. Cultures were monitored every week using darkfield microscopy. Contaminated and positive growth cultures were filtered through a 0.22 µm Millipore membrane.

DNA Analysis

Serum samples that were collected from cases and controls were tested for the presence of leptospiral DNA. DNA from each sample was extracted with QIAamp® DNA Blood Mini Kit Qiagen™ and we followed the manufacturer's instructions. Briefly, we took a 1.5 ml microcentrifuge tube and added proteinase K (600 mAU/ml solution), 200µL serum sample and 200µL Buffer AL. Immediately after that, the mix was vortexed and incubated at 56°C for 10 minutes to digest the cells from the sample, which after the digestion, was applied to a QIAamp Mini spin column. The column was centrifuged at 6,000 x *g* and the DNA trapped in the column was washed with buffers 500µL AW1 and 500µL AW2. Finally, the DNA from the column was eluted to a clean tube using 100µL buffer AE [21]. The concentration and quality of the extracted DNA was measured in a NANOVUE spectrophotometer (GE Healthcare Life Sciences). Finally, the product of this extraction was amplified by RT-PCR.

Real time PCR amplification was performed in CFX96 thermal cycler (Biorad, Hercules, USA) in a total reaction volume of 10µL. The reaction mixture included SsoFast™ EvaGreen® Supermix (BioRad, California, USA), 0,5µm of primer A and 0,5µm of primer B [6], and 2µL DNA from each serum sample. The first step was enzyme activation at 95°C for 3 min, followed by 44 amplification cycles (30 sec at 95°C, 30 sec at 62.5°C, 30 sec at 72°C). To detect the presence of A/B *rrs*-gene amplicon, a melting curve was performed and it was considered positive if that sample had a temperature around 79°C or higher. To increase the concentration of the amplicon, 2µL of a 1:10 dilution of the positive PCR-RT reaction mix was subjected to the second round of PCR amplification using the conventional PCR

protocol with the same set of primers [6]. To rule out accidental contamination of PCR reagents, all the reactions included a negative control (purified water for PCR). Amplicons of 81 samples were sent to Functional Biosciences; (http://functionalbio.com/web, Madison, Wisconsin, USA). MEGA 6 and GENEIOUS software's were used to examine the DNA sequences as described in previous studies [2,22-24]. Finally, to define *Leptospira* species was investigated by using BLAST program (National Center for Biotechnology Information). The selection criterion for positive results was a percentage of homology and identity over 99%.

Results

Leptospiral culture

From the fifty-three blood samples cultured from febrile patients only 1 was positive as it is shown in Table 2.2 and 2.4.

DNA Amplification

From the total 576 serum samples (513 from febrile patients and 63 from non-febrile patients), 122 (21.2%) samples were positive for PCR-RT, and 81 from 576 (14.1%) samples were positive for conventional PCR which were sequenced (see Table 2.1). There was only 1 (0.3%) febrile serum from the 567 samples that was positive for the presence of leptospiral DNA (see Table 2.1 and 2.4). From the remaining 80 sequences, 55 contained unreadable sequences and 25 belonged to either soil or gut bacteria as it is shown in Table 2.5.

From the 53 blood samples cultured from febrile patients only 1 (1.9%) was positive (see Table 2.2). The other 52 (98.1%) samples did not showed presence of

Leptospira after two months of cultivation. The DNA extraction was followed by a positive PCR-RT and conventional PCR. Finally, the amplicon of *rrs* gen was sent to be sequenced to identify the *Leptospira* species.

In total, *Leptospira* was detected in 2 out of 513 (0.4%) sera/blood from febrile patients whereas 0 out of 63 in non-febrile patients, as it is shown in Table 2.3

Nucleotide sequences analysis of both amplicons from human sera and the leptospiral isolate in BLAST indicated that there were two pathogenic *Leptospira, L. interrogans* (Accession: KU053947/GI: 952025539) and *L. santarosai* (Accession: HQ709387/GI: 317487630) respectively, in febrile patients as shown in Table 2.4; nevertheless there was no evidence of leptospirosis in sera from non-febrile patients.

Discussion

This study showed the presence of two *Leptospira* species *L. interrogans* (by PCR) and *L.santarosai* (by culture) in blood from febrile patients in two communities of Portoviejo city. These results suggest that more than two species of *Leptospira* could be causing human disease in the same region and during the same period time. These results are similar to other studies, such as Guayaquil (Ecuador) where they found *L. borgpetersenii* and *L.kirschneri* [2], and Mayotte (Indian Ocean) where they found three species of pathogenic *Leptospira* circulating in the same region and during the same period of time [25].

We found low incidence of human leptospirosis in years 2014 and 2015 which is in contrast with a previous study in this region in 2011 [2,26]. These results suggest that

the levels of pathogenic and intermediate *Leptospira* change from year to year in Manabí. This phenomenon may be explained by environmental changes like decreased rainfall which may reduce the presence of *Leptospira* [27,28].

Another reason for the low incidence of *Leptospira* DNA is the period of sampling. For a febrile patient was determined by a person with a 24 hour period or more with fever but the end time of sampling was not established. Not knowing exactly the interval were the samples were taken, there is uncertainty whether there was or not bacteria in blood, since *Leptospira* can only be found in the acute phase of the disease [5].

As in previous studies, the PCR protocol used here produced a large number of spurious products (DNA from environmental and intestinal bacteria) [2,22,26]. Even though these primers are very sensitive for *Leptospira* species detection for pathogenic and intermediate clusters [6,25,29-31], they also amplify DNA sequences from other bacteria [22,26]. It is recommended to change the protocol and make the PCR analysis more specific in the future, with the help of TaqMan or a nested PCR to avoid the spurious PCR products like it has been done in other studies [30,32] allowing therefore to justify the acquisition of more resources for the next *Leptospira* project.

Finally, one of the limitations of this study was the lack of epidemiological variables from patients (such as age, sex, occupation, symptoms, similar others) which were not possible to obtain due to lack of resources and the study design. This data may have provided information about risk factors associated to leptospirosis in this region.

Additionally the low number of leptospirosis cases plus low number of non-febrile samples hampered the possibility to test the association of fever with the presence of leptospiral DNA in sera.

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Author's contributions: Ana María Salinas conducted the molecular tests of the serum samples, extracted DNA from non-febrile serum, cultivated blood samples in EMJH medium, analyzed the DNA sequences and wrote the first manuscript. Gabriel Trueba made de study design, supervised the research project and reviewed the manuscript. Jorge Chiriboga gave training for the molecular analysis, recollected the samples, extracted the DNA from the febrile serum samples and helped with the blood cultures. All authors read and approved the final manuscript.

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Tables and figures

Table 2.1: Results from samples of human serum Febrile and Non-Febrile analyzed for leptospiral DNA collected between 2014 and 2015 in Santa Ana and Calderón communities (Portoviejo city)

Location	Samples Analyzed	Positive for real time PCR	Positive for conventional PCR	Positive for sequencing	Spurious PCR products
	n (%)	n (%)	n (%)	n (%)	n (%)
Calderón	411 (100.0)	71 (17.3)	49 (11.9)	1 (0.2)	48 (11.7)
Santa Ana	165 (100.0)	51 (30.9)	32 (19.4)	0 (0.0)	32 (19.4)
Total	576 (100.0)	122 (21.2)	81 (14.1)	1 (0.2)	80 (13.9)

Table 2.2: Results from culture of human blood samples from febrile patients to detect possible *Leptospira* collected between 2014 and 2015 in Santa Ana and Calderón communities (Portoviejo city)

Location	Samples cultured	Positive for culture	Spurious cultured products	Positive for real time PCR	Positive for conventional PCR	Positive for sequencing
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Calderón	23 (100.0)	1 (4.3)	22 (95.7)	1 (4.3)	1 (4.3)	1 (4.3)
Santa Ana	30 (100.0)	0 (0.0)	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	53 (100.0)	1 (1.9)	52 (98.1)	1 (1.9)	1 (1.9)	1 (1.9)

Table 2.3: Samples of human serum analyzed for Leptospiral DNA collected between 2014 and 2015 in Santa Ana and Calderón communities positive for *Leptospira* DNA. *Sample positive by culture, not by PCR

Patients	**Location**	**Samples n (%)**	**Samples Positive *Leptospira***	
			n (%)	**Group**
Febrile	Calderón	372 (72.5)	2 (0.5)	*L. interrogans* *L.santarosai**
	Santa Ana	141 (27.5)	0 (0.0)	-
	Total	513 (100.0)	2 (0.4)	
Non-febrile	Calderón	39 (61.9)	0 (0.0)	-
	Santa Ana	24 (38.1)	0 (0.0)	-
	Total	63 (100.0)	0 (0.0)	

Table 2.4: *Leptospira* species found in this study

N°	**Method**	**Species Highest Hit Genbank (Short Name)**	**Sequence ID Genbank**	**Sequence Size /Length of aligned nucleotides**	**Homology**	**Identity**
			GI	**nt**	**%**	**%**
1	PCR	*Leptospira interrogans* serovar Icterohaemorrhagiae	952025539	223/223	100	100
2	CULTURE	*Leptospira santarosai* strain Calderon	317487630	328/328	100	100

Table 2.5: Comparison of false positive sequences for *Leptospira* with general bacteria according to BLAST (+Sequences with the highest percentage of similitude/the sequence was amplified but it was not *Leptospira* sp.) (^Sequences with fragments of Leptospira DNA)

			HIGHEST HIT FOR LEPTOSPIRA IDENTITY^					HIGHEST HIT FOR GENERAL BACTERIA+				
N°	SEQUENCE SIZE (nt)	TYPE FEBRILE: 1 NOT FEBRILE: 0	SPECIES HIGHEST HIT GENBANK (SHORT NAME)	SEQUENCE ID GENBANK (GI)	LENGTH OF ALIGNED NUCLEOTIDES (nt)	HOMOLOGY %	IDENTITY %	SPECIES HIGHEST HIT GENBANK (SHORT NAME)	SEQUENCE ID GENBANK (GI)	LENGTH OF ALIGNED NUCLEOTIDES (nt)	HOMOLOGY %	IDENTITY %
1	228	1	*Leptospira terpstrae* serovar Hualin	636559237	112	49	95	Uncultured *Peptoniphilus* sp.	262478448	228	100	98
2	268	1	*Leptospira meyeri* serovar Ranarum	343202656	134	50	94	*Peptoniphilus* sp.	512134443	268	100	99
3	276	1	*Leptospira meyeri* serovar Ranarum	343202656	132	48	93	*Peptoniphilus coxii*	645320324	276	100	99
4	186	1	*Leptospira interrogans* serovar Copenhageni	444304100	87	47	92	Uncultured alpha proteobacterium	371786412	186	100	95
5	220	0	*Leptospira meyeri* serovar Ranarum	343202656	180	82	90	Uncultured Acidobacteria bacterium clone	325148201	220	100	95
6	227	1	*Leptospira licerasiae* serovar Varillal	343205852	129	57	88	*Brevundimonas* sp.	307716224	218	96	100
7	238	1	*Leptospira kmetyi* serovar Malaysia	343200857	112	47	87	Uncultured *Rubritalea* sp.	998644635	119	50	90
8	257	1	*Leptospira interrogans* serovar Lai	444304057	193	75	87	Uncultured delta proteobacterium clone	927354213	257	100	94
9	277	0	*Leptospira biflexa* serovar Patoc	526641927	36	13	86	*Collinsella aerofaciens*	745628433	271	98	86
10	219	0	*Leptospira inadai* serovar Lyme	636559239	184	84	84	Uncultured Acidobacteria bacterium clone	679036703	219	100	96
11	200	0	*Leptospira inadai* serovar Lyme	636559239	162	81	84	Uncultured Acidobacteria bacterium clone	110754206	200	100	99
12	321	1	*Leptospira mayottensis*	961555099	202	63	83	Uncultured candidate division OP10 bacterium clone	156121571	318	99	94
13	222	1	*Leptospira licerasiae* serovar Varillal	343205852	133	60	83	Uncultured Acidobacteriaceae bacterium	672229820	222	100	89
14	225	1	*Leptospira inadai* serovar Lyme	636559239	189	84	83	Uncultured Acidobacteria bacterium clone	679036703	225	100	99
15	330	1	*Leptospira broomii*	343202760	281	85	78	Uncultured actinobacterium clone	110753120	330	100	95
16	333	1	*Leptospira broomii*	343202760	283	85	77	Uncultured *Aciditerrimonas* sp.	409182285	323	97	95
17	240	1	*Leptospira idonii*	631253138	226	94	77	Uncultured Acidobacteria bacterium clone	389547097	233	97	94
18	275	1	*Leptospira interrogans*	636559177	220	80	76	Uncultured *Leptothrix* sp.	308053158	94	34	88
19	198	1	*Leptospira borgpetersenii* serovar Hardjo-bovis	444304102	190	96	76	Uncultured Acidobacteria bacterium clone	375112274	198	100	100
20	232	1	*Leptospira borgpetersenii* serovar Hardjo-bovis	444304102	225	97	76	*Turneriella parva*	444304020	232	100	98
21	233	1	*Leptospira idonii*	631253138	221	95	76	Uncultured Acidobacteriales bacterium	444740135	226	97	92
22	322	1	*Leptospira mayottensis*	961555099	312	97	75	Uncultured candidate division OP10 bacterium clone	156121571	312	97	97
23	238	1	*Leptospira borgpetersenii* serovar Hardjo-bovis	444304102	224	94	75	Uncultured Oxalobacteraceae bacterium clone	283979594	159	67	88
24	241	1	*Leptospira licerasiae* serovar Varillal	343205852	239	99	75	Uncultured actinobacterium clone	110753501	241	100	98
25	278	0	*Leptospira inadai* serovar Lyme	636559239	247	89	69	*Olsenella* sp.	285198779	228	82	91