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Detection of Leptospirosis and Dengue in Patients with Acute Undifferentiated Febrile Illness in the Northern Coast of Ecuador.

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Detection of Leptospirosis and Dengue in Patients with Acute Undifferentiated Febrile Illness in the Northern Coast of Ecuador.

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Contenido

PART I: General introduction.

1. DENGUE VIRUS

1.1. Introduction

Dengue fever is a serious health problem worldwide, the global distribution is estimated to be over 100 countries (Slifka, 2014). In 2009 the World Health Organization (WHO) estimated 50 million cases per year reported in 2009 (WHO W. H., 2009), however recent studies have suggested global infection at closer to 390 million infections per year (Bhatt S., 2013). This phenomenon may be related to the vector´s adaptation to different climates and ecosystems (Gubler, 1998).

The causative agent of dengue fever is dengue virus belonging to the genus *Flavivirus*, family Flaviviridae, there are four dengue serotypes appointed from Dengue-1 to Dengue-4 (Gubler, 1998). These serotypes differ based on the envelope protein (E) and different serotypes vary approximately 30% in the polyprotein (Tuiskunen A., 2013).

Infection with dengue virus may be asymptomatic or present varied symptoms as a flu-like syndrome and correspond to dengue fever (DF), severe cases present coagulopathy, increased vascular fragility and permeability and correspond to dengue hemorrhagic fever (DHF). Some cases progress to hypovolemic shock and result in dengue shock syndrome (DSS) (Martina B., 2009).

1.1. Structure and replication cycle.

The virus consists of a single strand of RNA, approximately 11Kb, this nucleic acid is surrounded by isometric icosahedral nucleocapsid of approximately 30nm in diameter. The envelope around 10nm in diameter and completing a diameter of about 50nm. The virus comprises structural proteins such as core or nucleocapsid protein (C), membrane

associated proteins (M) and envelope proteins (E) (Henchal R., 1990). Proteins come from a single polypeptide precursor. Seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) are products of viral translation. (Fig 3) (Martins S., 2012).

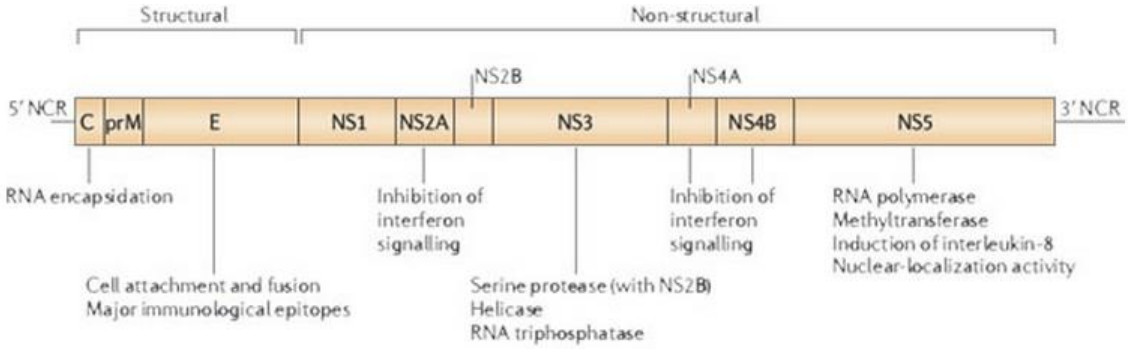

Figure 3: Scheme of dengue virus genome (Vasilakis N., 2011).

The genome consists of two terminal untranslated regions at the 5` and 3` ends of the genome. These have secondary structures that confer different functional structures and have high sequence conservation between different dengue serotypes. The cellcycle of the virus starts with viral attachment to cell surface receptors and entry by endocytosis. Due to the pH of the endosome, viral glycoproteins fuse to the endosome membrane and release viral RNA in the cytoplasm. The viral RNA (as mRNA) is translated directly and produces a polyprotein. Non-structural proteins allow viral genome replication. Virus assembly occurs in the endoplasmic reticulum to form immature particles, where they are carried through the acidic environment of the Golgi network. The prM protein leads to the maturation of the virus and is released from the cell (Tuiskunen A., 2013).

1.2. Epidemiology

It is thought likely that major epidemics occurred in Asia, Africa and North America in 1779 and 1780. However, old records describe cases clinically compatible with dengue in China 610 A.D. An ecological disorganization was observed in the areas of Southeast Asia and Pacific during and after World War II, where appropriate conditions for the transmission of mosquito-borne diseases initiated global pandemic of dengue. In the 1970s cases were observed in the Pacific Islands and subsequently in the Americas.

In the, 1980-1990 period there was a global increase of dengue cases enlargement of the geographical distribution and mosquito populations, and icreases in virus diverstity and disease severity (Gubler, 1998).

Considering the data from 2010, from 96 million dengue infections, 66.8 million were from Asia, 15.7 from Africa and 13.3 million were from Americas (Table1) (Bhatt S., 2013).

Table 1: Estimated burden of dengue in 2010, by continent (Bhatt S., 2013).

	Apparent	**Unapparent**
	Millions (credible interval)	**Millions (credible interval)**
Africa	15.7 (10.5-22.5)	48.4 (34.3-65.2)
Asia	66.8 (47.0-94.4)	204.4 (151.8-273.0)
Americas	13.3 (9.5-18.5)	40.5 (30.5-53.3)
Oceania	0.18 (0.11-0.28)	0.55 (0.35-0.82)
Global	**96 (67.1-135.6)**	**293.9 (217.0-392.3)**

The earliest records of dengue in Ecuador is evidence in 1980, along with Brazil, Bolivia, Paraguay and Peru, explosive epidemics of DENV-1 were presented. After the introduction of DENV-1 major epidemics in Brazil, Ecuador and Peru are recorded (Pinheiro F., 1997). In the years 2000-2010 presented an increase in cases of Dengue in the Americas, with the four serotypes. In 2000 Ecuador presents an outbreak of 22,937 cases with 6,117 cases of dengue hemorrhagic fever (Fig. 1) (Brathwaite Dick O., 2012).

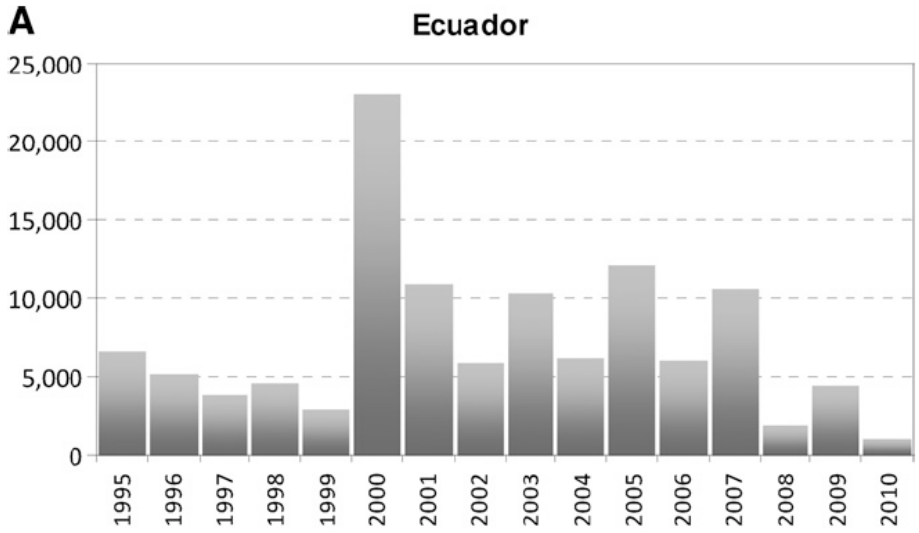

Figure 1: Number of dengue cases Ecuador in 1995–2010 (Brathwaite Dick O., 2012).

In Ecuador, there was a reduction in cases of approximately 27% between 2012 and 2013. In 2013, 11,662 new cases of dengue were confirmed, of which, 10,560 (90.6%) were dengue without warning signs were recorded (defined according to the MSP patients with symptoms of "dengue fever": fever, headache, pain behind the eyes, muscle aches, decay and eventual redness and itching of the skin); 1,042 (9%) were cases of dengue with warning signs (defined according to the MSP patients with symptoms like: severe abdominal pain, difficulty breathing, persistent vomiting, minor bleeding, lethargy or irritability) and 60 (0.52%) cases with severe dengue (defined according to the MSP patients with drop in blood pressure to the shock, fluid accumulation in the lungs, severe bleeding and damage to various organs) (MSP, www.salud.gob.ec, 2013). It should be noted that many asymptomatic cases are not reported and do not appear in the statistics of the Ministry of Public Health.

Currently, cases of dengue are distributed mostly along the Ecuadorian coast. In 2014, the highest number of cases found in the province of Guayas (Fig. 2) (MSP, www.salud.gob.ec, 2014) (MSP, www.salud.gob.ec, 2014).

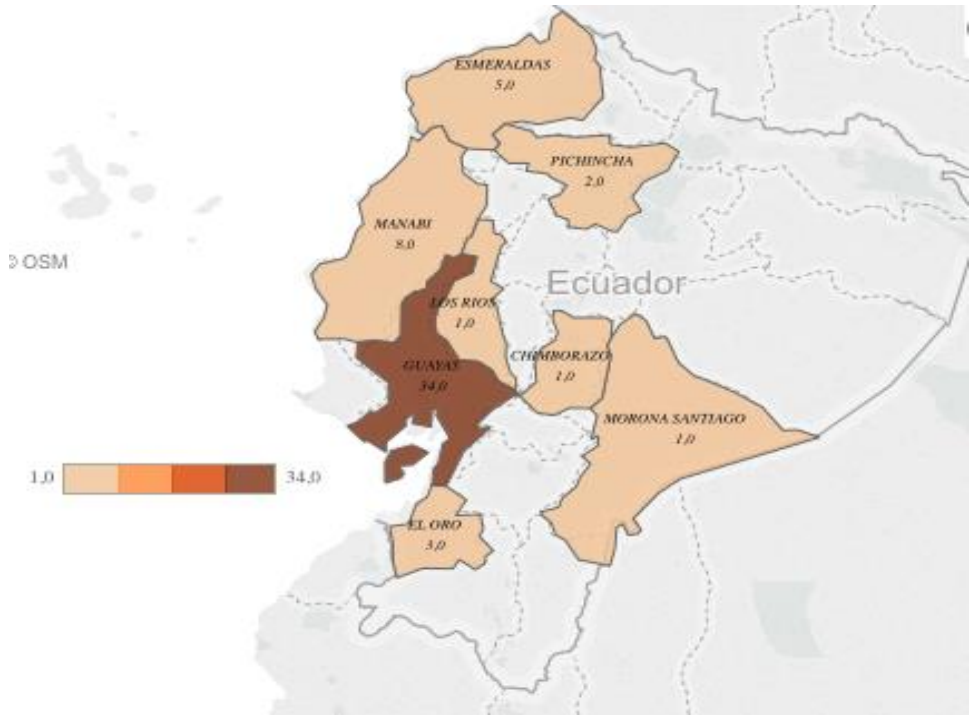

Figure 2: Map of severe dengue distributed by provinces in 2014 (MSP, www.salud.gob.ec, 2014).

1.3. Transmission

Dengue virus (DENV) is transmitted by the bite of the mosquito, *Aedes aegypti*. Cases of dengue fever have been recorded following the bite of *Aedes albopictus* y *Aedes polynesiensis* mosquitoes, the spread of the disease depends very much on the distribution of the vector. In tropical climates the mosquito develops in places where water is stagnant for several weeks, especially after accumulation of rainwater. It has also been observed that eggs can remain viable for several months in the absence of

water (WHO W. H., Prevention and Control of Dengue and Dengue Haemorrhagic Fever, 2009).

After an infected adult female bites a human, the virus is introduced and the symptoms will start after an incubation period of 3-14 days, the disease include fever and nonspecific symptoms. During fever spikes, the virus is circulating in peripheral blood and could be the source of infection of another mosquito feeding on the patient (Gubler, 1998). Once blood is ingested, it reaches the midgut of the mosquito, the virus binds to receptors of the midgut cells where it replicates and the new viruses pass to the hemocoel and spreads to the salivary glands from where it is shed with the saliva during the blood-meals (Carrington L., 2014). Many factors influence a successful transmission of the virus, the level of viremia is a determining factor of virus transmission. It has been observed that patients with higher viral loads have a greater window of infectivity compared to those with low levels of viremia. (Nguyena N., 2013). It is thought that cases 290 million each year are presented in asymptomatic way, suggesting that contribute to the continued transmission of dengue, especially in endemic areas (Carrington L., 2014).

1.2. Pathogenesis and Virulence

Several investigations have been carried out in order to understand why some patients develop classical dengue while others develop a life-threatening hemorrhagic disease. Many studies have shown that pre-existing humoral immunity enhance dengue dissemination. This process is called antibody-dependent enhancement (ADE), it increases the number of infected cells (Flipse J., 2013).

The cell tropism of dengue virus plays an important role in the pathogenesis of the disease. Initially the virus is injected into the epidermis/dermis, therefore the first

infected cells are Langerhans cells (epidermal dendritic cells –DC-) and keratinocytes. These infected cells migrate to lymph nodes, where other cells become infected (monocytes and macrophages). The infection spreads through a wide variety of infected immune cells. Some postmortem studies of patients that died from dengue fever have presence of dengue virus in in skin, liver, lymph node, spleen, kidney, thymus, lung bone marrow and brain. The virus can also be isolated from liver and peripheral blood mononuclear cells. Apoptosis was detected in endothelial cell in intestinal and lung tissues in cases of DHF/DSS. Perhaps this explains profound plasma leakage in peritoneal and pleural cavities (Martina B., 2009).

Different virulence has been associated to dengue serotypes and other genetic differences among dengue viruses. It has been observed that the emergence of new circulating serotypes was associated with severe cases of dengue (Martina B., 2009).

It was possible to observe a relationship between virulence and geography. Based on these cases, it was determined that increases virulence was connected with more pathogenic DENV strains circulating in specific populations, and the ability to infect specific cell types such as macrophages of dendritic cells (DC). For example titers of DENV-2 in human DCs is higher in South East Asian viruses than American ones (Tuiskunen A., 2013) and the ability of DC to interact with the replication of the virus may be associated with the development of DHF or DSS. It has been observed that dengue virus non-structural proteins NS5, NS4B and NS2B-NS3 complex can inhibit type I IFN (cytokine that can be produced by DC infected with dengue), which compromises the innate immune response. Chemokine produced by infected DC can contribute to the plasma leakage and local inflammation and it cans contribute to the vascular leakage with secreted metalloproteinases (Martins S., 2012)

It has been observed that dengue virus influences activation of apoptosis has an important role in the viral life cycle and may contribute to dengue pathogenesis. (Ghosh R., 2014). In summary, virulence may vary according to viral phenotype and host immunity status (Martina B., 2009).

1.3. Host immune factors

The immune response plays an important role in development of the disease in at DH and DSS role. (Tuiskunen A., 2013). Mediators including cytokines tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-2, IL-6, platelet-activating factor (PAF), complement activation products C3a and C5a, and histamine are all thought to be involved in of the severity of the disease. CD41 T lymphocytes produce cytokines such as interferon gamma (IFN-g), IL-2, IL-4, IL-5, IL-6, IL-10, and lymphotoxin. These mediators and cytokines induce the production of other cytokines, which can result in vascular permeability effects (Gubler, 1998).

NSI is thought to activate complement protein and fewer anti NS3 and NS5 protein antibodies. However, the spread of the disease can also thank antibodies, especially when another serotype subsequently infects a previous infection, increasing the risk of serious illness, because as was explained in ADE, these antibodies bind to partially another serotype antigen, rather allowing free access to more cells and increase the infection (Tuiskunen A., 2013).

Dengue antibodies can activate complement fixation as a favorable virions and inhibition of viral infection. However, again this can be detrimental because the increase of complement in the endothelial surfaces may contribute to vascular leak and this action has been related to plasma loss relating complement as a trigger factor for DHF (Tuiskunen A., 2013).

As regards the cellular response is also crucial in the pathogenesis of dengue, similarly that the humoral response is considered protective or deleterious in some cases. The dengue virus can infect both CD4 T cells and CD8, in the case of low affinity for the current infecting virus and high affinity for a previous infection, results in altered between protection and disease pathology balance (Tuiskunen A., 2013).

1.4. Diagnosis

Detection of viral RNA by RT- PCR in whole blood, serum, plasma or cellular material is useful only in the early stages of disease. This technique can be easily developed in comparison with cell culture, however care must be taken in the collection and maintenance of the samples. Detection of anti-DENV antibodies can acts as a tool in places where access technologies such as PCR is not possible and when the patient is no longer viremic. IgM antibodies can be detected in the acute phase of the disease and IgG for several years later. The limitation is false positive results due to cross-react with other flaviviruses, such as patients vaccinated against yellow fever virus (Tuiskunen A., 2013). There are also methods of diagnosis by enzyme-linked immunosorbent assay (ELISA) for detection of the NS1 protein up to 9 days after infection. The amount of NS1 in serum is relative to dengue viremia. It has proven a useful assay in patients where IgM is not detectable or RT-PCR is not available. The sensitivity of this test depends on the time of completion of the test, and whether serotype DENV infection is primary or secondary (Tuiskunen A., 2013).

1.5. Vaccine

There are several risks in the development of a vaccine for dengue, one is that immunity to some dengue virus serotypes may enhances the severity of the disease caused by

other serotypes. The vaccine should be protective for the four serotypes of dengue and should generate an effective immune response to all four serotypes simultaneously. It is important to assess vaccine effectiveness in patients who have been infected with other flaviviruses and those who have not had contact with flavivirus, it is particularly important in areas were dengue and other flaviviruses are endemic (Thisyakorn U, 2014). It was observed that ADE may intervene in the effectiveness of attenuated vaccines interfering especially when increased levels of viremia after immunization. Also has been observed that different levels of neutralizing antibodies against different serotypes are generated. Therefore development of live vaccines is also a challenge for health systems, especially in developing countries, to reach the right dose at the time of vaccination (Ghosh A., 2015). There are currently several challenges for the development of an effective vaccine for all four serotypes, in the following

Currently there are different candidate dengue vaccines: live attenuated virus vaccines, live chimeric virus vaccines, inactivated virus vaccines, live recombinant, viral-vectors DENV antigens, subunit vaccines and DNA. The development of these vaccines is vital to combat the disease whereas vector control has had limited success in reducing the transmission of dengue (Ghosh A., 2015).

Table 2: Current development of vaccines for dengue (Ghosh A., 2015).

Type of vaccine	**Phase of clinical trial**	**Developer/Manufacturer**
Cell culture passage based lived attenuated viruses (Tetravalent vaccine)	II	Walter Reed Army Institute of Research (WRAIR), GlaxoSmithKline Biologicals
Yellow fever-DENV chimeric viruses (Tetravalent vaccine)	III	National Institutes of Health (NIH) and St. Louis University Health Science Centre, Sanofi Pasteur
Mixture of cell culture passage based attenuated virus and dengue-dengue intertypic chimeric viruses (Tetravalent vaccine)	II	Inviragen Inc.
Mixture of targeted mutagenesis	I	National Institute of Allergy and Infectious

based attenuated viruses and dengue-dengue intertypic chimeric viruses (Tetravalent vaccine)		Disease (NIAID), National Institutes of Health (NIH), Bustantan Institute
Purified inactivated dengue vaccine (Tetravalent vaccine)	I	WRAIR, GlaxoSmithKline Biologicals, Oswaldo Cruz Foundation Merck and Co
Recombinant subunit vaccine (Monovalent vaccine)	I	Merck and Co.
DNA vaccine expressing prM and E protein (Monovalent vaccine)	I	Naval Medical Research Centre, Walter Reed Army Institute of Research (WRAIR)

Finally, there are many challenges in developing an effective vaccine for the four serotypes of dengue, and if necessary the existence of a new serotype. Through a series of regulations can be determined one vaccination as a preventive measure and control strategies vectors could resist the annual global burden of dengue (Ghosh A., 2015).

2.1. Treatment

Symptoms of dengue fever are resolved in most cases from 3 to 7 days post infection, however when it comes to severe dengue, which can be a hypovolemic shock or hemorrhagic, patient management includes vasopressors and replacement electrolytes to take care of fluid loss by vascular permeability, and in some cases use of blood products is required. There is a need to develop a therapies so that development of the disease and severe complications are reduced: this can be achieved by treatments or antivirals. However the major challenge is the complexity of dengue, especially in endemic areas (Whitehorn J., 2014).

2. LEPTOSPIRAL INFECTION

2.1. Introduction

Leptospires are spirochetes, 0.1μm by 6 to 0.1 by 20μm. The lipopolysaccharide has a low endotoxic activity. *Leptospira* are aerobic they are catalase and oxidase positive, and grow in laboratory from 28 to 30ºC.

Leptospirosis is a zoonosis distributed worldwide, especially in developing countries. Infection occurs by contact with infected animal urine or water contaminated with urine from domestic or wild animals. The bacterial entry to the host occurs through skin cuts, conjunctives, skin submerged in water for long time. The bacteria is maintained in nature in the renal tubules of animal carriers which are mammals like rats, dogs, etc. There is an occupational risk for leptospirosis in people who are in direct contact with animal or animal tissues. However infection in the urban environment occurs through rodents, especially in developing countries where sanitary infrastructure is inadequate (Levett, 2001).

2.2. Epidemiology

Leptospirosis is common in tropical and subtropical areas, and the presence of the disease has more impact with high rainfall. According to Leptospirosis Burden Epidemiology Reference Group (LERG) at the World Health Organization estimated 500,000 cases of leptospirosis per year. However, the poor surveillance and difficult diagnosis do not estimate a real number of cases (Lehmann J., 2014).

The incidence estimated is 0.1-1 per 100,000 per year in temperate climates and 10-100 per 100,000 in the humid tropics. In high risk groups or during outbreaks the incidence may reach over 100 per 100,000 (WHO W. H., Human leptospirosis : guidance for diagnosis, surveillance and control., 2003)

In Ecuador leptospirosis behaves as an endemic disease with epidemic outbreaks, being observed in urban and rural areas. One of the first reports of *L. noguchi* was in 1918, since it is reported *Leptospira* second time in 1932 (Quinga L., 2010). Outbreaks have occurred in 1998, in 2012 and 2013 (Fig. 4 and 5) (MSP, www.salud.gob.ec, 2014).

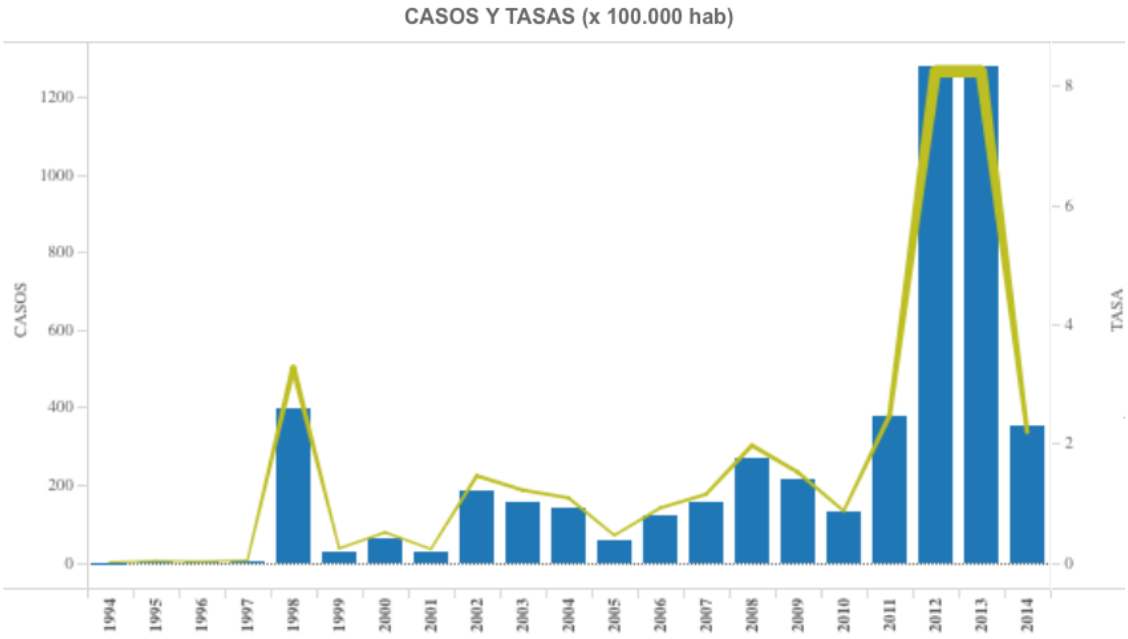

Figure 4: Number of leptospirosis cases Ecuador in 1994–2014 (MSP, www.salud.gob.ec, 2014).

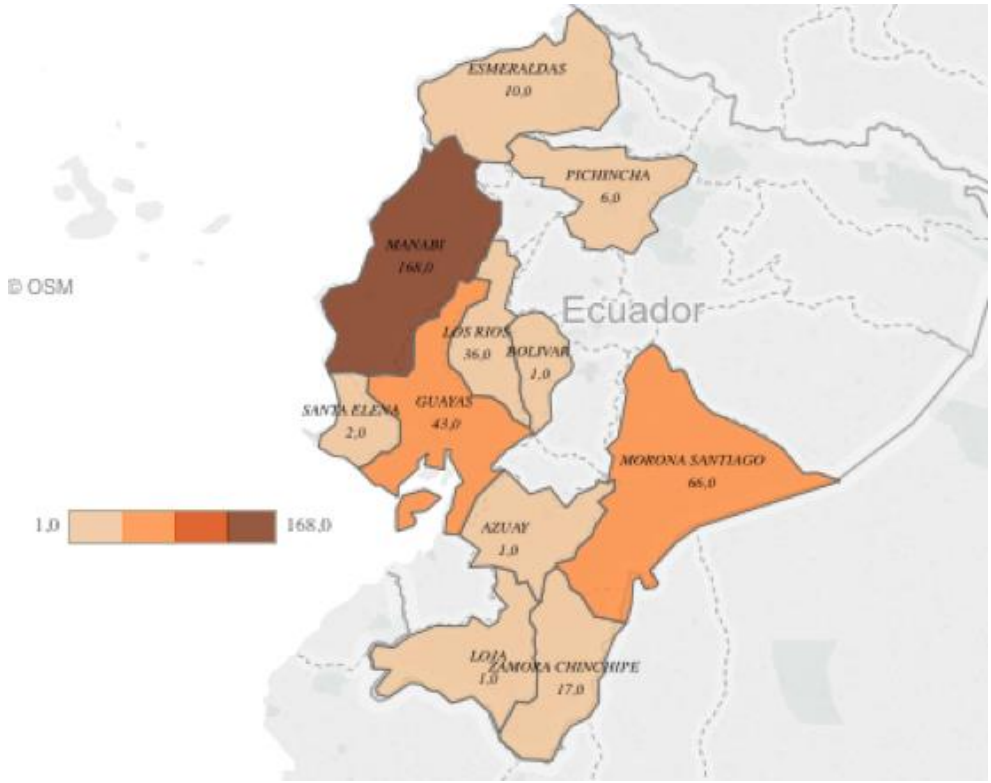

Figure 5: Map of leptospirosis cases by province in 2014 (MSP, www.salud.gob.ec, 2014).

2.3. Diagnosis

The typical symptoms of leptospirosis are fever, chills, headache, abdominal pain, conjunctival effusion and these representing an undifferentiated febrile illness. The most frequent disease is anicteric leptospirosis which shows elevated erythrocyte sedimentation and a small elevation of aminotransferases, bilirubin and alkaline phosphatase. The disease can be fatal with renal failure, jaundice hemorrhage (particularly in the lungs) and vascular collapse. In urine samples can be observed proteinuria, pyuria and microscopically observed hematuria (Lehmann J., 2014).

Leptospira can be observed in dark-field microscopy, but require large numbers of leptospires per optical field in order to be detected. Direct inmunofluorescense can be used in urine, water, soil and blood. Histophatological techniques include immnoperoxidase (immune-histochemistry) and silver stain. During the first 5 days of the disease leptospires can be found in blood (Levett, 2001).

For the detection of antigens was evaluated radioimmunoassay (detection of 10^4 to 10^5 leptospires/ml) and ELISA (detection of 10^5 leptospires/ml). For cultures, samples should be processed as soon as possible, leptospires survive a few days and rarely recover after the initial symptoms. Also we can grow urine from the second week of symptomatic disease (Levett, 2001).

Within 5 to 7 days after the onset of symptoms, the diseases can be diagnosed by serology and the gold standard technique is microscopic agglutination test (MAT); for this technique patient serum reacts with suspensions of different strains of *Leptospira* and examined microscopically for agglutination. This method is complex and requires experienced personnel additionally the maintenance of live cultures involves the risk of

infection. Agglutination results are affected by the culture medium where the antigens grow. There is cross-reactivity (among leptospire serovars) in the acute phase of the disease, and increased specificity during the convalescent phase. The MAT detects both IgM and IgG antibodies. In most places, detection of antileptospiral IgM is carried out by ELISA. Other techniques are less used such as immunoflurescence, RIA, counterimmunoelectrophoresis and thin-layer immunoassay (Levett, 2001).

2.4. Genetic characteristics and microbiological

Leptospira genome size is 5000 kb approximately, two parts comprising the genome, one chromosome size is 4400 kb and one smaller size is 350 kb. *Leptospira* was observed two sets of genes for 16s and 23s RNA and a set of genes for 5s. It also has insertion sequences (IS) encoding transposases (Levett, 2001).

Initially leptospires are grouped into two species *L. interrogans* sensu lato(pathogenic strains) and *L. biflexa* sensu lato (saprophytic strains). Based on agglutination reaction with specific antigens leptospires are divided into numerous serovars, 60 serovars of *L. biflexa* and more than 200 serovars of *L. interrogans* (sensu lato) were identified (Levett, 2001).

Additionally, leptospires are grouped into serogroups all antigenically related serovars and this classification into serogroups are useful for more than taxonomic epidemiological understanding (Levett, 2001).

Based on core genes phylogeny, the genus *Leptospira* in 21 species which form three groups: pathogens, intermediate pathogens and saprophytes; pathogens and intermediates are associated to animal infections (Fig. 6) (Lehmann J., 2014).

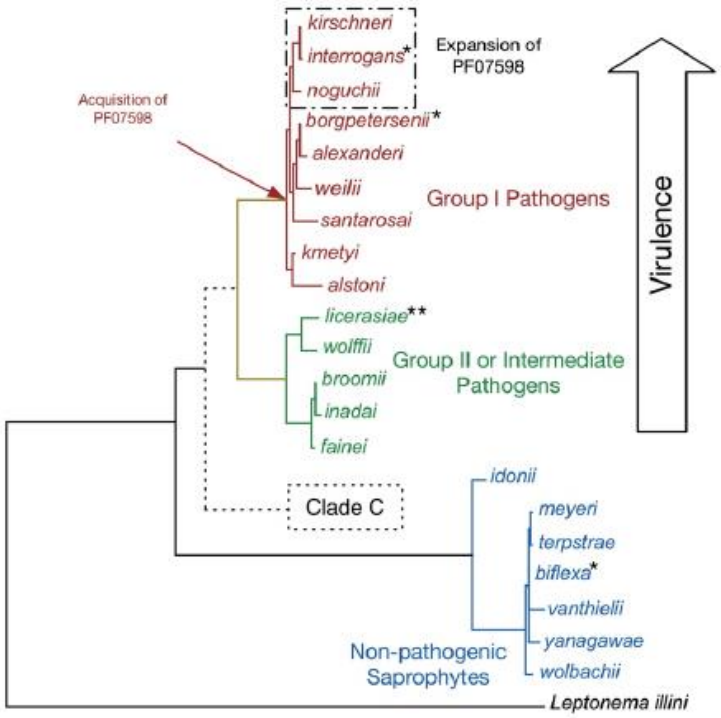

Figure 6: Phylogenetic tree representing the taxonomy of the genus *Leptospira* (Lehmann J., 2014).

2.5. Pathogenesis and virulence

Animal reservoirs are colonized (and may remain infected for life) by *Leptospira* in the renal tubules and this bacterium is excreted via urine to the environment. However, infected animals including humans can remain colonized by *Leptospira* for a few months, even a year or more in the case of pathogenic and intermediate *Leptospira* (Lehmann J., 2014).

The gain or loss of genes are important for understanding virulence and pathogenesis of bacteria. Among the factors that increase the virulence of *Leptospira* are the mobile genetic elements. Some examples are found in *Leptospira*: transposons, prophages, genomic islands, insertion sequence (IS) elements. These elements can encode important molecules for colonization, invasion of niches, use of substrates, or evasion of the host immune response. In *Leptospira* not much is known about the mechanisms of

pathogenicity. Few factors have been associated to virulence such as LPS, flagellum, heme-oxygenase, adhesion molecules and the OmpA-like protein Loa22. Specifically sphingomyelinases and hemolysins have an important role during infection (Lehmann J., 2014).

Leptospira genes acquired through prophage were observed and have an influence on the differentiation of pathogenic leptospires. The prophage LE1 (BB-LiZ_VAR010-LE1) found in *L. licerasiae,* encode an efflux pumps, these proteins are homologous to those found in pathogenic leptospires and are absent in saprophytic leptospires like *L. biflexa*; the presence of these genes may suggest the adaptation to mammalian host (Lehmann J., 2014).

Within elements that are transferred via plasmids found the type II and type III toxin-antitoxin systems (TAS), to which have been attributed various functions ranging from; no functional roles (remaining of chromosomes, transposons and bacteriophages) and functional roles (gene regulation, programmed cell death and anti-phage activity). The mobile genetic elements may be associated with unknown ecological adaptations of *Leptospira*. For example; *L. licerasiae* has three types of unique type III TAS in this genus. *L. interrogans* has five TASs compared to *L. biflexa*, it should be emphasized that these five TASs, four are unique to *L. interrogans* (Lehmann J., 2014).

When analyzing the O-antigen (*rfb*) shows an important idea of the determining factors for adaptation in the host. Understanding the evolution of O-antigen serotypes could clarify emergence and transmission cycles (Lehmann J., 2014).

Genomic comparison between *Leptospira* species show that *L. biflexa* has few transposable elements, and reduced metabolic functions solute transport systems when *L. borgpetersenii*, is compared *L. interrogans*, *L. interrogans* has more signal transduction systems, metabolic genes, transcriptional factors and solute transport

systems, all this be related to survival in the environment, which facilitates transmission (Lehmann J., 2014).

Based on functional content and amino acid sequences shows that *L. licerasiae* (intermedia group) is genetically more related to pathogenic leptospires than saprophytic *Leptospira* (Fig.7) (Lehmann J., 2014).

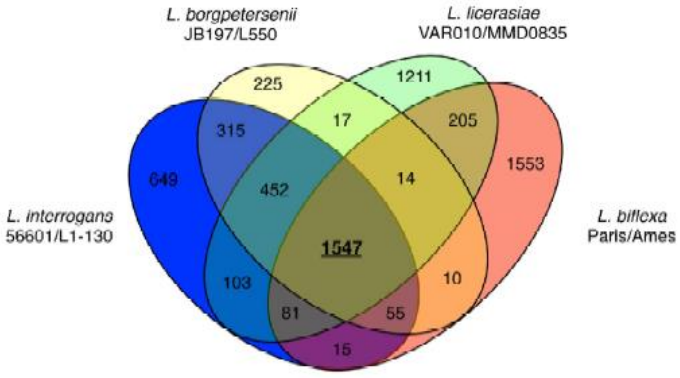

Figure 7: Proteins diagram between different species of *Leptospira* (Lehmann J., 2014).

2.6. Vaccines

Until now there has not been one a vaccine for humans. Some proteins are candidates for vaccine development, for example: proteins of adhesion to I and IV collagens, laminin, fibronectin and fibrinogen. The latter are important in certain bacterial infections, are widely conserved can be important for each candidate vaccine development (Lehmann J., 2014).

2.7. Treatment

The treatment of leptospirosis in the first like-flu symptoms do not require further treatment, however patients with more severe disease, who are jaundiced may require hospitalization. With doxycycline for seven days have been good results. By way of prophylaxis prescription doxycycline 200 mg for a week (Levett, 2001).

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PART II: Detection of Leptospirosis and Dengue in Patients with Acute Undifferentiated Febrile Illness in the Northern Coast of Ecuador.

ABSTRACT

In this study the presence of two important developmental diseases in tropical areas are detected, such as: Dengue and Leptospirosis. A total of 251 was collected in serum and blood spots on filter paper between February 2011 and March 2013. Just over half (52.78%) of serum samples from febrile patients who sought medical attention in local hospital were PCR positive for leptospirosis, 4.62% were positive for dengue alone, 26.86% were positive for both leptospirosis and dengue, and 15.74% were negative for both agents.
Blood samples were obtained from 102 febrile patients were 78,43% positive for Leptospira, 1.96% were PCR positive for both dengue and leptospirosis, 19 60% were negative and no patient was positive for dengue alone.
Our results showed that DENV-1, DENV-2 and DENV-3 serotypes were present in the area. One hundred forty five potential leptospiral amplicons were sequenced, 3 products belonged to pathogenic *Leptospira noguchii*, 136 were identified as *Leptospira wolfii*, and 6 were undetermined.

Keywords: Dengue, intermediate *Leptospira*, ELISA, RT-PCR, qPCR, Serotype.

RESUMEN

En el presente estudio se detectó la presencia de dos enfermedades de importante desarrollo en áreas tropicales, como son: Dengue y Leptospirosis. Se recolectó un total de 251 en suero y manchas de sangre en papel filtro entre febrero de 2011 y marzo de 2013. Poco más de la mitad (52,78%) de las muestras de suero de pacientes febriles que buscaron atención médica en el "Hospital Civil de Borbón", fueron PCR positiva para la leptospirosis, 4,62% fueron positivos para dengue solo, 26,86% fueron positivas tanto para la leptospirosis y el dengue, y 15.74% fueron negativos para ambos agentes. Muestras de sangre en papel filtro de 102 pacientes febriles fueron 78,43% positivos para leptospirosis, 1,96% positivas para el dengue y *Leptospira*, 19,60% fueron negativas para los dos agentes y ningún paciente fue positivo únicamente para el dengue.
Las reacciones de RT-PCR mostró que DENV-1, DENV-2 y DENV-3 serotipos estaban presentes en la zona.
Ciento cuarenta y cinco productos de PCR para Leptospira fueron secuenciados; tres productos pertenecían a *Leptospira noguchii*, 136 fueron identificados como *Leptospira wolfii*.

Palabras clave: Dengue, *Leptospira* intermedia, ELISA, RT-PCR, qPCR, Serotipo.

INTRODUCTION

Dengue fever is a mosquito-borne disease that has rapidly distributed throughout the world. An estimated 50 million dengue infections occur annually and approximately 2,5 billion people live in dengue endemic countries. Dengue fever is caused by one of the four serotypes of dengue virus (serotypes 1-4). Humans, lower primates and *Aedes* mosquitoes are host of dengue virus (McBridea W., 2000).

The history of dengue in Ecuador started in 1988 when DENV 1 was detected for the fist time, in 1990 DENV 2, in 1993 DENV 4 and in 2000 DENV 3 (Alava A, 2005). From 2001 to 2007, the countries present in Andes zone accounted for 19% of all case of dengue in the Americas. It is the subregion with the highest number of dengue hemorrhagic fever (DHF) cases in the Americas (WHO W. H., 2009).

Leptospirosis is a bacterial zoonosis caused by a pathogenic spirochete from the genus *Lesptospira*. The disease is maintained in nature through the chronic asymptomatic renal infection of several mammals and the bacterium is shed in their urine to the environment. Human infection results from either direct contact with the urine of an infected animal or indirectly through contact with contaminated water or soil (Ellis T., 2008). Annually, tens of millions of human cases occur worldwide, with case fatality rates ranging as high as 20% - 25% in some regions (Ganoza C, 2006).

Leptospirosis and dengue often have similar clinical manifestation and are among the most common diagnostic dilemmas. Both typically occur during the rainy season, and rapid laboratory confirmation of the infecting pathogens is not available. Several studies have shown that leptospirosis is often confused with dengue and underdiagnosed in endemic regions (Libraty D., 2007) (Navarrete J., 2006) (Levett P., 2000) (Behera B.,

2012). The aim of this study was to assess the frequency of dengue and leptospirosis in rural communities of the northern coast of Ecuador.

MATERIALS AND METHODS

Febrile patients of a broad range of ages from 49 communities in the northern Coast of Ecuador were part of the research (Figure 1 and 2). All the participants accepted an oral informed consent approved by Institutional Review Board of University of Michigan and bioethics committee of Universidad San Francisco de Quito.

A total of 251 sera (108) and blood spots (143) samples from febrile patients were collected between February 2011 and March 2013, 111 patients (44.22%) were male, 109 (43.42%) were female and 31 (12.35%) were samples in which gender information was not provided. Age distribution of the patients was as follows: 23.9% 0 to 15 years old, 28.29% were 16 to 30 years old, 15.14% were 31 to 45 years old, 8.37% were 46 to 60 years old, 3.59% older than 60 years old and 20.72% no age data was obtained (Figure 3).

Sera were tested by RT-PCR and IgM ELISA rapid test for dengue (Panbio,USA). ELISA was carried out by laboratory personnel at local hospital. While, blood spots samples were analyzed by RT-PCR for dengue only. Both sera and blood spots were analyzed for *Leptospira* by PCR (Table 1 - 2).

The serum samples and blood spots, from febrile patients were collected by the Borbón Hospital laboratory and Health Ministry personnel, respectively. Serum samples were stored at -20ºC, then transported in liquid nitrogen to Quito. Blood spots from febrile patients were collected on filter paper (Whatman 93 Specimen Collection Paper), dried at room temperature and stored at -20ºC in a zipper bag.

RNA extraction

For blood spots, 2-6 punches (6mm diameter) were collected in a 1.5 ml microcentrifuge tube and eluted with 400 µl of RNase free water at 37ºC for 30 min; 250 µl of human serum samples or eluate from blood spots were mixed with 750 µl of Trizol (Ambion by life technologies, Cat. 10296-010) and 200 µl of chloroform and kept at -20ºC for 10 min. The mixture was centrifuged at 4ºC for 15 min at 15000 x g and the aqueous phase (400 µl) was removed and combined with an equal volume of isopropanol to precipitate the RNA. The mixture was kept at -20ºC for 10 min and centrifuged at 4ºC for 25 min at 15000 x g. After centrifugation, the resulting RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 4ºC for 5 min at 15000 x g. The pellet was dried for 1 hour and dissolved in RNase free water (25-30 µl) and stored -80ºC before used. For controls we used cell culture supernatant of DENV-1-4, the RNA extraction was made with 50 µl of supernatant.

DNA extraction

DNA was extracted using QIAamp DNA Mini Kit following instructions from Blood Mini Handbook (QIAGEN, USA), 2-6 blood spots punches were incubated with 180 µl of ATL buffer for 10 minutes at 85ºC, all the liquid was placed in a new 1.5 ml microcentrifuge.

For serum samples 200 µl were centrifuged for 15 min at 15000 x g (Queipo M., 2008), the supernatant was discarded and the pellet was resuspended with 180 µl of buffer ATL.

Eluates from blood spots and the resuspended serum pellet were mixed with 20 µl of proteinase K stock solution and incubate at 56ºC for 3 hours in shaking water bath. Then 200 µl of buffer AL was added and mixed and incubated at 70ºC for 10 min.

Finally, 200 µl of ethanol was added and the mixture was applied to the spin column. Wash steps were performed according to the manufacturer's instructions by using AW1 and AW2 buffers (Quiagen, USA). DNA was eluted with 50 µl of AE buffer and divided in 5 aliquots. Eluted DNAs were stored at -80ºC until use.

DNA Amplification by real-time PCR

The reaction mixture consisted of 5 µl IQ^{TM}SYBR Green supermix, 0,5 uM of each primer AB/CD, 3 µl of DNase-free water (GIBCO), 1 µl of DNA extract, in a final volume of 10 µl. Primer sequences are as follows: A, 5'-GGC GGC GCG TCT TAA ACA TG; B, 5'-TTC CCC CCA TTG AGC AAG ATT; amplification products are 333bp and C, 5'-CAA GTC AAG CGG AGT AGC AA; D 5'-CTT AAC CTG CTG CCT CCC GTA; amplification products are 292bp (Merien F., 1992)

PCR was performed in a CFX96 BioRad Hercules USA termal cycler. The first cycle consisted of initial denaturation at 95°C for 3 min. The next 45 cycles consisted of denaturation at 95°C for 30 s, annealing at 62.5°C for 30s and extension at 72°C for 30s), an additional 30 s was included for final extension. Finally a melting curve was generated by reducing from 65°C to 95°C with a ramp of 0.5°C/5s.

Reverse Transcription and PCR Amplification (RT-PCR)

The reverse transcription reaction contained 12.5 µl of reaction mix 2x (SuperScript III One-Step RT-PCR System, Invitrogen Life Technologies), 0.2 mM of each primer, 4U/µl Taq Platinum (SuperScript®III RT/Platinum®Taq Mix, Invitrogen Life Technologies), 4.2 µl of RNA extract, in a final volume of 25 µl. Primer sequences are as follows: D1, 5'-TCA ATA TGC TGA AAC GCG CGA GAA ACC G; TS1, 5'-CGT CTC AGT GAT CCG GGG G; TS2, 5'-CGC CAC AAG GGC CAT GAA CAG; TS3,

5’-TAA CAT CAT CAT GAG ACA GAG C; and DEN4, 5’-TGT TGT CTT AAA CAA GAG AGG TC (Harris E., 1998).

Reverse transcription was conducted at 52ºC for 1 hour for reverse transcription was followed by 40 cycles of 94ºC for 30 s, 55ºC for 1 min, 72ºC for 2 min, with a final extension at 72ºC for 5 min. The expected sizes of the amplification products are 482 bp (DENV-1), 119 bp (DENV-2), 290 (DENV-3) and 389 (DENV-4) (Harris E., 1998). RNA samples were also tested using a housekeeping by β–actin gene amplification.

DNA Amplification by conventional PCR

Amplification of DNA was performed in a total volume of 25 µl, the reaction mixture consisted of 1.5 mM of $MgCl_2$, 0.1 mM dNTP mix, 1x Colorless GoTaq® Reaction Buffer (Promega, USA), 0.4 µM each oligonucleotide primer AB/CD, 0.02 U/µl of GoTaq® DNA Polymerase (Promega, USA) and 0.2 µl of positive PCR products from real time PCR were submitted for conventional PCR.

The cycle consisted of initial denaturation at 94ºC for 3 min. The next 30 cycles consisted of denaturation at 94°C for 1 min, annealing at 63°C for 1,5 min and extension at 72°C for 2 min, an additional 10 min was included for final extension. The expected PCR products are 333 bp (primer AB) and 292 bp (primer CD).

Visualization of the PCR products was carried out using a 1.6% agarose gel with ethidium bromide (0,2 ul/100ml) under UV light.

Sequencing

Amplicons from conventional PCR were sequenced at Functional Biosciences (Madison, Wisconsin, USA) and analyzed using MEGA5.0 and compared with Basic Local Alignment nucleotide tool (Blast).

RESULTS:

Over half (52.78%) of serum samples from febrile patients who sought medical attention at local hospital were PCR positive for leptospirosis, 4.62 % were positive for dengue alone, 26.86% were positive for both leptospirosis and dengue, and 15.74% were negative for both agents (Table 1).

Blood spot samples were collected from 102 febrile patients, 78.43% were positive for *Leptospira*, 1.96% were PCR positive for both dengue and *Leptospira*, 19.60% were negative and no patient was positive for dengue alone (Table 2).

Serum samples were 28.7% ELISA positive for dengue, 1.9% of these were also positive by RT-PCR, 0.9% were positive by ELISA and RT-PCR, and 68.5% were negative (Table 3). The RT-PCR results showed the presence of three dengue serotypes; DENV-1, DENV-2 and DENV-3, within the sampling period in this part of the north coast (Table 4) (Figure 4).

Of the 108 serum and 102 blood spots samples, 169 tested positive *Leptospira* by PCR, 145 were sent for DNA sequencing (Functional Biosciences, USA), three products showed high homology to pathogenic *Leptospira noguchii*, 136 were identified as *Leptospira wolfii* species, and 6 were undetermined (Table 4).

DISCUSSION:

In the present study more than 80% of the patients were positive for *Leptospira*. Previous studies have reported cases of leptospirosis or dengue in patients with acute

undifferentiated febrile illness in Ecuador and in other parts of the world (Manock, 2009) (Acestor N., 2012). Of all samples submitted to sequencing more than 90% were identified as *Leptospira wolffii*, which is part of a leptospiral group mainly associated with mild febrile disease (Matthias M., 2008).

A smaller number of samples (31.3%) showed evidence of dengue virus infection. This report shows that DENV-1, DENV-2 and DENV-3 were present in the northern coast of Ecuador in 2011, 2012 and 2013. We detected DENV-3, a serotype that has been absent in other parts of Ecuador since 2010; but it was detected in the same region in 2010-2011 (Cifuentes S., 2013). The Colombian Health Ministry has detected DENV-3 during the time of this study in the southern region (Valderrama, 2013). Therefore we speculate that DENV-3 entered Ecuador through the border with Colombia. Human travel plays an important role in the geographic distribution of the disease because mosquitoes tend to fly short distance (Gubler, Dengue and dengue hemorrhagic fever, 1997) (Harrington, 2005) (Getis, 2003) (Reiner R., 2014) (Stoddarda S., 2013).

Identification of DENV-3 on the north coast, highlights the importance of identifying serotypes circulating in our country, especially in border areas. There are studies in which a relationship between the appearance of a new serotype and increased cases of DHF or DSS is observed. The authors argue that the appearance of DENV-3 in Africa and Latin America is related to outbreaks of DHF, it appears that the endemic genotypes of Central and South America cause mild disease, while Asians genotypes produce epidemics of DHF (Messer W., 2003). Lanciotti et al. have been identified geographically distributed DENV-3 subtypes, where DENV-3 subtype III has been found in East Africa, India and Latin America and it has been observed that the appearance of this subtype is associated with cases of DHF (Lanciotti RS, 1994).

In 2001 Ecuadorian government finished building a road linking the Coast of Ecuador with southern Coast of Colombia which may allow travel of dengue patients from Colombia to Ecuador and vice versa. Additionally the northern coast of Ecuador has been subjected to deforestation and intense ecological disruption (Eisenberg J. N. S., 2006). Other studies found that deforestation and road construction influenced the movement of the mosquitoes to remote regions (Vittor AY, 2006) (Dutta P, 1998).

In this study two patients were positive for dengue by RT-PCR and negative to IgM antibodies; RT-PCR is more sensitive to detect early infection of dengue before antibodies were generated. Detection of IgM antibodies sometimes does not indicate acute infection because this immunoglobulin persists for months (Prince HE, 2011), it makes it difficult to distinguish a new infection, and may crossreact with other flaviviruses (Senanayake S., 2006) (Teles F., 2005). In this study, 31/108 patients were positive for IgM dengue antibodies and negative for RT-PCR.

The evidence of co-infection (leptospirosis and dengue) has been reported previously (Levett P., 2000) (Navarrete J., 2006) (Michael G., 2010) (Rele M., 2001) (Crociati L., 2010) (Behera B., 2012) (Kaur H., 2002). In the present study one patient (0,47%) was PCR positive for *Leptospira noguchii* and had anti-dengue IgM antibodies, 28 patients (13,3%) were positive for *Leptospira wolffii* and dengue (IgM or RT-PCR), these results show that co-infections may be common in remote communities. Existence of simultaneous infection has been described in immuno-compromised patients, however it was found to be uncommon in immune-competent individuals (Behera B., 2012).

High presence of leptospirosis in this area may reflect the high reliance on rivers to obtain water, personal hygiene and transportation. Other studies showed that it was possible to identify high-risk areas for leptospirosis based on environmental variables such as deficient sanitary infrastructure, contact with sources of fresh water and flooding risk (Lau C., 2012) (Reis R., 2008).

There was no relationship between start of the rainfall and the onset of dengue cases (Figure 6). A better relationship was found between rainfall and *Leptospira* DNA, in the blood of febrile patients, the highest peaks of cases of leptospirosis were associated with months with heavier rainfall (January to March) (Figure 7). Similar to other studies, leptospirosis cases seemed to peak with rainfall (Chadsuthi S., 2012). Rainy seasons are known to promote *Leptospira* infections due to removal of animal urine from soil and contamination water sources (Wasinski B., 2013). Several studies have found a direct relationship between rainfall and presence of the disease in warm climates (Johansson M., 2009) (Chua K., 2005) (Díaz F., 2008) (Senanayake S., 2006).

In this study, 136 out of 145 febrile patients samples had DNA with high similarity to leptospires of the intermediate cluster (*L. wolffii*) and three had DNA with high similarity to leptospires of pathogenic cluster (*L. noguchii*). Another intermediate species were found to cause febrile disease in humans (Matthias M., 2008) (Slack AT, 2008) (Levett PN, 2006) (Arzouni JP, 2002). In our study we did not find any leptospiral DNA in samples from non-febrile patients suggesting the involvement of intermediate *Leptospira* in fever. Genome association studies are being conducted to identify important genetic differences for the occurrence of species of *Leptospira* in febrile patients (Figure 8) (Lehmann J., 2014) (Slack AT, 2008).

In this study *Leptospira* and dengue seemed to be important etiological agents of fever in northern Ecuador; this findings are in agreement with previous studies (Manock, 2009).

RECOMMENDATIONS

- To run a study with the highest number of febrile and non-febrile patients (controls) in order to determine the prevalence and incidence of *Leptospira* and dengue in Borbón.
- To do molecular studies on dengue genotypes circulating in the area in order to know the origin of DENV-3.
- To run a study in patients, animals and environmental samples to determine the circulating strains of *Leptospira* in northern coast of Ecuador.
- To do geographical epidemiological studies in order to anticipate possible outbreaks of dengue and *Leptospira* in this area.

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TABLES AND FIGURES:

TABLE 1**: Reactivity of serum samples for Dengue and *Leptospira*, from febrile patients*.**

	Positive *Leptospira* (PCR)	Negative *Leptospira*
Dengue positive (IgM or PCR)	29 (26.86%)	5 (4.62%)
Dengue negative	57 (52.87%)	17 (15.74%)
Total	87	21

* Patients from Hospital Civil of Borbón.

TABLE 2**: Reactivity of blood spots for Dengue and *Leptospira*, from febrile patients*.**

	Positive *Leptospira* (PCR)	Negative *Leptospira*
Dengue positive (PCR)	2 (1.96%)	0
Dengue negative	80 (78.43%)	20 (19.60%)
Total	82	20

*Patients who were collected by "SNEM" for malaria diagnostic test.

TABLE 3**: Positive RT-PCR and ELISA dengue cases in serum samples*.**

		RT-PCR		
		Positive	Negative	
ELISA**	Positive	1 (0.9%)	31 (28.7%)	
	Negativa	2 (1.9%)	74 (68.5%)	
				Total 108

*Patiens from Hospital Civil of Borbón.
**ELISA test was carried out by HCB laboratory personnel.

TABLE 4**: Identification of serotypes of Dengue and year.**

	POSITIVE RT-PCR/qPCR	Dengue 1	Dengue 2	Dengue 3	Dengue 4
Borbón Hospital patients	5	0	3 (2012, 2013)	2 (2011, 2013)	0
SNEM patients	2	1 (2012)	0	1 (2012)	0
	7	**1**	**3**	**3**	**0**

Table 5**.- Identification de *Leptospira*.**

Number of samples processed	210	
	Real time PCR positive	193
	Conventional PCR positive	169

Number of samples identifies by sequencing	145	
	Leptospira wolfii	136
	Leptospira noguchii	3
	Undetermined	6

FIGURE 1**: Study sites in the "Cantón Eloy Alfaro" communities and origin of the samples (yellow).**

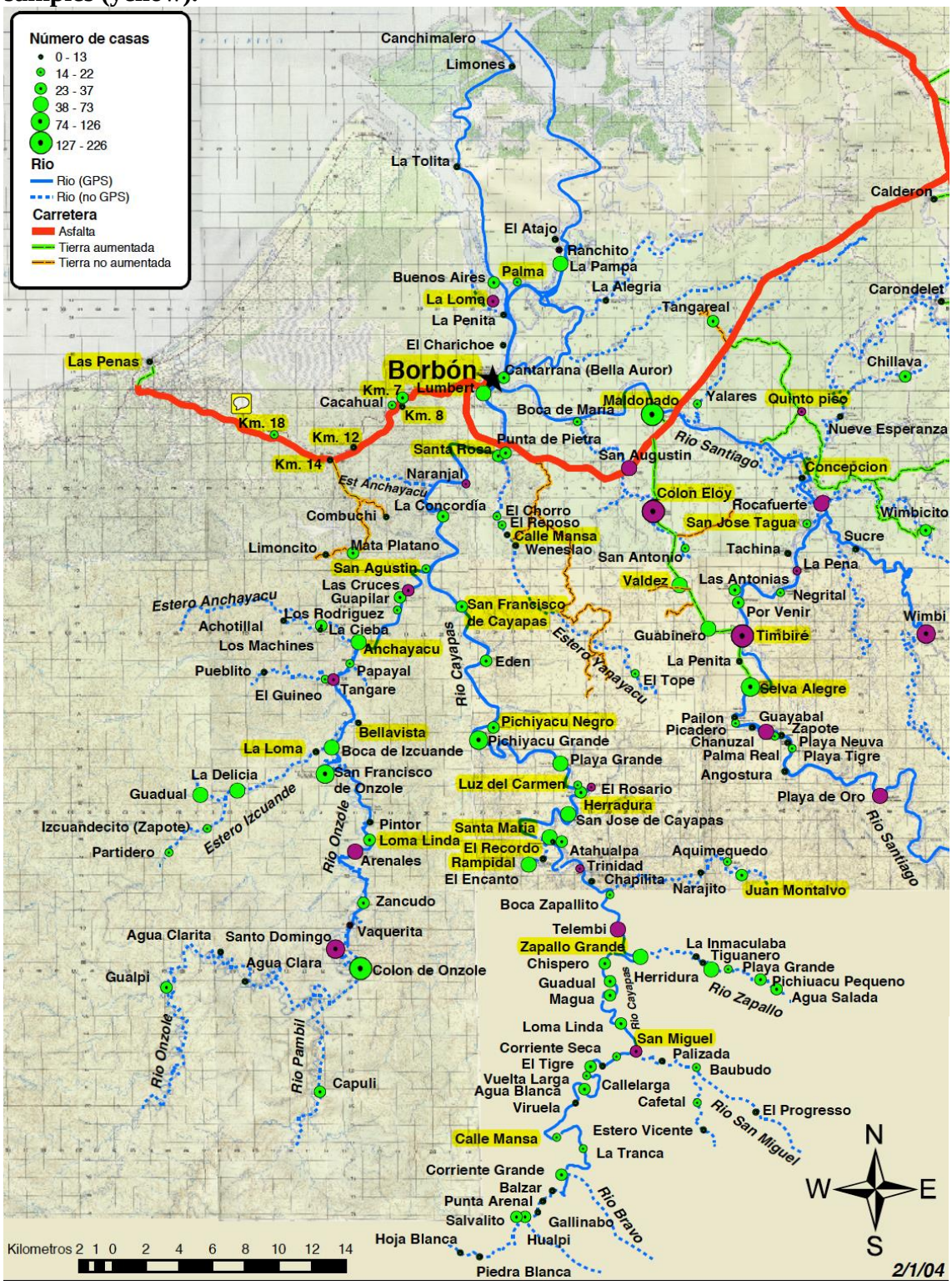

FIGURE 2**: Number of patients for each communities.**

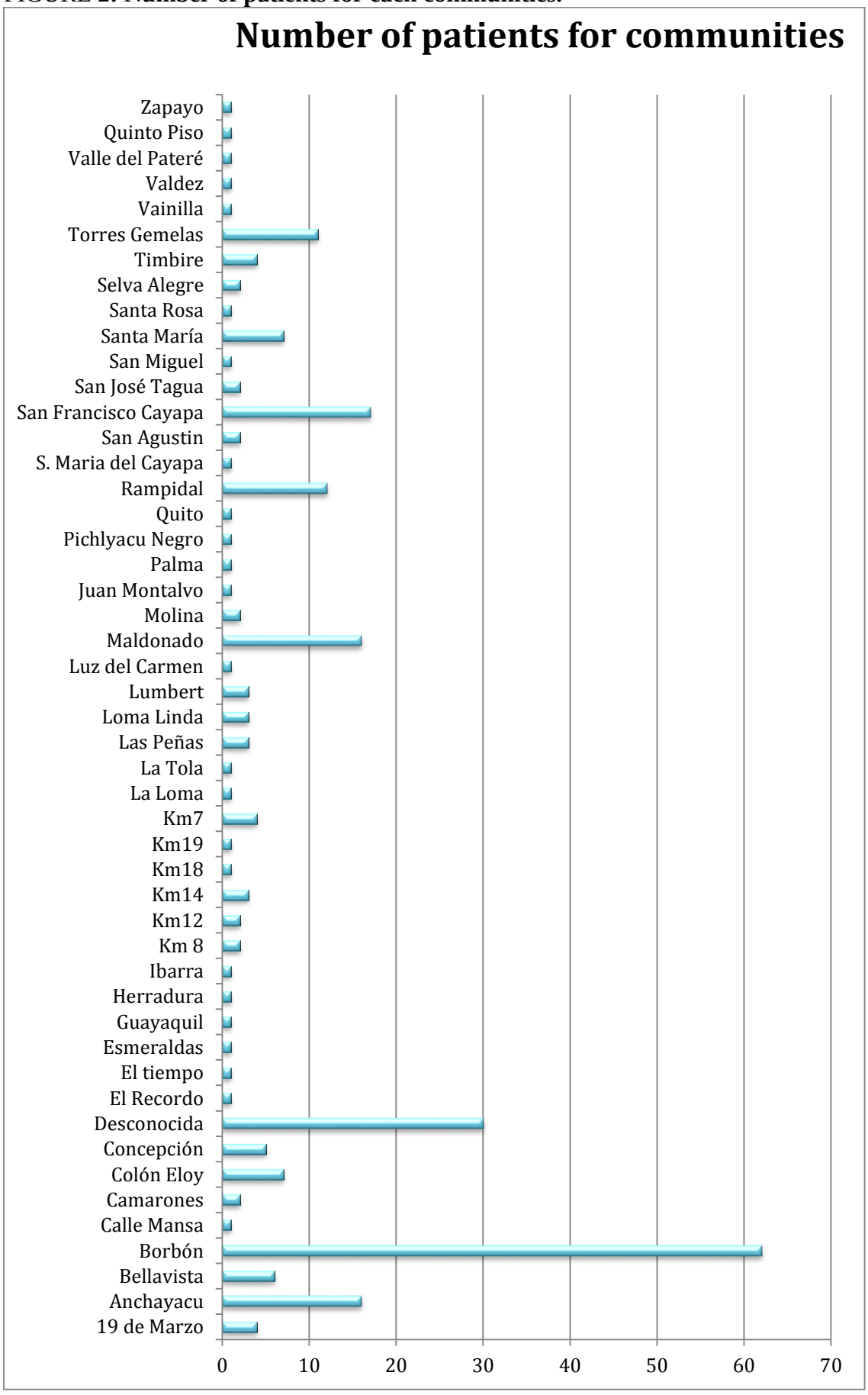

FIGURE 3**: Distribution of age and patients.**

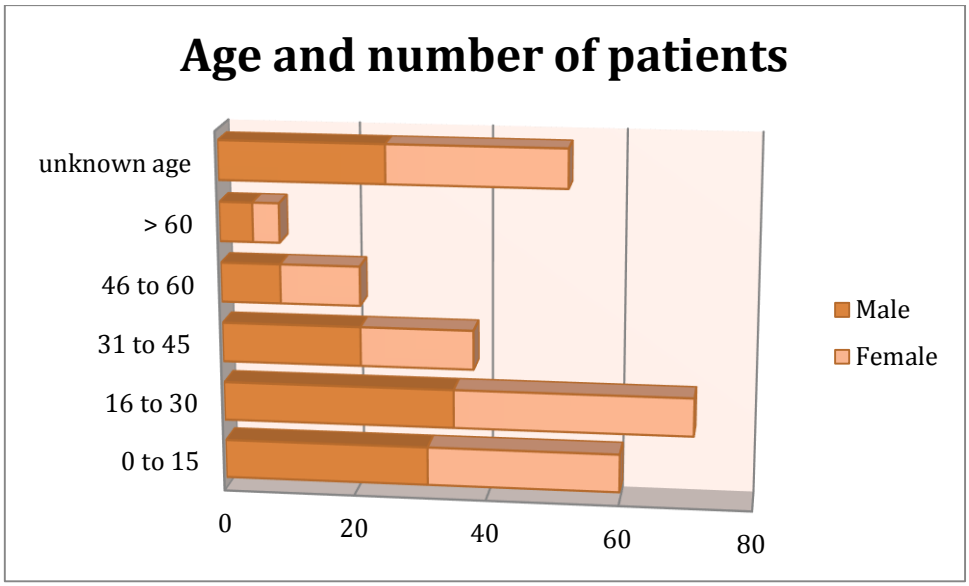

FIGURE 4**: Dengue positive cases (PAMBIO IgM / RT-PCR) in the "Cantón Eloy Alfaro" communities.**

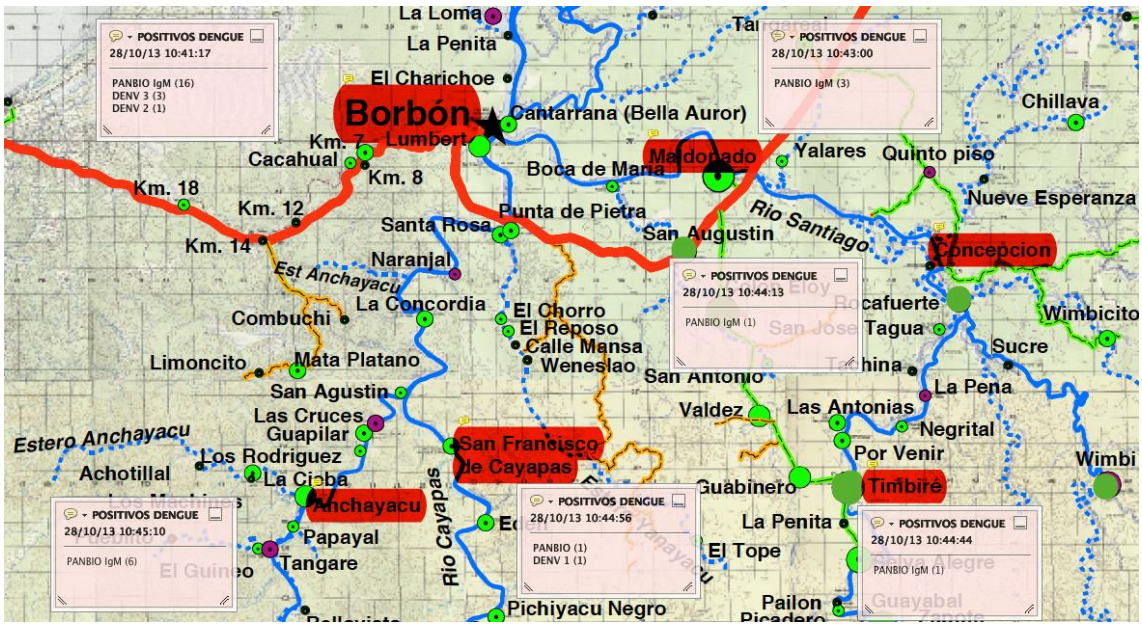

FIGURE 5**: Leptospira positive cases in the "Cantón Eloy Alfaro" communities (red=pathogenic Leptospira and blue= Intermediate Leptospira).**

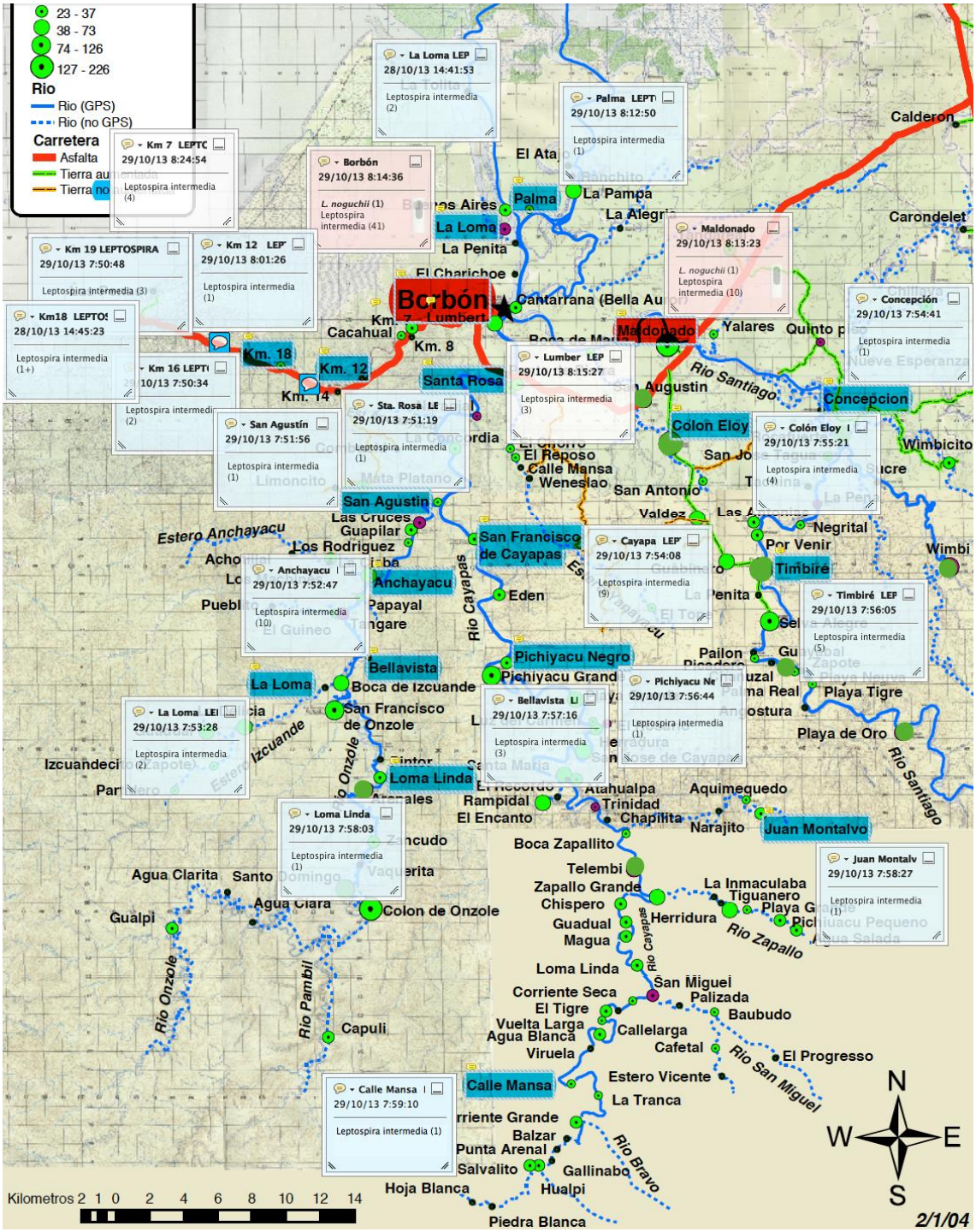

FIGURE 6**: Association between precipitation and cases of dengue during 2011 to 2013.**

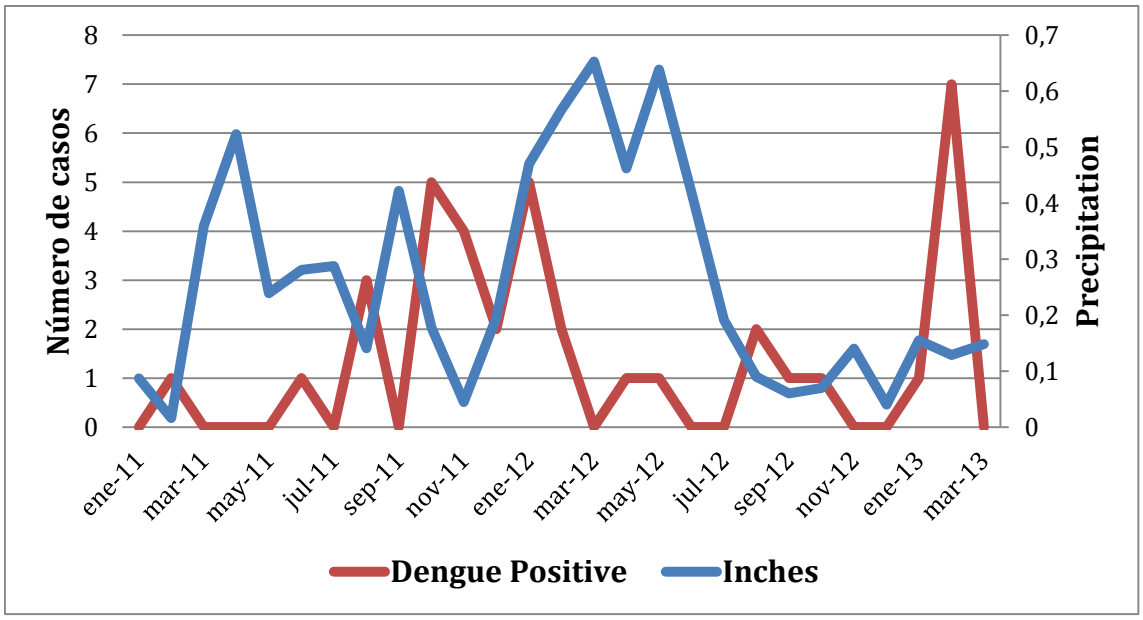

FIGURE 7**: Association between precipitation and cases of leptospirosis during 2011 to 2013**

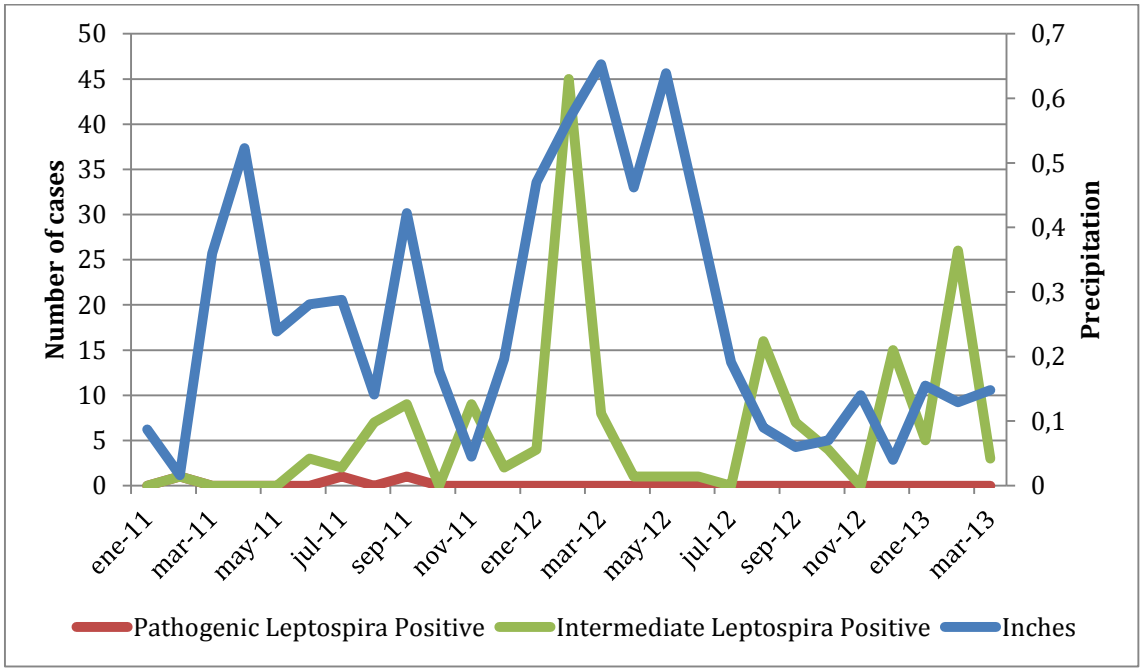

FIGURE 8**: Dendrogram based on 16S rRNA gene, which is shown *Leptospira* found in Esmeraldas, and representative *Leptospira* species. The neighbor-joining method, bootstrap 500 was used.**