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Vigilancia Molecular del Dengue en un Área Remota de la Costa Norte del Ecuador usando Muestras de Suero y Sangre Capilar Tomada en Papel Filtro

Molecular Surveillance of Dengue Fever in a Remote Area of the Northern Coast of Ecuador Using Serum and Capillary Blood Samples on Filter Paper

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**Molecular Surveillance of Dengue Fever in a Remote Area of the
Northern Coast of Ecuador Using Serum and Capillary Blood Samples on
Filter Paper**

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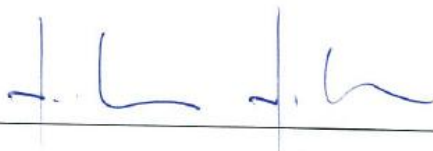
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ABSTRACT

In the last 20 years, dengue has suffered a geographic expansion from urban to rural settings. Currently, the methods for the diagnosis of dengue infections in the field include antibody detections with ELISA rapid test. The objective of this research was to apply a retrotranscriptase polymerase chain reaction (RT-PCR) technique in blood collected from febrile patients for the diagnosis and identification of dengue serotypes circulating in an endemic area in the Northern Coast of Ecuador. Two types of samples were collected and analyzed, serum and capillary blood on filter paper from febrile patients at Hospital Civil de Borbón (HCB) and National Service of Arthropod Borne Diseases Control (SNEM) respectively. A total of 77 samples (36 serum and 41 blood spots samples) were collected from July 2010 to February 2011. Six (17%) serum samples and 7 (17%) blood spots samples were positive for dengue infection by RT-PCR. Nucleotide sequences of the amplicons indicated the presence of DENV-2 and DENV-3 serotypes in these two types of samples. This is the first report of DENV-3 since 2009 in Ecuador. In addition, this is the first time that RNA from blood samples on filter paper has been used successfully to study dengue virus infection in Ecuador.

Keywords: Dengue, ELISA (Enzyme-linked immunosorbent assay), RT-PCR (Polymerase chain reaction- retrotranscriptase), blood spots on filter paper, surveillance, DENV-3.

RESUMEN

En los últimos 20 años el dengue ha sufrido una expansión geográfica desde zonas urbanas a zonas rurales. Actualmente, los métodos para el diagnóstico de infecciones por el virus del dengue en el campo incluyen pruebas rápidas de ELISA. El objetivo de ésta investigación fue aplicar una técnica de laboratorio por la técnica de Reacción en Cadena de la Polimerasa-Retrotranscriptasa (RT- PCR) en muestras de sangre colectadas de pacientes febriles para el diagnóstico e identificación de los serotipos circulantes del dengue en un área endémica en la costa norte del Ecuador. Dos tipos de muestras fueron obtenidas y analizadas, suero y sangre capilar en papel filtro de pacientes febriles del Hospital Civil de Borbón (HCB) y del Servicio Nacional de Control de Enfermedades Transmitidas por Artrópodos (SNEM) respectivamente. Un total de 77 muestras (36 muestras de suero y 41 de sangre capilar) fueron obtenidas entre Julio del 2010 a Febrero del 2011. Seis (17%) muestras de suero y 7 (17%) muestras de sangre capilar fueron positivas para la infección por el virus del dengue por RT-PCR. Las secuencias de nucleótidos de los amplicones indicaron la presencia de DENV-2 y DENV-3 en estos dos tipos de muestras. Este es el primer reporte de DENV-3 desde el 2009 en el Ecuador. Esta es la primera vez que el RNA de muestras de sangre en papel filtro ha sido utilizado exitosamente para estudiar infecciones por el virus del dengue en Ecuador.

Palabras clave: Dengue, ELISA (Enzyme-linked immunosorbent assay), RT-PCR (Polymerase chain reaction-retrotranscriptase), sangre capilar en papel filtro, vigilancia, DENV-3.

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PART I.

DENGUE VIRUS INFECTION

INTRODUCTION

Dengue fever (DF) is a vector borne disease with wide distribution in tropical and subtropical regions around the world. In the last 10 years, the geographic expansion of this disease has been from urban to rural settings (WHO, 2009). Currently, it is estimated that 50-100 million cases of DF occur annually worldwide, and approximately 40% of world population are at risk because they live in dengue endemic areas (WHO, 2009; Stephenson, 2005). Dengue virus (DENV) is member of *Flaviviridae* family, *Flavivirus* genus (Henchal & Putnak, 1990; Martina, Koraka, & Osterhaus, 2009). It consists of a single-stranded RNA genome surrounded by an icosahedral nucleocapsid covered by a lipid envelope, and the complete virion is about 50 nm in diameter (WHO, 2009; Henchal & Putnak, 1990). Four DENV serotypes were established based on neutralization assay data: DENV-1, DENV-2, DENV-3, and DENV-4 (Martina, Koraka, & Osterhaus, 2009). Infection with one of these four serotypes does not provide complete cross-protective immunity; this means that people who are living in a dengue endemic area can become infected with DENV four times in their lifetime (Gubler & Clark, 1995).

DENVS are transmitted among humans by *Aedes* mosquitoes (the only natural mosquito hosts), mainly *Aedes aegypti* and *Aedes albopictus* (Henchal & Putnak, 1990; Martina, Koraka, & Osterhaus, 2009). An important event related with the dissemination of the vector and the virus around the world was the Second World War, it produced economic disruption and human population migration during and after that conflict. DF

became disseminated beyond of common endemic areas (Stephenson, 2005). On the other hand, in the 1950s, 1960s, and most of the 1970s, the American region reported very few cases of DF because Central and South America had eradicated *Aedes aegypti*. However, in the 1970s the eradication program was discontinued, and the vector reached many areas in which the mosquito was not present before (Gluber, 1998). The big changes in the world population and humanity's life styles during the second half of the twentieth century increased the intercontinental movement of people, and decreased the public health measures worsening the situation about the control of this infectious disease (Stephenson, 2005).

EPIDEMIOLOGY

The first epidemics of dengue-like disease were recorded between 1779-80 in Asia, Africa and North America. It was not until the Second World War when the global pandemic of dengue in Southeast Asia began and with that the spread of the disease beyond its usual geographical locations. (Gluber, 1998; Stephenson, 2005). Outbreaks of classic DF occurred in the Caribbean and in the northern part of South America in 1963-64, 1968-69, 1972-75 and 1977-78 (WHO, 1997) Dengue hemorrhagic fever (DHF) was first described in the 1950s in the Philippines and Thailand, but it was not until an outbreak in Cuba in 1981 that DHF became a health problem in the Americas (CDC, 2010). During this epidemic, 344.203 cases of dengue were reported with 158 deaths. After that, other epidemic occurred in Venezuela in 1989 to 1993 where 11.260 cases were reported with 136 deaths. Three DENV serotypes were isolated during these events, DENV-1, 2 and 4 (WHO, 1997). In recent decades, DF/DHF has become a major public health problem in Ecuador. The first dengue epidemic in Ecuador occurred in 1988 and it was caused by DENV-1. DENV-2 was detected two years later,

DENV-4 appeared in 1993 and DENV-3 in the year 2000 (Regato, Mosquera, Coloma, Mosquera, & Alava, 2006). The first cases of DHF were reported in 2001. Year 2001 was the year with the highest level activity of DF ever recorded in the world (Stephenson, 2005). However, according to the information given by the Ministry of Public Health of Ecuador (personal contact), between 1998 and 2010, the peak observed in the major number of cases was in 2000 with 22.958 cases (Aguilar, 2010).

TRANSMISSION

Gluber has described three types of transmission cycles of dengue virus (Gluber, 1998): a) enzootic transmission cycle in the rain forest of Asia and Africa; b) rural epidemic transmission cycle; and c) urban endemic /epidemic cycle, the most important transmission cycle in public health. Different *Aedes (Stegomyia)* spp. may act as a vector in the enzootic and rural cycles; however, *A. aegypti* is the main vector in urban cycles. *A. aegypti* is a highly domesticated tropical mosquito; it lives closer to humans, and prefers to lay its eggs in artificial containers within and around homes. The adult mosquito prefers to rest indoors, this means that people, rather than mosquitoes, spread the virus between communities (WHO, 2009; Gluber, 1998). Female mosquitoes bite preferably 2 to 3 hours after daybreak and several hours before dark. Humans are infected with DENV when a female infective mosquito bites a susceptible person. Then, start the intrinsic incubation period of 3 to 14 days, after which, the person experience fever and other nonspecific symptoms and signs. While a person is in the febrile period, the circulating virus in the peripheral blood may be taken by the biting of other mosquito and start the extrinsic period of 8 to 12 days; after that, this mosquito can transmit the virus to other uninfected person (Gluber, 1998).

DENGUE CASE CLASSIFICATION

Classic DF can result in clinically silent infection and most of patients recover (Stephenson, 2005), it usually occurs as an acute febrile disease characterized by headaches, bone, joint, and muscular pain, rash and leucopenia. Epidemiological studies from endemic areas show that 14%-87% of all dengue infections manifest few or atypical clinical symptoms (Jelinek, et al., 2002). DHF is a potential complication of DF, and may have case fatality rates of 1% or higher, especially in infants and young children (Stephenson, 2005). It is manifested specially by high fever, hemorrhagic phenomena, frequently with hepatomegaly and signs of circulatory failure when the condition is severe. The patients with DHF can develop dengue shock syndrome (DSS) characterized by hypovolemic shock consequential from plasma leakage (WHO, 1997). Halstead found that DHF and DSS were 15–80 times more likely in secondary infections than in primary infections and were positively associated with pre-existing dengue-virus-specific antibodies; this shows an increased immune response (Halstead, 1982).

Traditionally the WHO has classified dengue infections into Dengue fever (DF) and dengue hemorrhagic fever (DHF). *Dengue Fever*: DF is characterized by the presence of acute febrile illness with two or more of the following manifestations, frontal headache, retro-ocular pain, muscle and joint pain, rash and hemorrhagic manifestations and leucopenia. These criteria are in agreement with the WHO and CDC guidelines (WHO, 1997; CDC, 2010). *Dengue Haemorrhagic Fever*: DHF is characterized by the presence of fever (or history of acute fever) lasting two to seven days; hemorrhagic signs (positive "Tourniquet

test"¹, petechiae, ecchymoses or purpura; bleeding from oral mucosa and gastrointestinal tract (haematemesis or melena), or other locations; thrombocytopenia (100 000 cells per mm³ or less); evidence of plasma leakage manifested by elevated haematocrit ($\geq 20\%$ above average for sex, age and population), serous effusion (pleural effusion, ascites and hypoproteinaemia. When DHF is accompanied of circulatory failure manifested by rapid and weak pulse, narrow pulse pressure (<20 mm Hg) or hypotension, cold skin, and restlessness the patient will be diagnosed of DSS (WHO, 1997). However, changes in the epidemiology of dengue have lead to problems with the use of the existing WHO classification (WHO, 2009). A systematic review (Bandyopadhyay, Lum, & Kroeger, 2006) has suggested that the current WHO case classification system should be reviewed and if necessary modified owing to difficulties in applying the criteria for DHF in the clinical situation, together with the increase in clinically severe dengue cases which did not fulfill the strict criteria of DHF.

According to a group of expert, the new suggested dengue classification is as: *non-severe* (with warning signs or without warning signs) and *severe dengue* (WHO, 2009). A probable dengue case is identified when a subjects presents a febrile disease with two or more of the following criteria: nausea, vomiting, rash, aches and pains, a positive “Tourniquet test”, leucopenia and any warning signs (abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, restlessness, liver enlargement and a laboratory test showing increase in hematocrit levels concurrent with rapid decrease in platelet count). Confirmation of dengue infection is done by laboratory diagnosis. Severe dengue is considered when the patient presents the following signs and symptoms: a) *severe plasma*

¹ **Positive Tourniquet test.** It is a clinical diagnostic method to assess fragility of capillary walls showing the presence of thrombocytopenia. A blood pressure cuffs is applied on the upper arm and inflated to a point between the systolic and diastolic blood pressures for five minutes. The test is positive if there are more than 20 petechiae per 2.5 cm area square are observed (Teixeira & Barreto, 2009; Cao, et al., 2002).

leakage that could lead to a Dengue Shock Syndrome (DSS) or a respiratory distress; b) *severe bleeding* and c) *severe organ involvement* (WHO, 2009; Teixeira & Barreto, 2009). However, currently the old WHO classification into DF/DHF/DSS continues to be widely used (WHO, 2009).

LABORATORY DIAGNOSIS

Dengue infection confirmatory diagnosis require three types of procedures: a) isolation of the virus, b) identification of the presence of dengue antibodies in serum, and c) identification of the presence of viral antigen or nucleic acids (RNA) in the serum or tissue (WHO, 2009; WHO, 2011). Techniques for virus isolation, RNA or antigen detection can be used for 4 to 5 days after the onset of the illness because the virus is circulating in peripheral blood. Once the acute phase of infection has finished, serology is the method of choice for diagnosis (WHO, 2011).

Lindergren et al., demonstrated that the combination of serology method as IgM ELISA and nucleic acid detection method as RT-PCR assays, dengue diagnosis can be identified in as many as 85% of single acute-phase samples (Lindergren, Vene, Lundkvist, & Falk, 2005)

SURVEILLANCE

The management of a dengue epidemic is carried out by the implementation of effective strategies involving prevention, control and timely response when emerge new dengue cases. These strategies are part of dengue surveillance system which must involve both epidemiological surveillance and entomological surveillance (WHO, 2009).

Epidemiological Surveillance

This type of surveillance involves an ongoing systematic collection, recording, analysis, interpretation and diffusion of data used as public health tools for dengue prevention and control (WHO, 2009; WHO/SEARO, 1999). The surveillance system has three components: passive surveillance, active surveillance and event-based surveillance. Passive surveillance involves reporting of dengue cases according to a standardized way of case identification (WHO classification into DF/DHF/DSS) by private physicians, clinics, health centers and hospitals that provides medical attention to the population at risk. On the other hand, active surveillance involves a proactive search of dengue infections, especially when they could be attributed to other infectious agents as, influenza or rubella in periods of low transmission. Such surveillance requires adequate laboratory diagnosis support (WHO, 2009). Finally, the event-based surveillance is conducted to investigate cases of fever of unknown etiology without a routine data collection, but requires interdisciplinary action of an epidemiologist, an entomologist and a microbiologist. The three described methods need laboratory support and should be used collectively for accurately predict epidemic activity (WHO, 2009; WHO, 2011). Laboratory support is a critical component in surveillance because should be able to identify the presence of dengue virus, the serotype, the severity of illness, and a primary or secondary infection. The prediction of epidemics should be based on the analysis of genetic sequence of the viruses circulating, both during and between epidemics (Ooi, Gluber, & Nam, 2007). Unfortunately, most endemic countries do not have effective surveillance system, neither a mosquito control program. A literature review about effectiveness of epidemiological surveillance systems in dengue endemic countries showed that Ecuador has a weak passive surveillance system, it does not have active surveillance

program, and the infrastructure and laboratory capabilities are rudimentary (Gubler D. , 2002).

Entomological Surveillance

Good measures for vector control are based on surveillance of *A. aegypti*.

Entomological surveillance allows health authorities to determine the distribution, population density, major larval habitats, and spatial and temporal risk factors related to dengue transmission, and levels of insecticide susceptibility or resistance. For vector control there are several methods for the detection and monitoring of larval and adult populations, for example, larval surveys, pupal/ demographic surveys, and adult surveys. The selection of an appropriate method depends on surveillance objectives, levels of infestation, and availability of resources (WHO, 2011; Ooi, Gluber, & Nam, 2007).

DEFICIENCIES OF DENGUE DIAGNOSIS IN ECUADORIAN REMOTE COMMUNITIES

Esmeraldas is a coastal province of Ecuador that is endemic for DF. There is limited information about the epidemiology of DF and DHF in this region. The largest number of DF cases in Esmeraldas was 680 in 2001, without DHF cases. In 2004, 1 case of DHF was reported for first time and, 4 cases of DHF in 2005, since then, there have not been any other report of DHF until 2009. In 2008, 110 DF cases were reported and in 2009, 231 cases were the two lowest numbers of cases reported in the last 5 years (Aguilar, 2010). However, a study conducted in summer 2007 in Borbón (Borbón-Esmeraldas located in a remote region of the northern coast of Ecuador near the Colombian border) established a 92% of seroprevalence

for dengue infection. Based on the recent serology data, dengue has a substantial presence in rural northern coastal Ecuador (Connors, Coloma, Beatty, Cevallos, & Eisenberg, 2008). The Hospital Civil de Borbón (HCB) in Borbón reflected the weakness in the passive surveillance system. For example, when a febrile patient was classified as a probable case of DF based on clinical symptoms, following a hospital protocol, blood samples should be collected for serum analysis. The serum samples were subjected to an IgM ELISA rapid test (Panbio, Cat. E-DEN01D). When the onset of fever and other nonspecific symptoms and signs occurred recently (less than a week), febrile patients were invited to provide a blood sample in a week time, but patients seldom comply the medical indication and the probable dengue case were lost (Unpublished data).

On the other hand, record keeping at the hospital level was deficient. Between July 2010 to February 2011 the HCB registered 5 DF cases, but only 3 were reported to the national surveillance system (Dirección Provincial de Salud de Esmeraldas, 2011). Additionally, in the same period of time, 10 of 36 serum samples analyzed by the laboratory of HCB were found positive for IgM ELISA rapid test (Panbio, Cat. E-DEN01D), showing a significant loss of data that could be reported to national surveillance system. Similar situation has been described in Bandung-Indonesia, where a study designed to evaluate the dengue reporting system found that only 31% of the 650 hospitalized DHF/DSS cases were reported to the Municipal Health Authorities. They recommended that DHF/DSS cases should be reported according to a diagnosis made during hospitalization sooner than a serological confirmation is obtained (Chairulfatah, Setiabudi, Agoes, van Sprundel, & Colebunders, 2001). In Texas, USA government undertook an assessment of under diagnosis of dengue through a review of medical records between 23 July and 20 August 1999. The data showed

significant and alarming results, 50% of suspected case-patient (a person aged ≥ 5 years with a temperature of ≥ 38.3 C° and rash of any duration or fever for ≥ 3 days without cough or diarrhea) had undiagnosed dengue infection (CDC, 2001). Furthermore, dengue infection cases could be missed if they appear in the setting of other febrile illnesses with similar signs or symptoms as rubella (Bustosa, Hamdan, Loroño, Montero, & Gómez, 1990), rickettsiosis (Zavala-Velazquez, Yu, & Walker, 1996), measles (Oliveira, et al., 2001), etc.

Therefore, it is important to establish a strong epidemiological surveillance system at HCB, and educate the medical personnel and administrative staff on the importance of an accurate and opportune report. Gubler has considered that most DF/DHF endemic countries including Ecuador, acknowledge the need to carry out surveillance programs of this disease, but not all have the capacity to develop a program planning owing to deficiencies in the laboratory capabilities and the lack of active surveillance (Gubler D. , 2002). However, it is possible to have good surveillance systems; Singapore has showed to be the only one endemic country where the surveillance is effective to plan, response, prevent and control a DF/DHF outbreak.

VACCINE DEVELOPMENT

The complexity of developing a dengue vaccine is attributed among other things to the distribution of serotype/genotype of dengue epidemics. Additionally there are serotype/genotype variants from year to year a phenomenon that is somewhat unpredictable (WHO, 2009). Despite or this, progress has been made in the development of a vaccine that immunize against all four virus serotypes simultaneously. Currently, there are six tetravalent candidate vaccines in Phase I–III trials; probably a new tetravalent vaccine will be licensed in few years (Gubler D. , 2011). According to Hombach, the type of candidate vaccines that are

in advanced stages of development are live attenuated, live recombinant, and subunit and inactivated vaccines (Hombach, 2007). The advantages in developing live attenuated vaccines for dengue are because they are based on other live flavivirus vaccines that have been previously successful as yellow fever vaccine. Other advantages are focused in that live attenuated viral vaccines have a track record of producing lasting antibody responses and protection, and the cost of production should be less than other vaccine technologies.

Until the availability of a licensed vaccine, it is important to focus in disease surveillance. Vector control remains the key strategy in dengue prevention and control. Unfortunately, the extensive and often indiscriminate use of insecticides has resulted in a global pandemic of insecticide resistance (Wilder-Smith, Ooi, Vasudevan, & Gubler, 2010; Ng, 2011).

PART II.

SHORT REPORT: MOLECULAR SURVEILLANCE OF DENGUE FEVER IN A REMOTE AREA OF THE NORTHERN COAST OF ECUADOR USING SERUM AND CAPILLARY BLOOD SAMPLES ON FILTER PAPER

INTRODUCTION

Dengue is the most important arthropod borne viral disease in the world. Approximately 2.5 billion people are at risk to get the infection. The World Health Organization (WHO) considers that dengue is a public health problem that not only affects urban groups as traditionally observed but also rural populations as seen in the last two decades (WHO, 2009). There are four serotypes that cause the disease and are antigenically related (DENV 1 – 4) (WHO, 2009; Teixeira & Barreto, 2009; Gubler D. , 1997).

Dengue is transmitted through the bite of infected female *Aedes spp.* mosquitoes (Gubler D. , 1997). Humans, lower primates, and mosquitoes are considered the only natural hosts for DENV infections (Henchal & Putnak, 1990). Clinical disease follows unpredictable clinical evolution ranging from unapparent or mild febrile to severe and fatal hemorrhagic disease. According to a group of experts, dengue is classified as: non-severe (with warning signs or without warning signs) and severe dengue (WHO, 2009). Severe dengue is considered when the patient presents the following signs and symptoms: a) *severe plasma*

leakage that could lead to a Dengue Shock Syndrome (DSS) or a respiratory distress; b) *severe bleeding* and c) *severe organ involvement* (WHO, 2009; Teixeira & Barreto, 2009).

Outbreaks of classic DF occurred in the Caribbean and, in the northern part of South America in 1963-64, 1968-69, 1972-75 and 1977-78 (WHO, 1997). DHF was first described in the 1950s in the Philippines and Thailand but, it was not until an outbreak in Cuba in 1981 that DHF became a health problem in the Americas. In recent decades, DF/DHF has become a major public health problem in Ecuador. The first dengue epidemic in Ecuador occurred in 1988 and it was caused by DENV-1. DENV-2 was detected two years later, DENV-4 appeared in 1993 and DENV-3 in the year 2000 (Regato, Mosquera, Coloma, Mosquera, & Alava, 2006) and the first cases of DHF were reported in 2001. Year 2001 was the year with the highest level activity of DF ever recorded in the world (Stephenson, 2005). It has been estimated that an increase in disease incidence will continue indefinitely in the future around the world owing to demographic, societal and technical changes (Gubler D. , 2002). Gubler has suggested implementing efficient prevention and control strategies to fight against the emergence of the more frequent and larger epidemics of DF/DHF.

Surveillance is an important support of any prevention and control program (Gubler & Casta-Valez, 1991). Ecuador as endemic country has neither an effective surveillance system nor an effective mosquito control program (Gubler D. , 2002). Ecuadorian surveillance system is based on passive surveillance. It means that health authorities wait until the medical community recognizes the dengue transmission. Unfortunately, this type of surveillance is very insensitive, because in the absence of epidemic transmission there is a low index of suspicion among physicians, and dengue is rarely diagnosed and generally is too late to

implement effective preventive measures that can throw back the course of the epidemic (Gubler & Casta-Valez, 1991).

In remote areas as Borbón (a northwest town of Esmeraldas) with poor economic and health services development, the passive surveillance is less effective and critical. A previous study conducted in the summer of 2007 in the same town, established a 92% of seroprevalence for dengue infection (Connors, Coloma, Beatty, Cevallos, & Eisenberg, 2008), indicating that dengue was endemic in this region and that the weakness in the surveillance system contribute to mask the real number of DF cases specially during inter-epidemic transmission. The objective of the present study was to establish a laboratory technique for RT-PCR using blood samples collected on filter paper from febrile patients for the diagnosis and identification of dengue serotypes circulating in an endemic area in the Northern Coast of Ecuador.

MATERIALS AND METHODS

Study Site and Sample Collection

The study was conducted at Hospital Civil de Borbón (HCB) and at the National Service of Arthropod Borne Diseases Control (SNEM) laboratory, in Borbón-Esmeraldas located in the Northern coast of Ecuador. Two types of samples were obtained to study the presence of dengue RNA. Serum samples and whole blood spotted on filter paper from febrile patients that attended HCB or SNEM laboratory respectively. Both groups of samples were from different sources of patients. On the one hand, part of the routine diagnosis of febrile patients at HCB is the collection of venous blood for clinical laboratory test, an aliquote of serum from these samples was used to look for dengue RNA. On the other hand, blood spots

were collected from subjects referred to or spontaneously seeking diagnosis for malaria at SNEM laboratories. All the participants accepted an oral informed consent approved by Bioethics committee USFQ and Institutional Review Board of University of Michigan.

Serum and blood samples

Venous blood was obtained from the arm, and sera were stored at -20°C on the HCB laboratory, and then transported on liquid nitrogen to the laboratory of USFQ and stored at the same conditions until analysis. Blood drops (2-4) were collected on filter paper (Whatman 903 Specimen Collection Paper, Model 10538017) from patients showing symptoms of malaria. Filter papers were left to dry over night and then stored at 0°C in a zipper bag to avoid humidity until their transport to the laboratory of USFQ.

RNA Extraction

Total RNA from blood spots on filter paper and from sera was isolated by the Trizol method (Ambion by life technologies, Cat.10296-010). To prepare the samples on the filter paper (Prado, et al., 2005), six punches (6mm diameter) were collected in a 1.5 ml microcentrifuge tube and eluted with 400ul of RNase-free water (GIBCO) at 37°C for 30 minutes. Subsequently, 250ul aliquot of the filter paper eluent or 250ul of serum were transferred to a 1.5 ml microcentrifuge tube, mixed with 750ul of Trizol and 200ul of chloroform and incubated at -20°C for 10 minutes. Samples were centrifuged at 4°C for 15 minutes at 18000g and 400ul of the aqueous phase was transferred to a new 1.5 ul microcentrifuge tube and treated with an equal volume of cold isopropyl alcohol. After 10 minutes incubation at -20°C , samples were centrifuged at 4°C for 25 minutes at 18000 g The

precipitate was washed with 1ml of cold 75% ethanol and then centrifuged at 4°C for 5 minutes at 18000 g. The pellet was dried for 1 hour and resuspended in 25 ul of RNase-free water and stored in liquid nitrogen until used.

Reverse Transcription and PCR Amplification (RT-PCR)

A 5ul aliquote of RNA extract was subjected to retrotranscriptase PCR using SuperScript III One-Step RT-PCR System with *Taq* DNA Polymerase (Invitrogen Life Technologies) following manufacturer instructions. The reaction mix contained 12.5ul of Reaction mix 2x (Invitrogen Life Technologies), 0.2mM primer D1, 0.2mM primer TS1, 0.2mM primer TS2, 0.2mM primer TS3, 0.2mM primer DEN4, SuperScript III RT/Platinum *Taq* Mix and, RNase-free water (GIBCO). Reverse transcription was conducted at 52°C for 60 min, followed by 40 amplification cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes and a final extension at 72°C for 5 min. The amplification protocol and primers used was previously described with some modifications (Harris E, 1998). The sequence primers and amplicon sizes are as follows: D1: 5'-TCA ATA TGC TGA AAC GCG CGA GAA ACC G, TS1: 5'-CGT CTC AGT GAT CCG GGG G (482 pb, D1-TS1), TS2: 5'-CGC CAC AAG GGC CAT GAA CAG (119 pb, D1-TS2), TS3: 5'-TAA CAT CAT CAT GAG ACA GAG C (290 pb, D1-TS3), DEN4: 5'-TGT TGT CTT AAA CAA GAG AGG TC (389 PB, D1-DENV4).

cDNA amplification products were tested by electrophoresis in a 1.5% agarose gels using 12ul of the reaction. Gels were stained with SYBR® Safe DNA Gel Stain (1:10,000) and evaluated under UV light. PCR products size was estimated according to the migration

patterns of a Trackit 100 bp DNA Ladder (Invitrogen Life Technologies). In addition, part of the PCR product was sent to Functional Biosciences (Wisconsin, USA) for sequencing.

As positive controls enriched media from Dengue cultures (DENV 1 to 4) were kindly donated by Instituto Nacional de Higiene, Leopoldo Izquieta Pérez, Guayaquil-Ecuador. RNA isolation and RT-PCR analysis from cell culture media were performed as indicated above.

Quality of RNA extracts was tested using β -actin gene amplification (primer forward, 5' CGG AAC CGC TCA TTG CC 3' and, reverse: 5' ACC CAC ACT GTG CCC ATC TA)

STATISTICAL ANALYSIS

PASW Statistics 18 (version 18.0, 2012) statistics software was used to determine the concordance and kappa index.

RESULTS

RT-PCR Detection and Typing of Dengue Virus

A total of 77 serum and blood spots samples from febrile patients were collected from July 2010 to February 2011. Thirty-six serum samples and 41 blood samples on filter paper were collected. Thirty-nine samples were from male and 38 were from female patients.

Positive PCR reactions were evident in 6 (17 %) of serum samples and seven (17 %) from blood samples (Table 1). Of the six positive serum samples 5 were from male patients and 1 from a female patient. Seven positive samples were from whole blood spots (3 men and 4 female). It was shown the amplification of DENV-2 and DENV-3 serotypes in the two types of samples, proved by sequencing. One patient showed PCR amplification of DENV-2 and DENV-3 sequences at the same time, probably owing to a co-infection. Samples of two

laboratories were from different patients, except two patients that assisted to both laboratories in the same day; both patients were negative for Malaria microscopy, but only one was positive for RT-PCR test.

It is important to note that it was possible to isolate total RNA from all blood samples from filter paper. To our knowledge, this is the first time that RNA from blood samples on filter paper has been used successfully to study dengue virus infection in Ecuador.

On the other hand, the HCB laboratory personnel carried out IgM ELISA rapid test (Panbio, Cat.E-DEN01D) using serum samples. Comparison of ELISA and RT-PCR using Cohen's kappa index (Table 2) showed that there was no concordance (Cohen's kappa index was 0.030, $p= 0.837$).

DISCUSSION

The use of an inexpensive transport method and a PCR procedure allowed it to detect DENV-2 and DENV-3 in blood samples of people from July 2010 to February 2011 in the northern coast of Ecuador. During the same period of time DENV 1, 2 and 4 were detected in other coastal cities located in the center and southern region of the Ecuadorian coast (Regato, Personal contact, 2010). This results showed the reintroduction of DENV-3 in Ecuador, which was not reported since 2009 (PAHO, 2012). Thus, during 2010 and 2011, 4 serotypes of dengue virus were circulating in the Ecuadorian coast.

In the present study, samples were collected from a remote region in the northern Ecuadorian Coast that limits with Colombia. The proximity of both regions has allowed the commerce and exchange of individuals and goods through time though waterway. Of

relevance for the present report is that during the time this research was carried out, Colombia reported the four serotypes of virus in its territory (PAHO, 2012); opening the possibility of an interchange of the different dengue viruses including DENV-3 between both countries. Mosquitoes frequently fly approximately 100 to 200 meters around the breeding house (Muir & Kay, 1998); therefore, DENV-3 could be introduced by people as reservoirs or by mosquitoes brought by transport medium. Alternatively, is important to consider the possibility of sylvatic transmission, because this region is surrounded by rain forest. Dengue viruses circulate in two types of cycles, a sylvatic cycle involving non-human primates and various species of *Aedes* mosquito (such as *Ae. furcifer*, *Ae. luteocephalus* and *Ae. taylori*), and in an urban and rural cycle involving humans which principal vector is *Aedes aegypti* (Cardosa, et al., 2009). However, only in Africa and Asia has been described this cycle (Wang, et al., 2000). Although, in Brazil for first time between 1999 and 2005 was reported the possibility that DENV-1 could be involved in a sylvatic cycle (Mario LG de Figueiredo, 2010).

Previous studies have identified a dynamic movement of dengue viruses in different regions of the world associated with more severe forms of dengue infection, particularly with the appearance of DENV-3. A study conducted in India, has shown that replacement of circulating DENV-2 (subtype IV) by a DENV-3 (subtype III) could be the reason for the increased incidence of DHF/DSS in that country (Dash, et al., 2006) . The authors of the study indicate that the major dengue outbreaks in northern India were the result of the appearance of DENV-3 (subtype III). Lanciotti, et al., hypothesized that a genetic shift in DENV-3 may have been responsible for the emergence of severe form of the disease in some countries in Africa, Asia and Puerto Rico in America between 1956 to 1992 (Lanciotti, Lewis, Gubler, &

Trent, 1994), however studies that associate DHF to the abundance of DENV-3 in an endemic region of Colombia did not show an increase in the severity of the disease. During 2007, DENV-3 was more abundant than DENV-1 and 2 and the presence of DHF was lower than in 2008 where DENV-1 and 2 were more common (Gomez, Villabona, Torres, Miranda, & Ocazonez, 2008). The authors of the study conclude that the genetic characteristics of dengue viruses determine their pathogenicity. In addition, competitive displacement between dengue strains may play an important role in the dynamics of dengue infection. Invading more virulent strains may compete with local strains and could infect and disseminate within the vector making it more efficient in the transmission of the virus (Hanley, Nelson, Schirtzinger, Whitehead, & Hanson, 2008). Thus, it is important to improve laboratory capabilities in dengue endemic areas to determine the dynamics of dengue infection and the identification of different serotypes and genotypes associated with dengue outbreaks. Good laboratory support is a critical component of dengue epidemiological surveillance, it allows the diagnosis and identification of dengue virus serotypes, their severity, the recurrence, the genetic characteristics, and could also help in the prediction of future epidemics (Ooi, Gluber, & Nam, 2007). Evidence of the importance of good laboratory capabilities was demonstrated in the present study when out of 41 samples that were taken only for the diagnosis of malaria, the 41 were diagnosed as free of malaria and 7 were positive for dengue infections The laboratory diagnosis allowed the identification of 7 cases of dengue that could have never been detected if molecular techniques would not have been used. . One reason for the lack of diagnosis of dengue infection could be owing to dengue's non-specific signs and symptoms and the low index of suspicious between health providers personnel. In addition, the community where this study was carried out has the perception that malaria and dengue are

the same disease. It is important to understand that a patient with an undifferentiated febrile illness in an endemic area of tropical diseases should be studied to rule out all possible infectious agents *Leptospira* , *Plasmodium* , *Rickettsia* , dengue fever, *Brucella*, and other viral infections including viral encephalitis (Manock, et al., 2009). Shirtcliffe et al., stated that dengue virus infection should be considered in all febrile travelers from developed countries who have recently returned from areas where the disease is endemic and in whom tests for malaria are negative (Shirtcliffe, Cameron, Nicholson, & Wiselka, 1998) .

An important method for dengue infection in the clinical setting is ELISA that identifies immunoglobulin M and G (in paired sample) as a marker of acute infection (WHO, 2009). Hospital records of ELISA analysis of serum samples also analyzed by RT-PCR in the present study identified 10 positive samples. Out of these 10 samples, only one sample was positive with both techniques, ELISA and RT-PCR. It is important to consider the natural course of dengue infection and the immune response. After the onset of illness, during the viremia for approximately 2 to 7 days that roughly corresponding to the period of fever (Shu & Huang, 2004) there are absence of specific antibodies in a primary infection. On the other hand, an important humoral, antibody mediated immune response appears only after 5 to 7 days of the onset of illness (WHO, 2009). These characteristics of dengue infection would explain the differences in the detection of infection by RT-PCR and ELISA in the present study. The detection of viral RNA by RT-PCR could be done between 3 to 6 days (data not shown in results), and the virus specific serum immunoglobulin M (IgM) was detected by ELISA between 5 to 13 days after the onset of fever (data not shown in results), it is according to previously described information (WHO, 2009). The individual that was positive for dengue infection by RT-PCR and ELISA presumably was re-infected at the time of the laboratory

analysis since the patient had viremia and had antibodies specific for dengue. It would be difficult to compare both techniques in a population that could be in different stages of dengue infection. This study identified that 3 (8.33%) serum samples that were negative for ELISA were positive to PCR, and 10 (27.77%) that were positive to ELISA were negative to PCR. Overall, using PCR and ELISA techniques together identified 13 positive samples in serum samples (Table 2). As indicated before, the clinical stages of the population studied would explain these results.

In agreement with the low kappa index showed by this study, Pok et al., indicated that there was moderate agreement between both methods shown by a kappa index of 0.5 (Pok, Lai, Sng, & Ng, 2010). The combination of ELISA and RT-PCR for the diagnosis of dengue could contribute to the control of this public health problem. Lindergren et al., demonstrated that the combination of IgM ELISA and RT-PCR assays, could detect up to 85% of individuals infected with dengue (Lindergren, Vene, Lundkvist, & Falk, 2005).

The cost-benefit of implementing both laboratory techniques depends on the purpose for which the test is intended, such as clinical use or for surveillance, or whether it is for use in the laboratory or at point of care (Peeling, et al., 2010). The World Health Organization (WHO) has indicated that the use of direct methods such as virus isolation, nucleic acid and antigen detection have more confidence than indirect methods as serology for detection of IgM or IgG. However, indirect methods are more accessible and unexpensive because they don't need complex laboratory capabilities, some of these methods provide results in less than one hour, but they can't confirm a dengue virus infection unless paired serum samples are analyzed (WHO, 2009).

CONCLUSIONS

This report describes the reintroduction of DENV-3 in the northern coast of Ecuador between 2010 and 2011, and the utility of an inexpensive transport method to stabilize RNA from blood spots samples to conduct RT-PCR procedures to detect dengue serotypes. This procedure may improve epidemiological surveillance in remote Ecuadorian communities where febrile patients are only tested for malaria (Chairulfatah, Setiabudi, Agoes, van Sprundel, & Colebunders, 2001; CDC, 2001). Blood collection on filter paper has improved the management of other tropical disease like malaria in many endemic countries (Taylor, et al., 2011; Al-Harhi & Jamjoom, 2008; Maeno, et al., 2008). Blood filter paper collection offers a safe to handle and easy to storage, and transport samples (Matheus & al., 2008; Matheus & al., 2012).

RECOMMENDATIONS

- To implement the blood filter paper collection to transport samples from remote areas to a reference laboratory as the Instituto Nacional de Higiene (INH) Leopoldo Izquieta Perez.
- To improve laboratory capabilities in dengue endemic areas to determine the dynamics of dengue infection and the identification of different serotypes and genotypes associated with dengue outbreaks.
- To study an undifferentiated febrile illness in an endemic area of tropical diseases to rule out all possible infectious agents as leptospirosis, malaria, rickettsioses, dengue fever, brucellosis, and other viral infections as encephalitis.

- To implement a strong epidemiological and entomological surveillance system in Ecuador to improve the way to control, to prevent and to response during dengue outbreaks.
- To carry out other studies with a bigger sample of people and the comparisons between a gold standard technique as virus isolation and RT-PCR and ELISA rapid test to test the sensitivity, specificity and predictive value of this procedures in the field.

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GLOSSARY

Active surveillance involves a proactive search of dengue infections, especially when they could be attributed to other infectious agents as, influenza or rubella in periods of low transmission. Such surveillance requires adequate laboratory diagnosis support.

Aedes mosquitoes are the largest genus of the subfamily Culicinae, they are found in all habitats, ranging from the tropics to the Arctic.

Antigen is any molecule that can bind specifically to an antibody. When an antigen can produce by itself the production of antibodies is called immunogen.

Ascites is pathologic fluid collection within the abdominal cavity.

Assay: An assay is an analysis done to determine the biological or pharmacological potency of a drug, or the presence of a substance and the amount of that substance.

Brucellosis is a highly contagious zoonosis caused by ingestion of *Brucella spp.* in unsterilized milk or meat from infected animals or close contact with their secretions.

Dengue hemorrhagic fever (DHF) is a potential complication of DF, and may have case fatality rates of 1% or higher, especially in infants and young children. It is manifested specially by high fever, hemorrhagic phenomena, frequently with hepatomegaly and signs of circulatory failure when the condition is severe. The patients with DHF can develop **dengue shock syndrome (DSS)** characterized by hypovolemic shock consequential from plasma leakage.

Dengue is also known as **Dengue fever (DF)**, an acute mosquito-borne viral disease of sudden onset that usually follows a benign course with fever and other signs and symptoms as headaches, bone, joint, and muscular pain, rash and leucopenia.

Ecchymoses is a subcutaneous purpura larger than 1 centimeter or hematoma.

Encephalitis is the inflammation of the brain.

Endemic is an epidemiologic term used to describe that a disease is present in a community at all times but in relatively low frequency.

Envelope in a virus is a coat typically derived from portions of the host cell membranes when the virus is maturing.

Enzootic is equivalent to endemic term, but in a non-human setting.

Enzyme-linked immunosorbent assay (ELISA), is a analytic biochemistry assay that uses one sub-type of heterogeneous, solid-phase **enzyme immunoassay (EIA)** to detect the presence of a substance (as proteins) in a liquid sample or wet sample.

Epidemic is the occurrence of more cases of a disease than would be expected in a community or region during a given time period.

Epidemiologic surveillance involves an ongoing systematic collection, recording, analysis, interpretation and diffusion of data used as public health tools for dengue prevention and control

Event-based surveillance is conducted to investigate cases of fever of unknown etiology without a routine data collection, but requires interdisciplinary action of an epidemiologist, an entomologist and a microbiologist.

Extrinsic incubation period in a vector, it is the time between entrance of an pathogen into the vector and the time when that vector can transmit the infection to other host.

Fever is any body temperature above 37°C. However, in practice a person is usually not considered to have a significant fever until the temperature is above 38°C.

Flaviviridae are a family of viruses that are primarily spread through arthropod vectors. *Flavus* means yellow in Latin.

Flavivirus is a genus of the family *Flaviviridae*, including West Nile virus, dengue virus, tick-borne encephalitis virus, yellow fever virus, and other virus that cause encephalitis.

Genotype is the genetic makeup of a cell, an organism, or an individual usually with reference to a specific character under consideration.

Haematemesis is the presence of blood in vomiting.

Haematocrit is the ratio of the volume occupied by red blood cells in the total volume of blood, expressed as a percentage.

Hepatomegaly is the enlargement of the liver.

Hypoproteinaemia reflex the abnormally low level of protein in the blood.

Hypotension is the blood pressure that is below the normal. This term is the opposite of hypertension (abnormally high blood pressure).

Hypovolemic shock refers to a medical or surgical condition in which rapid fluid loss results in multiple organ failure due to inadequate circulating volume and subsequent inadequate perfusion. Most often, it is secondary to rapid blood loss plasma leakage.

Immunity is the ability to resist to any infections. **Cross-protective immunity** is the reaction between an antibody and an antigen that differs from the immunogen.

Immunoglobulin, it is also known as antibody. It is a protein produced by plasma cells and B lymphocytes after activation. The classes of immunoglobulins are termed immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin D (IgD) and immunoglobulin E (IgE).

Influenza is a condition commonly referred to as the flu, and it is producer by Influenza virus. Influenza A, B, and C viruses are the only members of the Orthomyxoviridae family,

and only influenza A and B viruses cause significant human disease. The orthomyxoviruses are enveloped and have a segmented negative-sense RNA genome.

Insecticide is a pesticide used to combat insects.

Intrinsic incubation period is the time that takes to a pathogen multiply in a host, and then it can be transmitted to or infect to other host.

Leptospirosis is an infectious disease caused by a spirochete transmitted by rats as well as by skunks, opossums, raccoons, foxes, and other vermin.

Lethargy is the inability to continue functioning at the level of one's normal abilities.

Leucopenia describes the situation in which there are fewer leucocytes in the blood than normal.

Malaria is an infectious disease caused by parasites from the Plasmodium family that can be transmitted by the Anopheles mosquito or by a contaminated needle or transfusion.

Melena is the sign of black feces (digested blood) that are associated with gastrointestinal hemorrhage.

Neutralization is the process of inactivation of a virus or a toxin molecule through neutralizing antibodies.

Nucleic acids are biological molecules including DNA(deoxyribonucleic acid) and RNA (ribonucleic acid).

Nucleocapsid is a unit of viral structure, consisting of a capsid with the enclosed nucleic acid.

Outbreak is an epidemiologic term used to describe an occurrence of disease greater than would otherwise be expected at a particular time and place.

Pandemic is an epidemic of infectious disease that has spread through human populations across a large region; for instance multiple continents, or even worldwide.

Passive surveillance involves reporting of dengue cases according to a standardized way of case identification by private physicians, clinics, health centers and hospitals that provides medical attention to the population at risk.

Petechiae are a minor hemorrhage. It is small (1-2mm) red or purple spot on the body.

Platelet is a irregular, disc-shaped element in the blood that assists in blood clotting. They are fragments of large bone marrow cells called megakaryocytes.

Polymerase chain reaction (PCR) is a molecular technique designed to amplify a single or a few copies of a piece of DNA, generating thousands to millions of copies of a particular DNA sequence. **Retrotranscriptase (RT)** is a DNA polymerase enzyme that transcribes single-stranded RNA into single-stranded DNA.

Purpura is a small hemorrhage (> 3mm in diameter) in the skin, mucous membrane, or serosal surface.

Rash is an eruption of the skin, typically referred to as an exanthema.

Rickettsioses are severe infectious diseases caused by rickettsiae and rickettsia-like organisms. The best-known rickettsial diseases infect humans and are usually transmitted by parasitic arthropod vectors.

Ribonucleic acid (RNA) is a nucleic acid molecule similar to DNA but containing ribose rather than deoxyribose.

Sero-prevalence is the number of persons in a population who test positive for a specific disease based on serology specimens; often presented as a percent of the total specimens tested or as a proportion per 100,000 persons tested.

Serotype is the kind of microorganism characterized by serologic typing.

Serum is the clear liquid that can be separated from clotted blood.

Thrombocytopenia is the term for a reduced platelet count.

Tourniquet test is a clinical diagnostic method to assess fragility of capillary walls showing the presence of thrombocytopenia. A blood pressure cuffs is applied on the upper arm and inflated to a point between the systolic and diastolic blood pressures for five minutes. The test is positive if there are more than 20 petechiae per 2.5 cm area square are observed

Vaccination is the deliberate induction of adaptative immunity to a pathogen by injecting a **vaccine**, a dead or attenuated (non pathogenic) from the pathogen.

Vector borne disease A vector-borne disease is one in which the pathogenic microorganism is transmitted from an infected individual to another individual by an arthropod or other agent, sometimes with other animals serving as intermediary hosts.

Vector in epidemiology is any agent (person, animal or microorganism) that carries and transmits an infectious pathogen into another host.

Virus is a microorganism smaller than bacteria, they can replicate only in a living cell owing to metabolic machinery that viruses don't have. It is composed of a nucleic acid genome enclosed in a protein coat.

Yellow fever is an acute systemic illness caused by a Flavivirus. The viral infection causes a high fever, bleeding into the skin, and necrosis of cells in the kidney and liver. Severe jaundice gives the name to the disease.

APPENDIX

TABLE 1. SEROTYPE IDENTIFICATION BY RT-PCR IN SERUM AND BLOOD SPOTS ON FILTER PAPER SAMPLES.

Samples	Number	RT-PCR		DENV-1	DENV-2	DENV-3	DEN V-4
		Positive	(%)				
Serum	36	6	17	0	5	1	0
Blood spots	41	7*	17	0	3	5	0

* A patient shown co-infection with both serotypes

TABLE 2. CROSSTAB TO COMPARE ELISA AND RT-PCR RESULTS AGREEMENTS.

		RT-PCR		Total
		Positive	Negative	
ELISA**	Positive	1	9	10
	Negative	3	21	24
Total		4	30	34*

*Two samples were excluded from 36 serum samples, because didn't have complete information about positive or negative result in ELISA test.

** ELISA test was carried out by HCB laboratory personnel.