UNIVERSIDAD SAN FRANCISCO DE QUITO

Colegio de Postgrados

Dengue incidence in Northwestern Ecuador from 2013 to 2014

Julio César Carrera Montoya

Gabriel Trueba, Ph.D., Director de Tesis

Tesis de grado presentada como requisito
para la obtención del título de Magíster en Microbiología

Quito, julio de 2015

Universidad San Francisco de Quito
Colegio de Posgrados

HOJA DE APROBACION DE TESIS

Dengue incidence in Northwestern Ecuador from 2013 to 2014

JULIO CÉSAR CARRERA MONTOYA

Gabriel Trueba, Ph.D.,
Director de Tesis
Director de la Maestría en Microbiología ……..

Christopher Logue, Ph.D.,
Miembro del Comité de Tesis ……

Paulina Andrade, MSc.,
Miembro del Comité de Tesis ……

Stella de la Torre, Ph.D.,
Decano del Colegio de
Ciencias Biológicas y Ambientales ……

Victor Viteri Breedy, Ph.D.,
Decano del Colegio de Posgrados ……

Quito, julio de 2015

Firma: _____

Nombre: Julio César Carrera Montoya

C. I.: 1716685621

Lugar: Quito Fecha: julio de 2015

ACKNOWLEDGMENTS

To Gabriel Trueba, Paulina Andrade and Christopher Logue who have been the most important scientific and moral support for the realization of this work.

To Josefina Coloma and Joseph Eisenberg, who are the main managers of the Ecodess project of which this study was part.

To my family that has supported me in every possible way throughout my Master's degree.

To all my teachers at USFQ, who have been capable to transmit not only their knowledge and experience during the theoretical and practical classes of the Master's degree, but also their passion for research.

To Gardenia Freire, Irma Chilan and other laboratory technicians of the Hospital Civil of Borbon, who provided technical support in blood samples collection.

ABSTRACT

Dengue fever is a disease of importance for Public Health in Ecuador, especially in tropical and subtropical areas. This study was conducted in 48 rural communities in the Ecuadorian northwestern coast, where Dengue is endemic. Serum samples were collected from febrile patients and submitted to IgM ELISA and RT-PCR tests. We obtained 290 serum samples; positive RT- PCR reactions were evident in 37 (11 %) of the samples and the amplification of all the four serotypes was observed. DENV-1 was the predominant serotype present (more than 50% of the amplicons). The IgM ELISA rapid test was positive in 196 (67%) sera. Further, 81 serum samples from febrile patients were cultured in C6/36 cells and DENV-1 was isolated from 10 samples.

Keywords: Dengue, Ecuador, Panbio IgM, RT-PCR, cell culture, surveillance.

RESUMEN

La fiebre del dengue es una enfermedad de importancia para la Salud Pública en Ecuador, especialmente en zonas tropicales y subtropicales. Este estudio fue llevado a cabo en Borbón, la cual es una comunidad rural en la costa Noroeste de la provincia de Esmeraldas donde el dengue es endémico. Muestras de suero sanguíneo fueron recolectadas de pacientes febriles y sometidas a pruebas de IgM ELISA y RT-PCR. Los resultados de RT-PCR fueron positivos para 37 de las muestras (11%) y la presencia de los 4 serotipos fue observada. DENV-1 fue el serotipo predominante con más del 50% de los casos. La prueba de IgM ELISA fue positiva en un total de 196 casos (67%). Además, 81 muestras de suero fueron cultivadas en células C6/36, donde DENV-1 fue aislado de 10 muestras diferentes.

Palabras clave: Dengue, Ecuador, Panbio IgM, RT-PCR, cultivo celular, vigilancia epidemiológica.

TABLE OF CONTENTS

FIGURES AND TABLES

PART I

GENERAL INTRODUCTION

DENGUE VIRUS INFECTION

Introduction

Dengue fever is the most rapidly spreading tropical disease at present time, with 2.5 billion people living in dengue endemic areas and a 30-fold increase in geographic expansion during the last 50 years worldwide(WHO, 2009). The causative agent dengue virus (DENV), of which four different serotypes have been described to date (DENV1-4). It is transmitted by female mosquitoes of the *Aedes aegypti* and *Aedes albopictus* species, which feed on human blood and lay eggs in clean water containers (WHO, 2009; CDC, 2015). Poorly planned urbanization and population growth in tropical and subtropical areas around the world during the last decades, especially in developing countries, have tremendously multiplied the breeding sites for these mosquitoes and turned vector control increasingly problematic. In addition, global tire and bamboo trade has been a large source of dispersion of *Aedes* globally (Kyle & Harris, 2008).

Since there is no vaccine against dengue and treatment can be only palliative, prevention is currently the only way to avoid infections. Preventive measures include eliminating water containers for egg laying, placing mosquito nets on windows and beds, using repellent and long-sleeved clothes (Runge-Ranzinger, McCall, Kroeger, & Horstick, 2014).

Dengue virus

Dengue virus (DENV) is a positive-sense single-stranded RNA virus that belongs to the genus *Flavivirus* of the Flaviviridae family. The viral genome encodes for seven nonstructural (NS) proteins and three structural proteins (C, prM and E) which form the virus envelope and capsid (Mahy, 2009). The genome organization comprises 5'-CprM(M)-E-NS1-NS2A-NS2B-NS3-NS4ANS4B-NS5-3' and a polyprotein of about 3000 amino acids is synthetized and processed co-translationally and post-translationally by viral and host proteins (Stephenson & Warnes, 2011) (*Figure 1*).

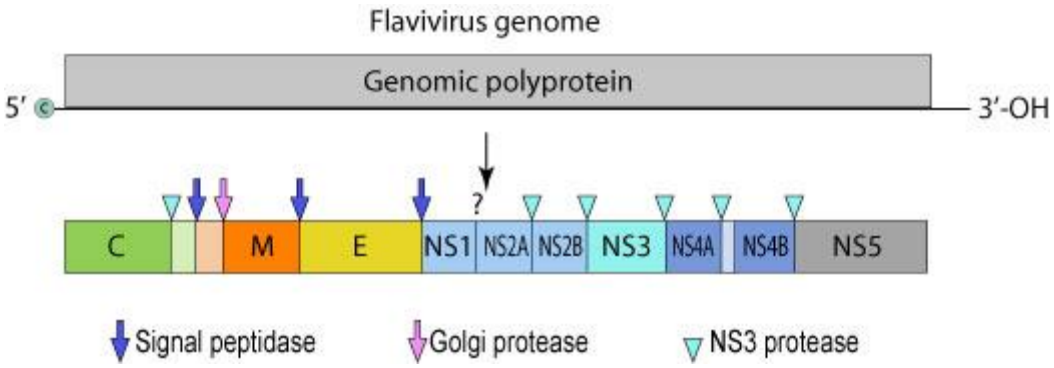

Figure 1. Genome organization of Dengue virus.
http://viralzone.expasy.org/all_by_species/43.htm

DENV genome is about 11,000 bases with two untranslated regions (5' and 3' UTR) as shown in *Figure 1*. The 5´-end forms an mRNA-like cap, where translation initiates in a cap-dependent manner, and the 3´-end forms a conserved stem-loop structure. The large loop structure contained within the 5´-end recruits the viral RNA-dependent RNA

polymerase (RdRp). Both UTRs are necessary for translation and replication and contain complimentary circularization sequences that mediate genome circularization (Bäck & Lundkvist, 2013).

The difference in neutralizing antibody binding for the four different serotypes (DENV1-4) is possibly due to 56 non-conserved amino acid residues in domain III of the protein E that is exposed on the external surface of the virion (Knipe & Howley, 2013). The existence of different genotypes may be the result of purifying selection against viruses unable to infect human or vector cells. Infection with one DENV serotype does not provide protection against the others, indeed, some DENV-2 and DENV-3 Asian genotypes of have been commonly associated with secondary infections causing severe dengue (Leitmeyer et al., 1999); (CDC, 2015). Apparently, the presence of subneutralizing concentrations of antibodies against DENV in the blood, due to previous infections by a different serotype of the virus, causes antibody-dependent enhancement (ADE), which leads to an increase in the quantity and types of infected cells, thereby causing a higher viraemia and severity of disease (Boonnak et al., 2008).

Transmission

DENV is transmitted by the bite of an infected mosquito *A. aegypti*, an anthropophilic species of mosquito that is likely to have been spread from the jungles of Africa throughout the rest of the world between the 17th and 19th century via the slave trade and maritime commerce. *A. albopictus* is a secondary vector of DENV, with a

geographical range stretching farther north than that of *A. aegypti* and for this reason it has gained much importance lately since it could become the cause of the emergence of dengue in the USA and Europe (Leisnham, Sala, & Juliano, 2008); (Kyle & Harris, 2008). Infection susceptibility differs among different strains of both *A. aegypti* and *A. albopictus* found in different geographical locations, suggesting that vector competence depends on genetic variation, and some specific quantitative trait loci (QTLs) have been found to be associated with the vector's natural barriers to infection (Lourenço de Oliveira, Vazeille, de Filippis, & Failloux, 2003; Bennett et al., 2002).

Three types of transmission cycles have been described: a) enzootic cycles involving the mosquito and lower primates in rain forests of Asia and Africa b) short epidemic cycles in immunologically naive populations and c) endemic/epidemic cycles in large urban tropical/subtropical areas involving a mosquito-human-mosquito character of transmission (Duane J. Gubler, 1998), and in very rare cases, dengue can be also transmitted via organ transplantation, blood transfusion or from an infected pregnant mother to the fetus (CDC, 2015).

Clinical evolution and case classification

Clinical evolution of dengue fever is unpredictable. Commonly, dengue cases have been classified in Dengue fever (DF) and Dengue hemorrhagic fever (DHF). In DF most of patients fully recover after a short non-severe clinical course characterized by acute febrile illness and two or more of the following symptoms: headache, retro-ocular pain,

joint pain, muscle pain, rash and leucopenia. A small proportion of cases progress to severe dengue with life threatening conditions (Dengue hemorrhagic fever – DHF) characterized by hemorrhagic or non-hemorrhagic plasma leakage, ecchymoses, haematemesis, thrombocytopenia and sometimes hypotension, weak pulse and cold skin (Dengue shock symdrome - DSS). Fatality can be reduced to less than 1% of these cases if an appropriate intravenous rehydration is implemented during treatment (WHO, 2009; PAHO, 2014; Bäck & Lundkvist, 2013). Dengue was previously classified under the above mentioned system (Magill, Strickland, Maguire, Ryan, & Solomon, 2012), but a simplified classification proposed by the Program for Research and Training in Tropical Diseases of the WHO grouped together DHF and DSS as “severe dengue”, to avoid false negative in DHF/DSS diagnosis (Bäck & Lundkvist, 2013).

An estimated 100 million infections take place every year around the world, but only 10-50% are symptomatic and only a portion of them are reported. Severe dengue appears much more frequently after secondary infections involving a different serotype to the primary infection (Kyle & Harris, 2008). The incubation period is 3-15 days (more commonly 5-8 days) with an abrupt onset of high fever. DF is commonly known as “break bone fever” due to the distressing muscular and bone pain which usually persist for up to 7 days (Bäck & Lundkvist, 2013).

Diagnosis

Dengue Fever (DF) treatment is presently only palliative as there are not effective antiviral drugs or vaccines; consequently timely diagnosis is a key factor for a good

clinical treatment and etiologic investigation for disease control. Laboratory diagnosis can be determined by virus isolation, viral antigen or antibody detection by Enzyme Linked Immunosorbent Assays (ELISA) and nucleic acid amplification by different PCR methods (Stephenson & Warnes, 2011).

Samples for viral isolation and nucleic acid amplification must be collected during the first 5 days after onset of symptoms. After day 5, antigens and viruses disappear from the blood as specific antibodies flood the bloodstream (*Figure 2*). Availability of dengue serologic tests in developing countries is much higher than direct virus detection assays (WHO, 2009). DENV is recommended to be handled in BSL2 conditions in endemic countries, but BSL3 is mandatory in countries where it is not (CDC, 2009).

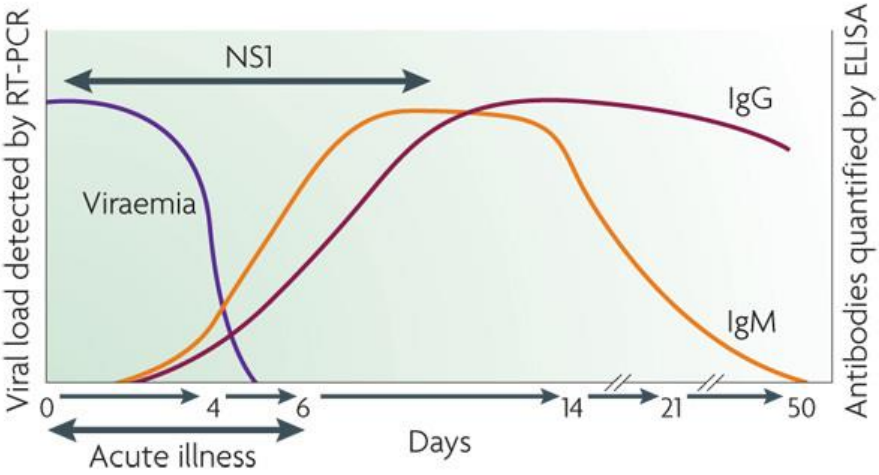

Figure 2. Immune response to dengue infection and effectiveness of diagnostic tools in a time-dependent manner. http://viralzone.expasy.org/all_by_species/43.html

DF can be easily confused with other illnesses such as yellow fever, Japanese encephalitis, St. Louis encephalitis, West Nile virus, several alphavirus infections, malaria, leptospirosis, typhoid, measles, enteroviruses, influenza and some hemorrhagic

fevers. DF cases must be confirmed by molecular methods (PCR) in addition to a the detection of specific antibodies (WHO, 2009); (Stephenson & Warnes, 2011).

Development of Vaccines

Antigenic differences between the four serotypes are considered enough to produce four monovalent vaccines, which can be theoretically mixed to get a tetravalent immunological response. Nevertheless, antibodies to dengue viruses may trigger an antibody-dependent enhancement (ADE) of disease (resulting in severe DHF) and this has hindered the obtaining an effective vaccine for decades (Rodriguez-Roche & Gould, 2013).

Some vaccine candidates are currently being evaluated in clinical trials and the one in a most developed stage is a live-attenuated tetravalent vaccine that has passed to the third stage of efficacy studies (Sanofi Pasteur). Several other vaccines, including some other live-attenuated, DNA and purified inactivated vaccines are also in development, but at earlier stages (Thisyakorn & Thisyakorn, 2014).

The most important concern in the development of a vaccine is the low level of neutralizing antibodies that can be reached sometime after vaccination and could be theoretically capable of starting ADE. Other important concerns are the spread of the attenuated vaccine virus by mosquitoes, neurovirulence and immunocompromised patients (Edelman & Hombach, 2008).

DNA vaccines containing dengue genes are currently considered as a potential method to bypass the problems of cross-reactive antibodies seen in multivalent live attenuated vaccines. However, concerns about the potential integration of these molecules in the host's genome and its consequences such as the inactivation of tumor suppressor genes and emergence of autoimmune diseases have hampered its development despite many promising results obtained in laboratory (Jechlinger, 2006; Raviprakash et al., 2006).

Epidemiology

The first records of well documented cases of dengue fever date back to 1779-1780 in China; but it was only after the World War II, when a global epidemic started in Southeast Asia, that Japanese and American scientists managed to isolate the virus. DENV1 and DENV2 were identified first during this period, DENV3 and DENV4 were described in 1954 during some outbreaks in Philippines and Thailand (D. J. Gubler & Clark, 1995).

Several major outbreaks of a dengue-like disease were described in the Americas between 1600 and 1946, but the modern era of its study began when the virus was first isolated and diagnostic tools became available during the forties (Brathwaite Dick et al., 2012). Mosquito eradication campaigns during the 60s and 70s in the Americas, decreased transmission of dengue virus, but as vector control measures were not

sustained, the virus has once again dramatically spread throughout the continent (Kyle & Harris, 2008).

Dengue in the Americas currently presents an endemo-epidemic pattern with important outbreaks every 3 to 5 years. Between 2008 and 2012 more than 1.2 million people were estimated to having been infected every year, 28, 233 of which were severe cases resulting in a total of 1000 deaths. In 2013 the highest burden of dengue in the region was registered, with an estimated total of 2.3 million cases, of which 37,898 were severe resulting in 1,318 deaths (PAHO, 2014). Dengue fever has also a very high social and economic impact, the estimated cost is 2.1 billion dollars per year (PAHO, 2014; WHO, 2009; Brathwaite Dick et al., 2012).

Between 2001 to 2007, 19% of the total cases in the Americas were presented in Andean countries. Interestingly, this is the region with the highest number of Dengue hemorrhagic fever cases 61,341 (58%) and Colombia was the country with most dengue deaths in the continent (73%). All four dengue virus serotypes have been identified in this area (WHO, 2009; PAHO, 2014)

Since the first cases of dengue were reported in Ecuador in 1988, malaria cases started to decline and a transition in the cause of acute fever in the tropical and subtropical areas of the country was seen. In 2000, 22,937 cases of dengue fever were reported, the largest outbreak in Ecuador to date with all four serotypes circulating at that time (Cifuentes et al., 2013). In 2014, 15,031 cases were reported, 13,453 without alarming

signs, 1,513 with severe symptoms and 65 fatalities (Ministry of Public Health of Ecuador, 2014).

Surveillance and early warning systems (EWS)

Dengue surveillance studies the circulation of the virus in human and mosquito populations (epidemiological and entomological surveillance) and therefore, it is a substantial component of the early warning systems (EWS) that seek to predict, prevent and timely respond to local outbreaks. EWS also incorporate environmental, socio-ecological, socio-economical and spatio-temporal data to generate prediction models that have been proved to be effective for many vector-borne diseases, including dengue (WHO, 2009; Stewart-Ibarra et al., 2014).

Epidemiological surveillance is the systematic collection of every type of relevant data that shows the health status of different communities in a given geographical area. It provides important information for risk assessment of transmission and prevention. Epidemiological surveillance seek to detect epidemics for timely intervention and weight severity to predict the socio-economic impact, monitor the success of control programs and define trends in the measuring parameters (WHO, 2009; Runge-Ranzinger et al., 2014).

Entomological surveillance aims to detect variations in the geographical distribution and population of vectors to evaluate vector control programs and plan new interventions.

Samplings of mosquitoes at different stages of development and in different environments are the most important tools to determine populations at higher risk. This type of surveillance is currently increasing linked to shifts in climate (quasi-global warming) allowing for more ecological niches to shelter invasive mosquito populations. (WHO, 2009; Runge-Ranzinger et al., 2014; Rodriguez-Roche & Gould, 2013; Kyle & Harris, 2008)

The study of dengue in Northwestern Ecuador: problems and prospective

DF is endemic in many tropical and subtropical areas of Ecuador and although there is a constant monitoring system, the limited scope of preventive measures in some remote areas and the socioeconomic characteristics of most of the communities where DF is endemic, has not made it possible to avoid constant epidemics of the disease since its appearance (Brathwaite Dick et al., 2012).

During recent years, a trend in which most of the cases are reported between the 10th and 32nd weeks of the year has been observed. During 2014, 15,446 confirmed cases of DF were reported in Ecuador (Guayas, Los Rios, Manabí, El Oro and Esmeraldas were the provinces where almost 86% of the cases was concentrated). Esmeraldas alone reported 1,051 cases, of which 110 presented severe symptoms and 9 were reported as severe dengue cases (Ministry of Public Health of Ecuador, 2014).

Based on serological and entomological data, communities in the Northwestern coast of Ecuador are endemic for DF and require an improvement in surveillance systems. Passive surveillance at the Hospital Civil de Borbon (HCB), which is a rural community close to the Colombian border, has proven insufficient for a correct assessment of the situation of the disease in this geographical area: patients rarely visit the hospital right after the onset of the symptoms and commonly only serological analysis are done, record keeping and report to the national surveillance systems are defective (Cifuentes S., 2012).

It is important to design and improve the dengue surveillance systems in Ecuador, especially in remote communities as Borbon, which in spite of being a rural community, is the center of development and trade for many surrounding communities. More health personnel training, improvement in their working conditions and infrastructure along with the optimization of the communication channels with the systems epidemiological surveillance are imperative for a better assessment of the situation of the disease in this area.

Bibliography of general introduction

1. Bäck, A. T., & Lundkvist, A. (2013). Dengue viruses - an overview. *Infection Ecology & Epidemiology*, *3*. http://doi.org/10.3402/iee.v3i0.19839

2. Bennett, K. E., Olson, K. E., Muñoz, M. de L., Fernandez-Salas, I., Farfan-Ale, J. A., Higgs, S., … Beaty, B. J. (2002). Variation in vector competence for dengue 2 virus among 24 collections of Aedes aegypti from Mexico and the United States. *The American Journal of Tropical Medicine and Hygiene*, *67*(1), 85–92.

3. Boonnak, K., Slike, B. M., Burgess, T. H., Mason, R. M., Wu, S.-J., Sun, P., … Marovich, M. A. (2008). Role of Dendritic Cells in Antibody-Dependent Enhancement of Dengue Virus Infection. *Journal of Virology*, *82*(8), 3939–3951. http://doi.org/10.1128/JVI.02484-07

4. Brathwaite Dick, O., San Martín, J. L., Montoya, R. H., del Diego, J., Zambrano, B., & Dayan, G. H. (2012). The history of dengue outbreaks in the Americas. *The American Journal of Tropical Medicine and Hygiene*, *87*(4), 584–593. http://doi.org/10.4269/ajtmh.2012.11-0770

5. CDC. (2009). CDC - Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition. Retrieved May 4, 2015, from http://www.cdc.gov/biosafety/publications/bmbl5/

6. CDC. (2015). CDC - Dengue. Retrieved March 5, 2015, from http://www.cdc.gov/dengue/

7. Cifuentes S. (2012). Master's Thesis: "Molecular Surveillance of Dengue Fever in a Remote Area of the Northern Coast of Ecuador Using Serum and Capillary Blood Samples on Filter Paper." Universidad San Francisco de Quito.

8. Cifuentes, S. G., Trostle, J., Trueba, G., Milbrath, M., Baldeón, M. E., Coloma, J., & Eisenberg, J. N. S. (2013). Transition in the cause of fever from malaria to dengue, Northwestern Ecuador, 1990-2011. *Emerging Infectious Diseases*, *19*(10), 1642–1645. http://doi.org/10.3201/eid1910.130137

9. Edelman, R., & Hombach, J. (2008). "Guidelines for the clinical evaluation of dengue vaccines in endemic areas": summary of a World Health Organization

Technical Consultation. *Vaccine*, *26*(33), 4113–4119. http://doi.org/10.1016/j.vaccine.2008.05.058

10. Gubler, D. J. (1998). Dengue and Dengue Hemorrhagic Fever. *Clinical Microbiology Reviews*, *11*(3), 480–496.

11. Gubler, D. J., & Clark, G. G. (1995). Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerging Infectious Diseases*, *1*(2), 55–57. http://doi.org/10.3201/eid0102.952004

12. Harris, E., Roberts, T. G., Smith, L., Selle, J., Kramer, L. D., Valle, S., … Balmaseda, A. (1998). Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *Journal of Clinical Microbiology*, *36*(9), 2634–2639.

13. Jechlinger, W. (2006). Optimization and delivery of plasmid DNA for vaccination. *Expert Review of Vaccines*, *5*(6), 803–825. http://doi.org/10.1586/14760584.5.6.803

14. Knipe, D. M., & Howley, P. (2013). *Fields Virology (Knipe, Fields Virology)-2 Volume Set* (Sixth, 2-volume set edition). Philadelphia, PA: LWW.

15. Kyle, J. L., & Harris, E. (2008). Global spread and persistence of dengue. *Annual Review of Microbiology*, *62*, 71–92. http://doi.org/10.1146/annurev.micro.62.081307.163005

16. Leisnham, P. T., Sala, L. M., & Juliano, S. A. (2008). Geographic variation in adult survival and reproductive tactics of the mosquito Aedes albopictus. *Journal of Medical Entomology*, *45*(2), 210–221.

17. Leitmeyer, K. C., Vaughn, D. W., Watts, D. M., Salas, R., Villalobos, I., Chacon, D., … Rico-Hesse, R. (1999). Dengue Virus Structural Differences That Correlate with Pathogenesis. *Journal of Virology*, *73*(6), 4738–4747.

18. Lourenço de Oliveira, R., Vazeille, M., de Filippis, A. M. B., & Failloux, A.-B. (2003). Large genetic differentiation and low variation in vector competence for dengue and yellow fever viruses of Aedes albopictus from Brazil, the United States, and the Cayman Islands. *The American Journal of Tropical Medicine and Hygiene*, *69*(1), 105–114.

19. Magill, A. J., Strickland, G. T., Maguire, J. H., Ryan, E. T., & Solomon, T. (2012). *Hunter's Tropical Medicine and Emerging Infectious Disease*. Elsevier Health Sciences.

20. Maher-Sturgess, S. L., Forrester, N. L., Wayper, P. J., Gould, E. A., Hall, R. A., Barnard, R. T., & Gibbs, M. J. (2008). Universal primers that amplify RNA from all three flavivirus subgroups. *Virology Journal*, *5*, 16. http://doi.org/10.1186/1743-422X-5-16

21. Mahy, B. W. J. (2009). *Desk Encyclopedia of Human and Medical Virology*. (M. H. V. van Regenmortel, Ed.) (1 edition). Oxford: Academic Press.

22. Manock, S. R., Jacobsen, K. H., Bravo, N. B. de, Russell, K. L., Negrete, M., Olson, J. G., … Kochel, T. (2009). Etiology of Acute Undifferentiated Febrile Illness in the Amazon Basin of Ecuador. *The American Journal of Tropical Medicine and Hygiene*, *81*(1), 146–151.

23. Ministry of Public Health of Ecuador. (2014). GACETA EPIDEMIOLÓGICA SEMANAL No. 51. MSP.

24. PAHO. (2014). State of the art in the Prevention and Control of Dengue in the Americas: Meeting report. PAHO.

25. Raviprakash, K., Apt, D., Brinkman, A., Skinner, C., Yang, S., Dawes, G., … Porter, K. (2006). A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques. *Virology*, *353*(1), 166–173. http://doi.org/10.1016/j.virol.2006.05.005

26. Rodriguez-Roche, R., & Gould, E. A. (2013). Understanding the dengue viruses and progress towards their control. *BioMed Research International*, *2013*, 690835. http://doi.org/10.1155/2013/690835

27. Runge-Ranzinger, S., McCall, P. J., Kroeger, A., & Horstick, O. (2014). Dengue disease surveillance: an updated systematic literature review. *Tropical Medicine & International Health*, *19*(9), 1116–1160. http://doi.org/10.1111/tmi.12333

28. Stephenson, J. R., & Warnes, A. (Eds.). (2011). Detection of Dengue Virus - Springer. Humana Press. Retrieved from http://link.springer.com/protocol/10.1007/978-1-60761-817-1_4

29. Stewart-Ibarra, A. M., Muñoz, Á. G., Ryan, S. J., Ayala, E. B., Borbor-Cordova, M. J., Finkelstein, J. L., … Rivero, K. (2014). Spatiotemporal clustering, climate periodicity, and social-ecological risk factors for dengue during an outbreak in Machala, Ecuador, in 2010. *BMC Infectious Diseases*, *14*(1), 610. http://doi.org/10.1186/s12879-014-0610-4

30. Thisyakorn, U., & Thisyakorn, C. (2014). Latest developments and future directions in dengue vaccines. *Therapeutic Advances in Vaccines*, *2*(1), 3–9. http://doi.org/10.1177/2051013613507862

31. WHO. (2009). *Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control*. World Health Organization.

PART II

Report: **Dengue incidence in Northwestern Ecuador from 2013 to 2014**

Introduction

Dengue fever (DF) is the most rapidly spreading vector-borne disease at present time, with 2.5 billion people living in dengue endemic areas and a 30-fold increase in geographic expansion during the last 50 years worldwide (WHO, 2009). About 100 million infections occur globally every year, with only 10-50% showing symptoms and only a fraction being reported. In DF most of patients fully recover after a short non-severe clinical course characterized by acute febrile illness, but a small proportion of cases progress to severe dengue with life threatening conditions such as plasma leakage, hypotension and death (WHO, 2009; PAHO, 2014; CDC, 2015). DF treatment is currently only palliative and consequently, timely diagnosis is a key factor for a good clinical treatment. Laboratory diagnosis can be achieved by virus isolation, ELISA (antigen or antibody) and PCR (Stephenson & Warnes, 2011).

In 2014, 15,446 confirmed Dengue cases (67 cases of severe dengue) were reported by the Ministry of Public Health of Ecuador. However, no surveys were carried out in the remote Northwestern region of Ecuador, where we observed additional genotypes of dengue viruses in 2010-2011 (Cifuentes S., 2012).The objective of our current study was to determine the genotypes of dengue virus circulating in the same region in the 2013-2014 period and compare the serotypes with those detected in rest of Ecuador. In addition, we sought to isolate the virus by cell culture methods.

Materials and methods

Study site and sample collection

The study was conducted at the Civil Hospital of Borbon (CHB). Borbon is located in the Northwestern Coast of Ecuador, Esmeraldas province, canton of Eloy Alfaro where Dengue fever is endemic. Serum samples were collected from patients coming from 48 different locations (Figure 3). Sampling was part of the routine diagnosis of febrile patients for the collection of venous blood for clinical laboratory test. All the participants accepted an oral informed consent previously approved by the bioethics committee of the USFQ. Venous blood samples were obtained from the arm and sera were stored and transported in liquid nitrogen to the USFQ for its further analysis. A total of 290 serum samples from febrile patients were collected between March 2013 and August 2014. 154 samples were from female patients and 136 were from male patients. All tested patients were hospitalized.

IgM analysis

A Panbio Dengue IgM Capture ELISA test for the qualitative detection of IgM antibodies to dengue antigen was run for every sample at the study site by the hospital staff according to the manufacturer's instructions (Panbio, USA).

Viral RNA Extraction

Viral RNA was isolated from 140µL of every serum or cell culture supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Netherlands) according to the manufacturer's

instructions. After the last centrifugation, the RNA was stored in two 35µL aliquots at -80ºC until used.

Reverse Transcription and PCR Amplification (RT-PCR)

For dengue virus detection and sero-identification, a 5µL aliquot of each RNA extract was subjected to RT-PCR. SuperScript III One-Step RT-PCR System; we used *Taq* DNA Polymerase (Invitrogen Life Technologies, USA) followed manufacturer's instructions. The reaction mix contained 12.5µL of 2X Reaction mix, 0.2mM of primers D1, TS1, TS2, TS3 and TS4, 80 U of SuperScript III RT/Platinum *Taq* Mix and 4.2 µL of RNase-free water to a final volume of 25 µL. The amplification protocol and primers used were previously described with some modifications from that of (Harris et al., 1998), these included: reverse transcription, annealing and extension temperatures; and primer concentrations. Reverse transcription was conducted at 52°C for 30 min, followed by a 2-min incubation at 94°C and 40 amplification cycles of 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 1 minute and a final extension at 72°C for 5 min. 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 bp, D1-TS1), TS2: 5´-CGC CAC AAG GGC CAT GAA CAG (119 bp, D2-TS2), TS3: 5´-TAA CAT CAT CAT GAG ACA GAG C (290 bp, D3-TS3), TS4: 5´-TGT TGT CTT AAA CAA GAG AGG TC (389 BP, D4-TS4).

For flavivirus detection of cell cultured sera, universal primers and PCR protocols were used as previously described (Maher-Sturgess et al., 2008). A 5µL aliquot of each RNA

extract was used for RT-PCR. SuperScript III One-Step RT-PCR System with *Taq* DNA Polymerase (Invitrogen Life Technologies, USA) was used following manufacturer's instructions. The reaction mix contained 25µL of 2X Reaction mix, 50 pmol of primers Flav100F and Flav200R, 100 U of SuperScript III RT/Platinum *Taq* Mix and 17 µL of RNase-free water to a final volume of 50 µL. The amplification protocol and primers used was as follows: a 40-min reverse transcription step was performed with four consecutive incubations of 10 min at each of 46°C, 50°C, 55°C and 60°C. *Taq* DNA Polymerase activation at 94°C for 15 min was followed by denaturation and extension steps at 94°C for 15 seconds and 68°C for 60 seconds, respectively. Annealing occurred for 30 seconds during each cycle, with a touch down stage at each of the following temperatures of 56°C, 54°C, 52°C, 50°C, 48°C, 46°C, 44°C and 42°C. After the touch down stage, 36 cycles with a 40°C annealing temperature were followed by a final extension for 10 min at 68°C. The reaction was held at 11°C until its processing or storing at -20°C. The expected size of the PCR products was 800 bp.

In both cases, cDNA amplification products were tested by electrophoresis in a 1.5% agarose gel using 5 µL of the PCR reaction. Gels were stained with EtBr and analyzed under UV light. The PCR products size was determined according to the migration pattern of a 100 bp DNA Ladder (Trackit, Invitrogen, USA)

As positive controls, enriched media from Dengue cultures (DENV-1 to 4) were kindly donated by Dr. Mary Regato at National Institute of Research in Public Health INSPI (Instituto Nacional de Investigación en Salud Pública), 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 with a PCR product size of 390bp (forward primer, 5' CGG AAC CGC TCA TTG CC 3' and, reverse primer: 5' ACC CAC ACT GTG CCC ATC TA).

C6/36 cells infection with sera from febrile patients

An 80-90% confluent cell monolayer of C6/36 cells was rinsed with 1X PBS and the serum was added in a 1:10 – 1:25 dilution (L15 medium, 2% FBS, Gibco, USA) in a total volume of 250µL for six-well plates and 500µL for T25 flasks. Plates were incubated for 60 min at 28°C, slowly rocking the culture every 10 min to ensure the monolayer was covered. Finally, culture medium (L15 medium, 5% FBS, Gibco, USA) was added to the infected cells and kept at 28°C for 7 days. Cells were monitored daily for cytopathic effects for 7 days after which, cells were detached using a cell scraper and pelleted by centrifugation for 5 min at 2000 x g, 4°C. Two 800µL aliquots of supernatant (with a final addition of 200µL of FBS) were stored at -80°C, and 140µL of supernatant were directly added to the lysis buffer (Qiagen, USA) for viral RNA extraction and RT-PCR detection and sero-identification.

Statistical analysis

Minitab® (version 17.0, 2013) statistics software was used to assess correlation through Cohen's kappa index.

Results

IgM, RT-PCR detection and sero-identification of Dengue Virus

RT-PCR positive reactions were detected in 37 (11 %) of the samples. Of the 37 positive samples, 16 were from female patients and 21 from male patients; all the four dengue serotypes were observed (*Table 1*). Since February 2014 to the end of the study in August 2014, only DENV-1 cases were observed . The IgM ELISA rapid test showed a total of 196 (67.5%) positive sera (*Table 2*). A poor correlation was observed between RT-PCR and IgM analysis with a Cohen´s kappa index of 0.34. Of the 37 RT-PCR positive samples, 24 were also positive for IgM.

Virus isolation

A total of 81 serum samples from febrile patients were cultured in C6/36 cells, including the 37 RT-PCR dengue positive sera; 10 of the 37 (27%) RT-PCR dengue positive sera were also culture positive for DENV-1. Six of the RT-PCR dengue positive cell cultures showed evident cytopathic effects (CPE) in C6/36 cells (massive cell detaching, syncytium-like structures, dark dotted zones). The remaining 44 cell culture samples tested negative for dengue by RT-PCR and were further analyzed with universal primers for the detection of other flaviviruses and all of them tested negative.

Discussion

This study was able to show that all the four serotypes of Dengue virus were circulating in this region during the period from March 2013 to July 2014 (*Table 1*). A survey carried out by the Ministry of Public Health of Ecuador detected only two serotypes in the Esmeraldas while all four serotypes were found in the rest of the Ecuadorian territory (Ministry of Public Health of Ecuador, 2014). Our results indicated that the Northwestern region shows different infectious patters than the rest of Ecuador. Similar results were described in a previous survey carried out 2010-2011(Cifuentes S., 2012). This finding is relevant because it may indicate that this people in the remote Northwestern region of Ecuador have greater risks of dengue infection than other regions of Ecuador and may also constitute a source of infection for other regions in Ecuador, where all the four serotypes are also found, but in different zones of the country.

It is important to note that the communities are located very close to the border with Colombia, where all four serotypes were also reported during this period (PAHO, 2014). It is possible that the constant circulation of people and goods through land and river trade routes along the Mataje river, in addition to a possible sylvatic cycle involving non-human primates and possibly other species of *Aedes* mosquito, maintains a constant interchange of the virus between both countries (Cifuentes et al., 2013). In addition, isolated communities along the coast, where education and information about preventive measures for vector control are practically inexistent, may also significantly contribute to the maintenance and re-emergence of the different serotypes of the virus.

In this study, positive RT- PCR reactions were evident in 37 (11 %) of the samples and the amplification of all the four serotypes was observed in very different proportions (*Table 1*). DENV-1 was the predominant serotype with more than 50% of the cases. The IgM ELISA rapid test was positive for a total of 196 (68%, *Table 2*). The correlation of positive results between molecular (RT-PCR) and serological analysis (IgM) was only 8% (poor agreement, Cohen´s kappa index= 0.34). This could have occurred, because viraemia commonly lasts until the fourth or fifth day after the onset of fever and its conclusion coincides with the increase in titer of IgM in blood, therefore, patients referred to the hospital after a fourth-fifth day of fever, were less likely to test positive for the virus (*Figures 3 and 5*). However, 7 (18%) out of the 37 positive results by RT-PCR were detected after a fifth day of fever (*Figure 5*) and 13 (35%) of the RT-PCR positive results were negative by the IgM ELISA rapid test, all of them up to a fifth day of fever (Table 2); this could be explained by an incorrect report on the days of fever by the patients, or the long lasting presence of IgM in the blood after, possibly, a previous infection. Most of the samples (122, 42%) were taken at the fifth day after the onset of fever and interestingly, most of the positive RT-PCR positive samples (18/37, 50%) corresponded to this group (*Figure 5*).

The presence of almost a 30% of negative results for both tests, but corresponding to patients with several days of fever, suggests the presence of other infectious agents (*Table 2*). In fact, it is widely suggested that patients with undifferentiated febrile illness in or coming from a tropical or subtropical area where endemic diseases are common, should be analyzed for infectious agents such as *Leptospira*, *Plasmodium*, *Rickettsia*,

dengue virus, *Brucella*, (Manock et al., 2009) and other viral infections including alphavirus infections such as Chikungunya virus. However, since IgM generally declines to undetectable levels over 2–3 months after infection (WHO, 2009), a new infection with a different serotype of the virus, which are known to be circulating in the zone, is also possible as well as an atypical course of infection in some patients.

Bibliography

1. CDC. (2015). CDC - Dengue. Retrieved March 5, 2015, from http://www.cdc.gov/dengue/
2. Cifuentes, S. (2012). Master's thesis "Molecular Surveillance of Dengue Fever in a Remote Area of the Northern Coast of Ecuador Using Serum and Capillary Blood Samples on Filter Paper." San Francisco de Quito University DSpace.
3. Cifuentes, S. G., Trostle, J., Trueba, G., Milbrath, M., Baldeón, M. E., Coloma, J., & Eisenberg, J. N. S. (2013). Transition in the cause of fever from malaria to dengue, Northwestern Ecuador, 1990-2011. *Emerging Infectious Diseases*, *19*(10), 1642–1645. http://doi.org/10.3201/eid1910.130137
4. Harris, E., Roberts, T. G., Smith, L., Selle, J., Kramer, L. D., Valle, S., … Balmaseda, A. (1998). Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *Journal of Clinical Microbiology*, *36*(9), 2634–2639.
5. Maher-Sturgess, S. L., Forrester, N. L., Wayper, P. J., Gould, E. A., Hall, R. A., Barnard, R. T., & Gibbs, M. J. (2008). Universal primers that amplify RNA from all three flavivirus subgroups. *Virology Journal*, *5*, 16. http://doi.org/10.1186/1743-422X-5-16.
6. Manock, S. R., Jacobsen, K. H., Bravo, N. B. de, Russell, K. L., Negrete, M., Olson, J. G., Kochel, T. (2009). Etiology of Acute Undifferentiated Febrile Illness in the Amazon Basin of Ecuador. *The American Journal of Tropical Medicine and Hygiene*, *81*(1), 146–151.
7. Ministry of Public Health of Ecuador. (2014). GACETA EPIDEMIOLÓGICA SEMANAL No. 51. MSP.
8. PAHO. (2014). State of the art in the Prevention and Control of Dengue in the Americas: Meeting report. PAHO.
9. Stephenson, J. R., & Warnes, A. (Eds.). (2011). Detection of Dengue Virus - Springer. Humana Press. Retrieved from http://link.springer.com/protocol/10.1007/978-1-60761-817-1_4
10. WHO. (2009). *Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control*. World Health Organization.

APPENDIX

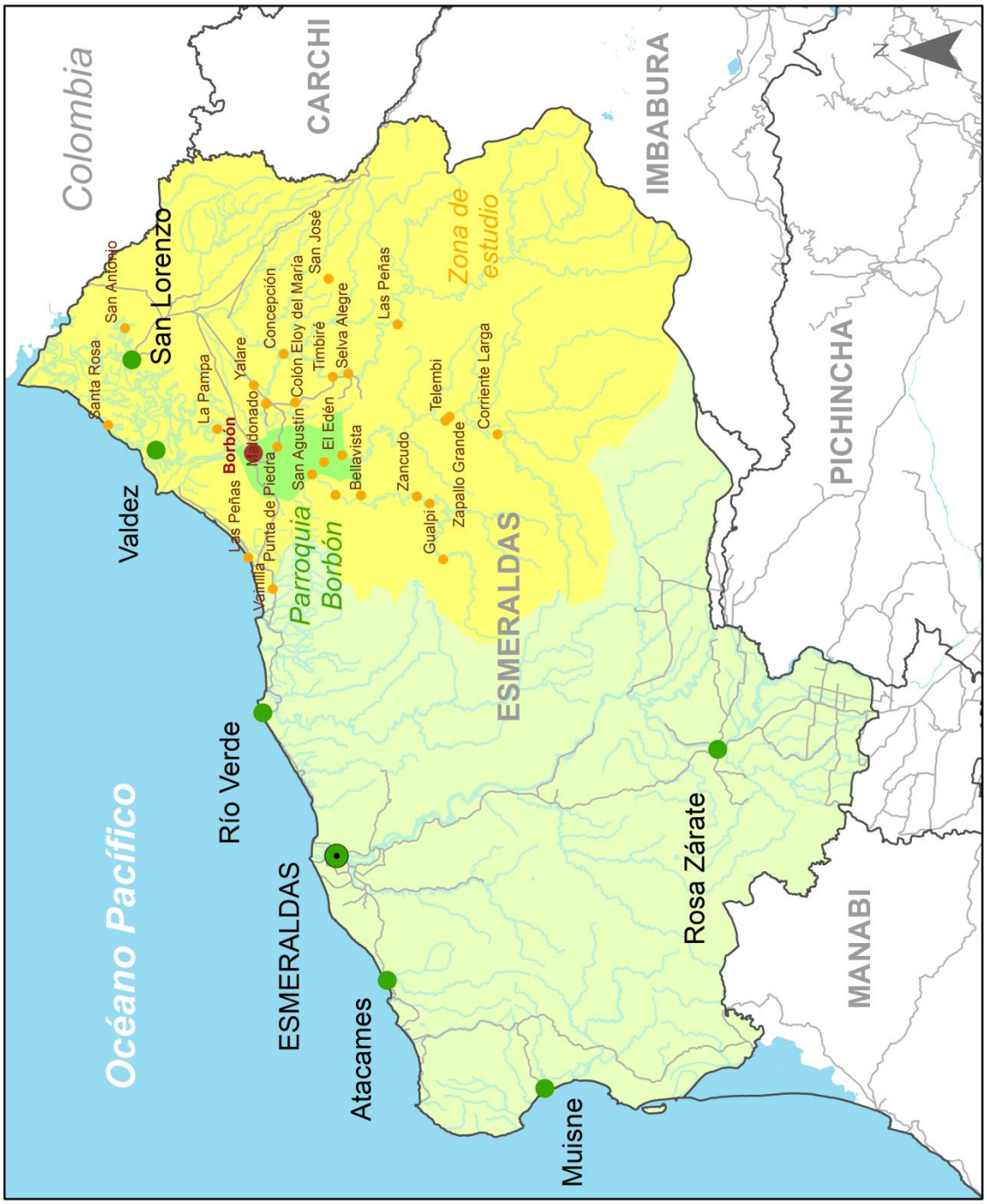

Figure 3. Map of the province of Esmeraldas and the zone of study. Credits: Ing. Gustavo Rueda, 2015. ArcMap 10.2. Basic cartographic information - 1:250000, SIGAgro – Ministry of Agriculture of Ecuador.

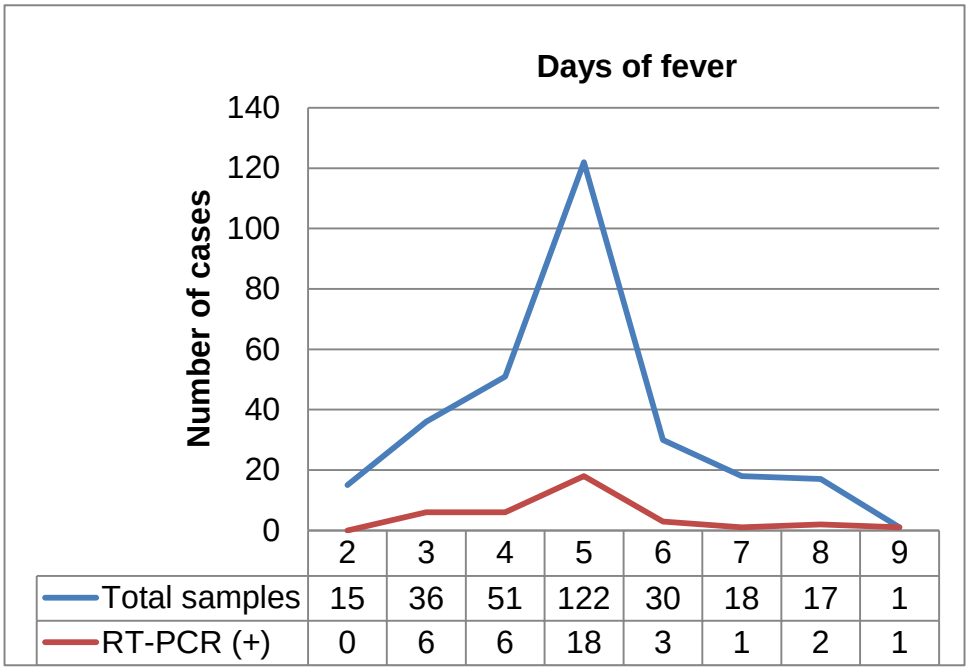

Figure 5. Number of cases reported by days after the onset of fever.

Table 1. (RT-PCR) vs serological analysis (IgM).

RT-PCR	IgM (+)	IgM(-)	Total
DENV1	18	2	20
DENV2	1	6	7
DENV3	4	4	8
DENV4	1	1	2
Negative	172	81	253
TOTAL	196	94	290

Table 2. Coincidence of serological (IgM) and molecular analyses (RT-PCR).

IgM vs PCR	#	%
IgM (+) & PCR (+)	24	8
IgM (-) & PCR (+)	13	4
IgM (+) & PCR (-)	172	60
IgM (-) & PCR (-)	81	28
TOTAL	290	100