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Active Surveillance and Incidence Rate of Dengue Infection in a Cohort of High Risk Population in Maracay, Venezuela.

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Active Surveillance and Incidence Rate of Dengue Infection in a Cohort of High Risk

Population in Maracay, Venezuela.

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Date of Approval:
December 16, 2009

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ACTIVE SURVEILLANCE AND INCIDENCE RATE OF DENGUE INFECTION IN
A COHORT OF HIGH RISK POPULATION IN MARACAY, VENEZUELA

Carlos Espino, MD., Ph.D. candidate

ABSTRACT

In the absence of an effective vaccine, vector control and surveillance of dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most important strategies currently used to reduce the impact of these diseases in affected population. The objectives of this study were to estimate the incidence of symptomatic and asymptomatic dengue cases, the prevalence of antidengue antibodies, and to evaluate the laboratory and clinical aspects related to an active surveillance of dengue cases. In this study, active surveillance was incorporated as a part of the study design. At total of 3,255 people from four high risk neighborhoods were followed in a two years prospective study whereby the participants' houses were visited three times a week. During these visits, dengue cases were characterized by identifying patients with fever as well as other symptoms that were compatible with dengue disease. In addition, a biannual blood sample was taken for each study participants, to establish the prevalence and six month incidence of dengue infection.

We found a crude incidence density (ID) of 3.24 by 100,000 person/days (p/d) which changed from 5.69 by 100,000 p/d for the first year of the study to 1.45 by 100.000 p/d in the second year. In both years, the months from July through September had the

highest ID of 8.81 by 100,000 p/d. Children displayed higher ID when compared to adults, RR: 3.92 (2.38 – 6.48).

The Plaque Reduction Neutralization Test was used to assay for the presence of antidengue antibody in 2,125 study participants (65.3% of total). The prevalence of anti dengue antibodies was found to be 86.6% (1,840 positives). The prevalence of anti DENV-1 was 74%, while 65.2 % of the participants had anti- DENV-1 and anti- DENV-2 simultaneously. The cumulative incidence of anti IgG dengue antibody in the negative participants (283 at the start of the study) was 30% in the first 6 months period, 29.6% in the second 6 months, and 23.8 in the third one.

The difference between the numbers of participants detected in the active surveillance, (270 confirmed and non confirmed dengue cases) with the numbers of people who showed sero-conversion to anti-IgG dengue antibody within a relatively short period of time suggested that there was a high number of asymptomatic dengue infections present in the population. Transmissibility of the virus, the surveillance of dengue, and vaccine implementation in the near future would all be affected by the large number of asymptomatic people in hyperendemic countries.

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Keywords: Prevalence, asymptomatic, sero-conversion, antibodies, epidemic.

CHAPTER ONE: THE STUDY PROBLEM

Introduction to the Problem

In the last forty years in the Americas, Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF) respectively have been considered a re-emergence and an emergence disease (OPS, 2000), (OPS, 2006). In the absence of an effective vaccine, mosquito control and surveillance of dengue disease have been the strategies used worldwide to prevent this infection. However, 40 years later, the combination of these strategies has not been totally effective in reducing the number of DF and DHF cases. Dengue virus (DENV) is spread in all Central and South American countries; all four DENV serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) are circulating, and the number of cases reported each year is increasing. A deficient vector control and the type of ineffective public health surveillance currently being used in Central and South American countries could partly explain the unsuccessful prevention and control of dengue (OPS, 1994), (Call et al 2006), (Ooil et al 2006). Usually surveillance is based only on the passive detection of symptomatic cases and thus does not provide information about the actual number of people infected, and the true proportion of the population at risk to get a secondary infection (which is a risk factor of DHF). Passive surveillance system cannot detect epidemics at a pace rapid enough to provide ample time for appropriate preventive actions. Furthermore, this type of passive surveillance system cannot provide an updated analysis related to the dynamic of the dengue disease. For example, the introduction of a new serotype or genotype could change the expected

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clinical presentation and epidemiological indicators in the population. Additionally, this type of information would be essential in the new era of dengue vaccine and necessary to understand the dynamic of the viral infection. Passive surveillance that provides only general information such as the number of symptomatic cases reported is not adequate; alternative options such as active, vector, sentinel and interepidemic surveillance will have to be globally implemented. (Gubler, 2002), (WHO,1997), (WHO,1999).

Incidence and Prevalence as measures of Dengue Infection.

In order to achieve a good system of disease surveillance, the epidemiology system requires specific indicators to be able to calculate and determine the frequency of the disease. Usually the selected measurement (and/or indicators) depends on the type of disease. That is how the measure of the dengue infection can be made, which is either to directly determine the occurrence of their new symptomatic cases (disease incidence) in a risk population within a given period, or indirectly, to calculate the prevalence of presence of anti-dengue antibodies in healthy people previously infected in a determined population. Estimating the infection incidence could be determined by computing symptomatic and asymptomatic cases of dengue. The disease incidence is the measure to establish what would be the magnitude of dengue epidemics; what would be the impact of the problem in terms of public health, and which should be the best way to proceed to reduce their consequences. The prevalence of antibodies permits us to know the magnitude of the transmission in a given period, how the virus is circulating, which age groups are more affected. Furthermore, having two or more prospective measures of antibody prevalence, we can also estimate the incidence of the

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infection. Through all of these possibilities, early prediction of new epidemics is reasonably the main goal of many public health programs. (Runge-Ranzinger et al, 2008), (Gordis & Saunders, 2000).

The simultaneous analysis of these two measures of disease and infection permits us to understand the dynamic of the dengue virus, determine the proportion of symptomatic and asymptomatic cases, and establish the risk and the factors associated with the disease.

Historically in dengue disease, Cumulative Incidence (CI) and serological prevalence of anti dengue antibodies have been the most frequent indicators used to determine the frequency of clinical dengue disease and dengue infection respectively. Since dengue is an acute disease, the incidence is the best way to determine its frequency. Since immunological conversion is a prevalent condition, sero prevalence of anti dengue antibodies is the best way to determine previous infection of DENV, both usually reported in surveillance dengue information and through epidemiological dengue researches (WHO-DENGUENET, 2009).

Considering incidence exclusively, Cumulative Incidence is a good indicator of risk and the only incidence indicator that can be used to compare temporal and geographical impact of dengue epidemics. However, its value is not enough to determine factors associated with the disease. Incidence Density (ID), defined as the number of cases over the sum of length of time at risk for each participant in a prospective study, is the best indicator to establish association between factors and disease. Besides, ID is more precise to estimate the rate of disease occurrence. Due to the fact that ID requires a

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close follow up on the studied population, relatively few studies of dengue have been reported with this indicator. (Kleimbaum et al, 1982).

In spite of the fact that Venezuela has had the highest incidence of DF and DHF from 1989 to 2005 in the Americas, this country has not studied prospectively exposed population. There are no reports available to date of dengue researches that use Incidence Density and also, the studies of sero prevalence of antibodies have been limited to school children and transversal evaluations (WHO-DENGUENET 2009), (Guzman, 1999).

<div align="center">

Surveillance of Dengue

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Public Health Surveillance has been defined by Thacker as: "*The ongoing systematic collection, analysis, and interpretation of outcome-specific data for use in the planning, implementation, and evaluation of public health practice*". In this definition, the goal is clear, the steps are logical; however, the procedures of each one of those parts in the definition can differ among countries, particularly between developing and developed countries, and can even differ within the same country when comparing data from different decades. In general, developed countries have better surveillance systems, with better laboratories for diagnosis, better integration between health service dependences, and better quality of the data. Gubler, analyzing dengue data generated prospectively, has been specific about these differences   (Gluber, 2002), (Teutsch and Churchill, 1994).

The choice of which types of surveillance system to use will depend on the characteristics of the disease, the magnitude of the problem caused by the disease in a specific country (or global region), and the resource and technology of each affected

country. In this respect, when analyzing all Latin American countries and Venezuela in particular, the areas most affected by dengue in the Americas could be considered developing countries, according to the World Bank classification.. Cuba and Puerto Rico have demonstrated superior efficacy to control and prevent dengue epidemics. In the case of Puerto Rico, this country has tried to develop a surveillance system during inter epidemic periods by implementing a system of the early detection of circulating viruses, before the epidemic has been recognized (Rigau-Perez, 2005), (Gubler, 2002). (Guzman, 2005). Venezuela has supported its surveillance system with the passive reporting of cases; when the number of cases reported is higher than the number of cases expected; the system can declare epidemic and activate the specific measure of control. This is the regular application of surveillance in many Latin American countries, and certainly that is better than nothing, because it has reduced the fatality from DHF in the last 25 years in the region. (Feldman, 2004).

Clinical symptoms and laboratory diagnosis of Dengue

Diagnosis based on clinical symptoms and laboratory diagnosis of dengue play two important roles that may be different somewhat. On the one hand, early recognition of dengue based on the clinical symptoms and early laboratory confirmation could make the difference between the life and the death of the patient; both of these contribute towards individual significance of surveillance. On the other hand, clinical symptoms and diagnosis are also indispensable to detect the presence of the disease and thus could help to predict and avoid an epidemic. If an epidemic is recognized early, control measures can be implemented immediately; this represents the public importance and is the point that is related to the public health surveillance system. (WHO, 1997)

Statement of the problem

Latin America and many other developing countries do not have a good surveillance system to detect cases of dengue that are less evident and less symptomatic, whereby infection of dengue does not yet produce epidemic but may very well be on its way to generating one.

Specific aspects that could be obtained in these cases include the number of asymptomatic cases, clinical variations and changes in the criteria for clinical and laboratory diagnosis of dengue. Additional information that can be acquired include differences between what regular passive surveillance system is detecting and what active surveillance system could detect, which and how other risk factors could affect incidence of dengue and its consideration in an active surveillance system.

Purpose of the Study

The purpose of this study was to establish those epidemiological characteristics, diagnostic and clinical aspects of dengue disease that could be related with the surveillance of dengue, and use these to understand how surveillance can be used as one strategy to prevent the effect of the disease caused by dengue infection. The goal of this study is to compare and contrast the findings with the traditional or regular dengue surveillance system. Epidemiological aspects are those indicators which usually are not considered in the passive surveillance systems but are affecting the dynamic of the disease in the populations. In this study, epidemiological aspects included are frequency of dengue, silent or asymptomatically infected people, and the proportion of people at risk to acquire DHF. Clinical and laboratory diagnostic aspects are those criteria which can improve the identification of the dengue cases. We considered and collected during

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the course of this study this information, the day of the physical exam was performed and the blood sample taken, as well as the specific time and type of diagnostic test used and the clinical symptoms presented. A surveillance system of dengue should have the capacity to collect and analyze this kind of information in order to prevent disease effects.

Aims of the Study

1. To estimate the incidence density of dengue disease in the population, by detecting the number of symptomatic cases during active surveillance, and sorting out and analyzing data by age groups, location and seasonal year period.

   1.1 To estimate the incidence of dengue disease in four neighborhoods.

   1.2 To estimate the incidence of dengue disease in children from 5 to 14 years old, and adults from 15 to 94 years old.

   1.3 To establish the Incidence density and compare the Relative Risk of dengue disease between children and adults.

2. To estimate the prevalence of antibody against dengue in the population using anti-dengue IgM MAC ELISA and anti-dengue IgG by Plaque Reduction Neutralization Test (PRNT) using biannual sero-prevalence surveys.

   2.1 To estimate the prevalence of antibody against dengue according to specific serotype and age group.

3. To estimate the proportion of dengue infection through the biannual seroprevalence surveys, by detecting asymptomatic and symptomatic dengue cases during each interval of blood sample collection.

3.1 To estimate the proportion of dengue infection in people with primary and secondary dengue infection.

3.2 To estimate the proportion of specific dengue serotypes in people tested negative in the first sero-prevalence sample of dengue antibody (Sample number1).

4. To identify and compare procedure in laboratory test used in the active surveillance, by considering viremic and immunological indicators at specific time points, which are categorized based on the number of days after onset of the symptoms, age group, neighborhoods and serotype from the active surveillance.

4.1 To describe the frequency of blood samples that were taken according to the days after onset of the symptoms.

4.2 To estimate the proportion of specific dengue serotype by age group of patients, and looking for possible association between these parameters.

4.3 To estimate the proportion of specific dengue serotype by four neighborhoods, looking for possible association between these parameters.

4.4 To estimate the number of cases detected by specific type of diagnostic test, and the day of sample taken after the onset of the symptoms.

4.5 To compare the results of confirmed dengue cases determined by Active Surveillance with the same samples tested using PRNT, and establishing the proportion of congruency between temporal and specific dengue serotype.

4.6 To compare the results of IgM MAC ELISA test in confirmed dengue cases by Active Surveillance with the same samples tested by PRNT, looking for the proportion of primary and secondary infection according to IgM test results.

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5. To identify clinical symptoms according to disease confirmation and age group in those people who were detected in the active surveillance.

    5.1 To describe the most frequent symptoms present in all people detected to be dengue positive by the active surveillance as designed in the study.

    5.2 To compare the most frequent symptoms between confirmed and non- confirmed dengue cases as detected by the active surveillance.

    5.3 To identify those symptoms and hematologic indicators associated with confirmed and non confirmed cases by the active surveillance.

    5.4 To identify hematologic indicators of dengue and anti-dengue IgM antibody in consecutives samples during acute phase of dengue disease in people detected by the active surveillance.

6. To identify the differences between the regular system of passive surveillance of dengue disease in the national and local department of health and the data obtained in this study, assuming this study represents a system of active surveillance.

    6.1 To determine diagnostic classification according WHO in the study data.

    6.2 To describe the cumulative incidence of dengue in 2007 related with the seasonal rainy months.

    6.3 To describe the cumulative incidence of dengue in 2007 related with the passive national and local surveillance of dengue.

Significance of the Study

In dengue, surveillance is a recognized preventive measure to control the disease. However, few studies have been conducted to determine which specific aspects of the

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dengue disease can be used to improve that surveillance system. This is particularly true in developing countries where the disease is hyperendemic, and where many control activities have had little impact in controlling the increasing numbers of epidemics. The goal of this study is to identify some of the aspects within an active surveillance design that can be applied in a real community, and which include children as well as adults as the study subjects. Apart from our study, we could not find any prospective study focusing on the communities that conducted weekly door to door visits and which take in consideration a broad range of age, when we reviewed more than 75 articles searched through PubMed that reported prospective studies of dengue. Most of these studies were conducted either in schools or workplaces, or using community health services. Besides, these studies were usually focalized in a specific age group, which are either children or adults, but not both.

   With dengue vaccines continually being developed in different phases of study but still elusive, the precise knowledge of virus transmission and early detection of cases is becoming extremely necessary in the effort to work towards prevention of dengue diseases.

   This study will contribute towards increasing the knowledge pertaining to dengue.

CHAPTER TWO: BACKGROUND AND LITERATURE REVIEW

Dengue Disease: General Characteristics.

Worldwide, dengue is the most important viral disease transmitted by mosquitoes and other arthropods, with an estimation of around 100 million cases and 20,000 deaths annually (Stephenson, 2005). Dengue affects tropical areas of Asia, Africa, Oceania and the Americas. The causal agent is the Dengue Virus (DENV), which belongs to the *Flavivirus genus*, and is transmitted mainly among humans by the species of mosquitoes *Aedes egypti and Aedes albopictus (Fields 2001),* (Kuno 1997). For more than 200 years, this infection has been recognized causing important epidemics in the Americas, Africa and Asia. However, Dengue Fever (DF) or also known as classical dengue was not considered a fatal disease until the 1950's when it appeared in South East Asia as a new, severe and fatal variant of dengue disease known as Dengue Hemorrhagic Fever (DHF). Even though a few hemorrhagic cases of dengue had been described before 1950, these cases had never occurred as an epidemic. Therefore since that time, DHF is considered an Emergence disease (WHO, 1997), (Isturiz et al, 2000), (Gubler 1995), (Pinheiro 1997).

Clinical presentation of dengue is described as an asymptomatic infection until severe and fatal Hemorrhagic and shock syndrome, passing by mild like viral syndrome and non fatal Dengue Fever. (WHO, 1997).

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Without an effective vaccine and antiviral treatment for dengue, control of the vector and surveillance are the only two most important public health strategies to prevent and control this disease and its consequences. (Stephenson, 2005), (WHO, 1999).

Supported by established theories, dengue disease could be considered as one of only a few viral human diseases where previous infection by heterologus dengue serotypes increases the risk to a developing a new, severe and sometimes fatal dengue virus infection. (Rico-Hesse et al, 1997), (Gubler, 2002).

Dengue Virus.

Dengue virus is a positive sense single strand RNA virus, belonging to the family flaviviridae. This family has three genera: Hepacivirus, Pestivirus and Flavivirus. DENV is a flavivirus which belongs to a genus with more than 70 viruses, including two phylogenetic groups: the mosquito borne with West Nile Virus (WNV), Saint Luis Virus (SLE), Yellow Fever Virus (YFV), Murray Valley encephalitis virus, Kunjin virus and Japanese Encephalitis Virus (JEV) and the tick borne with Powassan virus, central European encephalitis virus and Far Eastern encephalitis virus. (Fields, 2001), (Mukhopadhyay et al. 2005).

The dengue virion consists of an envelope and nucleocapsid, and is spherical with a diameter of 40-60 nm. The RNA complete sequence is around 10,700 nucleotides long. (ICTV, 2009). The genome encodes three structural proteins (capsid, pre-membrane and envelope) and seven non structural proteins (NS1–NS2A–NS2B-NS3-NS4A– NS4B-NS5). Due to polarity and exposition, the majority of the primers to identify and differentiate DENV to other flavivirus target the Envelope and NS1 regions. Primer

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regions usually are related with the pathogenesis and cross reactive immunity of the viruses (Kuhn et al, 2002).

Dengue virus is subdivided into antigenic types according to serological criteria and four serotypes have been recognized: Dengue virus 1 (DENV-1), Dengue virus 2 (DENV-2), Dengue Virus 3 (DENV-3), and Dengue Virus 4 (DENV-4). Each serotype produces lifetime immunity against homologous dengue viruses, and temporary and partial immunity against heterologous dengue viruses. (WHO, 1997), (Fields, 2001), (Kuno, 1997).

Depending on the area of the world, the cross reactivity among the flaviviruses plays a key role in specific antibody identification and surveillance system. This cross-reactivity among flavivirus species can be causative of false positive in serological test results. In Asia, Japanese encephalitis and West Nile infections are the main cause of cross reactive immunological response in dengue laboratory surveillance. Yellow fever has the same importance in Central and South America, while West Nile and Saint Luis Encephalitis viruses are more prevalent in North America (Mukhopadhyay et al 2005), (Koraka et al 2002). In addition, cross reactivity can be generated by vaccination of YFV live attenuated and JE inactivated virus. (Scharwtz et al 2000), (Vasquez et al, 2003).

Based on the nucleotides substitution rates estimation of Dengue virus, mean substitution rates are in a range from 4.55×10^{-4} (DENV-1) to 9.01×10^{-4} (DENV-3) substitutions per site, per year; therefore, the origin of DENV could be relatively recent, at around approximately 1,000 years ago.(Twiddy et al., 2003).

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Anti DENV antibodies have been found in non human primates in West Africa and Asia (Malaysian), supporting sylvatic transmission cycle idea for Dengue viruses. Indirectly, this provides evidence that dengue originated from monkeys and sylvatic mosquito vectors. At some point in time, the virus passes to the humans; this probably occurred approximately 200 or 300 years ago (Holmes, 2003), though othershave related the origin of dengue disease with former cases reported from China and French West Indies (Gubler,1998). Supposedly, the disease was effectively spread to the Americas when the virus and its mosquito vector (*Aedes* species) were transported in slave trading or other commercial trips from Africa either during the XVIII century or before. Even when two cycles (sylvatic and non-sylvatic) of the DENV transmission are recognized, clinically and epidemiologically, dengue transmission is considered a human to human infection disease. Therefore, in contrast to other vector-borne diseases and especially arthropod viral diseases, dengue does not have other hosts or reservoirs to facilitate or anticipate the action of public health surveillance. DENV can grow in monkeys and mice but these animal species do not exhibit symptoms of the disease as seen in humans (Holmes et al , 2003), (Pang, 2003), (CDC-DHHS, 1993).

Disease Transmission and Vector.

Dengue infection is transmitted from human to human through the bite of female mosquitoes. During the viremic phase, infected humans are capable of transmitting the virus to the mosquitoes taking in a blood meal. This viremic phase in human blood lasts approximately 5 days and takes place after one incubation period of 6-8 days; this is known as the "intrinsic period". After mosquito bites an infected human while taking a blood meal, the viruses propagate inside the vector during the "extrinsic incubation"

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period, which usually lasts 7 to 8 days. Finally the infected mosquito re-feeds and transmits the virus to a second susceptible human (WHO, 1997), (McBride et al, 2000). The virus human cycle is limited at 3 -5 days due to a cytopathic humans cells effect; this cytophatic effect does not occur in mosquito where the viruses can be propagated and accumulated in the salivary gland throughout the lifetime of the mosquito. (Fields, 2001). After a mosquito gets infected, it is capable of, and is effectives in transmitting the Dengue virus to the next human host and cause dengue infection.   In addition, experimental studies have demonstrated that mosquito to mosquito transmission by vertical transovarial infection is possible. One study demonstrated the capabilities of seven mosquito generations passing the virus. This could be a way to keep the virus in a place without the direct infection of humans (Joshi et al, 2002). Besides, Mourya found DENV in aedes aegypti eggs and the vertical transmission rate was higher in those eggs with more time to hatch. (Mourya et al, 2001).

   Aedes aegypti and *Aedes albopictus* are the two most important mosquitoes capable of transmitting the Dengue viruses and infecting humans. Both mosquito species are present in the Americas and Asia where dengue is a public health problem. However, *Aedes aegypti* has demonstrated more efficiency for dengue viral transmission. This advantage is related to the capacity of *A. aegypti* to live within the human communities, sharing resting areas and using the stored human consumption water as a feeding habitat. All the life stages of this mosquito can be developed within the human habitat; this behavior is different when compared to *A. albopictus* which is a more aggressive vector which sustains feeding habitats farther away from human houses. Consequently,

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A. aegypti has been considered to be the principal vector, and *A. albopictus* the secondary vector in the transmission of dengue (Kuno 1997), (Nagao et al, 2003).

In the absence of an effective vaccine against dengue, control of the mosquito vectors which is based o elimination of larvae and breeding ground sites more than the adult mosquitoes control, is the key to prevention of the disease and the epidemics. However, in many developing countries, the current vector control emphasis is in emergency response rather than prevention of epidemics. This is illustrated by the emphasis on adult mosquito elimination, which corresponds to an emergency response approach, versus larvae elimination which unfortunately is not perceived as a great control measure since the impact is not immediate.(Gubler, 1998). The best examples to illustrate the point above are represented by these countries where the focus of prevention has been the mosquito control; United State in the last century, Cuba after 1981, and Singapore in the last 25 years (Wilder-Smith et al, 2004), (Halstead, 2000). Of course, mosquito control by use of chemicals such as insecticide is not the only method for an effective vector control. Other approaches that could also be very important include ways such to improve the housing conditions, education of people at risk, and improvement of water system supply in the community so as to reach a definitive prevention of this disease (Heukelbach et al, 2001), (Gubler, 2002).

In addition to the control of vector to prevent disease, mosquitoes can also play a significant role in conducting the dengue disease surveillance. The measures of the larvae or mosquito density are indicators to identify areas of greater risk. Breteau index has been classically been one of the most used method to estimate relative densities of mosquito. Breteau index is defined as the number of positive containers (*Aedes aegypti*

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larvae that can be visually detected) per 100 houses inspected. House Index is another indicator that can be used to estimate the mosquito density; it is defined as the percentage of households infested with larvae or pupas in a specific urban or rural area. Another aspect of the dengue vector surveillance that poses a challenge is the fact that dengue does not have other hosts that can be used to detect or predict, during the early stages that the virus is circulating in the community. In other words, beyond knowing the density of the mosquitoes in terms of determining the entomological risk, and identification of dengue viruses and their specific serotypes in these mosquitoes; these would be the only invaluable measures that can provide information about circulating dengue viruses during the interepidemic periods. In the United State the mosquitoes surveillance for both purposes. However in developing countries, the mosquitoes' surveillance when it does take place is used only to determine the density of the mosquito (CDC- DHHS, 1993).

Transmissibility

The transmission dynamic in dengue disease is the result of many variables. This is due to a combination of actions by four independent serotypes, and the different ways the disease is presented into the variety of environmental conditions. The four serotypes have the capacity to circulate simultaneously in hyperendemic areas where secondary or tertiary infections can be potentiated by previous heterologous serotypes and non neutralizing antibodies. The majority of infections are unapparent or asymptomatic even though some clinical cases can be severe. Information from the literature does not describe if the asymptomatic cases have the same transmission capacity than patients presenting with dengue symptoms. This concern is expressed in this quote "*Another*

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problem rarely addressed in BRR determination is the impact of asymptomatic

infections" (BRR: Basic Reproductive Rate). (Kuno, 1997).

Basis Reproductive Rate (Ro/BRR) also known as Basis Reproductive Number, is the average number of new infections produced for each case of current infection. This indicator helps to determine how a disease infection can spread throughout the population. When Ro is higher than 1, the infection is able to spread in the population, and if Ro is less than 1, epidemiologically the infection will die out. In addition, BRR has public health importance as it can be used to establish the proportion of population that needs to be vaccinated to reach herd immunity. The higher the BRR, the higher the proportion of people that needs to be vaccinated. According to Kuno, the range of dengue BRR would be from 1.33 to 2.00, though recent studies show that the numbers can be greater. BRR is in direct relation with the number of contact per unit of time, transmission probability per contact and duration of infectiousness. (Kuno, 1997), (Massad, 2003).

Special situations can occur with DENV transmission; DHF cases could increase when vector prevention control partially reduces the mosquito density. In his study, Thamalato concluded that the negative relationship between DHF incidence and dengue transmission intensity implies that in regions of intense transmission, insufficient reduction of vector abundance may increase long-term DHF incidence. (Thamalato et al., 2008). Singapore provides another example of a situation of dengue transmission that is difficult to understand. This country has been successful in reducing the mosquito density; however, simultaneously they have had an increase of dengue fever cases in the last years. (Egger et al, 2008). In other viruses the infection transmissibility

could be explained easier but not so for dengue as it is almost like four diseases that is closely linked by a confused immunological system.

<p style="text-align:center">Herd Immunity</p>

Herd immunity is the resistance of a group or community to attack by an infectious disease due to the fact that a large proportion of the population is immune against the disease. This term is usually associated to vaccination effect; however, having the disease is a way to acquire "natural" immunity and theoretically acquire herd immunity. Herd immunity threshold is the maximum level of immunity beyond which transmission is eliminated (Kuno, 1997). There is not only a meaning to this concept, John and Samuel proposed one interesting differentiation: herd immunity as "the proportion of subjects with immunity in a given population" and "herd effect" defined as "the reduction of infection in the unimmunized segment as a result of immunizing a proportion of the population". (John & Samuel, 2000).

In any case, herd immunity is closely related to the Basic Reproductive Rate. Massad in Brazil showed 64 cities with dengue epidemic; all these cities had BRR greater than 1 with a range from of 2.74 to 11.57. The maximum value in Brazil is similar to the BRR of Measles infection viral disease which required between 83% and 94 % of vaccine coverage. Ferguson in Thailand obtained BRR to four serotypes using different methods; the range of values was from 1.39 to 7.73 but they did not find differences among serotypes. (Massad, 2003), (Ferguson, 1999).

When we check the different mathematic models proposed in studies to estimate the Basic Reproductive Rates, these are based on the number of cases that started the epidemics, known probabilities of human to mosquitoes contact, and the number of

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susceptible at a certain time. However, probable effect of asymptomatically infected people in the transmission is not clearly represented in these mathematical models. (Koopman and Longini, 1999), (Kuno, 1997).

Epidemiology and Surveillance of Dengue

Worldwide Distribution

Historically, there are at least three different sources providing report about the first dengue case; chronologically, a report from China provided the first description of a disease compatible with dengue in 610 A.C. (Gubler, 1998). The second report probably registered the source of dengue outbreak was in West French Indies in 1635 (Izturiz et al, 2000), (Gubler, 1998). Finally, and usually the most cited first dengue report was about dengue in Philadelphia in the summer of 1779 (Gubler, 1995), (Holmes, 2003).

There is disagreement about the the origin of the dengue virus, reported to be either from Africa or Asia. This is due to non-human primates that were detected with DENV in the sylvatic cycles in both continents; and is not clear which was the direction of the expansion. (Holmes, 2003).

There were at least 4 Dengue-like illness epidemics in the Americas that happened in the XVIII century occurred (Pinheiro et al, 1997). Asia had similar evidence of non-fatal disease with epidemics of 10-40 years intervals (Gubler, 1995). The negative effect of the World War Two in the South East Asia environment was probably the event which separated the benign dengue disease from the severe and sometime fatal dengue. Hyperendemicity and expanded DENV epidemics in the region were the prelude to the first confirmed epidemics worldwide of Dengue Hemorrhagic Fever, which later became established as an emerging global disease. The exact event that

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started all of this was thought to have occurred in Manila Philippines in 1953–1954, (Gubler, 1998). However, according to Halstead, non confirmed DHF epidemics had already previously appeared in Australia in 1897 and in Greece (western hemisphere) in 1928, (Halstead, 1980). After the 1953 epidemic of Manila, the DHF spread throughout all of Asia, beginning by the south east countries and extending to the rest of Asia.

The first report of an epidemic of Dengue Hemorrhagic Fever (DHF) in the Americas occurred in Cuba in 1981, whereby 158 people died and 10,300 DHF cases were registered. The second large epidemic took place in Venezuela in 1989 with 2,665 DHF cases, extending to 1990 with 3,325 DHF cases, whereby 18 and 52 people died respectively, in those two years of outbreak. According to WHO-DENGUENET, since 1960 to 1980, Cuba and Venezuela were the two countries with the most number of dengue fever reported to WHO; in Cuba the highest number of DF cases occurred in 1977 (477,440 cases) and in 1978 (75,692). Venezuela had three peaks of DF cases; 18,306 in 1964, 7,750 in 1966 and 100,000 in 1978. (WHO-DENGUENET, 2009). However, after the epidemics described above, Cuba and Venezuela followed different paths reporting different epidemiological histories. Cuba did not have cases reported again after the earlier epidemics until 1997, when Cubans suffered a second epidemic which characteristically affected only adults. On the contrary, after 1989, Venezuela became the Latin American and Caribbean country with the greatest number of DHF cases, whereby many people died from dengue disease. Other countries also have had higher incidence of dengue but none has had worst indicators in terms of magnitude (Kouri et al, 1998). According to the Antibody Dependent Enhancement theory, DHF could be directly correlated to hyperendemicity. This could explain the high number of

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DHF cases in Venezuela, specifically in Maracay which is the capital of the Aragua State, where geographical studies has demonstrated the simultaneous circulation of all dengue serotypes.(Rico-Hesse et al, 1997), (Barrera et al, 2000).

Incidence of Dengue

Incidence means new events or cases happening within a period of time. In epidemiology these new events are referred either in relation to the population at risk, or in relation to the population-time at risk. As a result, incidence of a disease could be measured by two ways. One way is determining the Cumulative Incidence (CI) which is estimated by calculating the proportion of people who developed the disease in a fixed and disease-free population at the beginning of the follow-up period. Frequently, the CI is the indicator used either by the regular system of surveillance, or by the public health programs inserted in a national or international communicable disease network. CI is obtained considering new cases detected in annual periods of time. Theoretically, Cumulative Incidence can measure the risk and predict risk in an individual level. The other way to obtain incidence is by determining the Incidence Density (ID) which is defined as the instantaneously change of disease status per unit of time. The ID determination requires the amount of population-time (PT) added by each person in the study. PT can be expressed interchangeably in years, months, days and hours. ID can estimate rate referred to a population thus it does not have direct interpretation on the individual level. (Kleinbaum, Kupper and Morgenstern, 1982) (Rothman, 1986). The Incidence Density is mostly used by Researchers who are looking for association among factors and the frequency of the disease.

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Through DENGUENET, World Heath Organization has the most complete historical database about the amount of dengue cases represented by the three more affected regions in the world: Pan American Health Organization (PAHO) in the Americas, South East Asia Region, and Western Pacific Region in Asia. (WHO-DENGUENET, 2009)

According to data of PAHO in the period from 2000 to 2005, the highest Cumulative Incidence of dengue (by 100,000 population) in the Americas has consecutively been in French Guiana (1,001), Costa Rica (343), Honduras (254), Barbados (221), Brazil (195), San Vincent (158), Suriname (154), Venezuela (143), Puerto Rico (92), Colombia (82), Paraguay (81), Ecuador (72) and El Salvador (68). Considering the period time from 1980 to 2005 and dividing into two sub periods: one from 1980 to 1996 and the other from 1997 to 2005, it is important to indicate that Brazil and Costa Rica have had more than 80 % of their cases after 1996, compared with Venezuela, Colombia and Honduras, which reported having similar number of cases in both the 1980 to 1996 period as well as in 1996 to 2005 period. (WHO-DENGUENET, 2009).

In the South East Region of Asia and in the same period from 2000 to 2005, the highest CI of dengue were in Maldives, Thailand, Sri Lanka and Indonesia with 113, 109, 37 and 26 dengue cases by 100,000 population. In Western Pacific Region, Palua, French Polynesia, Cook Island, Northern Mariana Island and American Samoa had more than 1,000 cases by 100,000 populations. Malaysia had CI of 82.8 while Singapore had 368, but only in 2005 (WHO-DENGUENET, 2009).

In dengue disease, one of the first prospective studies was performed by Burke et al. in Thailand in 1980. They followed 1,757 students between the ages of 4 to 16 years old, and obtained two blood samples within an interval period of six months. Febrile students

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were always tested in acute and convalescent stages. 103 (5.6%) were infected in the 7 month study period and 90 out of these were asymptomatic children. Seven students were hospitalized with DHF and all of them had previous antibodies against dengue. (Burke et. al.1988). A second follow up three year study was performed in Thailand in 2002; this time 2,214 school children made up the cohort. The overall incidence was 5.8 %, and out of them 3.1 % was in Asymptomatic dengue virus infection and 2.7% in symptomatic dengue. The incidence of dengue was gradually declining in the three year study from 7.9 % in 1998 to 6.5 % in 1999 and down to 2% in 2000. They found similar incidence between symptomatic and Asymptomatic infection, contrary to the conclusion in other studies.(Endy et al. 2002). Other incidence study was performed in adults in Bandung, Indonesia. Incidence Density was calculated; contrary to Endy et al conclusion, they found 56 per 1,000 person-day dengue infection in asymptomatic people versus 18 per 1,000 person-days in symptomatic cases. Even in areas without symptomatic cases, they found incidence density of 8 per 1,000 person-days in asymptomatic people. (Porter et al 2005). Other prospective study was performed in Indonesia in 1995, reporting an incidence of 29.2 % in the year of follow up. All febrile cases in this study were secondary infection (Graham, 1999). In Vietnam where a study on 977 school children was conducted, an annual incidence of 11.7 % of dengue primary infection was estimated by binary regression of the sero-prevalence by age. In a second part of the study two years later, the sero negative school population (831 children) was newly tested with 30.6 % of sero-converted, and then the true annual incidence of primary dengue was 17.5%. (Thai et al, 2005), (Thai et al, 2007).

In the western hemisphere less numbers of prospective studies have been reported. However, incidence of dengue has been published from different region of epidemics. The Puerto Rican 1994-1995 epidemic presented an incidence of 7.01 per 1,000 people increasing from 2.55 per 1,000 people annual average incidence in the last 4 years from 1991 to 1994 (Rigau-Perez et al, 2001). Teixeira in a prospective study in Salvador, Brazil reported an incidence of 70.6 % of dengue infection but it was significantly higher in those people previously tested positive with one serotype (83.0%) than those people previously tested negative (60.8%), (Teixeira et al, 2002). Balmaseda et al in Nicaragua in a two year follow up study found 12% of dengue incidence the first year, decreasing to 6 % in the second year (Balmaseda et al 2006). In Brazil, where the incidence of endemic disease included dengue, it was studied in the Amazon region. The average incidence of dengue from 2001 to 2005 was 185 per 100,000 populations, which is very similar to that registered by DENGUENET in all of Brazil (195 per 100,000 population) in a similar period from 2000 to 2005 (Penna et al 2009).

Sero-prevalence of Dengue.

Most of the epidemiological studies in dengue have been performed to determine which proportion of the population has been previously infected by the dengue virus, which age group is more affected and which dengue virus serotypes are present in the population. Single transversal sero-prevalence surveys and prospective sero-prevalence studies have been designed to reach those goals. In Indonesia in 1995, 1,837 children from 4 to 9 years old were studied. Dengue serotype antibodies prevalence was estimated twice during a one year interval. At the beginning of the study, 56.1 % of the children were positive to dengue antibodies, 12.0% were immune to DENV-1, 16.3% to DENV-2,

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2.2% to DENV-3, 3.8% to DENV-4, and 21.8% were immune to two or more serotypes. The prevalence of two or more serotypes increased from 37.2 % in the 4 year old children to 69.7 % in 9 year old children. At the end of the study, 26.8 % of the sero-negative children seroconverted; the children with primary infection in the cohort study (Graham et al 1999). Similar study was performed in Vietnam but with an age group of children from the ages of 7 to 14 year olds. The dengue serotype antibodies prevalence increased from 53 % for the 7 year old children to 88 % in the 13 year old children. (Thai et al, 2005). In Singapore, a cross sectional seroprevalence study was conducted to estimate the proportion of adult people (18 to 45 year old) with anti dengue antibodies 133 of 298 (45%) enrolled participants were tested positive. The prevalence increased with the age group from 17 % in the group of 18 to 25 year old, to 44% and 74% in the groups of 25 to 35 year old and 36 to 45 year old, respectively. Singapore is one of the few Asia countries which have been able to reduce the incidence of dengue cases. (Wilder-Smith et al, 2004).

The study of Teixeira in Brazil presented a seroprevalence of 68.7%. The lower value was 39.0% among 0 to 4 year old children and the greater value was 76.4 % among 30 to 39 year old adults. Also, this study showed variation from 16.2% to 97.6 % among 30 areas in Salvador, Brazil. (Teixeira et al 2001). In Dominican Republic, 98 % of the 1,008 adults recruited in blood bank and 56 % of the children less 10 year old visiting a Hospital in Santo Domingo were tested positive to IgG anti-dengue. Among children, the prevalence of antibodies increased by age (Yamashiro et al 2004). A seroprevalence of dengue in children less than 11 years old found a prevalence of 19.9% of dengue antibodies. They compared children living in coastal area with children living in inland

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area. The prevalence in coastal area was 36.9 % significantly higher than inland area which was 2.9% (Iturrino-Monge et al, 2006). The prospective study made by Balsameda et. al in Nicaragua presented one of the greatest prevalence of dengue antibodies in children (4 to 16 year old) of the Americas. The overall prevalence was 91%, increasing from 75% at age 4 to 100% at age 16. (Balmaseda et al, 2006). Some areas in the Americas show low prevalence of dengue antibodies; one study in Tabasco Mexico was performed by enrolling university students between the ages of 18 to 39 year old. The prevalence of dengue antibodies was 9.1 %. Interestingly, the prevalence of anti DENV-1 antibody was 20% and 100% and 68% of antibody against anti DENV-2 and DENV-4 respectively. (Sanchez et al, 2008). In Maracay, Venezuela, a prospective study was performed on 710 schoolchildren from 5 to 13 year old. The Prevalence of anti dengue antibody was 51 %.; 30.1 % were tested with immune response to one serotype, and 20.9% to two or more serotypes. The highest dengue type antibody prevalence was DENV-2 with 14.2% followed by DENV-1 with 13.4%. 25.6% of the children seroconverted among all previously sero-negative children, and 26% of the children seroconverted in children with secondary infection. (Comach et al. 2009).

Surveillance of Dengue

Public Health surveillance is defined, according to Thacker and Berkelman as: "*Public Health Surveillance is the ongoing systematic collection, analysis, and interpretation of outcome-specific data for use in the planning, implementation, and evaluation of public health practice.*" (Thacker & Berkelman, 1988). However, this concept has undergone several changes through the time, until the middle of the last century the emphasis was on the contacts of sick people with a communicable disease. Now surveillance is addressing

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to the risk factors and other health-related events like accidents, injuries, chemical exposure, and infection disease vectors. New features have also improved the concept, classifying the surveillance into the categories of passive and active. In a Passive Surveillance System, the disease case (or other health-related events) notification is dependent on information retrieved from inferior levels of public (or private) health institutions. In an Active Surveillance System, the central department of health contacts regularly the health services to ask about (or look directly for) disease cases or other health-related events. In accordance with Teutsch, surveillance data can be used in the following ways (among others):

"To estimate the magnitude of a health problem

To understand the natural history of the disease

To detect outbreaks or epidemics

To document the distribution and spread of a health event

To monitor changes in infection agents." (Teutsch & Churchill 1994) (CDC, 2001).

CDC in 2001 presented the Updated Guidelines for Evaluating Public Health Surveillance Systems with the purpose to ensure that public health problems are monitored efficiently. Also the CDC considers evaluation of traditional surveillance attributes including: simplicity, flexibility acceptability, sensitivity, predictive positive value, representativeness, timeliness and stability. However; depending on the highest priorities in the health, related event could change the importance of each attribute. Other interesting aspect in this document is the concept of Preventability defined as: *"From the perspective of surveillance, preventability reflects the potential for effective public health*

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intervention at any of these levels" (Primary, Secondary and Tertiary levels). (CDC 2001).

In dengue infection these theoretical concepts have been adapted to the disease characteristics. PAHO in its scientific publication No.548 links Active Surveillance with laboratory-based surveillance, in the effort to understand the low sensitivity of clinical dengue parameters in non epidemic periods. This document also defines different levels of surveillance depending on epidemiological situations: countries where no dengue cases have been detected but where the vectors are present, countries where dengue is endemic, and countries where dengue is epidemic. Viral laboratory support is most important where dengue is endemic and less important where there are no dengue cases (PAHO, 1994).

In health services, the final goal of any kind of surveillance is prevention. Depending on which level of the natural history of disease the health service wants to emphasize, it will represented by the type of surveillance action employed.   For example, if a country wants to totally prevent disease and eradicate cases, then that country has to focus on eliminating those risk factors which facilitates the disease as the first level of prevention. In dengue disease, this control could be achieved with the surveillance of density vectors, feeding vectors, water supply service and, human household conditions. This kind of surveillance or risk surveillance is exceptionally implemented in developing countries. These actions are regularly implemented in a developed country; even though dengue would not be the exclusive focus of the intervention effort.

In dengue endemic areas, other levels of surveillance would need to be set in place in preparation to detect any early increase circulation of the dengue virus, through increase

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in virus activity either within vectors or through detection of the first human cases during an interepidemic period. The goal would be to predict the incensement of the human cases to avoid epidemics. Active Surveillance would be the best way to reach this goal. In this case, early control measure will be applied and the epidemic would be avoided. Developed countries would not need this kind of surveillance but developing countries do, but only a few can maintain this system because it requires an advanced viral laboratory. (Gluber, 2002).

Also, in dengue endemic areas situated within a country that lacks organization and equipment to actively look for either early disease cases in interepidemic period or positive vectors to virus infection, the system has to wait passively for the dengue cases to be registered and analyzed. This approach is known as a Passive Surveillance system, whereby it would be too difficult to predict and avoid epidemics from happening. As a result, the goal has to shift towards to reducing impact of the epidemic as a secondary and tertiary level of prevention, reducing the Fatality Rate, preparing the health services and hospitals, eradicate the vector, and training health workers and population to handle the epidemic situation. This is the usual the approach adopted in many developing countries facing the threat of dengue. (Gluber 2002).

<div align="center">Laboratory and Diagnosis.</div>

The clinical manifestations of dengue are represented by symptoms that are specific to dengue make the diagnosis of this disease difficult. Additionally, in periods of no dengue epidemic, the sensitivity of the symptom identification is much lower. As a result, laboratory confirmed tests become necessary procedures and tools to diagnose dengue. Another important aspect of dengue laboratory is the high frequency of

asymptomatically infected cases, which are commonly detected by sero-prevalence surveys. Asymptomatic infections impact the knowledge of the virus transmissibility and new serotypes introduction. In surveillance, laboratory support is one of the cornerstones of the system; efficacy in the dengue diagnosis is closely related to the dynamic between the dengue viremia and the immune response in primary and secondary infections. Viremia is present in the blood patient at the moment of the onset of symptoms (usually fever) and could be present for more than three or five days which would be during the time the virus is detected. The end of the viremia usually marks the beginning of the immune response. The duration of the immunoglobulin in blood will depend on their type: IgM antibody will be in blood for 80 to 90 days and IgG antibody for life. (WHO, 1997).

Therefore, there are two basic procedures to establish laboratory dengue diagnosis: the detection of the virus and the detection of the antibody.

Viral Isolation.

Viral Isolation is the most sensitive way to detect DENV. Three techniques can be used: 1. Mosquitoes (pool of 15 to 20) are inoculated either with serum, or plasma, or pleural fluid or other sterile body fluid. Days after, virus infection is confirmed by immunofluorescence. This is the most sensitive isolation technique. 2. Inoculation of mammalian or insect cell cultures (usually C6/36) is other common used for viral isolation. The presence of the viruses is confirmed by cytoptathic effect or plaque formation assay. RNA detection and immunofluorescence also can confirm the infection. 3. Intracranial inoculation of sucking mice is the third way to isolate dengue virus; either encephalitis signs or antigen in brain tissue are evidence of infection. The higher

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limitation of the Viral Isolation is that this method ise time consuming, and also there is high cost associated with use of of the cell culture method. (WHO 1997).

Polymerase Chain Reaction and Dengue Virus.

A relatively new molecular technique, Polymerase Chain Reaction (PCR) was developed to amplify DNA fragments. Later, a variant of that technique has also permitted amplification of RNA through a cDNA intermediate; this advanced technique has had direct consequences in the diagnosis of RNA viruses. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) test has reduced the time of diagnosis of dengue disease, while keeping a good level of sensitivity for the purpose of detection. RT-PCR method requires for reaction to start with the reverse transcription from RNA to a complementary DNA (cDNA), using the enzyme reverse transcriptase. The PCR step that follows reverse-transcription has three parts all performed in a thermal cycling instrument (which would be the same as performing traditional PCR with DNA as the starting material): 1. Denaturation of the DNA, increasing the temperature to melt the double stranded DNA into single strands , 2. Annealing the target DNA with specific primers by decreasing to an optimal the temperature and finally, 3. Elongation is the extension of the DNA from the primers, increasing the temperature according to specific DNA polymerase. This step should be repeated between 20 to 40 times to get the ideal amplification of the DNA fragment. In dengue, RT-PCR was initially used with a nested two step protocol   (Lanciotti et al 1992). Later, an improved version of the test was designed which reduced reaction time, using a single tube multiplex RT-PCR (Harris et al 1998). In both cases, all four Dengue viruses can be detected. (McPherson et al, 1999), Raengsakulrach et al. in 2002 compared four RT-PCR procedures in Thailand where all

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serotypes of dengue are circulating and need to be detected and distinguished from one another. The method developed by Henchal et al. amplifies the 482 nucleotide sequence in the NS1 region of the dengue virus genome. The method of Morita et al, utilized four pairs of type-specific primers to detect simultaneously the dengue serotypes. And the methods of Lanciotti et al. and Yenchitsomanus et al. employed universal dengue primers followed by a type-specific nested PCR. The sensitivity, considering serologically confirmed cases, was 54, 52, 60, 79 % respectively with the indicated methods (Raengsakulrach et al, 2002).

A new advance in PCR and RT-PCR was the introduction of the real time analysis detecting amplified products during the process of the DNA amplification, using fluorescent based reporter chemistries. Comparative advantages comparing real time to conventional PCR and RT-PCR are; Real Time RT-PCR is less time consuming, is more sensitive and the viremia can be estimated quantitatively. Oliveira in Brazil compared RT-PCR with Real Time RT-PCR, finding better sensitivity in Real Time RT-PCR. They concluded that a good way to detect and diagnose dengue is by combining Real Time RT-PCR during the first days after onset of the symptoms with serological detection of anti IgM dengue antibody in later days of acute phase. (Oliveira et al, 2005). These are important advances in Public health surveillance system whereby laboratory diagnosis of dengue can be made from patient samples from any day during acute disease. To establish the viremia is important in studies to relate blood levels of viruses and pathogenesis through the different cells and cells products in infected people.

In 2004, Lemmer et al. showed a study of external quality assurance (EQA) of 13 laboratories which apply RT-PCR and Real Time RT-PCR in dengue diagnosis: only two

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laboratories received the maximum score of quality, and only four detected low dilution of RNA dengue in samples. In their recommendation, the authors said that eight of thirteen laboratories should improve the sensitivity and specificity of the test (Lemmer et al, 2004). Oliveira et al compared five RT-PCR kits (two Two-Step kits and three One-Step kits) and found clear advantage using One-Step kits to detect low dilution of viruses.

The problem of short duration of virus presence in the blood of patients would be resolved with the use of non-structural dengue antigens, because of the long persistence of these antigens in the blood (Schilling, 2004).

Serological Test.

The use of immunological test is based in the immunological response after being exposed to an external agent such as virus. For the purpose of laboratory diagnosis, humoral response detecting antibody would be the most important criteria to consider in diagnosing dengue infection. Immunoglobulin G (IgG) and immunoglobulin M (IgM), each one or in combination, are usually the key factors to be detected in the diagnosis either of past dengue infection (IgG) or acute primary and secondary infection (IgM and IgG). A definitive diagnosis of acute infection requires a pair of samples, which need to be collected during the acute and convalescence phase. In addition, a secondary infection can be demonstrated if the ratio IgM/IgG is less than 1.5. In dengue, this would be possible because each type of immunoglobulin levels can rise for two or four weeks after infection, depending on the kind of infection. If a patient had a primary contact with the virus, the IgM will be expected to present with higher levels in blood; while in a secondary infection the IgG in blood will be higher (WHO, 1997).

Haemagglutination Inhibition Assay (HI), Complement Fixation (CF), Neutralization Test (NT), Indirect IgG Enzyme Linked Immunosorbent Assay (ELISA) and IgM Monoclonal Antibody Capture Enzyme Linked Immunosorbent Assay (MAC-ELISA) are some of the tests used in serological dengue diagnosis. (WHO, 1997).

For many years HI was the most used test in the routine dengue diagnosis, due to its high sensitivity and relatively low cost. Characteristically however, this test does not distinguish between immunoglobulin isotypes. As a result, for HI, absence or low level of antibodies in the acute phase with increased level in convalescence phase is considered to be the indicator of primary infection in dengue disease. In addition to this shortcoming, HI is not a serotype specific test for dengue (Teles et al 2005).

NT is the test with highest specificity in dengue infection and contrary to HI, is capable of discriminating the virus serotype in primary dengue infection; though, this would be difficult to determine in secondary and tertiary infections (Morens et al, 1985). NT has an advantage related to its specificity and it is usually necessary in studies of sero-prevalence and prospective design. However, this test is laborious, expensive, and time consuming in those clinical scenerios where quick results are needed.

Since 1987, IgM capture ELISA (MAC-ELISA) test has been recommended by WHO to be used in the diagnosis of acute dengue disease. Having a slightly higher sensitivity than HI, MAC ELISA is also more specific with less cross reactivity to other flaviviruses. This test becomes positive five or six days after the onset of the symptoms. The day of conversion when the antibody could be detected is extended to more days after the onset of symptoms in secondary dengue infection, whereby also a smaller proportion of patients appear to be negative. (Gubler et al 1998).

Indirect IgG ELISA test is a sensitive test to determine the chronic prevalence of anti dengue antibodies but is less specific and with higher cross reactivity to other flaviviruses.

Guzman et al. showed the report for external control proficiency test of dengue serological diagnosis in the Region of the America in the period 1996-2001. Twenty seven laboratories received 54 serum panels. The result showed that 95.6 % of the antibody IgM tests were concordant with the result from the reference center. In conclusion, they summarized that the majority of the participating laboratories showed excellent performance for diagnostic capabilities (Guzman, 2003). Donoso et al. in 2002 evaluated laboratories in Europe, in a program of External Quality Assurance, with combination of different panels of IgG and IgM antibodies (+ and +, - and +, - and − ,etc.). They found correct results in 88% of the IgG-positive samples and for 100% of the IgG-negative samples, 91 % of the IgM-positive samples and 97% of the IgM-negative samples. (Donoso 2004). One of the problems of serological tests is the differentiation among flaviviruses which is very important in countries where many of these flaviviruses are co-circulating simultaneously with dengue virus. (Koraka et al, 2002), (Cuzzobbo et al, 2000), (Scharwtz et al, 2000). As a result, some studies compare ELISA techniques (Palmer et al, 1999) that could distinguish these different flaviviruses. Cuzzobbo et al. compared PanBio Dengue Duo Igm and IgG Capture ELISA and Venture Technologies Dengue IgM and IgG Dot Blot. One of his most important conclusions was that in countries with high prevalence of co-circulating flavivirus, PanBio ELISA performed better because this test was able to avoid false positive results. (Cuzzobbo, et al, 2000). On the other hand, those countries with low prevalence like the USA DotBlot ELISA

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would be a good choice as dengues test kit, because it can detect any case of dengue, thus avoiding false negatives. Another innovation with PanBio duo ELISA is to simultaneously measure IgG and IgM in the same test. According to Sang et al, the sensitivity, 99%; specificity, 96% of this technique was superior to the use of IgM alone or IgG alone, 88 % of sensitivity and 96 % of specificity, and 85% of sensitivity and 96% of specificity, respectively. (Sang et al, 1998). Oliveira found more sensitivity in MAC-ELISA compare to PCR when this test is performed in the 5 and 6 days after onset of the symptoms. In addition, Chanama et al. compared IgM antibodies in primary and secondary infection; specific IgM was detected in all the cases with primary dengue virus infection on disease day 9 or later. However, specific IgM cannot be detected in 28% (204 / 716) of the cases in secondary infections. They recommended other test for confirmation in all secondary infections (Chanama et al, 2004).

One of the weaknesses of serological test is the necessity of using two samples from the acute and convalescence phase of infection to confirm the disease. In addition, the use of invasive technique which is the need to take blood samples, would be another factor that is considered problematic. In this sense, it is of significant interest for some studies to apply less or use of a non- invasive procedures for sampling such as taking saliva samples. These studies have found a good correlation between IgM in saliva and IgM serum. One of these studies also included IgA in this saliva-serum comparison, but the result showed that these antibodies were better detected in serum. (Balmaseda et al, 2003), (Cuzzobbo et al, 1998).

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Clinical Manifestations.

The clinical characteristics of Dengue disease can be different according to the kind of disease presentation. The dengue infection could be asymptomatic as detected through expressed immunological response, or symptomatic. Symptomatic patients may have undifferentiated fever like a viral syndrome, Dengue Fever (DF) Syndrome (with or without hemorrhagic manifestations) and Dengue Hemorrhagic Fever (DHF). DHF with shock is also known as Dengue Shock Syndrome (PAHO, 1994).

According to WHO, a clinical case of Dengue Fever is an acute febrile illness with frontal headache, retroocular pain, muscle pain, joint pain, and rash; even though other signs and symptoms could also be presents (such as lymphadenopathy, petechiae, nausea, hepatomegaly, and different types of hemorrhagic). A probable case of DF is defined as cases with fever and two or more of the following manifestations: headache, retro-orbital pain, myalgias, arthralgias, rash, hemorrhagic manifestation and supportive serology (HI, IgG, IgM tests) or occurrence at the same location and time as other confirmed cases of dengue.   A confirmed case of DF is defined as cases with isolation of dengue virus for serum or autopsy sample; or demonstration of fourfold change in reciprocal IgG or IgM antibody titers in paired serum samples; or demonstration of dengue virus antigen in autopsy tissue, serum or cerebro-spinal fluid samples by immunohistochemestry, immunofluorescence or ELISA; or detection of virus genome sequence in in autopsy tissue, serum or cerebro-spinal fluid samples by RT-PCR. Hemorrhagic Dengue Fever case has to have all the four following criteria: Fever, hemorrhagic tendencies (including tourniquet test), thrombocytopenia (100,000 per mm^3 or less) and plasma leakage

(hematocrit >20%, signs of plasma leakage: pleural effusion, ascites and hypo-proteinemia) (PAHO,1994), (WHO, 1997).

<div align="center">Pathogenesis of Dengue Hemorrhagic Fever.</div>

The pathogenesis of hemorrhagic dengue fever that has been described has many implications in the understanding and control of this disease, as it relates to efforts to identify people at risk. Basically, at least two theories have been presented to explain the severity of DHF. (McBride et al, 2000), (Halstead et al 1977): 1. According to the first theory of Antibody dependent enhanced (ADE), previous antibodies of a specific serotype of dengue virus bind to different dengue virus serotype producing non neutralized antibody–virus complexes. These complexes bind to macrophages which lead to activation of T cells from previous presentation to Major Histocompatibility Complex molecules (MHC). Cytokine production then becomes the a consequence of T cells activation: Interferon-γ, Tumor Necrosis Factor, IL1, IL6, IL8 (Yang et al, 2001), Fernandez et al 2004), (Huang et al, 2000), (Talavera et al 2004). Additionally, Vascular Endothelial Growth Factor (VEGF) is produced by monocytes and endothelial cells (Tseng et al, 2005). Among other effects, it produces changes in the wall of capillaries with hemorrhagic symptoms and leakage of fluid in the body cavities that could end in death of the patient. 2. According to the second theory, specific genotypes of dengue virus which are more virulent could cause of more severe symptoms of dengue (Rico-Hesse et al, 1997). Probably both theories are correct and complement each other. Epidemiological studies show the relationship between severity of epidemics and previous serological conditions of the population affected. Sequence of infection with DENV-1 virus followed by DENV-2 has been demonstrated to correlate

with high rates of DHF (WHO, 1999). However, differences in the severity and number of DHF cases have been demonstrated when the strain of the DENV-2 virus is Asian or American, with the latter one being less virulent (Rico-Hesse, 1990).

<center>Prevention and Control.</center>

Worldwide, one of the challenges of the public health is the search for effective methods to control dengue disease. For decades, either in the Americas or Asia, WHO has made recommendations about the best way to prevent dengue epidemic. Throughout this time, the essence of the WHO's effort is the same, though the strategies to reach this goal have continually changed (WHO, 1997), (PAHO, 1994), (WHO, 1999), (Parks et al, 2004).   Unfortunately, most of these of strategies have only generated very poor results (Gubler, 2005), (Calisher, 2005), (Gubler, 2002).

There are three approaches to prevent dengue disease and dengue epidemic: 1. To block the virus action inside the human hosts with use of vaccine to induce specific immunization. 2. To halt the infectious chain reducing or eliminating the vector**:** mosquito control. 3. To detect the cases and the virus in early periods of viral activity by epidemiological surveillance either to reduce or prevent the impact of the epidemic (PAHO, 1994).

These three activities should not work independently. However, the focus and intensity of each application can depend on the moment of infection, the location (world region or country), and the budget in health and the specific type of disease infection. For example: the focus of Yellow Fever prevention in South America is the vaccine and surveillance of human cases and monkeys, and secondarily, the mosquito control. For one specific disease, the same sentinel animal could not be good in all areas of one

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country or region (PAHO, 1994), (CDC, 1993). In addition, these three strategies can be (more or less, depending of each one) affected by other important factors such as education, family income, housing conditions and social services (water supply and waste disposal). For example: housing condition (without window screen) affects more the mosquito control than a massive vaccination campaign.

The uncontrollable worldwide activity of dengue virus and its vector deserves a short analysis of each strategy of prevention. In dengue, the vaccine is already being developed. However, an effective vaccine is yet far from being available for public use. In general terms, this challenge of an effective vaccine selection is due to the presence of multiple serotypes and their interactions with the heterotypic antibodies. In particular, it has been difficult to measure the amount of virus and its immunological response in different situations. Serum Neutralization, a very specific and sensible test to make these measurements, has presented with problems to standardize procedure using these four different serotypes. Many authors have always been worried about the risk that the dengue vaccine could be the prime vaccine for development of DHF by Antibody Dependent Enhancement (ADE) which makes the infection worse, instead of providing protection. Furthermore, a good animal model for dengue is lacking whereby in many cases, in the existing animal model systems showed that these animals can be infected but do not develop the symptoms of the disease (Stephenson, 2005), Pang, 2003), (Calisher, 2005).

Mosquito control has been shown to be the best strategy to prevent dengue epidemic. This was evident during the time when the Americas was free of dengue in the period (1940's and 1950's decades) of control and almost eradication of *Aedes aegypti*, as a

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plan of yellow fever control. Re-infestation in the sixties, of *Aedes aegypti* permitted entry of new dengue virus serotypes in the Americas (PAHO, 1994). Other historical example was the campaign against the mosquitoes and their breeding sites carried out in Cuba after the huge epidemic of 1981(Guzman et al, 2005), (Kouri et al, 1998) which permitted for a period of 16 years without dengue to take place in that island. Singapore is the Asian example of the same result with the same strategy; this small but rich country could reduce the index of *Aedes aegypti* to levels apparently secure, and reduce almost totally the cases of dengue (Wilder-Smith et al, 2004). This strategy is based on the reduction of breeding sources (Essentials as storage water and Non essentials as tires and waste), larvae and adult mosquito control, surveillance of vector, community education and personal protection. These two countries which represent two opposite political models have been the best examples of successful mosquito control strategy. However, this strategy though good, has not been perfect in any of these countries. As an example,n 2002 Singapore had a dengue epidemic with unthinkable low density levels of mosquitoes, and with a high number of risk population with low level of herd immunity as a result of many years without epidemics. A similar situation occurred in Cuba in 1997 when DHF affected only adult population who had been in contact to dengue virus in 1981, or before. No child born after 1981 was reported with DHF in the 1997 epidemic. This finding could explain two aspects of dengue situation in Cuba; the relationship between secondary infection and DHF, and the success in the control of Aedes aegypti for more than 15 years. (Gubler, 2002), (Kouri et al, 1998). Something however is true in that neither Singapore nor Cuba could avoid a new epidemic of

dengue. Both events indicate that the dynamic of dengue virus and its vector is complex and cannot be reduced to only one strategy or a single program in Public Health.

Recognizing the little advance in the control of dengue, the last WHO's recommendations focused on community participation in the programs of prevention and mosquito control in dengue (Parks et al, 2004), (Suhaili et al, 2004). However, we can see how this suggestion was so general in 1995, more specific in 2002 and very specific in 2004 when WHO includes COMBI (Communication for Behavioral Impact) plan and textual analysis say: "*Knowledge is not enough, Evaluation researchers have noted that, despite growing levels of knowledge and awareness about dengue and mosquitoes, many people are still not taking action.*" And "*Many programs continue to focus only on changing people's knowledge and on raising awareness, believing that behavior will change.*"

The success of this strategy depends on the continuity of its implementation, either in the period of epidemic or during the inter-epidemic periods. In many developing countries, several of these activities of control are activated in the periods of epidemic, acting as tertiary prevention but not as primary prevention in public health, like a disease control focused on emergency response. Gubler stated that emphasis is on emergency response rather than more on prevention (Gubler, 1998).

The third general strategy in the prevention and control of dengue is the surveillance. In many developing countries, surveillance is part of the epidemiology department in the services of public health. However, most of these countries have the passive system of surveillance (Gubler, 2002), (WHO, 1999. It means that the case of dengue is registered when the patient goes to the health service center. However, if for any reason

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the patients with dengue fever or DHF do not go to look for medical attention, they will never be recognized by the system. It is clear that in passive surveillance the cases with diagnostic will be those with more obvious and florid disease. Asymptomatic cases or mild cases could not be detected (Endy et al, 2002). Developing countries with passive system of surveillance use this system to detect epidemic and activate measures of control and attention of patients in hospitals. For active surveillance, the goals can be different. The objective of active surveillance is to predict the epidemic instead to detect it. In active surveillance, is important to detect any case of dengue and not only the most florid case. In addition, the role of active surveillance is more important in inter-epidemic periods where laboratory confirmation is essential (clinical criteria can be enough in epidemic stage) (Rigau-Perez, 2001), (Gubler, 2002). According to Gubler, few countries in the world have capacity to do an active surveillance; he summarized these countries in this category: Singapore in Asia and Puerto Rico and Brazil in the Americas, which are countries with adequate laboratory resources to sustain an active surveillance system. Active surveillance in Puerto Rico has permitted the prediction of all the epidemics since 1998 with only one wrong prediction in 2003. Gubler says that in Puerto Rico the rainy cyclical population of Aedes aegypti has not been historically affected by programs of mosquito control, as a result, this characteristic supports the emphasis that Puerto Rico has created and sustained the active surveillance system of dengue (Gubler, 2002). In an infectious disease as dengue without other animals or host to detect early the circulation of the virus and consequently to activate epidemiological alarms, the system has to be very sensitive to detect the first human cases. Ideally, the virus needs to be detected two, three or four weeks before the evident ascend of the

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epidemic. At this time the identification of the dengue case has to be very precise, and any gap or doubt in the case definition could affect the success of the active surveillance. The study of Endy et al in Thailand showed around 50 % of the cases of dengue could be asymptomatic and others are mild symptomatic (Endy et al, 2002). This is important if we see that World Health Organization promotes very specific criteria to define a dengue case (WHO, 1997). These criteria would leave out many asymptomatic and mild cases of dengue. Besides, confused procedures recommended by WHO avoid a better detection of cases. WHO recommends taking two blood samples: one in the acute phase and one in the convalescent phase, marking 10 days as the ideal time between the two blood samples. We have shown in un-published data how more than 90 % of the cases in a regular surveillance system have access to only one sample, with low percentage of confirmed cases of dengue. We have also shown that the tendency in the last years is to reduce the days to take the blood sample after the onset of the symptoms, from 6 in 1998-1999 to 4.5-5 in 2000-2002. Maybe this tendency has been promoted for the interest in taking blood sample in the times when viruses can be detected. The big problem is that 4 to 5 days could be too late to detect RNA from the virus and too early to detect immunological response. (CDC Puerto Rican branch). Also, few patients would return for a second blood sample, reducing highly the number of case confirmation because a hypothetical second sample could identify elevations of antibodies from nothing in the first one (WHO, 1997). Therefore, it could be reasonable to recommend taking two samples in the acute phase: the first sample in the first three days of fever to detect the RNA and the second one after day number 6 after onset of symptoms to detect the immunological response. With this

condition, if RNA is detected, the case is confirmed. However, if RNA is not detected, two samples after day 6 looking for immune response will be necessary.

We can summarize the limitations of a regular passive system of surveillance as such: 1.Passive surveillance can estimate incidence of symptomatic cases of dengue but not incidence of infection (symptomatic, mild symptomatic and asymptomatic cases). It has particular importance because a first exposure to dengue virus is a significant risk factor to get HDF.   2. Passive surveillance waits for the cases, usually florid cases; some cases will never be detected if the system does not look to detect for these cases. 3. Passive surveillance is not concerned about the prediction of an epidemic of dengue with a prudent time to avoid it; the role would be to detect the cases over the epidemic to activate inter-sectors responsibilities: mosquito control, hospital, media and community. 4. Passive surveillance is not concerned about the identification of the prevalence to specific serotypes of dengue virus in population at risk to a new epidemic with new serotype or genotype. 5. Passive surveillance cannot identify changes or variant in the clinical presentation of the cases that could be affected for the introduction of new serotypes or genotypes.

CHAPTER THREE: METHODOLOGY

Design

In order to achieve our objectives, a Longitudinal Panel Study Design. (Hybrid Studies) (Woodward M. 2005) was developed and implemented. A close tracking of people at risk for dengue was required to detect information that is usually hidden in the natural dynamic of the dengue disease. One strategy employed was routine visits of families recruited in the study, and inquiring about fever and other risk factors that could be related with dengue so as to determine the best way to describe unknown characteristics of this disease. Individuals who were recruited to participate in this study were from different four neighborhoods and lived in their designated family houses. The cohort was followed after the initial demographic questionnaires were completed, and determination of sero-prevalence of dengue antibodies was made. Three visits a week were made by nurses who asked participants about fever events experienced by individuals studied. If any case of fever was detected, a clinical and laboratory evaluation was performed by physicians. This portion was named the active surveillance in the study design. Besides, each six months sero-prevalence surveys of dengue antibodies were repeated in a procedure that was called biannual blood samples. A smaller or sub-cohort of people with clinic diagnosis of dengue determined according to the WHO criteria, were invited to participate in a special part of this study describing clinical and laboratory changes during the period of acute disease. This part of the study also required having

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blood samples taken at 24, 48 and 72 hours of fever period, and 24 and 48 hours after fever disappears, but only in those confirmed dengue cases.

All dengue cases were described monthly within the two years and three months study period. This duration of study period permitted us to compare the incidence in this study with the state and national incidence of dengue disease. Simultaneously, we were also able to compare the traditional passive model of the state and national surveillance with our design which, in some sense, reflected an active surveillance system to detect dengue disease.

Incidence Density (ID)

With the purpose of obtaining the Incidence Density, we computed the number of days per months for each individual who participated in the study. We obtained the total of person-days either per months or trimester or year. The report of days began the day that each person is included in the study, and was completed when he or she left the study by any of these three possible causes: 1.When the person died. 2. When the person left the study before it is over, and 3. When the person left the study at the time the study is over. The number of person-days was also distributed by neighborhood, by age group, and by gender. The Incidence Density was calculated dividing the number of confirmed dengue cases by the total number of person-days, in general, or by specific groups. This number was then multiplied by 100,000, and expressed by 100,000 person-days.

ID Relative Risk was calculated to age groups and gender, by dividing ID in children less than 15 year old by ID in adult people equal or higher than 15 year old, and dividing ID in females by ID in males.

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In order to compare our data with the state and national surveillance, we had to calculate the Cumulative Incidence in 2007 because this is the measure used in passive surveillance. We divided the number of dengue cases detected in the active surveillance during 2007 by the total number of people who began and finished the study within the 365 days of 2007.

Population and selecting a sample.

For this study, Venezuela was the reference country. In 1989 Venezuela had, after Cuba, the second largest epidemic of DF and DHF in the Americas. Aragua state, where this study was performed, was one of the most affected areas in Venezuela. Aragua state is located in the central north of Venezuela, with the Maracay city as its capital. (Appendix A and Appendix B). This study was conducted in four neighborhoods located within the city of Maracay. In Maracay, the range of temperature is between 25 and 35 ° Celsius, and for the most part, the city is situated around four hundred fifty meters over the sea. The mean total precipitation in Maracay is between 3.5 mm in the dry months to 179 mm in the rainy months. (Appendix C). The target population included in the regular surveillance system is represented by approximately one million six hundred thousand people. From this population, around thirty six percent (576,000) are less than fifteen years old. In Maracay and its metropolitan area, there are about one million people. Aragua state has eighteen municipalities and six of these are parts of Maracay's urban and suburban areas. Two hundred and nine local primary health centers are distributed in all of the state, and these are the first places utilized for dengue cases detection. When the dengue case is suspected, the specific characteristics of dengue disease according to the WHO recommendation are recorded, and then one

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blood sample is taken for each case. Each epidemiological municipality center collects the information and reports to the epidemiological department of the state health service, and sends the sample to the central laboratory of infections disease in the state (LARDIDEV). The Laboratory performs specific tests for dengue case confirmation according to recommendation of the World Health Organization. The highest risk areas of dengue are in the urban and suburban neighborhoods of Maracay. Four neighborhoods (known as "Barrios" in Spanish) with the highest incidence of dengue cases in the last years were selected for this study. In the next step, each neighborhood was divided into blocks or squares of approximately 125 houses or families, and then one of these blocks in each neighborhood was randomly selected. The total number of families ended being around 500 with a total of approximately 2,500 subjects. According to previous studies conducted in Maracay, we were expecting a 14 % lost of subjects per year.

The sample size of 2,509 subjects was obtained by assuming a hypothetical high incidence of 15 %, and an estimation error of 3 % from the target population, with a confidence interval higher than 95 % (alpha 0.05). This calculation was made using EPIINFO program, to determine sample size for population survey. We established a number of around 500 individuals by each neighborhood except in the highest neighborhood called "Cana de Azucar" where we selected 1,000 individuals with 500 participants in each two separated sections of this neighborhood. The individuals lost during the study were replaced by 746 people who entered into the cohort in 2007 and 2008 (Table 1). The criteria to replace people were either someone who is a family

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member of the study population, or neighbors living in the same blocks of the people studied.

Table 1.

People enrolled in the cohort study during 2006 and 2007 according to their Neighborhoods and age group.

Neighborhoods (Barrios)	Age Group	People enrolled in the initial Cohort in 2006	People enrolled in 2007	Total
23 de Enero	<15	100	93	193
	≥15	402	98	500
Caña de Azúcar	<15	212	82	294
	≥15	794	211	1005
Cooperativa	<15	134	41	175
	≥15	367	86	453
Piñonal	<15	108	42	150
	≥15	392	93	485
The Four Neighborhoods	<15	554	258	812
	≥15	1955	488	2443
Total		2509	746	3255

Collecting data

In the block of houses of every neighborhood, we started by visiting each family inviting them to participate in the study. If the family wished to be in the study, one informed consent had to be signed for each member after a clear explanation of the study was provided. Each member of the family was free to participate independently of the other member's decision. When every family had its first visit, and the demographic and risk factor questionnaires were completed the first blood sample was taken from each member of the family. After this first visit and during the duration of two years and three months, the family was visited three days weekly, whereby they are asked about fever incidence of any family member. If any family member had fever, a blood sample was taken and a clinical evaluation was made. Blood Sample collection

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was performed as follows; venipuncture was performed by an experienced phlebotomist followed by drawing 5 ml of whole venous blood from an antecubital vein from each adult, and 3 ml from each child ages 5-17. The blood was collected in one Vacutainer® collection tube (red top) without anticoagulant. Sera were separated by centrifugation at 2,500 rpm, transferred to cryo-vials and stored at -20ºC.

This weekly activity looking for febrile cases was named the active surveillance component in the study.

Persons presenting signs and symptoms consistent with dengue disease were invited to participate in a sub sample which was subjected to higher numbers of hematologic test. If the specimen was RT-PCR positive for dengue virus, additional blood samples were collected at 24, 48, 72 and 24-48 hours post fever defervescence, and 30 days post initial sample. Serology was tested in all sample and RT-PCR from 0 to 72 specimens. Ten ml of whole venous blood were obtained from each patient, collecting in one Vacutainer® collection tube (purple top) with anticoagulant.

In the same period of the study, we were collecting the information of dengue cases reported in the regular surveillance system from the health department of Aragua state. This data will be compared with the level of the neighborhood, the municipalities and the state.

<div align="center">Instruments of data collection.</div>

1. Questionnaire of the First Visit: this instrument will include:

  1.1. Demographic Information: Age, gender, number of members by family.

2. Serology, blood Sample taken to detect antibodies. (IgM MAC ELISA and IgG by PRNT). (Appendix D).

2.1. In the first visit: Time zero.

2.2. Following biannual visits: Each 6 month.

3. Card Family Visit (three times a week) in Active Surveillance component.

3.1. Registering family members with fever.

3.2. If somebody has fever: blood sample was taken for diagnosis of dengue and

clinical

evaluation.

4. Sub-cohort card of symptomatic cases of dengue, invited to participate in this study

component.

4.1. Blood samples of dengue diagnosis: 0, 24, 48 and 72 hours during fever period

and 24 and 48 hours after fever defervescence, and 30 days post initial sampling..

4.2. Clinical description.

Laboratory Analysis

IgM MAC ELISA: Sera were tested for anti-dengue antibodies. The ELISA anti-

human IgM antibodies were coated onto 96-well microtriter plates. Aliquots of diluted

serum were added to each of the anti-human IgM antibody coated cells, followed by

one hour incubation.

PRNT: Test sera were diluted two fold in the media (EMEM) from 1:5 to 1:5,120.

200 µl media with 40 to 80 PFU of assay virus was mixed with 200 µl diluted test

serum and then incubated at 4º C for 15 hours. In triplicate, 100 µl virus-sera mixture

were added to 0.5 ml media containing 1.5×10^5 BHK21 cells and then added to a 24

well tissue culture plate, and incubated at 37 º with 5% CO_2 for three hours. The cells

were then overlaid with overlay media and incubated at 37 º with 5 % CO_2 for 5 days.

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Following incubation, the media were removed and the cells were stained with stain solution Naphthol Blue Black, sodium acetate and Glacial Acetic Acid by 30 minutes. Stain was removed and the plaques were counted. The results were expressed as the serum dilution that reduced the number of plaques by 70% compared to that of normal human serum at the same dilution.

RT-PCR: Viral RNA was prepared fro 140 μl sera using QIAamp Viral RNA Mini Kits following the manufacture's instructions. Nested dengue virus RT-PCR was performed following the protocol of Lanciotti et al.

Processing Data

In order to analyze and describe the result, the data was distributed by each neighborhood. Initially the age of the people was classified in three ways: 1. Eighteen age groups of 5 year intervals, from 5-9 to 90-94. 2. Three age groups; less than 15 year old, equal or higher than 15 and less than 50 year old and, higher than 50 year old, and 3. Two age groups: less than 15 (called children in this study) and equal and higher than 15 year old (called adults in this study). The first two age distribution was made to acilitate demographic comparisons. However, due to the fact that most of the studies have made their age distribution similar to the children - adults' classification, most of the analyses were made almost exclusively with the two age groups distribution. We use the femininity ratio (RT) to see the gender distribution according to the age groups and the four neighborhoods in the study.

In order to describe the exact number of days of each participant and their type of permanency in the study, the participants were classified in five groups. Type I: people who never left the cohort. Type II: people who only left the Bi-annual blood sample to

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obtain prevalence of antibodies but not left the active surveillance. Type III: People who left and came back to the cohort. Type IV: people who definitively left the cohort, active surveillance and Bi-annual sample. Type V: people who died for other causes, but not due to dengue. This approach was taken to see the impact of the different causes that made the people leave the cohort according to their frequency, age groups, gender, residence, and person days in each months of the study.

The crude Incidence Density was attained in the two year period and was adjusted (direct method) by age (children/adults) and gender of the Maracay city population. ID was also calculated by each year in the study and by months and trimester of each year. We obtained ID by neighborhoods, age-group and gender simultaneously.

Incidence Density Relative Risk of dengue was calculated in the two year study, associating age-groups (children versus adults), and gender (female versus male).

IgM anti-dengue antibody was tested (MAC ELISA) each six months in all study individuals to establish its prevalence as a percentage. This percentage was obtained with the positive number of people to IgM, dividing by the number of people tested.

IgG anti-dengue antibody was tested by PRNT. All prevalence were calculated by determining the percentages of monotypic antibody (for each four serotypes), and multitypic antibodies with two, tree and four antibodies simultaneously, and all combination of serotypes detected. The percentages of people with negative results were also obtained. These results were analyzed according to the year (2006-2007) and age-group (children/adults and each 5 year intervals). The percentages of sero-conversion were also calculated into the three intervals of the four biannual samples: {between the first sample (S1) and the second sample (S2)}, {between the second

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sample (S2) and the third sample (S3)} and {between the third sample (S3) and the fourth sample (S4)}. Simple Proportion test was applied to establish the difference in the percentage of infection among negatives and infected people, and monotypic infected and multytipic infected people Simple proportion test was also applied to compare difference between samples in each group of negatives, monotypic and multytipic infected people.

A model of Nominal (Binary) Logistic Regression was made to estimate the relation between the infection by dengue virus in a period of six month, considering the sample 2 (S2) as a dependent variable and as independent variables or factors: previous infection in time cero (the first sample at the beginning of the study) and sample 1 (S1) at the first six months, age, sex, residence (neighborhoods), beginning of the study (cohort 2006 or 2007), number of serotypes in time cero and sample 1 and interaction variables between age and sex, residence and age, cohort and age and cohort and sex, with a confidence level of 95% The infection in sample 3 (S3) was not used as a dependent variable because the number of lost people was higher that last sample.

In the patients detected by active surveillance, confirmed cases were defined as those patients with clinical manifestation of dengue according to WHO and positive RT-PCR; probable cases as those patients with clinical manifestation of dengue, positives IgM and negative RT-PCR. Finally, negative cases were those patients with clinical manifestations of dengue but negative in RT-PCR and IgM.

The people detected by active surveillance were analyzed by case definition, residence, serotype, age-group, and days of diagnosis after the onset of the symptom.

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Independence among categorical variables was tested with Chi square test with a confidence level of 95%.

Percentage of specific symptoms and signs were obtained in people detected in active surveillance distributed by age group, and by confirmed and non confirmed cases. Independence among categorical variables was tested with Chi square test with a confidence level of 95%. To confirm the statistical results by bi-variable test and adjust them by age group, gender and residency, logistic regression analysis was applied with the symptoms and signs of the people detected in the active surveillance.

Additional tests were done in a sub sample of patients during the acute and convalescence phase of the dengue disease, including: hemoglobin, hematocrit, platelets, white blood cells, lymphocytes and neutrophils. Statistical differences among these hematologic quantitative variables were tested with Student T test and F test when was compared between confirmed and non confirmed cases, and Student T matched test when consecutive samples were made.

Acute and convalescence sero-conversion to IgM anti-dengue antibody was evaluated in those patients confirmed with dengue by RT-PCR.

To do comparable analysis of the active surveillance in this study with the passive surveillance in the local and national system, we redefined the terms of confirmed cases of dengue. In this part of the analysis people detected in active surveillance with clinical manifestation of dengue and positive IgM sero-converted in the convalescence phase of the dengue disease were considered confirmed cases. Besides, for similar reasons, cumulative incidence was calculated in the sample studied. The comparison between both surveillance systems was made in the year 2007 exclusively.

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<center>Ethical consideration.</center>

The confidentiality of all participants will be maintained throughout the study. All forms with identifiers will be maintained in a limited access office at the BIOMED. In the reporting of the laboratory results, names were used, but the information was only provided to the head of the household or the attending physician. All demographic, clinical, epidemiological, and laboratory data on each sample were entered into a database by the unique identification number. The risks of infection with venipuncture sampling were minimized by using only trained personnel to perform the venipuncture procedures using sterile, single use needles, alcohol/betadine wipes and bandages. All official protocol files (protocol, IRB minutes, and approvals) were maintained at the NMRCD under password protection. All consent forms and questionnaires will be maintained in the BIOMED, Maracay, with copies provided to NMRCD and stored under limited access.

Informed Consent Process: Wellness visits/longitudinal cohort: in selected Barrios, a study representative was knock on prospective participant's doors, identified them self and described the study. If the head of household was interested in participating, they gave a blank consent form and the representative returned in 3-5 days, allowing time for the household members to discuss the study, to enroll the household if the household members agree to participate. The enrollment process included reading the informed consent document (ICD) to the potential subjects followed with the potential subjects reading the ICD and signing it, for children and adults. (Appendix E and Appendix F).

CHAPTER FOUR: RESULTS

Demographic Description:

A total of 3,255 people aged 5 to 94 years old were recruited to participate in longitudinal study. Enrolled people were followed during two years in four neighborhoods (Barrios) of Maracay, Venezuela. The barrios selected were: "23 de Enero" including 693 (21.3%) people, "Caña de Azucar" with 1299 (39.9%) people, "La Cooperativa" 628 (19.3%), and "Piñonal" 635 (19.5%). The goals were to detect acute dengue cases in an active surveillance of fever and dengue symptoms, and to identify antibodies anti-dengue virus with biannual blood samples. According to the age group dividing in children and adults, 784 people (24.1%) were children (among 5 and 15 years old), 1,721 (53.9%) adults equal and older than 15 and less than 50 years old, and 750 (23.0%) were adults equal and older than 50 years old. (Table 2).

Table 2.

Demographic Features of the enrolled people, distributed by Barrios, gender and age groups < 15 year of age, ≥ 15 < 50 and ≥ 50 years of age.

| Neighborhoods (Barrios) | Gender | Age Groups in Years | | | |
		<15	≥15<50	≥50	Total
	Female	73	263	108	444
23 de Enero	Male	92	113	44	249
	Sub total	165	376	152	693
	Female	144	459	240	843
Caña de Azúcar	Male	150	222	84	456
	Sub total	294	681	324	1299
	Female	94	218	100	412
La Cooperativa	Male	81	92	43	216
	Sub total	175	310	143	628
	Female	68	221	86	375
Piñonal	Male	82	133	45	260
	Sub total	150	354	131	635
Total		784	1721	750	3255

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The number of females was higher than the number of males in the study with 2,074 women (63.7%) and 1,181 men (36.3%). The femininity ratio (FR) was 1.76, being higher in older people, from 15 to 94 than young people from 5 to 15; 2.18 and 0.94 respectively. This tendency also can be seen in age group by 5 years from 5 to 9 to 90 to 94. (Table 3).

Table 3

Distribution of the people studied by age group, gender and Femininity Ratio (FR).

Age Groups in years	Female	Male	Total	Femininity Ratio (FR)
5-9	176	206	382	0.85
10-14	203	199	402	1.02
15-19	197	135	332	1.46
20-24	184	78	262	2.36
25-29	188	92	280	2.04
30-34	191	90	281	2.12
35-39	150	54	204	2.78
40-44	140	51	191	2.75
45-49	111	60	171	1.85
50-54	144	54	198	2.67
55-59	136	40	176	3.40
60-64	76	45	121	1.69
65-69	78	30	108	2.60
70-74	52	24	76	2.17
75-79	23	15	38	1.53
80-84	14	5	19	2.80
85-89	8	1	9	8.00
90-94	3	2	5	1.50
Total	2074	1181	3255	1.76

These femininity ratios by age groups were similar in the four barrios of Maracay, and comparing with the total. (Table 4).

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Table 4

Distribution of Age Group, Gender and Femininity Ratio in the Four Neighborhoods in the study. Maracay 2006-2008.

Neighborhoods (Barrios)	Age Group	Female	Male	Total	Femininity Ratio
23 de Enero	<15	73	92	165	0.79
	≥15	371	157	528	2.36
Caña de Azúcar	<15	144	150	294	0.96
	≥15	699	306	1005	2.28
Cooperativa	<15	94	81	175	1.16
	≥15	318	135	453	2.36
Piñonal	<15	68	82	150	0.83
	≥15	307	178	485	1.72
The Four Neighborhoods	<15	379	405	784	0.94
	≥15	1695	776	2471	2.18
Total		2074	1181	3255	1.76

The study of the cohort began in September 2006 with 2,509 people. During the first year of follow-up, until September 2007; 556 people were added in the cohort. In the second year of the study, 190 people were added, from October 2007 to December 2008.

The people lost in the follow-up were 471 in the first year (15.4%) and 239 (8.6%) in the second one. These people were lost in both groups: on the active surveillance of febrile cases of dengue and in the bi-annual detection of specific IgG antibody anti-dengue. However, 178 people rejected, exclusively, to participate in the bi-annual blood sample to detect specific IgG antibody anti-dengue so they kept in the active surveillance of febrile cases of dengue, (66 people in the first year and 112 in the second one).

In the total period of two years 710 people (21.8%) were lost in the follow-up on active surveillance and 888 (27.3%) in both the active surveillance and bi-annual IgG anti-dengue antibody detection.

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Table 5.

Distribution of the people according to their type of permanency in the study. Maracay 2006-2008.

Group of Age and sex	People who never left the Cohort Group I	People who only left the Bi-annual blood sample but not the active surveillance. Group II	People who Left and came back to the Cohort Group III	People who left the cohort. (Did not die) Group IV		People Who Died (Other causes, not dengue) Group V	**Total**
< 15	622	38	9	115	(14.7%)	0	784
> 15	1745	140	21	544	(22.0%)	21	2471
Sub-Total	2367	178	30	659	(20.2%)	21	3255
Females	823	86	12	247	(20.9%)	13	1181
Males	1544	92	18	412	(19.9%)	8	2074
Sub-Total	2367	178	30	659	(20.2%)	21	
Total	2367	178	30	659		21	3255

From these 710 people lost in the active surveillance, 21 died by causes different than dengue (13 in the first year and 8 in the second one). 30 left and came back to the study and 659 left definitely the study.

According to the type of permanence of the people in the study, there were five groups: Type I, integrated by 2,367 (72.7%) people who never left the study. Type II, it had 178 (5.5%) people who only left the Bi-annual blood sample component to detect IgG specific antibody anti dengue virus but they did not leave the active surveillance component. Type III, formed by 30 (0.9%) people who left and returned to the study before it was over. Type IV, they were 659 (20.2%) people who left definitely the study in both components (active surveillance and biannual blood sample). Type V: 21 (0.7%) people who died during the period of the study. The Types I,II and III were always in the

active surveillance part of the study and only the Type I was in the bi-annual blood sample part of the study to detect IgG specific antibody anti dengue virus. (Table 5).

"23 de Enero" and "Piñonal" were the Barrios with the highest number of people lost in the study, 25.1% and 23.3% respectively, and "Cana de Azúcar" and "La Cooperativa" had 19.6% and 16.4% of people lost, respectively. The proportion of death by no dengue cause was similar in the four barrios, being the fewer in "Piñonal" with 0.5% and higher in "La Cooperativa" with 0.8%.

The percentage of people lost in the follow-up was small in young people (5 to 15) with 14.7% respect older people (>15%) with 22.9%. The male sex, lost in the study, was little higher than female; 22.0% and 20.3% respectively. All people who died were older than 15 (0.8%) and the proportion was higher in males than females (1.1% vs. 0.4%).

Table 5.

Distribution of the people according to their type of permanency in the study. By neighborhoods, Maracay 2006-2008.

Neighborhoods (Barrios)	People who never left the Cohort Type I	People who only left the Bi-annual blood sample Type II	People who Left and come back Type III	People who left the cohort. (Did not die) Type IV	People who Died Type V	Total
23deEnero	468	37	14	169 (24.4%)	5 (0.7%)	693
Caña de Azucar	984	55	5	247 (19.0%)	8 (0.6%)	1299
Cooperativa	474	42	9	98 (15.6%)	5 (0.8%)	628
Piñonal	441	44	2	145 (22.8%)	3 (0.5%)	635
Total	2367	178	30	659 (20.2%)	21 (0.7%)	3255

Incidence Density

The people in the study (3,255) contributed with 1,914,496 person-days (p/d) during the two years of follow-up. The period from Sep. 06, 2006 to Sep. 30, 2007 (2006-2007) had 808,339 p/d and the period from Oct. 01, 2007 to Dec. 08, 2008 (2007-2008) had 1,106,157 p/d. Dividing the study period in nine trimesters, the first one had 132,396 p/d and the last one 177,453. Every other trimester had values among 218,000 p/d and 239,000 p/d. In this study period were also considered the number of person days by each month, with a total of 28 months. September 2006 had 528 p/d, October 2006 had 18,388 p/d and November 2006 had 41,566 p/d. From December 2006 until November 2008 all amounts of person-day were among values of 70,000 and 81,000. Finally, December 2008 had 20,592 p/d.

	Oct-06	Nov-06	Dec-06	Jan-07	Feb-07	Mar-07	Apr-07	May-07	Jun-07	July-07	Aug-07	Sep-07	Oct-07	Nov-07	Dec-07	Jan-08	Feb-08	Mar-08	Apr-08	May-08	Jun-08	July-08	Aug-08	Sep-08	Oct-08	Nov-08	Dec-08
Cases	1	4	4	3	4	2	3	0	4	9	9	3	2	5	0	0	0	0	0	2	2	3	0	0	2	0	0
Incidence	5.44	9.62	5.56	3.92	5.82	2.71	4.36	0.00	5.25	11.2	11.2	3.87	2.51	6.55	0.00	0.00	0.00	0.00	0.00	2.57	2.60	3.76	0.00	0.00	2.51	0.00	0.00
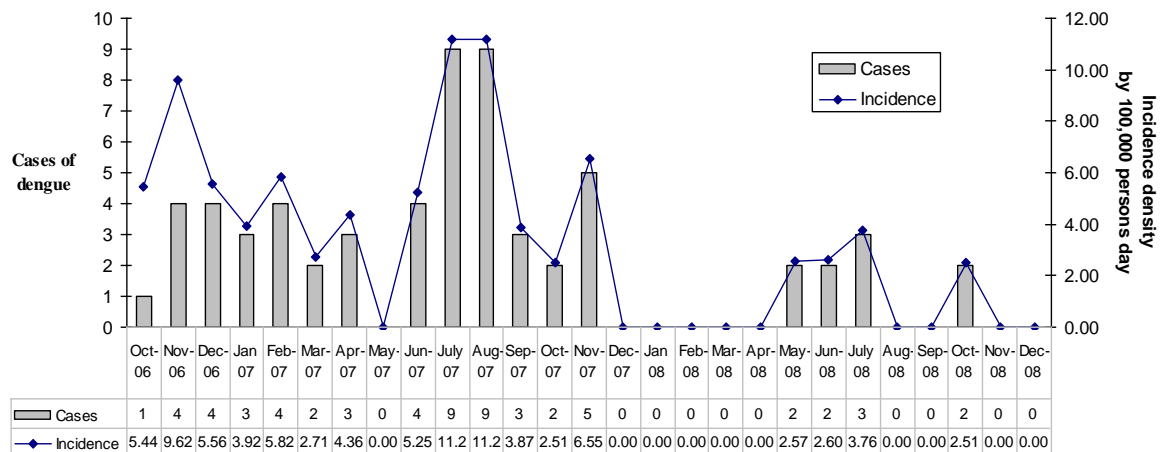
Figure 1 Incidence Density and Number of dengue cases by month, Maracay. 2006-2007

According to the kind of permanence in the cohort, the people from the Type I, who never left the cohort, contributed with 1,634,162 p/d: 627,566 in the first year (2006-2007) and 1,006,596 in the second one (2007-2008). The people lost (Type IV) gave

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117,594 p/d in the period 2006-2007 and 14,438 in the period 2007-2008. Dead and reincorporated people had a little impact in the number of person days. . (Table 7).

Children, 5 to 15, contributed with 475,764 p/d and adults (>15) added 1,438,732 p/d in the study. Males and females between 5 and15 had similar number of person days, 243,438 and 232,326 respectively. However, adult females had 1,009,188 p/d versus 429,544 p/d of adult males. (Table 8).

Table 7.

Number of person/days (p/d) by type of permanency in the study and by month in two years of follow-up. Maracay 2006-2008

2006-2007	Oct-06	Nov-06	Dic-06	Jan-07	Feb-07	Mar-07	Abr-07	May-07	Jun-07	July 07	Ago-07	Sep-07
Type I*	334	13549	29997	50719	53754	48552	53754	52500	58720	64115	67921	67921
Type II	23	817	2041	4390	4898	4424	4898	4762	5038	5133	5394	5394
Type III	0	131	369	613	651	588	590	479	491	494	527	527
Type IV	163	3784	8871	15675	16685	14753	14229	10621	8770	6011	6076	6076
Type V	8	107	288	517	525	422	465	450	470	450	443	374
Total Person/Days	528	18388	41566	71914	76513	68739	73936	68812	73489	76203	80361	80292

2007-2008	Oct-07	Nov-07	Dic-07	Jan-08	Feb-08	Mar-08	Abr-08	May-08	Jun-08	July 08	Ago-08	Sep-08
Type I	65730	67921	65730	69913	71610	66990	69300	71610	71666	70769	73377	73377
Type II	5220	5394	5220	5463	5518	5162	5518	5340	5518	5340	5518	5518
Type III	510	499	526	307	186	174	186	162	244	597	682	682
Type IV	5880	5762	4639	1606	372	339	372	360	372	254	93	93
Type V	258	222	195	134	124	116	124	93	93	85	62	47
Total Person/Days	77598	79798	76310	77423	77810	72781	75500	77565	77893	77045	79732	79717

2007-2008	Oct-08	Nov-08	Dic-08	Total p/d
Type I	71010	73377	18936	1634162
Type II	5340	5518	1424	129563
Type III	660	700	232	12587
Type IV	90	78	0	132032
Type V	30	31	0	6152
Total Person/Days	77130	79704	20592	1914496

* Type I: **People who never left the Cohort.** Type II: **People who only left the Bi-annual blood sample**
Type III: **People who Left and come back to the study.** Type IV: **People who left the cohort. (Did not die).** Type V: **People who died.**

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The barrio "23 de Enero" had 381,295 p/d from 693 participants, "Cana de Azucar" contributed with 780,521 p/d from 1,299 people. "La Cooperativa" added 389,825 p/d with 628 people in the study and finally "Pinonal" had 362,855 p/d and 635 people in follow-up.

Table 8.

Number of person/days (p/d) by neighborhoods, age group and gender Maracay 2006-2008.

Neighborhoods (Barrios)	Children less than 15 years old			Adults equal and higher than 15 years old			Total
	Females	Males	Subtotal	Females	Males	Subtotal	
23deEnero	38733	54286	93019	209081	79195	288276	381295
Caña de Azucar	91551	94697	186248	416868	177405	594273	780521
La Cooperativa	61331	50747	112078	203811	73936	277747	389825
Piñonal	40711	43708	84419	179428	99008	278436	362855
Total	232326	243438	475764	1009188	429544	1438732	1914496

The crude incidence density of the cohort during two years was 3.24 by 100,000 p/d (2.01 by 100,000 p/d adjusted by age group and gender of Maracay population): The first year (from Sep 2006 to Sep 2007) the ID was 5.69 p/d and 1.45 p/d in the second year (Oct. 2007 to Dec. 2008). The trimester with higher incidence density in both years of study was from July 2007 to September 2007 with 8.81 p/d. All trimesters in the first year had higher ID than second one. The months with higher ID were July 2007 (11.20 p/d) and August 2007 (11.21 p/d) (Figure 1).

In two years of study (2006-2008), "Cana de Azucar" was the barrio with the highest Incidence Density: 4.23 p/d, followed by "La Cooperativa": 3.59 p/d. However, in the 2006-2007 period "La Cooperativa" had 7.92 p/d of Incidence Density and "Cana de Azucar" had 6.67 p/d. In all Barrios, the ID was always higher in the first year of the study; 2006-2007. (Figure 2).

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Table 9.

Number of cases, person days (p/d) and Incidence density (ID) distributed by neighborhoods, gender and age groups, Maracay 2006-2008.

Neighborhoods		Males <15	Females <15	Total <15	Males >15	Females >15	Total >15	Total
23 de Enero	Cases	2	4	6	1	3	4	10
	p/d	54286	38733	93019	79195	209081	288276	381295
	ID	3,68	10,33	6,45	1,26	1,43	1,39	2,62
Cana de Azucar	Cases	7	10	17	3	13	16	33
	p/d	94697	91551	186248	177405	416868	594273	780521
	ID	7,39	10,92	9,13	1,69	3,12	2,69	4,23
Cooperativa	Cases	8	2	10	2	2	4	14
	p/d	50747	61331	112078	73936	203811	277747	389825
	ID	15,76	3,26	8,92	2,71	0,98	1,80	3,59
Pinonal	Cases	1	1	2	0	3	3	5
	p/d	43708	40711	84419	99008	179428	278436	362855
	ID	2,29	2,46	2,37	0,00	1,67	1,08	1,38
Total	Cases	18	17	35	6	21	27	62
	p/d	243438	232326	475764	429544	1009188	1438732	1914496
	ID	7.39	7.31	7.36	1.40	2.08	1.88	3.24

The incidence density during the two years study was higher in infants from 5 to 15 years old with 7.36 p/d than adults equal and higher than 15 years old with 1.88 p/d. This difference was in both years of the study, but it was much evident in 2006-2007. (Table 9). In the period 2006-2007 and in the four barrios the incidence densities were always higher in infants but in 2008 in two barrios the Incidence density were a little higher in two of the four barrios. (Piñonal and La Cooperativa) (Figure 2).

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	First Year 2007		Second Year 2008	
	Children	Adults	Children	Adults
■ Cana de Azucar	14.71	4.31	5.78	1.59
□ Cooperativa	21.64	2.54	0.00	0.67
■ 23 de Enero	13.92	2.40	1.89	0.66
■ Pinonal	5.91	1.67	0.00	0.68
■ Total	14.68	3.08	2.64	1.05
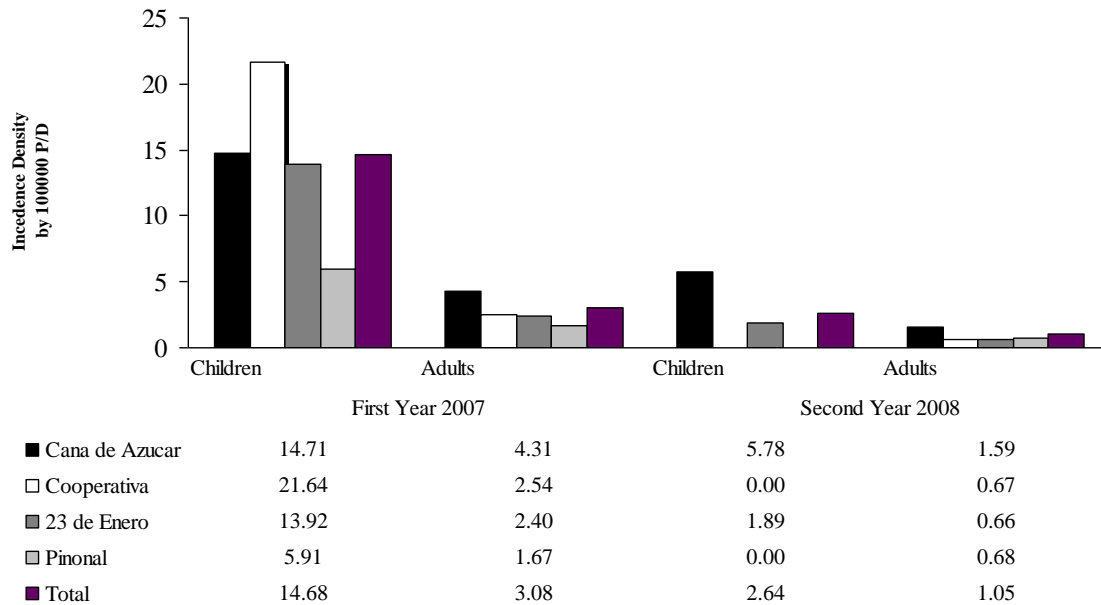
Figure 2. Incidence Density of confirmed dengue cases by Neighborhoods, age group and year of follow-up. Maracay 2006-2008

Relative Risk in Age Groups

In the two of years study (2006-2008), the Relative risk (RR) in confirmed infant cases respect to adults was 3.92 (95% IC 2.38 – 6.48) being 4.77 (95% IC 2.66 – 8.54) in 2006-2007 and 2.52 (95% IC 0.91 – 6.94) in 2007-2008. According to gender, in females between 5 to 15 the ID was 7.32 by 100,000 person-days and 2.08 in females higher than 15. RR 3.52 (95% IC 1.86 – 6.66) In males 5 to 15 the ID was 7.39 by 100,000 person-days and 1.40 by 100,000 person-days in males higher than 15. RR 5.28 (95% IC 2.10 – 13.33). In the period 2006 to 2007 the Relative Risk in females was 5.13 by 100,000 person-days; 15.01 by 100,000 p/d in females from 5 to 15 and 3.00 by 100,000 p/d in older female. In the same period, the ID in male 5 to 15 was 14.37 by 100,000 p/d and 2.71 by 100,000 p/d in male older or equal than 15. In the period 2007-2008, the ID was reduced similarly in both sex and age: 2.16 by 100,000 p/d in young women and 1.39 by

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100,000 p/d in the older ones and 2.74 by 100,000 p/d and 0.41 by 100,000 p/d in young

and older men respectively (Table 10).

Table 10

Relative Risk ID of age group and gender by years of the study.

	2006-2008		2006-2007		2007-2008	
	RR	IC	RR	IC	RR	IC
Age Group (Children/Adults)	3.92	2.38-6.48*	4.77	2.38-6.48*	2.52	0.92-6.94
Female	3.52	1.86-6.66*	5.00	2.35-10.64*	1.55	0.41-5.86
Male	5.29	2.10-13.3*	5.30	1.91-14.78*	6.72	0.75-60.11
Gender (female/male)	0.86	0.51-143	0.76	0.42-1.37	1.20	0.42-3.46
Children	0.99	0.51-1.92	1.04	0.50-2.19	0.79	0.18-3.52
Adults	1.22	0.49-2.52	1.11	0.39-3.10	3.41	0.43-27.23

* Significant Relative Risk.

   During the two years study, the Incidence Density in female was 3.06 by 100,000

person-days and 3.56 by 100,000 persons-days in males: RR female/male: 0.86 (95% IC

0.51 – 1.43). In females <15 the ID was 7.32 by 100,000 person-days and 7.39 in males:

RR 0.99 (95% IC 0.51 – 1.92). In female >15 the ID was 2.08 versus 1.40 in women: RR

1.22 (95% IC 0.49 – 2.52).

 Prevalence of antibody anti dengue virus and Incidence of infection by prospective sero-

prevalence of antibodies: IgM anti-antidengue antibody.

   The first biannual sample (at the beginning of the study) was made in all the 3,255

participants in different times of the study, depending when they were included in the

cohort. Out of them, 75 (2.3%) were IgM positive, 21 children and 54 adults. In the

second biannual sample 2,622 people were tested and 86 were IgM positive (3.28%) 4

of them had been detected with dengue infection by the Active Surveillance few weeks

before (with a limit of 13 weeks), either by IgM or RT-PCR. In the third biannual sample

116 people were IgM positive and 11 were detected with dengue infection by active

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surveillance in a period from zero to 13 weeks before the biannual sample. 2,402 people were participating in this third test so 105 (4.4%) were asymptomatic. In the fourth biannual sample 69 people were IgM positive, only one of them had been detected by the active surveillance system. It represented 3.24% of 2,129 people. The fifth and last biannual sample had 61 IgM positive and two of them were detected previously by the active surveillance system. 1,599 people were tested.

<center>Bi-annual samples, Plaque Reduction Neutralization Test.</center>

The Plaque Reduction Neutralization Test (PRNT) was applied in 2125 people; 65.3% from all people in the cohort. Four samples from 1,684 people who began the study cohort in 2006 and three samples from 441 people who began the study cohort in 2007. In the first sample (S1) in both years of the cohort, the PRNT detected at least one of the four anti DENV antibodies in 1,840 people (86.6%), it was negative in 283 (13.4%) people and 2 participants were not tested in their S1. All the anti DENV antibodies were detected either alone or in combination with other serotypes. Anti DENV-1 antibody was positive in 1,573 (74%) people but only in 157 (7.4%) this antibody was found alone. Anti DENV-1 and DENV-2 antibodies were positives simultaneously in 1,386 (65.2%) people. Three anti DENV serotypes were positive in 401 people and anti DENV 1, DENV-2 and DENV-3 antibodies were the most frequent combination, it occurred in 371 (92.5 %) people. The four antibodies were present in 31 (1.5 %) people. (Table 11).

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Table 11

Results of Plaque Reduction Neutralization Test in the first sample of people studied; distributed by number of serotypes detected in each participant

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Negatives and number of Serotypes detected in each individual.	Frequency	% of the subtotal	% of the total
One Serotype	401	N/A	18.9
DENV-1	157	39.2	7.4
DENV-2	166	41.4	7.8
DENV-3	70	17.4	3.3
DENV-4	8	2.0	0.4
Two serotypes	1007	N/A	47.4
DENV-1 DENV-2	955	94.8	44.9
DENV-1 DENV-3	23	2.3	1.1
DENV-2 DENV-3	16	1.6	0.8
DENV-1 DENV-4	6	0.6	0.3
DENV-2 DENV-4	2	0.2	0.1
DENV-3 DENV-4	5	0.5	0.2
Three Serotypes	401	N/A	18.9
DENV-1 DENV-2 DENV-3	371	92.5	17.5
DENV-1 DENV-2 DENV-4	29	7.2	1.4
DENV-1 DENV-3 DENV-4	1	0.3	0.05
Four Serotypes DENV-1 DENV-2 DENV-3 DENV-4	31	N/A	1.5
Negatives	283	N/A	13.3
NR	2	N/A	0.1
Total	2125		100

According to the age group, from 5 to 9 years old (y/o) interval had the less proportion of positive antibodies in S1 with 127 children (47.39%), in the next group from 10 to 14 y/o, 202 children were positive (75.01%). The number of positives were increasing in correspondence with the groups of higher age: 89.34% in the group from 15 to 19; 94.34% in the group from 20 to 24; in the group of 45 to 49, 99.12% were positive.

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Separating into two age groups: less than 15 (children) and higher than or equal to15 (adults), the proportion of negatives was significantly higher in children: 34.45 % versus 4.72 % in adults. This difference was consistent and significant (p value < 0.01) in the two years when the people started the study (cohorts of 2006 and 2007). The proportion of antibodies against serotype 1 and 2 were higher in people > 15; 85.45 % and 87.34% versus 24.31% and 25.51 % in people < 15 respectively. On contrary, people <15 had lightly higher proportion of serotypes 3 and 4, but this difference was not significant. (Figure 3).

	2007		2008		2007-2008	
	Adults	Children	Adults	Children	Adults	Chlidren
■ DENV-1	86.7	38.3	79.6	46.2	85.45	40.22
■ DENV-2	88.9	39.6	80.9	45.5	87.34	34.26
□ DENV-3	25.8	24.9	18.1	27.3	24.31	25.51
□ DENV-4	3.9	4.5	1.3	6.1	3.40	5.96
▥ Negatives	3.2	43	11	25.5	4.72	34.45
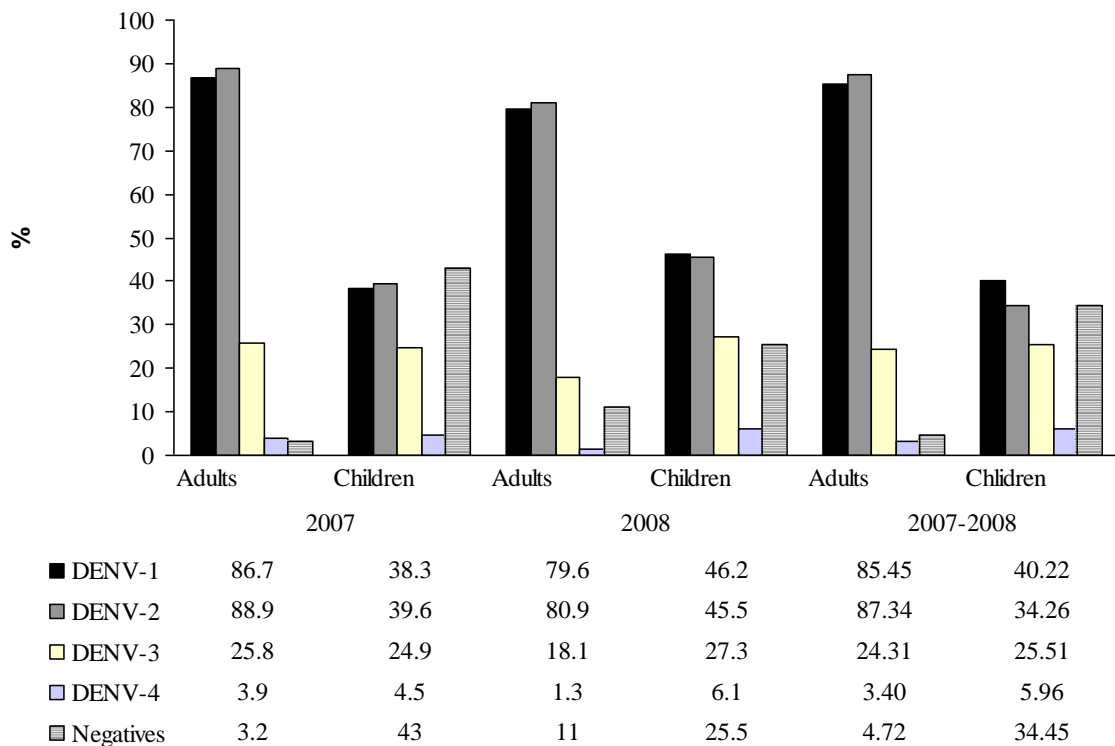
Figure 3 Percentage of people with antibodies according to serotype and negative results distributed by age groups and years when they started the study. Maracay 2006 - 2008.

Figure 4 showed the most frequent results of the PRNT. DENV-1 and DENV-2 were in four of the five first more frequent results, representing 70.6%, in contrast DENV-4 was in 3.95% of all possible results and DENV-3 in 24.45%.

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If we compare the prevalence of the three most frequent results in the PRNT, which included DENV-1 – DENV-2, DENV-1 – DENV-2 –DENV-3 and negatives according to age groups each 5 years, we can see how the proportions descend in negative results with the age groups and ascend with the serotype positive results. (Figure 5).

%
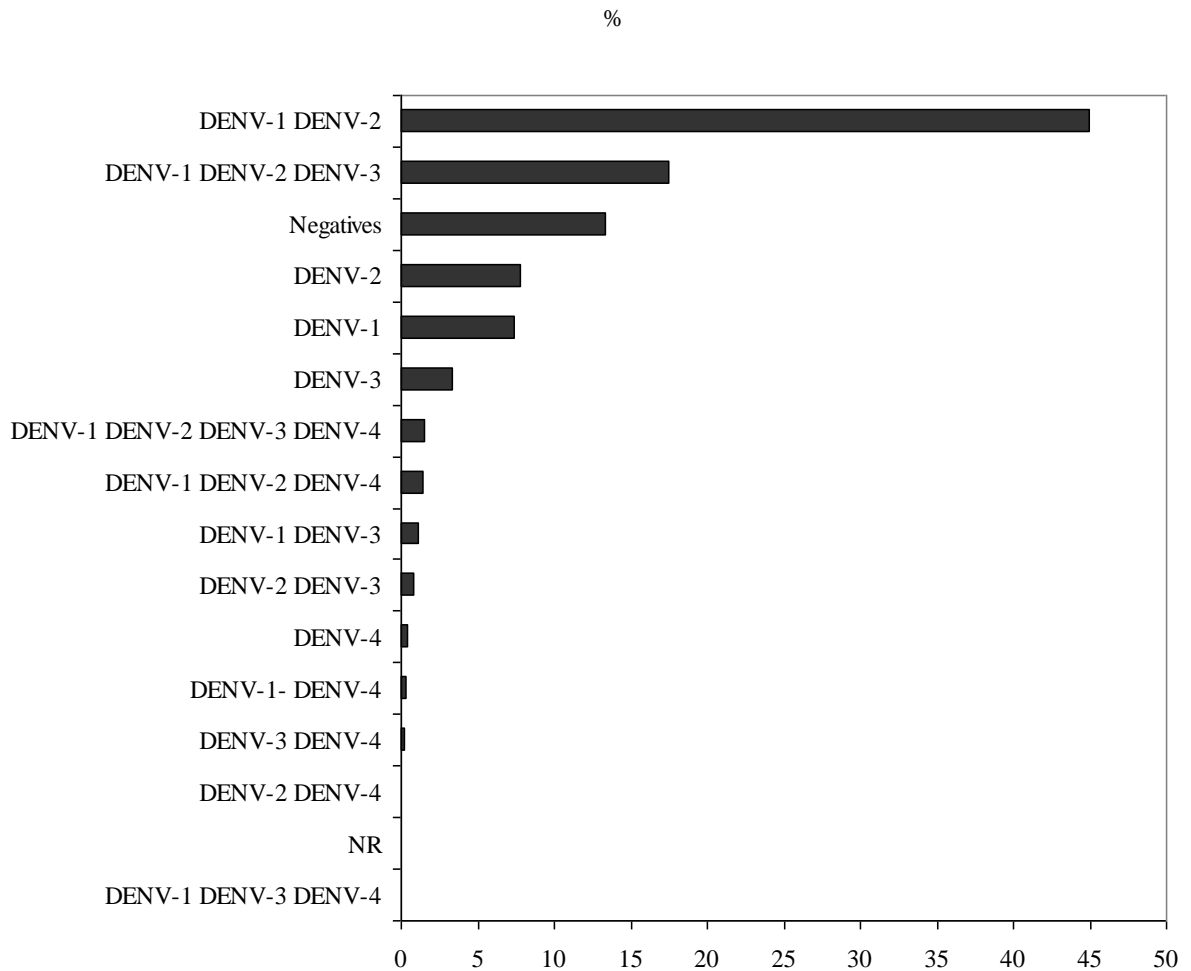
Figure 4 Plaque Reduction Neutralization Test in the first sample of 2,125 people sorted by most frequent results. Maracay 2006- 2008.

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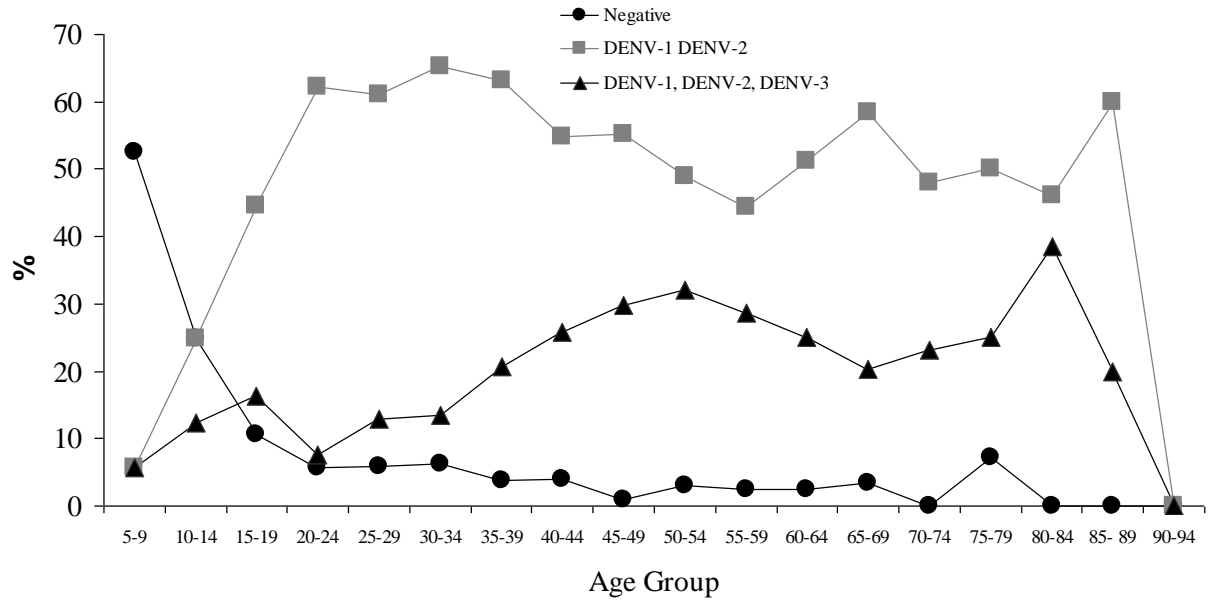
Figure 5 Percentage of the three most frequent results of PRNT in the first sample of people in the study by age group. Maracay 2006 – 2008.

Thirty percent of the negative people in the first sample were positive either to one or more than one serotype six months later in the second sample (S2). In the third sample (S3), it was 29.6% of positives and 23.8 % in the last sample (S4). The sero conversion in people with previous detection of anti anti-dengue antibody serotype 2 was the highest percentage in the second sample with 58.1 %. In the third sample (S3) people with previous anti anti-dengue antibody serotype 4 was the highest with 50.0 % and finally anti anti-dengue antibody serotype 3 was the highest in the last sample with 34.1%. (Figure 6). The same analysis but considering the people with negative results and the most frequent positive results (DENV-1 DENV-2, DENV-1 DENV-2 DENV-3, and DENV-1 DENV-2 DENV-4) showed the people with no antibody had the highest percentage of sero-conversion in the second sample (S2): 30.1%. In the third sample, the people with DENV-1 DENV-2 and DENV-1 DENV-2 DENV-4 combination in the

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second sample had the highest percentage of sero-conversion with 36.7 and 35.7

respectively. (Figure 7).
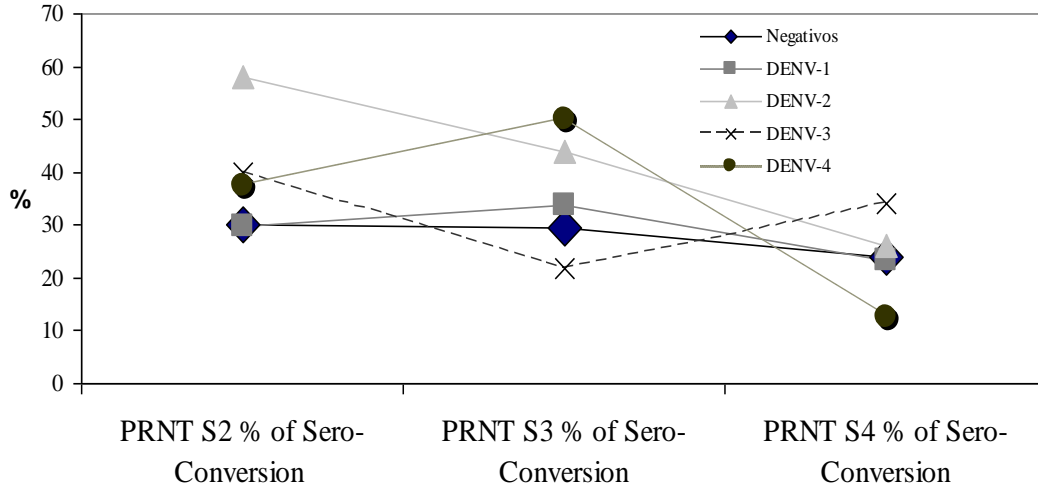
Figure 6 PRNT, percentage of sero conversion according to results of the previous PRNT in the second (S2), third (S3) and fourth (S4) samples. Maracay 2006 – 2008.
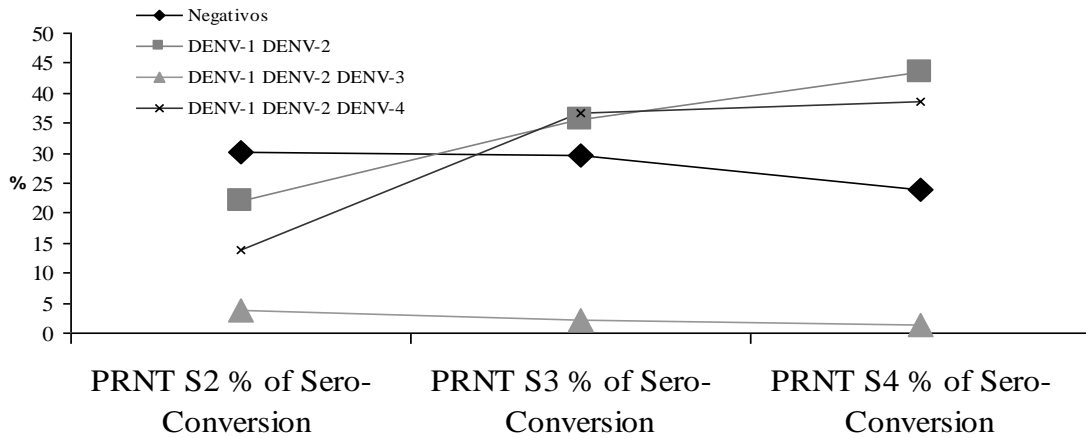
Figure 7 PRNT, percentage of sero conversion according to the most frequent results of the previous PRNT in the second (S2), third (S3) and fourth (S4) samples. Maracay 2006 – 2008.

When we compared the sero-conversion proportion of negative people with that

proportion of people with previous monotypic antibody detection in the three samples

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(S1, S2 and S3); the proportion of sero-conversion was always higher in the people with

monotypic antibody. These differences were statistically significant. The people with

multitypic antibodies detected had always less proportion of sero conversion respect to

either people negative or people with monotypic antibody. (Table 12).

Table 12

PRNT, percentage of sero conversion according to results of the previous PRNT in the second (S2), third (S3) and fourth (S4) samples by monotypic and multitypic antibodies. Maracay 2006 – 2008.

	Positives / Number of people in Sample 2	*PRNT Sample 2 % of Sero-Conversion*	*Positives / Number of people in sample 3*	*PRNT Sample 3 % of Sero-Conversion*	*Positives / Number of people in sample 4*	*PRNT Sample 4 % of Sero-Conversion*
DENV-1 *	47/157	29,9	45/133	33,8	20/86	23,3
DENV-2 *	86/166	58,1	39/89	43,8	12/46	26,1
DENV-3 *	28/70	40,0	10/46	21,8	15/29	34,1
DENV-4 *	5/8	37,5	5/10	50,0	1/8	12,5
DENV-1 ** DENV-2	174/955	22,0	319/894	35,7	13/4818	43,6
DENV-1 ** DENV-2 DENV-3	10/371	3,8	13/574	2,3	10/125	1,4
DENV-1 ** DENV-2 DENV-4	4/29	13,8	18/49	36,7	10/26	38,5
Monotypic	166/401	41,4 ***	99/278	35,6 ***	48/169	28,4 ***
Multitypic	188/1355	13,9 ***	350/1517	23,1 ***	158/1232	12,8 ***
Negatives	86/283	30.1 ***	58/156	29,6 ***	29/122	23,8 ***

* Monotypic antibodies   ** Multitypic antibodies
*** P value < 0.05

The Logistical Regression model with the new infection of dengue in the sample 3,

after the second six months of the study, as a dependent variable and previous infection in

time cero (the first sample at the beginning of the study) and sample 1 (S1) at the first six

months, age, sex, residence (neighborhoods), time of the beginning of the study (cohort

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2006 or 2007), number of serotypes in time cero and sample 1 and interaction variables between age and sex, residence and age, cohort and age and cohort and sex as a factors or independent variables showed a significant (P value less than 0.05) relation with age, and number of serotypes in the sample 1 (sample previous to the new infection). The difference was significant between 2 and 3 serotype. In conclusion, younger people and two anti dengue antibodies in blood sample were the most important factors to predict a new infection of dengue by PRNT.

Active Surveillance and Laboratory Diagnostic of dengue

270 people with possible dengue infection were detected by the surveillance action in the prospective study; all of them had fever either equal or higher than 38º Celsius, at the moment of the evaluation. The blood samples were taken the same day of the surveillance detection and that occurred between the first and the eighth day post onset of the symptoms. 137 (50.74 %) were less than 15 year old and 156 (57.78 %) were female. The neighborhood "Pinonal" had the highest percentage of people detected in the surveillance with 10.39% (66 from 635 people) and "La Cooperativa" had the lowest one with 5.25%. (Table 13).

The highest proportion of blood samples were taken in the second and third day with 37% and 28% respectively and 96.68 % of the samples were taken at the fifth day, or less, after the onset of the symptoms. (Table 14).

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Table 13

Number of possible cases of dengue detected in the active surveillance, distributed by Neighborhood and their population, age-group and gender. Maracay 2006 2008

Neighborhoods (Barrios) and their population.	Children less than 15 years old			Adults equal and higher than 15 years old			Total (% of population)
	F	M	Subtotal	F	M	Subtotal	
23deEnero 693	12	13	25	14	7	21	46 (6.64)
Caña de Azucar 1,299	25	31	56	49	18	67	123 (9.47)
Cooperativa 628	5	17	22	9	4	13	35 (5.25)
Piñonal 635	20	14	34	22	10	32	66 (10.39)
Total Population 3,255	62	75	137	94	39	133	270 (8.29)

Table14

Number of cases, percentage and cumulative percentage by day of detection in active surveillance. Maracay, 2006 2008.

Day of detection and blood sample	Number of cases	%	Cumulative %
1	18	6.67	6.67
2	101	37.41	44.08
3	77	28.52	72.60
4	44	16.30	88.90
5	21	7.78	96.68
6	5	1.85	98.53
7	2	0.74	99.27
8	2	0.74	100.00
	270	100.00	

The dengue infection was confirmed in 62 (22.96%) patients by RT-PCR. In

addition, probable dengue cases were estimated by detection of anti anti-dengue IgM

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antibody, with ELISA. For this test, two samples were taken: the first in the acute phase and the second one in the convalescence phase of the disease. 34 (54.84%) confirmed dengue cases were people less than 15 years old and 28 (45.16%) in older participants. The four serotypes were in both age groups. DENV-1 was more detected in <15 people and DENV-2 DENV-3 and DENV-4 in >15 people. (P value <0.01, Chi^2 test). DENV-1 and DENV-2 were 67.74 % of the total confirmed dengue cases. (Table 15).

Table 15

Number and percentage of confirmed dengue cases by RT-PCR in the active surveillance at four neighborhoods of Maracay, distributed by age-group and serotype. Maracay, 2006 - 2008.

Serotype	Children less than 15 years old (%)	Adults equal and higher than 15 years old (%)	Total
DENV-1	17 (50.00)	4 (14.29)	21 (33.87)
DENV-2	10 (29.41)	11 (39.29)	21 (33.87)
DENV-3	3 (8.82)	4 (14.29)	7 (11.29)
DENV-4	4 (11.76)	9 (32.14)	13 (20.97)
Total	34	28	62

Three of the four neighborhoods had all dengue serotypes; only "Pinonal" had only three dengue serotypes. There were not significant differences among the proportion of serotypes among neighborhoods. Figure 8.

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	Pinonal	La Cooperativa	Cana de Azucar	23 de Enero	Total
□ DENV-4	0	2	9	1	12
□ DENV-3	1	3	3	1	8
■ DENV-2	3	2	13	3	21
■ DENV-1	1	7	8	5	21
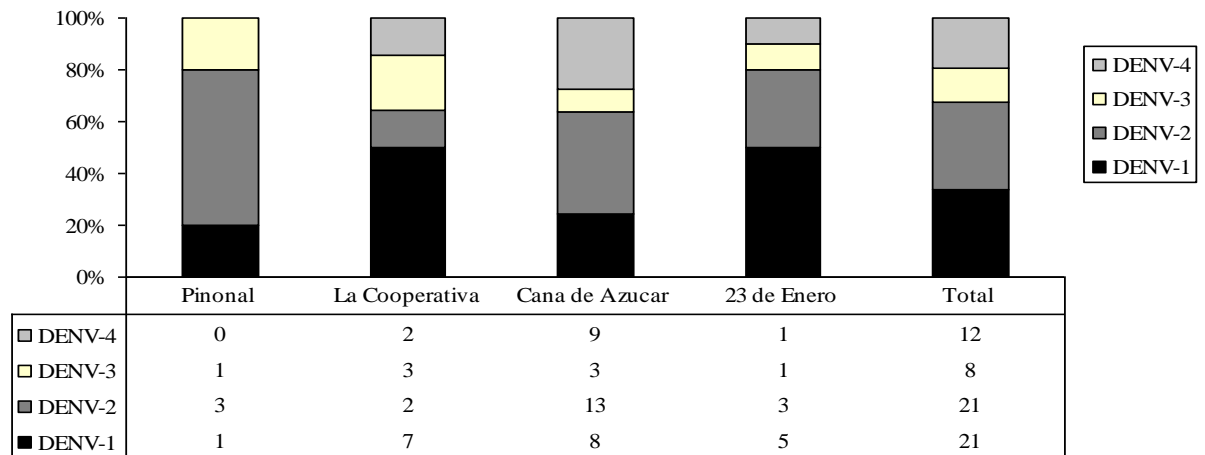
Figure 8. Percentage and number of dengue cases by serotypes detected in each neighborhood. Maracay, 2006–2008

73.48% of the confirmed cases by RT-PCR were detected in the first three days after onset of the symptoms and 24.19% in the days fourth and fifth. (Table 16). DENV-2 was the most detected serotype in the first two days after onset of the symptoms with 44.44% of 27 people with DENV detected in these two days. DENV-1 was the most detected serotype in the third, fourth and fifth days with 42.42%. However, these differences were not statistically significant. (p value > 0.05, Chi^2 test). 50 (18.5%) patients were negative the days fourth and fifth after onset of the symptoms.

In the acute period (the first to seventh days after onset the symptoms) of the 270 people, the IgM MAC ELISA test was positive in 29 patients and positive in 52 patients during the convalescence period (30 days after onset of the symptoms). In the acute period IgM was positive in 7 (12.5%) of the 62 Confirmed cases and positive in 23 (38.33 %) of 60 confirmed cases in the convalescence period. Six cases (10%) were positives in both periods (Table 17).

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Table 16

Number and percentage of cases detected by active surveillance according to DENV specific serotype identified by RT-PCR in each day after onset of the symptoms, Maracay 2006 – 2008.

Serotype	Day of detection after onset of the symptoms. Number of people detected and percentage (%).							
	1	2	3	4	5	6	7-8	
D1	1 (4.76)	6 (28.57)	7 (33.33)	4 (19.05)	3 (14.29)	0 (0.00)	0 (0.00)	21 (100.00)
D2	2 (9.52)	10 (47.62)	3 (14.29)	3 (14.29)	1 (4.76)	1 (4.76)	1 (4.76)	21 (100.00)
D3	0 (0.00)	1 (14.29)	4 (57.14)	0 (0.00)	2 (28.57)	0 (0.00)	0 (0.00)	7 (100.00)
D4	1 (7.69)	6 (46.15)	4 (30.77)	2 (15.38)	0 (0.00)	0 (0.00)	0 (0.00)	13 (100.00)
Total Positives	4 (6.45)	23 (38.00)	18 (29.03)	9 (14.52)	6 (9.67)	1 (1.61)	1 (1.61)	62 (100.00)
Negatives	14 (6.73	78 (37.50)	59 (28.37)	35 (16.83)	15 (7.21)	4 (1.92)	3 (1.44)	208 (100.00)
Total	18 (6.66)	101 (37.41)	77 (28.52	44 (16.30)	21 (7.77)	5 (1.85)	4 (1.48)	270 (100.00)

Table 17

Number and percentage of patient results of IgM MAC ELISA test according to day of detection by active surveillance. Maracay, 2006-2008.

IgM	Day of detection after onset of the symptoms Number of people detected and percentage (%).								Total
	1	2	3	4	5	6	7	8	
IgM +	2 (11.11)	1 (0.99)	8 (10.39)	6 (13.64)	9 (42.86)	3 (60.00)	0 (0.00)	0 (0.00)	29 (10.74)
IgM -	16 (88.89)	100 (99.01)	69 (89.61)	38 (86.36)	12 (57.14)	2 (40.00)	2 (100.00)	2 (100.00)	241 (89.26)
Total	18 (100.00)	101 (100.00)	77 (100.00)	44 (100.00)	21 (100.00)	5 (100.00)	2 (100.00)	2 (100.00)	270 (100.00)

P value Chi 2 0.0001

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The IgM MAC ELISA test was positive in 11 of 196 (5.61%) people who were tested in the first three days, and 18 of 70 (25.71%) were positives in those people who were tested between day 4 and day 6. In one hand, from 29 patients with IgM positive in the acute phase 7 (11.29%) were also RT-PCR positive. In the other hand, 23 of 50 (38.33 %) people were IgM positive in convalescence period. (p value 0.09, Chi^2 test). (Table 18).

Table 18

IgM MAC ELISA test in Acute and convalescence phase of the disease according to results of RT-PCR. Maracay, 2006-2008.

	IgM+ in acute phase	N	% IgM + in acute phase	IgM+ in conv. phase.	N	% IgM + in conv. Phase
RT-PCR +	7	62	11.29	23	60	38.33
RT-PCR -	22	208	10.58	27	162	16.67
Total	29	270	10.74	50	222	22.52

Based in the positives RT-PCR of 62 people in active surveillance, we compared the dynamic of the virus infection by the specific serotype PRNT results, detecting those congruent and logical results between both tests. We look for: 1. temporal congruency between confirmed dengue case in active surveillance and new infection dengue case detected in the biannual sample to PRNT. 2. Specific serotype congruency between confirmed dengue case in active surveillance and PRNT results. From 62 confirmed cases by active surveillance: five were not tested by PRNT, four were detected by active surveillance after the last biannual sample was taken. Ten PRNT results were incongruent with confirmed dengue cases in active surveillance. 43 had temporal infection congruency between active surveillance and PRNT biannual test, and 35 out of them had also specific serotype congruency. Five of ten cases without congruency between RT-

PCR and PRNT were sero-negatives by neutralization, in three cases they were DENV-4

and DENV-3 in two, it does not happen with DENV-1 and DENV-2. Table 19.

Table 19

*Congruency between confirmed dengue cases detected in Active Surveillance
and results of PRNT in biannual samples. Maracay, 2006-2008.*

	Number	%
Not tested by PRNT	5	8.1
Detected by Act. Surveillance after last biannual sample. PRNT.	4	6.5
Neither temporal nor serotype infection congruency.	10	16.1
Temporal Infection Congruency Between AS and PRNT. *	43	69.3
Total Confirmed Dengue cases by AS	62	100.0

AS: Active Surveillance
 * 35 out of 43 confirmed cases were also congruent with the serotype reported by PRNT.

   From the 35 confirmed dengue cases by active surveillance congruent with serotype

specific PRNT, DENV-1 has the best congruency with 88 % (15/17), DENV-3 has 78 %

(7/9), and DENV-2 69 % (11/16). DENV-4 has the lower proportion of congruency with

17 % (2/12).

   The relation between primary and secondary infection detected by PRNT was

described with the IgM MAC ELISA test results of 62 confirmed dengue cases in active

surveillance, looking for any association between them. From 62 confirmed cases by RT-

PCR, 23 had IgM antibody sero-conversion, 39 had not IgM antibody sero-conversion in

either acute or convalescence phase of the disease. We compared the primary and

secondary infection in the 62 people by PRNT with both kinds of IgM MAC ELISA

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results. From 23 confirmed dengue cases who sero-converted, 61,1 % were primary
infection according to PRNT and from 39 confirmed dengue cases who did not sero-
convert, 15,4 % were primary infection by PRNT. This difference was statistically
significant. Chi square p value < 0.0006.

Active Surveillance and clinical manifestation.

In the two years of study, 270 people were detected with fever and classified as
probable dengue fever by active surveillance, 62 were confirmed by RT-PCR. Headache
was the most frequent symptom, being referred by 94.9% of the people. Body pain,
shiver, ocular pain and joint pain were also referred for more than 50% of the people.
These percentages were affected significantly in the symptoms body pain, ocular pain,
joint pain and abdominal pain depending on the age-group of the people. (Table 20).

Table 20

*Number and percentage of people with symptoms of dengue detected in the active
surveillance. Maracay, 2006-2008.*

Symptoms	Number of people with symptoms.	% N=270	% in patients <15 N=137	% in patients >15 N=133	Chi^2 P value
Headache	256	94.82	92.70	96.99	NS
Body pain	206	76.30	65.69	87.22	S
Shivers	196	72.59	68.61	76.69	NS
Ocular pain	186	68.89	61.31	76.69	S
Joint pain	159	58.89	45.99	72.18	S
Nauseas – Vomits	128	47.41	46.72	48.12	NS
Abdominal Pain	108	40.00	32.85	47.37	S
Asthenia	93	34.44	35.04	33.84	NS
Rash	66	24.44	25.55	23.31	NS
Hyporeflexia	62	22.96	21.17	24.81	NS
Tourniquet Test	21	7.78	8.03	7.52	NS
Petechia	11	4.01	2.19	6.02	NS

When we compared the frequency of symptoms according to the dengue disease
confirmation by RT-PCR, headache was present in 96.9% of confirmed cases and 94.2 %

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in no confirmed cases. It difference was not significant (NS) statistically with a

probability value (p) higher than 0.05 (> 0.05). Ocular pain was showed by 68.89% of

the people; 79.03% in confirmed cases and 67.3% in no confirmed cases. It difference

was significant (S). Rash also had a significant difference between confirmed and non

confirmed cases with 37.10% and 20.67 % respectively. Body pain was positive in 75%

of the people but the difference between confirmed and no confirmed case was NS.

Similarly, shiver was referred by 71% of the people and the difference, between of

percentage of confirmed and no confirmed cases, was NS. In confirmed cases, joint pain,

abdominal pain, asthenia and nauseas were positive in 54.84, 46.77%, 30.65% and

53.23% respectively, without any significant difference with non confirmed cases. The

percentage of petechias and tourniquet test were always referred by less than 10 % of the

patients and there are no significant differences between confirmed cases and no

confirmed cases. (Table 21).

Table 21

Percentage of people with symptoms of dengue detected in the active surveillance by confirmed and non confirmed dengue cases. Maracay, 2006- 2008.

Symptoms	% of people with symptoms. Totals.	% Confirmed Cases of dengue. N=62	% Non Confirmed cases of dengue N=208	Chi^2. P value
Headache	94.82	96.77	94.23	NS
Body pain	76.30	72.58	77.40	NS
Shivers	72.59	74.19	72.12	NS
Ocular pain	68.89	79.03	65.87	S
Joint pain	58.89	54.84	60.10	NS
Nauseas and vomits	47.41	53.23	45.67	NS
Abdominal Pain	40.00	46.77	37.98	NS
Asthenia	34.44	30.65	35.58	NS
Rash	24.44	37.10	20.67	S
Hyporeflexia	22.96	22.58	23.08	NS
Tourniquet test	7.78	9.68	7.21	NS
Petechia	4.01	8.02	2.89	NS

Table 22 shows the results of a Logistic regression to estimate which symptoms could be associated with dengue confirmed cases. It was adjusted by age-group, gender and residency. Rash and ocular pain were associated with confirmed dengue.

Table 22

Symptoms associated with confirmed dengue cases, applying Logistic Regression Maracay 2006 – 2008.

Variables	Odds Ratio	95% C.I.		Z-Statistic	P-Value
Age-group	0,6091	0,262	1,4159	-1,152	0,2493
Gender	0,4507	0,0596	3,4084	-0,7721	0,4401
Age-group * gender (M/F)	1,2688	0,3277	4,9124	0,3447	0,7303
Neighborhood (2/1)	1,9177	0,801	4,5908	1,4619	0,1438
Neighborhood (3/1)	2,9112	0,9992	8,4818	1,9585	0,0502
Neighborhood (4/1)	0,2838	0,0787	1,0232	-1,9249	0,0542
Asthenia	0,5765	0,2771	1,1993	-1,4736	0,1406
Abdominal Pain	1,3865	0,6888	2,7907	0,9155	0,3599
Joint Pain	0,8475	0,3657	1,9641	-0,3857	0,6997
Headache	1,1204	0,206	6,0943	0,1316	0,8953
Body Pain	0,5717	0,2257	1,4481	-1,1791	0,2383
Hyporeflexia	1,0686	0,4727	2,4157	0,1595	0,8733
Shiver	1,0694	0,5001	2,2867	0,1729	0,8627
Nauseas Vomits	1,2743	0,6555	2,4772	0,7147	0,4748
Tourniquet Test	1,7111	0,4105	7,1333	0,7374	0,4609
Petechia	1,8569	0,3645	9,4607	0,745	0,4563
Ocular Pain	2,2507	1,0035	5,0481	1,9684	0,049 *
Rash	2,1038	1,0146	4,3623	1,9989	0,0456 *
CONSTANT	*	*	*	-1,0243	0,3057

Note: * Significant p value.

From the people detected in the active surveillance, a sub sample of patients was invited to participate in a special group of follow up. Several blood samples in the acute and one in convalescence phase of the disease were obtained to see the dynamic of some hematologic and serologic changes in those patients confirmed with dengue. From 270 people only 50 accepted to participate and out of them 17 were RT-PCR positive. Hemoglobin, hematocrit, platelets, white blood cells, lymphocytes and neutrophils in the

first sample were compared in confirmed and non confirmed dengue cases (Table 23). In addition, only in the confirmed cases the hematologic and serologic indicators were repeated at least three times in the acute phase of the disease Platelets and white blood cells showed significant changes in that short period; both decrease in the second sample and increase in the third one. (Table 24). Platelets and white blood cells also had been less than values in non confirmed cases of dengue.

Table 23

Hematologic indicators of confirmed and non confirmed dengue cases at the moment of the detection by the active surveillance. Maracay 2006 2008.

Hematologic Indicator	Confirmed Cases N=17 Means	Non Confirmed Cases N=33 Means	T test[2]. P value
Hemoglobin	13.28	13.03	NS
Hematocrit	41.67	40.18	NS
Platelets	179 (51.4)	188,8 (51.7)	NS
White blood cells	5.51	7.39	S
Lymphocytes	39.8	40.93	NS
Neutrophils	57.6	57.5	NS

Table 24

Hematologic indicators of 17 confirmed cases in three consecutives blood samples in different days of acute phase of dengue disease. Maracay 2006 2008.

Hematologic Indicator	Sample 1	Sample 2	Sample 3	T paired test between S2-S1 and S3-S2 P value
Hemoglobin	13.28 (1.59)	13.26 (1.30)	13.5(1.43)	NS
Hematocrit	41.67 (5,91)	41.26 (4.75)	41.34 (4.67)	NS
Platelets	176 (51.4)	165 (46.1)	188 (70.3)	S
White blood cells	5.51 (2.21)	4.73 (2.05)	5.00 (1.57)	S
Lymphocytes	39.81(13.6)	49.6 (13.7)	55.29 (14.4)	NS
Neutrophils	57.6 (14.9)	46.4 (16.7)	41.8 (13.6)	NS

IgM MAC ELISA test was positive in 3 of 17 patients at the moment of their detection by the active surveillance. The average of days after onset of the symptoms to take the blood sample was 2.65 in the first day, 3.29 in the second day, and 3.88, 5.0, and 6.7 in the third, fourth and fifth day respectively. All convalesce sample were obtained between 31 and 47 days after the onset of the symptoms. Four sero-conversions occurred in the third, fourth and fifth samples and one negative patient in the acute phase converted in positive in the convalescence sample. Finally, 8 (47%) patients never showed sero-conversion in the period of acute and convalescence phase of the dengue disease and four out of them were RT-PCR positive to DENV-4. (Table 25).

Comparing passive surveillance from regular system with active surveillance in this study.

For all 270 people in the two years of the study, 76 (28.1%) were confirmed with dengue disease by RT-PCR and IgM positive in sero-converted convalescence sample; 23 (8.5%) probable dengue cases and 171 (63.3%) negative cases were also identified. Proportion of confirmed dengue cases was similar by age group, gender, days after the onset of the symptoms, and clinical presentation. Proportion of probable cases was higher in those people who were tested four or more days after onset of the symptoms (pvalue < 0.001) Figure 9.

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Table 25

Serological conversion to IgM anti-dengue antibody in 17 confirmed dengue cases in the acute phase of the disease. Maracay 2006 2008.

Patient	Age	Sex	DV	First Sample DAOS	IgM1	Second Sample DAOS	IgM2	Third Sample DAOS	IgM3	Fourth Sample DAOS	IgM4	Fifth Sample DAOS	IgM5	Sixth Sample DAOS	IgM6
1	20	F	D1	2	-	3	-	4	-	5	-	8	-	44	+
2	5	M	D1	3	-	/	/	5	+	/	/	7	/	37	+
3	16	F	D1	3	-	4	-	/	/	/	/	6	-	35	-
4	21	F	D1	3	-	4	-	5	-	6	-	9	-	45	+
5	22	F	D2	1	-	2	-	3	-	/	/	5	-	33	-
6	10	F	D2	2	-	3	-	3	+	/	/	6	/	35	-
7	15	M	D2	2	-	3	-	/	/	5	-	8	-	45	-
8	26	F	D2	2	-	3	-	4	-	5	-	9	-	38	-
9	32	F	D2	3	+	4	/	/	/	/	/	6	/	31	+
10	10	M	D3	1	-	2	-	3	/	4	-	6	+	32	+
11	15	M	D3	5	+	/	/	/	/	/	/	7	/	39	+
12	31	F	D3	5	+	/	/	/	/	/	/	7	/	37	+
13	11	M	D4	2	-	3	-	4	-	5	+	7	/	38	-
14	18	F	D4	2	-	3	-	/	/	/	/	5	-	36	-
15	19	M	D4	2	-	3	-	/	/	/	/	5	-	46	-
16	9	F	D4	3	-	4	-	/	/	/	/	6	-	42	-
17	15	M	D4	4	-	5	-	/	/	/	/	7	-	47	-

Note: DAOS: Days after onset of the symptoms.
DV: Dengue Virus serotype.
/ : No made

	<15	>=15	Female	Males	<4 days	>=4 days	HDF	DF	Total
Negatives	79	93	103	69	137	35	1	171	172
Probable	15	7	8	14	7	15	3	19	22
Confirmed	43	33	45	31	52	24	3	73	76
	Age group		Gender		DAOS		Clinical Manifestation		Total
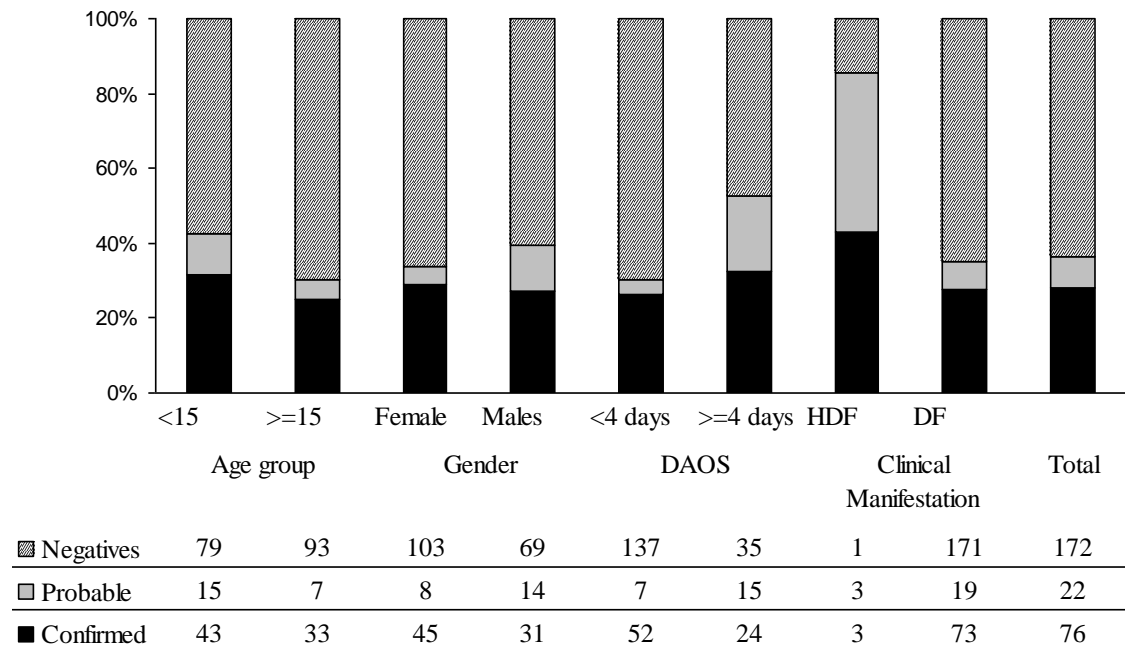
Figure 9 Dengue case definitions by age group, gender, days after the onset of the symptoms and clinical presentation in the people detected in the active surveillance. Maracay, 2006-2008.

2007 was a national dengue epidemic year, for that reason we compared this specific year with our data. We had 47 confirmed dengue cases in 2007 and the Cumulative Incidence was 1,873 per 100,000 populations. The months with highest CI were July, November and August. From January to May can see a reduction in the number of cases which is zero in May. Figure 10.

Figure 11 shows the index of pluviosity by months in Venezuela compared with the cumulative incidence of dengue disease by months per 100,000 populations. The months with higher pluviosity are almost coincident with the months with where the cumulative incidence is greater.

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	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
CI 2007	199.3	199.3	79.7	119.6	0.0	159.4	398.6	199.3	79.7	79.7	279.0	79.7

Figure 10 Cumulative Incidence by months per 100,000 populations in the study cohort, during 2007. Maracay, 2006-2008.
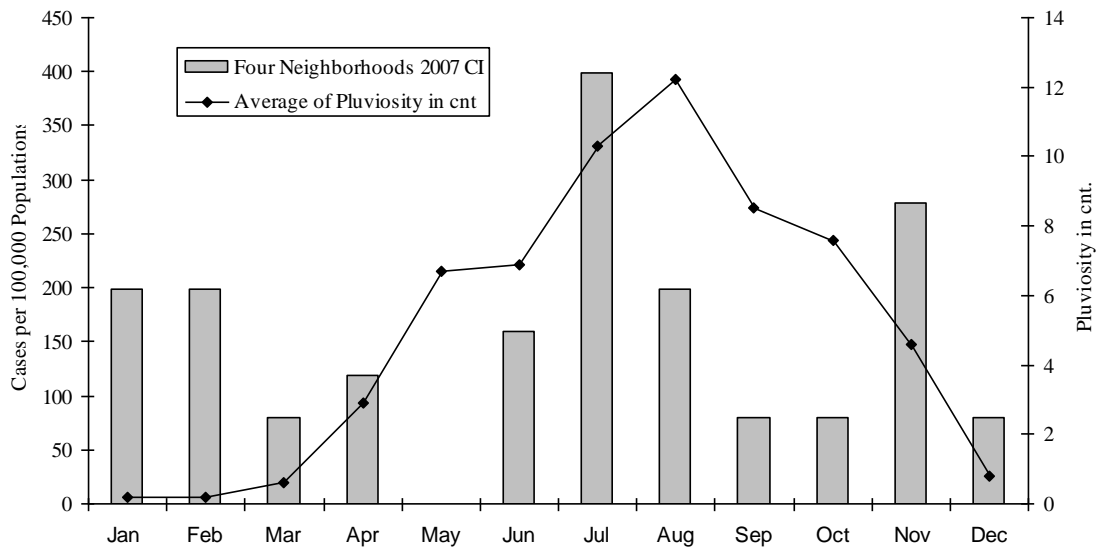
Figure 11. Cumulative incidences of dengue disease by months per 100,000 populations in the study cohort and pluviosity in centimeters during 2007 Maracay, 2006-2008.

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The 2007 cumulative incidence of dengue disease in the cohort study (1,873 per 100,000 populations) was higher than cumulative incidence in the state of Aragua (496 per 100,000 populations) in the same year. Analyzing the cumulative incidence per months in 2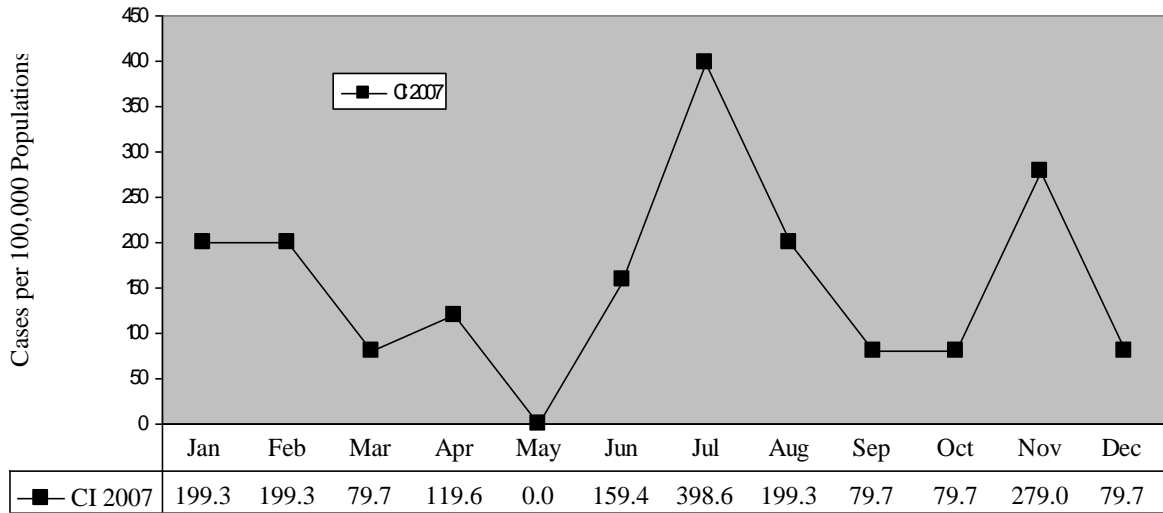007, except May in all months the cumulative incidence of dengue of the cohort study was higher than the state of Aragua. Figure 12.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
▨ "Barrios" in the cohort study	199.3	199.3	79.7	119.6	0.0	159.4	398.6	199.3	79.7	79.7	279.0	79.7
■ "Barrios" by Regular Surveillance	67.3	43.3	38.5	29.8	17.3	35.6	56.8	91.4	46.2	75	79.8	35.6
☐ Aragua State	29.8	22.1	17.5	13.1	18.2	30.6	54.1	76.4	60.5	70.3	64.5	38.8
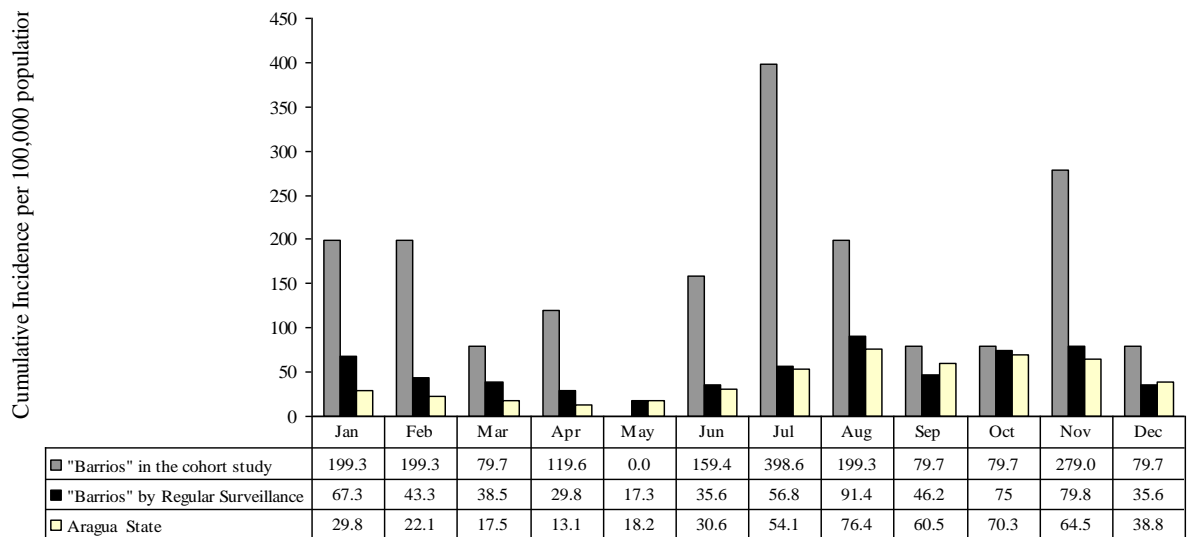
Figure 12. Cumulative incidence of dengue disease by month. Ccomparison between study cohort and regular surveillance in the same barrios of the study and in the state of Aragua in 2007. Maracay 2006-2008

Figure 13 shows the endemic levels of dengue in Venezuela and the cumulative incidence of dengue disease in 2005, 2006 and 2007. The cumulative incidence of dengue in 2005 increased in the rainy months from June to September but this CI never passed the limit from endemic to epidemic (up to one standard deviation of the average) instead in 2006 the CI became epidemic in September and it was maintained in epidemic phase from October 2006 until December 2007.

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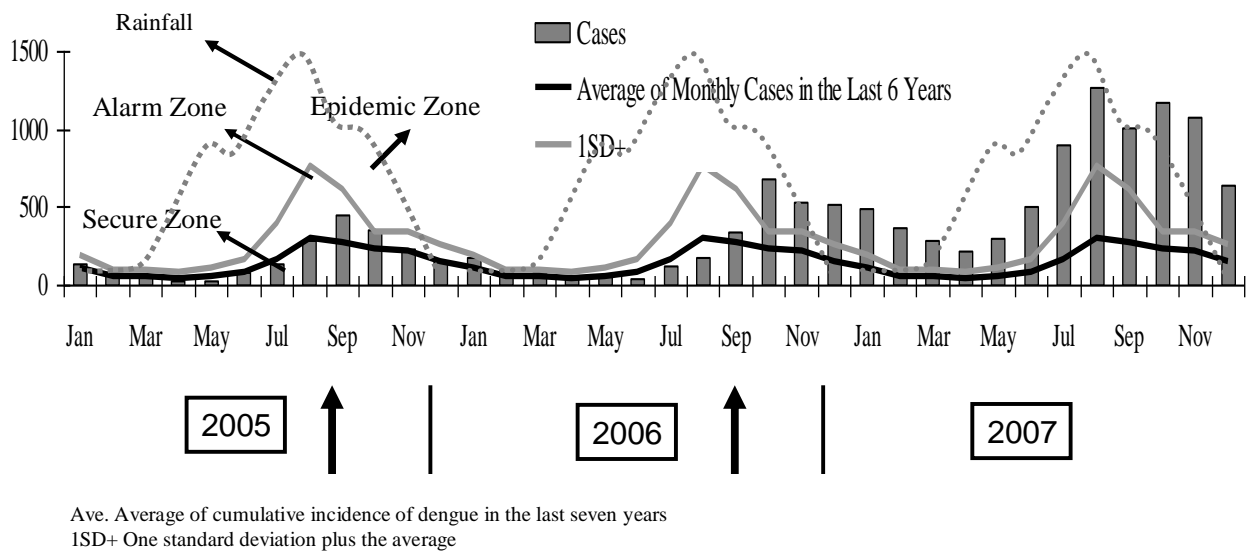
Figure 13. Endemic levels of dengue in Venezuela and the cumulative incidence of dengue disease in Venezuela in 2005, 2006 and 2007. Maracay, 2006–2008.

CHAPTER FIVE: DISCUSSION

In general terms the center of this study was to identify in a sample of one dengue disease endemic population those aspects that cannot be seen and understood in a regular public health surveillance system. The goal in a traditional passive surveillance is to detect a non expected number of cases in a specific period of time, assuming that any frequency of sick people can be correlated with the dynamic of the infection disease.

Many times, the high number of detected disease cases cannot be equated to infectious disease intensity; the real strength of the infection could be masked if it is considered as a lineal relation. Silent or unapparent cases might not be important in other viral infections; on contrary; it could be a way to get immunity. However, dengue disease does not follow the same pattern. A previous benign infection could be a risk to get a secondary severe and sometimes fatal disease. In addition, people that are not recognized as sick patients, can be a source of potential transmission without a preventive action to reduce it.

In this study design one of the main ideas was to know exactly how many people were clinically sick. Visiting their houses three times a week was the way to avoid the non-registration of febrile people who could not have felt sick enough to go to the health service which is inserted in a traditional passive surveillance system. Additionally, this permanent contact with the people permitted us to know those healthy people who have had dengue antibody sero-conversion during the study; people whom neither active nor passive surveillance would have been able to detect as infected ones.

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Even considering these previous aspects, it is important to discuss that active surveillance is designed not only to call or visit the place looking for cases, but also it could include special monitoring systems in specific samples from the population, of course, it always depends on the characteristics of the disease. Hypothetically in a Public Health Service, biannual sample to sero-prevalence test combined with active surveillance in a little sample of people could be a monitoring strategy to estimate, with a reasonable confidence level, the silent dynamic of the infection in the population.

In one sense our results support the idea that new monitoring methods have to be implemented, beyond to the traditional and passive strategies to respond against dengue disease. With some exceptions most of the developing countries where dengue is a real problem, these new strategies are needed. The focus of this discussion was oriented to relate analysis results with a hypothetic Public Health Surveillance System. In another sense, our results can be discussed in particular terms, and each part of the study can add information to the knowledge of dengue disease.

In this study the frequency of dengue disease and dengue infection were measured by two ways. On the one hand, the disease was directly estimated by the incidence of the dengue cases in the active surveillance and prospective design. Moreover, the infection was indirectly estimated by the sero-prevalence of the antibodies against dengue virus, which were obtained in fixed biannual blood samples. Two kinds of antibodies were tested: IgM and IgG. anti-dengue antibodies. IgM MAC ELISA test detected antibodies which are circulating in blood around three months after dengue infection, consequently IgM could not have been detected in a half of the total people with IgM sero-conversion; however, it could be a good indicator of silent infection. IgG anti dengue antibody was

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measured by Plaque Reduction Neutralization Test. With this technique IgG anti dengue antibody can be detected 50 years after infection. However, PRNT is mainly a sensitive procedure to detect dengue primary infection been also a perfect test to identify serotypes.

The present project is one of the few prospective dengue studies where the people were followed-up by door to door visits three times a week, asking for febrile cases. In the bibliographic review we checked 75 articles in PubMed of NCBI, using the key words: Dengue, prospective, incidence and prevalence to look for dengue prospective studies. We used the following descriptor: (Dengue and prospective) or (dengue and incidence) or (dengue and prevalence). The major numbers of prospective studies were made in community schools, being the children absence to class the alarm to investigate the cause. Other prospective studies were based in the use of health care services; when the people in the follow-up felt sick, they should have gone there. In another prospective adult studies, the workplace was the center of the follow-up. According to our review, none of the papers evaluated were made with a strategy of house visits. Our study, with three visits a week, gave us direct information about the patients and verified when exactly the onset of the symptoms occurred.

In this study, the general incidence density in two years was 3.24 per 100,000 p/d. Few studies have reported Incidence Rate as incidence indicator; most of them have used Cumulative Incidence, being difficult to do a direct comparison with our results. Porter et al,. in 2005, have been one of the authors who reported Incidence Density. However, their study was made in adults and the ID was expressed in person/years (p/y). Eighteen cases per 1,000 p/y was the ID in 2,536 workers from two textile factories in Bandung,

Indonesia in two years study from 2000 to 2002. Expressed in person/days, that means an ID of 4.9 per 100,000 person/days higher than 1.9 p/d adults ID in our study. In dengue, this kind of difference could express two contrary situations. In one sense, if both places had the same structure of viral transmissibility with similar inter-epidemic periods, similar sero-prevalence of anti-dengue antibody by age groups, and similar number of serotypes circulating; probably, we could say that either for some unknown reason (like human genetic variation, or virulence, or environmental factors) or for specific risk in the work place (textile factory) the adults ID in Bandung is grater than adults ID in Maracay. In another sense, if each city has different endemic or hyperendemic history and the sero-prevalence of antibodies by age groups are dissimilar, the situation analysis would be also different. Comparing the entire population cumulative incidence in similar periods in both countries where these cities exist; we can see that in Venezuela (where Maracay city is) historically has had four o five times more annual CI than Indonesia, (where Bandung city is): 143 and 26 per 100,000 populations respectively (six years average from 2000 to 2005). The same conclusion can be made comparing cumulative incidence reported between Bandung and Maracay. From 1995 to 1998, Bandung had CI of 44, 54.7 and 31.7 per 100,000 populations and Maracay, from 1996 to 1998, had CI of 73, 155 and 157 per 100,000 populations, respectively. According to these studies, in Maracay city respect to Bandung city, adults would have less susceptibility to dengue disease, being a place where the disease is probably, in terms of risk, a higher problem of public health. (Porter et al 2005), (Barrera et al 2002).

Saddiqui et al in Karachi (Pakistan) reported an Incidence Rate of 0.5 cases per 100,000 person/days in children in a 30 months study period. They obtained incidence

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rate of 0.4 cases per 100,000 person/days in age group from 5 to 10 year old and 1.6 cases per 100,000 person/days in age group from 11 to 15 year old. This values are low compared with the ID of 7.4 cases by 100,000 p/d found in our study in two years of study. This big difference could be explained because Pakistan has begun their dengue high epidemics in 1994 to 1996, and the second big epidemic occurred in 2006; 4 years after the study of Siddiqui et al was made. (Siddiqui et al 2009). In contrast, Venezuela has had several big epidemics from 1989 until 2008 when our study was completed.

The difference between the study in Bandung and Karachi respect to our study is that children in endemic areas are always the target of dengue infection and dengue disease, making it easier to compare the Karachi city children study with our study. There are logical exceptions like Cuban dengue disease epidemic in 1997, where adults were almost exclusively affected, even though the dengue infection should have affected all population. (Guzman 2005).

Prospective incidence and sero-prevalence studies should always consider children and adult people in their samples, to have a better perception about the viral dynamic in the people. In a monitoring surveillance of sample or sentinel population it would be indispensable.

Although most of the prospective studies of dengue have reported cumulative incidence instead of incidence rate, when we assume that population is stable in the time (in size and age distribution) the person/time can be calculated multiplying the size of the disease free population by the actual duration of follow-up (Morgenstern, 1980), even considering that is difficult because of withdrawals from the study cohort.

Acording to the last paragraph, we estimated the differences and similarities between our results and the other worldwide incidence studies.

Historically, two of the first prospective dengue studies were completed in Thailand in the 80's, both based in the 1980 epidemic. One of them was done in Rayong city (Sangkawibha et al 1984) and the other in Bangkok city (Burke et al 1988). However, disease incidence was reported only in the Bangkok study; it was completed in an exact period of 7 months and based in the children school absenteeism. Due to the finalization of the study by all the children, a close approximation to person time can be made. In seven months, the study reported 5.6 % of incidence, around 26 cases per 100,000 person/days clearly higher to 7.4 cases per 100,000 p/d incidence density that we found in Maracay. The importance to compare our study with the Bangkok study, in Thailand, was because this city has reported dengue cases from 1958, and Thailand with Philippines were the first countries where DHF outbreaks occurred, sharing with Venezuela hyperendemicity, age population structure, weather conditions, similar dengue cumulative incidence, and incidence of DHF. In some aspects of dengue, Thailand is to South East Asia, as Venezuela is to Latin America.

Eighteen years after Burke's study, Endy et al. repeated a similar study in schools situated in a district close to Bangkok. This time, the incidence of the dengue disease was 3.6% in 1998, approximately an Incidence Density of 9.9 cases per 100,000 p/d, 3.3% in1999, ID :9.0 cases per 100,000 p/d. and 0.8% in 2000, ID:2.2 cases per 100,000 p/d. The overall incidence in the three years study was 2.7% with an estimated ID of 7.4 cases per 100,000 p/d, the same values of our results of 7.4 cases per 100,000 p/d. This study was probably more accurate than Burke's study 18 years before, the dengue disease and

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dengue infection were better defined. Contrary to the other study of Bangkok, it was also designed to evaluate any children with one or more days of absence. One advantage in our study, in Maracay, was that we were usually able to make the medical evolution the first or second day after the onset of the symptom, and that day was exactly registered. We will get back to this point in the discussion of the clinical part of the study. (Endy et al, 2002).

In the Americas few studies of incidence of dengue disease have been made, Balsameda et al in Nicaragua reported an annual incidence of 8.5 cases per 1,000 schoolchildren in the first year of follow-up, and 8.3 per 1,000 schoolchildren in the second one; assuming the study did not have any lost, it means approximately 2.33 and 2.27 cases per 100,000 p/d respectively. (Balmaseda et al. 2006)

One limitation in many of the previous discussed studies is that they could not measure the incidence of the disease associated to age groups (children and adults), because those studies have been made either in children (usually in schools) or in adults. Working directly in the community permitted us to evaluate simultaneously the incidence in both groups and estimate the risk association. Its Relative Risk was 3.92 in children respect to adults, in the two years of the study; however, this risk was significant only the first year of follow-up, when the big Venezuelan dengue epidemic was occurring in 2007. This RR was consistent in females and males, in the overall two years study. This result could indicate that in endemic period the risk between children and adults tends to be similar, being children clearly affected in epidemic periods.

The incidence of dengue disease was close related with two external factors, the year of the study and the rainy months. The incidence density in the first year study (2007)

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was affected by the second highest epidemic in Venezuela in the last twenty years. The Venezuelan cumulative incidence in 2007 was 304 per 100,000 populations; since 2002 no annual CI in Venezuela was higher than 160 per 100,000 populations. In Aragua state, where this study was made, the number of dengue cases and the cumulative incidence in 2007 has been the highest in the last twenty years. The other factor associated with the incidence density was the rainy months. The months with highest pluviosity: July, August and September were the months with the highest number of dengue cases. This pattern was similar to the 2007 national and regional reports of dengue cases. Even though, this result was less evident in 2008; dengue cases were reported in the first months of raining (May and June), without dengue cases reported in the months with less precipitations (January, February, March and April).This study with a cohort of 3,255 people in four neighborhoods of Maracay reflected the national and regional epidemic and how the rising of dengue cases are related with the rainy period and the vector activity.

The incidence of the dengue disease has another advantage; it is the best way to identify specific serotypes in areas of hyperendemicity. Plaque Reduction Neutralization Test is the best test to estimate specific serotype in primary dengue infection, but it is not the same situation in secondary and tertiary ones.

According to our results, we could not establish different spatial distribution of the serotypes among the four barrios in Maracay city. Even though, these neighborhoods are separated by at least one mile among them. Except "Pinonal" which had serotypes of DENV-1, DENV-2 and DENV-3, the other neighborhood had the four serotypes. Endy et al found specific serotypes as a focus of transmission in their prospective school based

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study, giving to schools and community similar role in the dengue transmission. (Endy et al 2002). However, Mammen et al. studying spatial and temporal clustering virus transmission found that community was more important than schools as a source of the dengue infection transmissibility. (Mammen et al, 2008).

The proportion of DENV-1 serotype, into all children confirmed dengue cases, was significantly higher than that proportion in adults. DENV-2 and DENV-3 proportions were similar in children and adults, DENV-4 proportion was higher in adults but this difference was not significant. Long history of exposure to endemic infection of dengue can explain in adults the low proportion of DENV-1 which has been reported in the last 20 years in Venezuela. Similar proportion of DENV-3 in children and adults can be explained because this serotype was hardly introduced in Venezuela in 2001 and simultaneously many children and adults were infected. We could have expected with DENV-2 a similar behavior to DENV-1, due to long time adult exposition to that serotype.

As well incidence of dengue disease is a good indicator of imminent public health problems like epidemics; incidence of dengue infection is the way to know how the dynamic of the virus is and probably the way to predict disease events in the close future.

The sero-prevalence of IgM anti-dengue antibody in the first biannual sample was 2.3%, it could be considered low, thinking in a hyperendemic population with high rate of infection. However, Gluber in 1998 reported that IgM titers in primary infection are significantly higher than in secondary infections (Gluber, 1998). Then, it is possible to deduce the less sensitivity of IgM CAPTURE ELISA detecting acute infections in population (like ours) with high prevalence of secondary and tertiary infections. Besides,

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the lifetime of IgM antibody in people infected is around three months, and people infected before that time would not be detected. 2.3 % or little more percentage, even in a three months prevalence period, could indicate a high number of people who were silently infected. In this study, that was impossible to know in the first biannual sample but in a second biannual sample we were in capacity to know exactly which people had been symptomatic and who had not, because they have been identified in the active surveillance. In this context, the IgM sero-prevalence in the second biannual sample was 3.28%, 86 positive people in 2,622 populations tested. Out of them, only 4 people were detected with fever in the active surveillance design, indicating that 84 (95 %) people were probably asymptomatic or lightly symptomatic dengue cases. In the third biannual sample, 116 people were IgM positive and 11 (9.5 %) out of them had been detected in the active surveillance, a higher amount of people. In the fourth and fifth biannual sample the percentage of possible asymptomatic people were 98 % and 97 % respectively out of all positives IgM.

Sero-prevalence IgM test surveys in a sample population could be a quick and relative low cost way to estimate the proportion of unapparent dengue infection. However, reducing from 6 to 3 months period inter sero-prevalence samples would be a best way to adapt this strategy to the immunological dynamic of IgM antibodies.

Any advantage that could have IgM MAC ELISA test will never have the accuracy of PRNT to detect dengue infection and dengue specific serotype. Even though this test is also affected by secondary infections reducing its capacity to identify specific dengue serotype in samples from infected people with multiple dengue serotypes, PRNT continues being the main test to detect dengue virus.

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We tested 2,125 people with PRNT, 65.3 % of the complete cohort. It was a significant amount, thinking in this laborious and time consuming test. The overall sero-prevalence of anti-dengue IgG antibodies was 86% in the first biannual survey, 65.6 % in children less than 15 and 95.3% in adults. 66 % were positive to two or more dengue serotypes. Negatives results were found in 283 (13%) people where 34.5% were children and 4.7% adults. In Salvador city, Brazil, Texeira at al reported one of the few studies where they are considering sero-prevalence simultaneously in children and adults. They showed overall seroprevalence of 68.7%, 76.1% in adults and 57.4% in children. Similar to our study, Salvador is a complex city with poor areas and socio economic variations. However, its comparative lower seroprevalence could be explained because this study was made in 1998 – 1999 when Brazil was not yet in its worst period of epidemic after 2001.(Texeira etal, 2002). Contrary to Texeira study but close to ours, Balsameda in Nicaragua has showed seroprevalence in children of 91% from 75% at age 4 to 100% at age 16. Historically, dengue epidemics in Nicaragua are similar to Venezuela and could explain the closer dynamic of the virus in both countries. (Balmaseda et al, 2006). In Veracruz, Mexico 2003, Navarrete et al found IgG dengue antibody sero-prevalence of 79.6% in 500 samples from all age, reporting an increase of prevalence from 17 % at 1 year old to 94 % in people equal or higher than 65 years old.   Interestingly, sero-prevalence in Texas Mexico-border, the authors reported prevalence of 40 % in Brownsville and 80 % in Matamoros, increasing the percentage according to age stratus.

In the Maracay city, Comach et al in 2001 found a prevalence of 51 % in schoolchildren, even though this study was made in the same city to our study, this lower sero prevalence could be a main specific focus of dengue antibody prevalence and

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transmission and not necessarily antibody prevalence in geographic communities. Mammen et al is recently showing difference between virus transmission in schools and communities. (Comach et al 2008), (Mammen et al, 2009).

Asia, with more than 60 years of dengue epidemic history, had in Singapore an example were the dengue sero-prevalence is relatively low but the public health problem is yet important. Wilder in 2004 reported a sero-prevalence of 45 % in people from 18 to 45 years old, in a country were paradoxically Goh et al in 1997 demonstrated high incidence of dengue cases with low density of mosquitoes. (Wilder et al 2004), (Goh et al 1997). At that age group our prevalence was over 90 %.

In Vietnam, Thai et al reported sero-prevalence of 65.7 % in schoolchildren from 7 to 14 years old. This age group and its dengue antibody prevalence were very similar to our results in a similar group of children. (Thai et al, 2006). Graham et al in Indonesia, doing one of the few dengue antibody sero-prevalence larger studies by PRNT, showed the results from 1,837 children between 4 to 9 years old. Interestingly, they, like in our study, focalized many of their results in the proportion of specific serotypes, at that age group DENV-1 (12%) and DENV-2 (16%) were the most frequent serotypes, being 3% in DENV-3 and 4 % in DENV-4. In contrast with our study, we were able to compare the PRNT with the RT-PCRT results obtained in the active surveillance; we will retake this point ahead in the discussion. Other difference with Graham's study was that we described the specific serotypes combination in those people results with two or more serotypes. DENV-1 and DENV-2 was the most frequent (45%) mixture in our study followed to DENV-1, DENV-2 and DENV-3 (17.5%) these combinations could show that DENV-1 and DENV-2 are the viruses which have had not only more contact with

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Maracay population but also many people in Maracay would have less probability to get sick with dangerous virus infection sequences seen in HDF. It could be an incomplete herd immunity but enough to avoid many of the possible HDF. However, the same data shows that an important proportion (18.9%) of the study population, and probably in the total population of Maracay, is at risk to get HDF.

In advantage to have prospective sero-prevalence surveys through the biannual samples, we have been able to obtain indirectly, cumulative incidence of dengue infection from the sero-conversion detected inter each sample. In that sense, people with monotypic sero-prevalence had the highest six month period incidence of dengue infection. This position was repeated in the three inter biannual period of samples. Understandably, people with multitypic sero-prevalence had less incidence of dengue infection. For some systematic reason, people tested negative had less propensity to a new dengue infection than people with one serotype. The people in this study shared similar social conditions, water supply and houses characteristics so it was not our interest to identify social aspect associated to dengue disease. However, probably particular and personal habits could be related with this result. It is a good question to investigate in the future. Is it an action of the age stratification? Or, do those people have less personal risky conditions to be negative and to be less prospectively infected than monotipyc people?

The six month incidence around 30 % was found exactly in a period of national dengue epidemic, it could explain these huge values. The reduction to 23 % in the third inter biannual sample is also coincident with the end of the national epidemic. Low values of incidence of infection has been reported by Balmaseda et al in Nicaragua, with similar

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values between primary and secondary infection, probably because they did not discriminate between people with monotypic and multitypic sero-prevalence. (Balmaseda et al, 2006).

It has been reported the limitation that PRNT has to identify specific anti dengue antibody serotypes when there are multiple serotypes in the sera because of cross reactivity among them. In order to determine the precision of PRNT results in hyperendemic population, we compared the results of confirmed dengue cases in active surveillance by RT-PCR with the results of PRNT of each patient in the next biannual sample. We found that PRNT has a good sensitivity to detect DENV-1 (88%), DENV-3 (78%0 and DENV-2 (69%). However, it had poor capacity to detect DENV-4 (17%). Additionally, in other part of this study when we compare the day of IgM sero-conversion in patients early confirmed by RT-PCR, it is not occurred neither in three samples of acute phase nor convalescence phase in four from five DENV-4 infected patients, even though it could not happen exclusively with DENV-4. New studies could be necessary to ratify this result or to look new ways to improve the detection of DENV-4.

The fact of being almost permanently close to the house of the studied people and visiting them three or more times a week permitted us to have more than 70% of blood samples from sick people taken before or at the third day after the onset of the symptoms, and 97% at the fifth day. We did not find any study made in communities with those statistics. With this information, we were able to see the proportion of confirmed dengue cases by RT-PCR by day of sample. It did not change in the first five days, being it around 25%. However, according to many opinions, the specificity is good until the third

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day; after that many false negative could be registered. Some surveillance systems use RT-PCR as diagnostic method until the fifth day after the onset of the symptoms. We found that 20% of the sample on sixth and seventh days can be positive by RT-PCR, and it agrees with the initial authors who describe this technique. The question is: could be RT-PCR used in routinely recommended to days six and seven. This question makes a connection with other of our results. In conditions of low endemicity, a good combination between RT-PCR from the first to fifth day and IgM MAC ELISA test after the fifth day would be enough. However, we suspected and now confirmed that IgM test could reduce its sensitivity in hyperendemic conditions and it could be so important in surveillance of inter-epidemic periods. In our results some confirmed dengue cases never sero-converted through the IgM MAC ELISA test, being more important in adult people. We suspected that when the proportion of secondary infection (respect to primary infection) was higher in IgM negative people having positive RT-PCR test than in IgM positive people in equal condition. Supporting our assumption, several of the 17 confirmed dengue cases with four samples tested in acute and convalescence phase of the disease never had IgM sero-conversion.

Describe the clinical and hematological results considering the days in which the symptoms and hematological changes occurred is also, in our criteria, a small contribution of this study. Why? In the literature we can see different and sometimes contrary results when they are reporting symptoms. In our opinion it could be caused because those studies were made evaluating people in different moments of the disease. Any of the reviewed papers discriminate in this single aspect. We can say that our results are based in people who were asked about their symptoms the first days after they

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became sick. Many studies were made in people who went to the health services, and the main symptoms in that moment could have been different to initial symptoms of the disease. Similar cases can occur in retrospective studies, describing those main symptoms, in terms of patient perception or medical interest, instead either the initial, or more frequent or more specific symptoms.

Although, it is not specific, headache is a sensitive symptom in confirmed people in initial days. Ocular pain and rash were symptoms statistically associated with dengue disease and probably more specific than any other in the first days of the disease. We were not in capacity to describe the clinic days after of the dengue disease evolution in the studied people when probably other type of symptoms appeared but we can say with security that these were the most important symptoms at the beginning of the dengue disease. Health workers in a surveillance of dengue have to be in capacity to recognize these differences.

In a sub sample of 50 voluntary people who were previously detected in the active surveillance, we were able to expand the number of hematologic indicators. However, after that, only 17 of them, those who were confirmed with dengue disease, were followed with repeated blood samples in the short period of the disease.

Hematologic results only showed a significant reduction in the white blood cells in confirmed people (17) compared with non confirmed people (33). We have to insist that these samples were taken in the first days of the disease although they were enough to illustrate that some hematologic parameters can be similar to non confirmed dengue cases in early stages of the disease. The repeated samples in the 17 confirmed cases showed the dynamic of the parameters although only changes in platelets and white blood cells

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were statistically significant. Interestingly, the proportion of neutrophils in relation to lymphocytes was not reasonably higher in the sample number 1, and according to that we could think in a bacterial infection. Nevertheless, immediately (1 or 2 days) this proportion can change in the logical sense of a viral disease. Sometimes in stressful situation a physician, influenced by these small clinic aspects, could change a medical diagnostic. For that reason each small detail should be discussed in a surveillance program.

   The last part of this discussion is related with the real surveillance of the state and country where this design was applied. In the Aragua state and similarly in Venezuela the surveillance system is structured by the classical three level of attention: Primary, secondary and tertiary levels. Primary level is the first step where the people have to go when they are sick. When physicians detect possible dengue cases they report the case to the state central health service, called CORPOSALUD in the Aragua state and simultaneously send either the patients or their blood sample and a card with the patient clinical and epidemiological information to LARDIDEV which is the state center to viral diagnosis. According to the laboratory results, based mainly in IgM MAC ELISA test and secondarily in RT-PCR and viral isolation made to a partial number of cases, LARDIDEV classifies the patients as confirmed, probably, negative and undetermined dengue cases. This information is reported to the epidemiological services at state and national level. The national level of health ministry makes a public report through the Weekly Epidemiological Bulletin. The goal of the state and national health level is to analyze weekly the dynamic of the dengue cases, comparing the number of week cases with an average of week cases in the last seven years. With the average and its standard

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deviation (SD) is built the endemic channel or endemic levels to establish zone of security (between average and – 1 SD), zone of alarm (between average and + 1 SD) and zone of epidemic (up to the 1 + SD). The expected cases in the Venezuelan endemic channel are always higher in the rainy months (June, July, August, September, October and November). For that reason, it is always a question if the initial increase of cases in May and June is either the beginning of the seasonal period or the beginning of one epidemic.

In order to compare our data with the state surveillance in this part of the study, we defined a confirmed case as that case either with positive result of RT-PCR or viral isolation or with sero-converted IgM dengue antibody in the period between the acute and convalescence phase of the dengue disease.

We did not have cumulative incidence from previous years in the neighborhoods included in the study so we decided to compare with the state and national CI in 2007. We selected 2007 to do the comparison because it was an epidemic year, perhaps the second huge epidemic in Venezuela in the last twenty years. In our study population, the CI was at least 1,873 cases per 100,000 populations (47 cases in 2509 people); we said at least 1,873 cases because we included the people who left the cohort. In other words, the CI could be higher in our results but not less because people who left the study could have had dengue after they were gone in the same 2007. In 2007 the Aragua state CI was 496 cases per 100,000 populations and 293 per 100,000 populations in Venezuela.

The question is if this epidemic could have been predicted. Puerto Rico has been considered with high capacity to do it. (Gubler) (Perez-Rigau), reporting a sensitivity of 66 % (two predictions in three epidemics) and they have been able to do the prediction

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from 4 to 8 weeks before the epidemic is installed. Puerto Rican system based this analysis in the proportion of cases detected by virus isolation in May because they consider May as a key month in the prediction system, being this month the beginning of the rainy season. In Venezuela and the Aragua state, the epidemic had really begun in October 2006 but comparing CI tendencies between 2005 and 2006 in September, it was practically impossible to know why in 2005 the CI decreased and in 2006 increased becoming an epidemic.

Even though our study had much more detected cases and CI in 2006 and 2007, it had also high sample error. For instance: The study did not detect cases in May 2007. Based in our data, we could have thought the 2007 epidemic was ending.

We thought that a prediction 8 weeks before the epidemic is good enough to activate health service mechanisms but not to avoid it. Current active surveillance in Puerto Rico and most of the countries with passive surveillance system are structured to predict (Puerto Rican situation) or identify epidemics but any of these surveillance system has been thought to understand the virus transmission, different levels of people at risk, and which could be the impact of a new epidemic in a specific population. Consequently, it is also important to make surveillance of factors and interventions in endemic populations. Since epidemics in Singapore has been related with low densities of vector, other studies have found evidence that partial successful programs of mosquito control could have unexpected and contrary effects, increasing the incidence of severe forms of dengue disease. Partially, it explains the complex mechanism of the virus transmission which should be also followed-up by surveillance programs. In addition, the Basic Reproductive Number (BRN) has been considered an essential element to be applied in

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disease control and evaluation of vaccination programs. However, it is uncertain what the role of unapparent infeccion people could have to establish the BRN. To respond the question could be very important in a new dengue vaccine era. How many or what percentage of individuals should be vaccinated to obtain successful dengue herd immunity?

Summarizing the Defense

One of the aspects more discussed in the defense of the dissertation was that in a high proportion of confirmed dengue cases the sero-conversion of IgM anti dengue antibody never was reached. This result was supported either by the active surveillance or by the biannual sample. The importance of this point is because the IgM is considered the angular stone in the surveillance of dengue epidemic in a population. In addition, it has clinical implication due to the probable low specificity of this test. Its result could be explained by the huge proportion of secondary infection of dengue in the study population where the IgG anti dengue antibody could veiled the IgM response. For any explanation this outcome suggests the necessity of early detection of dengue cases using RT-PCR especially in interepidemic periods where the confirmation of the cases is very important.

Other significant issue discussed in the defense was the coincidence between the surveillance in the study design with the real surveillance in the city of Maracay in the same period of time. The real dengue epidemic in Maracay city in 2007 was reflected similarly in the study in the four sample population of the "Barrios". In 2007 the peak of cases and incidence of dengue occurred in the months of higher rain (July and August) similarly to the historic increase of cases in Aragua state from 2005 to 2007. However,

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either in the study or in the regular surveillance it is difficult to predict when the increase of the case will be caused by the epidemic or by the rain effect.

One question was related about the people lost in the study, around 22 % in the two years of the study, being this lost higher in adults with 22 % than children with 14 %. This study was made in poor neighborhoods where there is a high mobility of the adults looking for better economic incomes.

Other question in the defense was why DENV-1 was more present in adults and why DENV-3 was similar in adults and children. Since 1989, when the first big epidemic of Dengue Hemorrhagic Fever was described in Venezuela, DENV-1 always has been present; therefore, many adults have actually antibodies against this serotype and children have been less exposed to DENV-1. In the other hand, epidemic of DENV-3 had not been described in Venezuela until 2001 when a huge epidemic occurred, as a result children and adults were exposed similarly and it is the probable reason that DENV-3 has similar proportion of infection in both age groups.

How the results of this study could impact the surveillance system in the state of Aragua?

The passive surveillance is the current system in Aragua state and it is useful only to detect the initial growth of the epidemic and prepare the health services to reduce its impact. This study, made in small areas of population and communities, could be a model of sentinel surveillance to detect the real impact of the virus circulation, detecting the real number of asymptomatic people. This people could be an important factor in the transmission of the disease. In terms of control, the surveillance of the vector along with the human surveillance is a necessary strategy to improve the impact of the disease. The

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only reduction of the vector index is not enough to reduce the risk of DHF because simultaneously it could increase the number of susceptible people. The control of the vector has to be almost one hundred percent effective, permanent and integrated with the active surveillance if we really want to avoid worst situations of dengue epidemic in the future.

This sentinel groups from specific endemic areas could also identify people with different number of antibodies anti dengue and people without antibodies anti dengue to determine where the risk of DHF is higher.

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CHAPTER SIX: CONCLUSIONS

This was the first follow-up study of dengue disease made in Venezuela since it re-emerged in 1989. There have been other prospective dengue studies in this country but based in repeated surveys of seroprevalence. Similarly, few studies in the Americas have been designed to establish dengue incidence, identifying and following previously the healthy people at risk. It is also the first time in Venezuela and probably in Latin America that the Incidence Density is established in a high risk population of dengue. Other studies from the Americas and Asia have described the frequency of unapparent dengue infection, estimating the disease incidence based in cases reported from schools, workplaces or health service centers but very few of them have obtained the people information directly from their houses and communities. It permitted us to reduce the time when the sick people was recognized and registered. For example to know exactly if a person was really asymptomatic when he or she was infected by the dengue virus.

This study analyzed three particular aspects of dengue disease: epidemiology, diagnosis, and clinical of the people in the follow-up. All of them were considered in the framework of the public health surveillance.

From the epidemiological point of view we described the dengue disease according to temporal variable, determining the disease frequency in years and months. Personal factors were studied through the age groups, gender and immunological history of

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infection variables. Finally, the space variable was described through the four hyperendemic neighborhoods in the endemic city of Maracay.

Laboratory diagnosis was analyzed according to: the dynamic of the surveillance, type of test, the day of the diagnosis and its interrelation with personal factors, mainly age groups and immunological status. In the same way, some clinical aspects were analyzed trying to link them with the surveillance strategy.

We obtained the first ID values of dengue disease in sample population from a hyperendemic city in Venezuela. 5.69 per 100,000 p/d in the first year of study (Sep. 2006 to Sep. 2007), being this years coincident with a national dengue epidemic, and 1.45 per 100,000 p/d in the second year (Sept 2007 Sep. 2008) when the epidemic disappeared. In addition, for the first time we obtained a direct and significant higher Relative Risk in children. In the first year the RR was 4.77 and 2.52 in the second one. All these results were valuables for us because we had an important validity aspect in the study; we were able to demonstrate when the people were really symptomatic; even though sample error had to be present in our results. Comparing with the state massive and passive surveillance system, in this study the advantage that we lost in precision was probably earned in validity.

Cumulative Incidence and Incidence Density are different measures and have different goals. However, thinking how to visualize the ID values found in this study, we showed this example: 274 people followed-up by 365 days (one year) represents around 100,000 person days of follow-up. Then, if all 274 finished a supposed study period, we could obtain ID and CI independently. In one year, 2 cases of any disease will correspond not only with a CI of 730 per 100,000 populations but also with an ID of 2 cases per 100,000

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p/d. In our study we did not have fixed population but this example permitted us to have an idea in terms of magnitude about what an ID of 1 or 2 or 15 means.

We had two important limitations in this part of the study. The first one, we did not include children less than 5 years old. Future studies have to include this special age group of risk, by no published reports we know that a high proportion of children was born with mother IgG dengue antibody and it should be affecting the crude incidence of the disease. The second limitation was that we studied only hyperendemic communities; we should have had comparative non hyperendemic communities to see how different the incidence among them could be.

In our opinion, public health surveillance, in a big city as Maracay, should have at least two samples of population (with high and low incidence), like sentinel surveillance. It could detect new changes in the virus transmission, changes in the expected incidence and validate information obtained in massive and passive surveillance system.

We were able to demonstrate that either IgM sero-prevalence or PRNT sero-prevalence evidenced high proportion of asymptomatic infection of dengue. Thereby, systematic surveys of IgM sero-prevalence in supposed healthy people, in a complementary surveillance strategy, could be a less time consuming and cheaper way to track and estimate the virus dynamic in the entire population. Smaller sub samples tested by PRNT would be used to validate this information.

Even supposing IgM seroprevalence could be a good indicator of unapparent infection, we also demonstrated that IgM was not able to detect an important number of infected people by dengue virus when they were secondarily infected. Considering hyperendemic population in a surveillance system with high proportion of secondary infection, precise

laboratory strategies should be implemented. We propose to investigate a little expansion in the days to test RT-PCR; it could improve the surveillance sensitivity by confirmed dengue cases.

The analysis of serotype infection by age groups permits us to conclude that DENV-1 and DNEV-2 have been the two specific causes of dengue infection in the last 20 or 30 years in the sample studied and probably a good estimation of the hyperendemic neighborhoods of Maracay city. As a result, children have shown to be less protected against those serotypes and then higher proportions of those dengue serotypes cases were detected in them. In a surveillance system historical registrations of these proportions and values could be appreciated to detect changes by the action of new serotypes and potential new genotypes. We cannot demonstrate a spatial distribution of specific serotypes, and again our study limitation having only high endemic areas kept the question if there would be different distribution of serotypes depending on the incidence level of the community.

Another remarkable conclusion is that 86% of studied people have been infected by dengue virus and 68% have been infected by two or more serotypes, finally, 63% of the total study population was infected by DENV-1 and DENV-2.

Our sample size was big enough to estimate the DF cases but it was relatively small to have a significant number of HDF cases. Unfortunately we cannot look for association between number and serotypes sequences of previous dengue infection and clinic severity. On the other hand, fortunately enough percentage of multitypic infection could have protected the people against severe forms of dengue.

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By two different results, we found similar inconsistency whit DENV-4 serotype identification. Contrary with the others serotypes, PRNT results detecting DENV-4 were incongruent with the RT-PCR made in active surveillance to detect disease incidence. Additionally, four from five RT-PCR DENV-4 positive cases did not sero-converted in three acute samples and one convalescence sample tested by IgM MAC ELISA. These preliminary results could be a sufficient argument to investigate the DENV-4 immunological behavior.

Reasonably, a person with one serotype should have less probability (3 from 4 serotypes) to get a new dengue infection than those people with negative results to anti-dengue antibodies at risk to get 4 from 4 serotypes. However, consistently, our results showed that people with one serotype were more frequent infected by a second dengue serotype than people that have never been in contact with the virus. This is a conclusion but is also a question to be answered in a next investigation.

Our results, according to clinical symptoms associated with dengue confirmed cases, have to be understood in the framework of the short period in which the cases were detected and confirmed. That means that 72 % of our cases were detected by active surveillance in the third day or before after the onset of the symptoms, and 96 % equal or before to the fifth day after the onset of the symptoms. Therefore, we made emphasis to describe those early symptoms of dengue disease. Those symptoms which should appear in the late acute phase of the disease were not described in this study.

On one hand headache was the most sensitive symptom present in more than 94 % of the confirmed cases. On the other hand, ocular pain and rash were independently associated with dengue confirmed cases.

In the surveillance system health workers should be ready to differentiate the symptoms when the patients are coming in early or in late acute phase of the dengue disease.

The incidence of dengue cases detected by this study in the last trimester of 2006 and 2007 was higher than the incidence of 2008. This incidence dynamic was similar to the national incidence and Aragua state incidence which identified the epidemic in 2006 and 2007 hence we were also able to identify the 2006-2007 epidemic in our study population. However, the study sample was probably affected by the sample error and we could not see cases in May 2007 so it could have been understood like the epidemic was ending. In conclusion a sample size around 3,000 people could not be enough to have a monthly number of dengue cases to detect changes in an epidemic dynamic. Passive surveillance in the country or state is working with the total population therefore the sample error is practically zero and good indicator of the incidence dynamic. We believe the use of small sample size like sentinel group of surveillance can help but is not the fundamental point in the system to identify or predict epidemics. Smaller groups of sentinel active surveillance may help in those aspects where massive procedure cannot identify clue elements of the analysis.

A parallel discussion is the capacity to predict or identify epidemics. We thought that passive surveillance system can only identify but not predict epidemic. Therefore, it is in these cases of prediction and prevention where close active and sentinel surveillance of key and usually hidden aspects would be necessary.

Unfortunately, the dengue surveillance systems in Latin America and probably in other developing countries in the world are not structured to either prevent dengue epidemic or

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improve endemic situations. Basically their function and goals are to detect epidemics in the initial stage to prepare hospitals, health workers and communities in response to that situation. However, real advantages of new surveillance strategies are not being well-spent to improve the control and prevention of dengue disease.

In a near future we have to include new procedures in the surveillance of dengue and this study was oriented to help in that sense.

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APPENDICES

APENDIX A: Map of Venezuela, Aragua State and their Municipalities.

The two municipalities in the study.
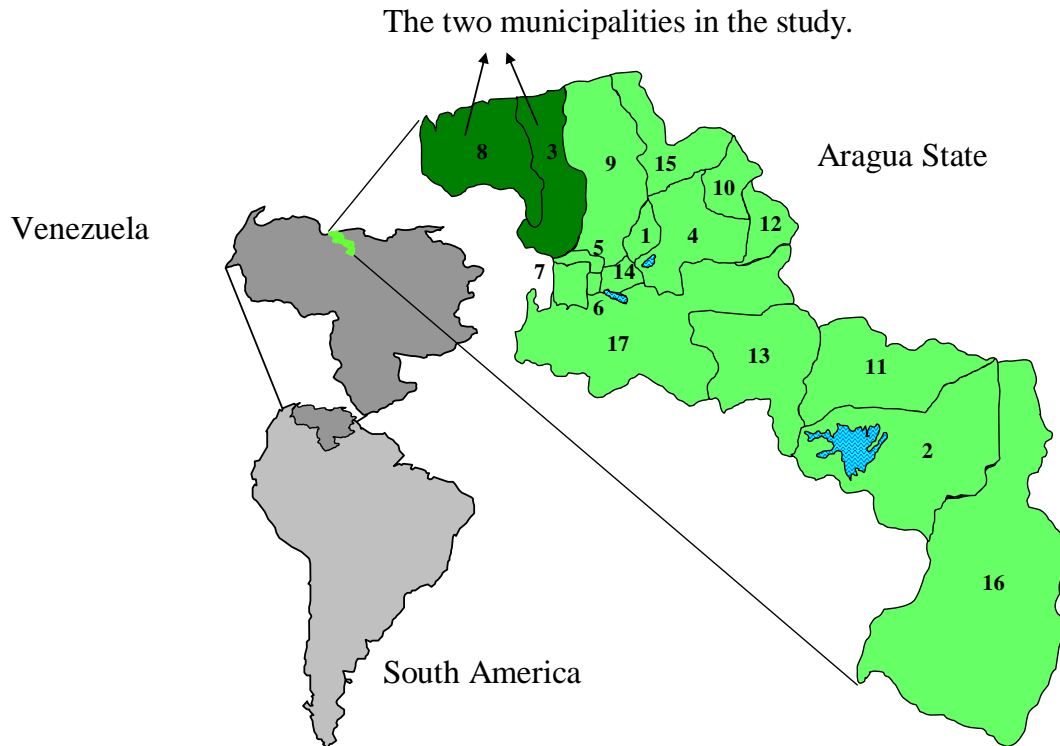
Municipalities: Bolivar (1), Camatagua (2), Girardot (3), J.F. Rivas (4), L. Alcántara (5), Lamas (6), Libertador (7), M.B. Iaragorry (8), Mariño (9), Revenga (10), San Casimiro (11), S. Michelena (12), S. Sebastián (13), Sucre (14), Tovar (15), Urdaneta (16) y Zamora (17).

APENDIX B: Maracay city and the Four Barrios:

Population: 12,722
    Sample: 628

Population: 16,996
    Sample: 635

"Piñonal"

"La Cooperativa"

Total Population in the four
"Barrios": 103,958
Total sample: 3,225

"23 de Enero"

Population: 15,718
    Sample: 693

"Caña de Azucar"

Population: 58,522
    Sample: 1,299

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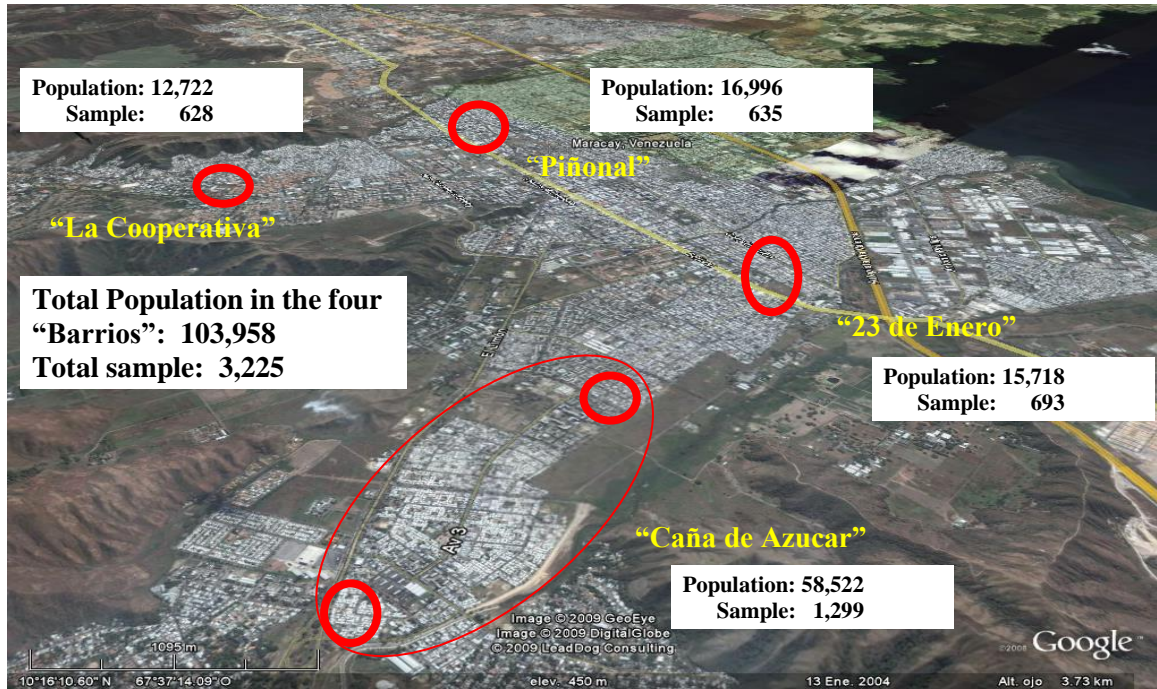
World Weather Information Service

Venezuela

Venezuela Air Force Weather Service

Weather Information for
Maracay

MAP

Climatological Information

Month	Mean Temperature ºC		Mean Total Precipitation (mm)	Mean Number of Precipitation Days
	Daily Minimum	Daily Maximum		
Jan	15.5	34.0	3.5	2
Feb	16.3	35.0	4.5	1
Mar	17.0	36.0	7.0	2
Apr	19.0	35.5	45.2	6
May	20.4	33.7	105.5	13
Jun	19.5	32.9	133.3	16
Jul	19.0	31.6	129.1	17
Aug	19.2	32.3	172.4	18
Sep	19.3	32.1	135.2	16
Oct	19.0	32.5	99.0	14
Nov	18.6	32.8	51.5	10
Dec	15.4	32.9	15.3	4

>> Click here for temperatures in ºF

Remarks:
* *Climatological information is based on monthly averages for the 30-year period 1961-1990.*
* *Mean number of precipitation days = Mean number of days with at least 1 mm of precipitation.*
* *Precipitation includes both rain and snow.*
* *Attention: Please note that the averaging period for climatological information and the definition of "Mean Number of Precipitation/Rain Days" quoted in this web site may be different for different countries. Hence, care should be taken when city climatologies are compared.*

APPENDIX D: Clinic epidemiologic card of febrile syndrome.

EPIDEMIOLOGICAL CLINICAL INVESTIGATION OF FEBRILE SYNDROME

Interview date ___/___/___   Epidemiological week _____

( ) ACUTE SAMPLE (complete the questionaire-collect convalescent sample two/three weeks after onset of symptoms)

( ) CONVALESCENT SAMPLE: Total duration of symptoms _____ still symptomatic ( ) if it is convalescent, APPLY CODE ON ACUTE SAMPLE:_____

Demographic Data

Last name

First name

Date:	Age:	Sex:
___/___/___		1M( ) 2F( )

Health center

Actual address

District	Province	Department.

Phone number

PAST HISTORY

Ocupation _____

Vaccines received:

Yellow Fever:	Hepatitis:
NO ( ) YES ( )	NO ( ) YES ( )
Date ___/___/___	Date ___/___/___

Place of residence for the last 60 days:
Locality: _____
District: _____

Trips in the last 30 days:
Locality: _____
District: _____

CLINICAL DATA

Clinical History Number _____

Date of symptom onset: ___/___/___

Date of second evaluation: ___/___/___

ANSWER YES OR NOT ON EACH CASE	1st Eval.	2nd Eval.
Temperature	( )___°C	( )___°C
Shivers	(Yes) (NO)	(Yes) (NO)
General Malaise	(Yes) (NO)	(Yes) (NO)
Hiporrhexia	(Yes) (NO)	(Yes) (NO)
Asthenia	(Yes) (NO)	(Yes) (NO)
Prostracion	(Yes) (NO)	(Yes) (NO)
Weight loss	(Yes) (NO)	(Yes) (NO)
Palor	(Yes) (NO)	(Yes) (NO)
Conjuntival Injection	(Yes) (NO)	(Yes) (NO)
Epistaxis	(Yes) (NO)	(Yes) (NO)
Gingivorragia	(Yes) (NO)	(Yes) (NO)
Ecchymosis	(Yes) (NO)	(Yes) (NO)
Petequiae	(Yes) (NO)	(Yes) (NO)
Purpura	(Yes) (NO)	(Yes) (NO)
Vaginal bleeding	(Yes) (NO)	(Yes) (NO)
Maculopapular Rash	(Yes) (NO)	(Yes) (NO)
Facial Erythema	(Yes) (NO)	(Yes) (NO)
Central Rash	(Yes) (NO)	(Yes) (NO)
Distal Rash	(Yes) (NO)	(Yes) (NO)
Vesicles	(Yes) (NO)	(Yes) (NO)
Subcutaneous nodules	(Yes) (NO)	(Yes) (NO)
Facial edema	(Yes) (NO)	(Yes) (NO)
Lower limbs edema	(Yes) (NO)	(Yes) (NO)
Soft tisues edema	(Yes) (NO)	(Yes) (NO)
Joint inflamation	(Yes) (NO)	(Yes) (NO)
Arthralgias	(Yes) (NO)	(Yes) (NO)
Myalgias	(Yes) (NO)	(Yes) (NO)
Bone pain	(Yes) (NO)	(Yes) (NO)
Joint function incapacity	(Yes) (NO)	(Yes) (NO)
Adenopathy	(Yes) (NO)	(Yes) (NO)

Digestive System/Abdomen	1° Eval.	2° Eval.
Abdominal pain	(Yes) (NO)	(Yes) (NO)
Diarrhea	(Yes) (NO)	(Yes) (NO)
Nausea	(Yes) (NO)	(Yes) (NO)
Vomiting	(Yes) (NO)	(Yes) (NO)
Melena	(Yes) (NO)	(Yes) (NO)
Hematochezia	(Yes) (NO)	(Yes) (NO)
Ascites	(Yes) (NO)	(Yes) (NO)
Abdominal distention	(Yes) (NO)	(Yes) (NO)
Hepatomegaly	(Yes) (NO)	(Yes) (NO)
Splenomegaly	(Yes) (NO)	(Yes) (NO)
Icterus	(Yes) (NO)	(Yes) (NO)
Hepatojugular Reflux	(Yes) (NO)	(Yes) (NO)

Cardio-Respiratory System	1° Eval.	2° Eval.
Congestiva Pharynx	(Yes) (NO)	(Yes) (NO)
Rhinorrhea	(Yes) (NO)	(Yes) (NO)
Coughing	(Yes) (NO)	(Yes) (NO)
Expectoration	(Yes) (NO)	(Yes) (NO)
Polipnea	(Yes) (NO)	(Yes) (NO)
Dyspnea	(Yes) (NO)	(Yes) (NO)
Wheezing	(Yes) (NO)	(Yes) (NO)
Cyanosis	(Yes) (NO)	(Yes) (NO)
Roncors	(Yes) (NO)	(Yes) (NO)
Crepitus	(Yes) (NO)	(Yes) (NO)
Pulmonar murmur	(Yes) (NO)	(Yes) (NO)
Pulmonary murmur	(Yes) (NO)	(Yes) (NO)
Cardiac murmur	(Yes) (NO)	(Yes) (NO)
Jugular regurgitation	(Yes) (NO)	(Yes) (NO)
Gallop	(Yes) (NO)	(Yes) (NO)
Arritmia	(Yes) (NO)	(Yes) (NO)

Urogenital System	1° Eval.	2° Eval.
Dysuria	(Yes) (NO)	(Yes) (NO)
Oliguria	(Yes) (NO)	(Yes) (NO)
Polyuria	(Yes) (NO)	(Yes) (NO)
Constipation	(Yes) (NO)	(Yes) (NO)
Urinary urgency		
PPL	(Yes) (NO)	(Yes) (NO)
Cervix tender to movement	(Yes) (NO)	(Yes) (NO)
Leukorrhea	(Yes) (NO)	(Yes) (NO)
Pelvic pain	(Yes) (NO)	(Yes) (NO)
Concrete mass	(Yes) (NO)	(Yes) (NO)

Nervous system		
Conciousness disorder	(Yes) (NO)	(Yes) (NO)
Headache	(Yes) (NO)	(Yes) (NO)
Seizures	(Yes) (NO)	(Yes) (NO)
Neck stiffness	(Yes) (NO)	(Yes) (NO)
Focalizing signs	(Yes) (NO)	(Yes) (NO)

Others		
Retroocular pain	(Yes) (NO)	(Yes) (NO)
Otorrhea	(Yes) (NO)	(Yes) (NO)
Ear pain	(Yes) (NO)	(Yes) (NO)

OUTCOME

CURED

RECOVERED

    DEAD (Date) ___/___/___

Possible diagnosis

Name of health provider:

_____

ADULT CONSENT FORM
Longitudinal Serology Survey

CONSENT FORM

Laboratorio Regional de Diagnostico e Investigacion del Dengue y otras Enfermedades
Virales y el Centro de Investigaciones Biomedicas de la Universidad de Carabobo,
Maracay, Venezuela (LARDIDEV/BIOMED-UC)

Naval Medical Research Center Detachment, Lima, Peru (NMRCD)
Walter Reed Army Institute of Research, USA (WRAIR)
Persons of 18 years or older
Consent to Participate in a Research Study

Project Title: Active Dengue Surveillance and Predictors of Disease Severity in Maracay, Venezuela.

Project House Code: _____   Participant ID No. _____

1. PURPOSE: LARDIDEV/BIOMED-UC, NMRCD and theWRAIR are carrying out a research study called "Active Dengue Surveillance and Predictors of Disease Severity in Maracay, Venezuela."   The purpose of this study is to determine what type of dengue virus is transmitted in Maracay and how severe is the disease presentation with that virus type.   We would like to ask you to volunteer to take part in this research project, which will include about 3500 people and it will last about 3 years.

2. PROCEDURES: If you decide to participate in the study, there are two possible levels of participation from which you may choose. The first option is that we will ask you for a small sample of blood every six months for the next 3 years and allow us to visit to your house 3 times a week to ask if anyone in the house has a fever or other illness. The second option is to simply allow us to visit to your house 3 times a week to ask if anyone in the house has a fever or other illness.

An experienced laboratory technician or a study physician will take the blood samples. The six-month blood samples will be 5 ml (1.25 teaspoons). The first sample will be used to determine if you have had dengue in the past and if you have had dengue which type of virus it was. The subsequent samples will be used to determine if you have had dengue (and if so what type of virus) during the last sampling period.

Study personnel will visit your house 3 times each week to ask if anyone in the house has a fever or other illness. If anyone with fever or history of fever is found, you/they will be invited to participate in an additional study where a blood sample (5 ml, 1.25 teaspoons) will be taken from your/their arm (venipuncture), and a physician free of charge will examine you/them. A study worker will visit your house daily until you/they are well. During the daily visits a study worker will take your/their temperature, vital signs, ask some questions about how you/they feel, and carry out a tourniquet test. The tourniquet test is used to look for signs of serious illness and applies pressure to the upper arm using a blood pressure cuff. A final sample of blood will be requested 10 to 21 days later. Your/their blood will be used to attempt to isolate virus from the first sample and to identify dengue antibodies from both the first and last specimens.

3. RISK TO PARTICIPANT: Blood will be drawn from your arm with a needle by an experienced laboratory technician or physician. The risk that you may be injured during collection of blood is minimal, but it is possible that there may be some pain and discomfort when the blood is removed from your arm; afterwards there may be some bruising or swelling and a very small possibility of infection at the site where the blood was collected. You may feel faint when the sample is taken but this is uncommon and the feeling will pass quickly. The dengue tourniquet test can cause arm pain in some people while the blood pressure cuff is inflated. This pain goes away when the cuff is deflated. While the cuff is inflated the skin on your arm and hand below the cuff may appear discolored (red, blue, purple). This discoloration will clear up after the cuff is deflated. You may develop a rash on your forearm appearing as many small red dots. This rash will disappear after a few days.

4. POTENTIAL BENEFITS: The possible benefits to you from taking part in this study include: the blood samples you give will tell you if you have had dengue (but will not prevent the disease directly) and the wellness visits to your home by a study physician at the time of illness may provide you with timely referral for medical care.

5. COST AND COMPENSATION: There is no cost to you to participate in the study.

6. MEDICAL CARE FOR RESEARCH RELATED INJURY: If you are injured as a direct result of taking part in this research project, you will be given medical care for that injury. This will be given to you at no cost to you. You will not receive any injury compensation, only medical care. You should discuss this issue thoroughly with the study personnel before you enroll in this study. Signing this document does not limit your rights to seek legal remedies through the Venezuelan legal system.

7. SUBJECT CONFIDENTIALITY: All the information related to this project will be confidential. The documents of this research study will be kept at the LARDIDEV/BIOMED-UC office in Maracay and at the NMRCD office in Lima. The data may be reviewed by the Institutional Review Board of the Naval Medical Research Center, Silver Spring, Maryland, USA and by the BIOMED-UC Investigation Ethics Committee. We will keep them private to the extent legally possible.

8. VOLUNTARY PARTICIPATION: You can decide not to take part in this study or you can leave this study at anytime without any negative consequences.

9. POINTS OF CONTACT: If you want to talk to someone about this study or if you have been injured from taking part in this study, please contact: Dr. Guillermo Comach at LARDIDEV/BIOMED-UC at 0-416-543-2116. If you have any questions about your rights as a participant, contact Dr. Silvia Montano, of NMRCD-Peru, at 011-511-561-2733.

10. ADULT CONSENT: Signing below indicates that the study has been explained to you and that you agree to take part at no cost to you or your family, or others living in the house. Additionally, your signature indicates that you have had the chance to ask questions. You should know that any questions that you may have in the future will be answered by one of the study investigators, and that you have informed all adult members of the household about the study. You will be given a copy of the consent form so that you have this information.

I agree to have blood samples taken _____.   I prefer not to give blood samples _____.
                                    Initials   Initials
It is possible that after we have completed the laboratory tests on your blood samples that there will be some leftover. What do you what us to do with your leftover blood samples? Initial only one option.
_____ After the study is completed destroy all remaining specimens.
Initials
_____ After the study is complete the remaining specimens can be used for any scientific purpose
Initials   provided that the scientific purpose is approved by an Institutional Review Board and that my
             specimen will not be identified by my name but only by a number.   I also understand that there will be
             no compensation for the future use of my specimen(s).

If you change your mind, at any time, and would like your leftover blood samples destroyed contact Dr. Guillermo Comach at 0-416-543-2116.

11. ADULT HEAD OF HOUSEHOLD CONSENT: Signing below indicates that the study has been explained to you and that you agree to take part at no cost to you or your family, or others living in the house. Additionally, your signature indicates that you have had the chance to ask questions. You should know that any questions that you may have in the future will be answered by one of the study investigators, and that you have informed all adult members of the household about the study. You will be given a copy of the consent form so that you have this information.

I agree that my household will participate in the project _____.
                                    Initials
Name of Participant: _____ Age_____

Signature of Participant: _____ Date _____
If the Participant is illiterate an adult must witness the consent process.

Name of Witness: _____ Age_____
Signature of Witness _____ Date _____
Name of Investigator: _____
Signature of Investigator _____ Date _____

APPENDIX F: Inform Consent Children

PARENTAL CONSENT FORM (Longitudinal Serology Survey)

CONSENT FORM

**Laboratorio Regional de Diagnostico e Investigacion del Dengue y otras Enfermedades
Virales y el Centro de Investigaciones Biomedicas de la Universidad de Carabobo,
Maracay, Venezuela (LARDIDEV/BIOMED-UC)**

Naval Medical Research Center Detachment, Lima, Peru (NMRCD)

Walter Reed Army Institute of Research, USA (WRAIR)
Parental Consent for children 5-17 years of age
Consent to Participate in a Research Study

Project Title: Active Dengue Surveillance and Predictors of Disease Severity in Maracay, Venezuela.

Project House Code: _____   Participant ID No. _____

1. PURPOSE: LARDIDEV/BIOMED-UC, NMRCD and the WRAIR are carrying out a research study called "Active Dengue Surveillance and Predictors of Disease Severity in Maracay, Venezuela."   The purpose of this study is to determine what type of dengue virus is transmitted in Maracay and how severe is the disease presentation with that virus type.   We would like to ask your child/children to volunteer to take part in this research project, which will include about 3500 people and it will last about 3 years.

2. PROCEDURES: If you agree to your child's/children's participating in this study, we will ask your child/children for a small sample of blood every six months for the next 3 years and allow us to visit to your house 3 times a week to ask if anyone in the house has a fever or other illness.

An experienced laboratory technician or a study physician will take the blood samples. The six-month blood samples will be 3 ml (0.6 teaspoons). The first sample will be used to determine if your child/children has/have had dengue in the past and if your child/children has/have had dengue which type of virus it was. The subsequent samples will be used to determine if your child/children has/have had dengue (and if so what type of virus) during the last sampling period.

Study personnel will visit your house 3 times each week to ask if anyone in the house has a fever or other illness. If your child/children is with fever or history of fever is found, your child/children will be invited to participate in an additional study where a blood sample (3 ml, 0.6 teaspoons) will be taken from their arm (venipuncture), and a physician will examine them free of charge. A study worker will visit your house daily until they are well. During the daily visits a study worker will take their temperature, vital signs, ask some questions about how they feel, and carry out a tourniquet test. The tourniquet test is used to look for signs of serious illness and applies pressure to the upper arm using a blood pressure cuff. A final sample of blood will be requested 10 to 21 days later. Their blood will be used to attempt to isolate virus from the first sample and to identify dengue antibodies from both the first and last specimens.

3. RISK TO PARTICIPANT: Blood will be drawn from your child's/children's arm with a needle by an experienced laboratory technician or a physician. The risk that they may be injured during collection of blood is minimal, but it is possible that there may be some pain and discomfort when the blood is removed from their arm; afterwards there may be some bruising or swelling and a very small possibility of infection at the site where the blood was collected. They may feel faint when the sample is taken but this is uncommon and the feeling will pass quickly. The dengue tourniquet test can cause arm pain in some people while the blood pressure cuff is inflated. This pain goes away when the cuff is deflated. While the cuff is inflated the skin on their arm and hand below the cuff may appear discolored (red, blue, purple). This discoloration will clear up after the cuff is deflated. They may develop a rash on their forearm appearing as many small red dots. This rash will disappear after a few days.

4. POTENTIAL BENEFITS: The possible benefits to your child/children from taking part in this study include: the blood samples they give will tell them if they have had dengue (but will not prevent the disease directly) and the wellness visits to your home by a study physician at the time of illness may provide you with timely referral of your child/children for medical care.

5. COST AND COMPENSATION: There is no cost to you or to your child/children to participate in the study.

6. MEDICAL CARE FOR RESEARCH RELATED INJURY: If your child/children is/are injured as a direct result of taking part in this research project, they will be given medical care for that injury. This will be given to them at no cost to you. They will not receive any injury compensation, only medical care. You should discuss this issue thoroughly with the study personnel before you enroll your child/children in this study. Signing this document does not limit your/their rights to seek legal remedies through the Venezuelan legal system.

7. SUBJECT CONFIDENTIALITY: All the information related to this project will be confidential. The documents of this research study will be kept at the LARDIDEV/BIOMED-UC office in Maracay and at the NMRCD office in Lima. The data may be reviewed by the Institutional Review Board of the Naval Medical Research Center, Silver Spring, Maryland, USA and by the BIOMED-UC Investigation Ethics Committee. We will keep them private to the extent legally possible.

8. VOLUNTARY PARTICIPATION: Your child/children can decide not to take part in this study or they can leave this study at anytime without any negative consequences.

9. POINTS OF CONTACT: If you want to talk to someone about this study or if your child/children has been injured from taking part in this study, please contact: Dr. Guillermo Comach at LARDIDEV/BIOMED-UC at 0-416-543-2116. If you have any questions about your rights as a participant, contact Dr. Silvia Montano, of NMRCD-Peru, at 011-511-561-2733.

10. CONSENT: THE PROJECT HAS BEEN EXPLAINED TO YOUR CHILD/CHILDREN IN YOUR PRESENCE IN A LANGUAGE AND LEVEL HE/SHE/THEY CAN UNDERSTAND. HE/SHE/THEY HAS/HAVE BEEN ENCOURAGED TO ASK QUESTIONS ABOUT THE RESEARCH STUDY. YOUR SIGNATURE BELOW WILL SHOW THAT YOU HAVE CONSENTED TO LET YOUR CHILD/CHILDREN VOLUNTEER TO TAKE PART IN THIS STUDY.

1 _____
   Name of child   Age of Child   Signature of Parent or Guardian

2 _____
   Name of child   Age of Child   Signature of Parent or Guardian

3 _____
   Name of child   Age of Child   Signature of Parent or Guardian

4 _____
   Name of child   Age of Child   Signature of Parent or Guardian

It is possible that after we have completed the laboratory tests on your child's/children's blood samples that there will be some leftover. What do you want us to do with their leftover blood samples? Initial only one option.

_____ After the study is completed destroy all remaining specimens.
Initials

_____ After the study is complete the remaining specimens can be used for any scientific purpose
Initials   provided that the scientific purpose is approved by an Institutional Review Board and that my
         child's/children's specimen will not be identified by their name but only by a number.
         I also understand that there will be no compensation for the future use of their specimen(s).

If you change your mind, at any time, and would like their leftover blood samples destroyed contact Dr. Guillermo Comach at 0-416-543-2116.

If the parent or guardian is illiterate an adult must witness the consent process.
Name of Witness: _____ Age_____
Signature of Witness _____ Date _____
Name of Investigator: _____
Signature of Investigator _____ Date _____

About the Author

Carlos Espino received a Medical degree from the University of Carabobo, Venezuela in 1985 and a Master of Science in Occupational Health at the same university in 1994. In 2004 he received a Master of Science Diploma in Public Health at the University of South Florida. He is a full professor in the Public Health Department at the University of Carabobo since 1992, where he was Department chief in 1998.

Since 2006, Espino is part of the team of the Institute of Biomedical Research (BIOMED) at the University of Carabobo, in the laboratory of dengue and other viral diseases.