Out of the
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Phylogenetic, epidemiological and clinical studies on dengue and dengue virus in Vitória, Espírito Santo state, Brazil

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Dengue, dengue virus, *Aedes*, phylogeny, epidemic, social class, severe dengue, signs and symptoms, age groups, gender and health.

Abstract

Background: Vitória is an endemic area in Brazil for dengue. This thesis presents the phylogeny of dengue virus serotype 4, the evaluation of dengue dispersion and the influence of serotypes and demographics to severe dengue outcomes in Vitória.

Methods: Sequences of envelope (n = 8) and NS1 (n = 4) gene of dengue virus serotype 4 were used to construct phylogenetic trees (2013). Spatial variation in temporal trends was analyzed (2012-2013). Cross-sectional studies were performed to assess associations of serotypes (2009-2013) and demographics (2007-2013) with severe outcomes.

Results: Dengue virus serotype 4 genotype I (n = 2) related to a strain from Bahia, and genotype II (n = 8) related to strains from Roraima, Mato Grosso and São Paulo were detected. Five space-time clusters with lower Time Trend Increase presented higher risk for dengue transmission and lower income than the six space-time clusters with higher Time Trend Increase. In 6,703 dengue cases, 11.3% presented severe dengue, which was significantly higher among males (13.0%) than females (10.0%), and among elderlies (15.5%) than children (8.8%), adolescents (12.5%), and adults (10.5%). Children with severe dengue presented hemorrhage (68.8%-86.4%) and plasma leakage (52.4%-62.5%) in a higher proportion than other age groups. Serotype was determined for 485 cases and severe dengue affected 6.6% of them. Severe dengue occurred at a significantly higher frequency in infections caused by dengue virus serotype 2 (32.3%) than in those caused by dengue virus serotype 4 (6.4%) or by dengue virus serotype 1 (4.5%).

Conclusion: Apparently, Vitória is not an international route for dengue introduction in Brazil. Living in low-income areas increased the chance of dengue infection. Dengue virus serotype 2 was associated with an elevated occurrence of severe dengue, which also affected more males and elderlies. Manifestations of severe dengue were worse in children than in other age group.

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Abbreviations

A: Adenine

A.D.: Anno Domini

Apr: April

Aug: August

C: Cytosine

C1: Space-time cluster 1

C2: Space-time cluster 2

C3: Space-time cluster 3

C4: Space-time cluster 4

C5: Space-time cluster 5

C6: Space-time cluster 6

C7: Space-time cluster 7

C8: Space-time cluster 8

C9: Space-time cluster 9

C10: Space-time cluster 10

C11: Space-time cluster 11

cDNA: Complementary deoxyribonucleic acid

CI: Confidence interval

Dec: December

DENV-1: Dengue virus serotype 1

DENV-2: Dengue virus serotype 2

DENV-3: Dengue virus serotype 3

DENV-4: Dengue virus serotype 4

DENV-5: Dengue virus serotype 5

DHF: Dengue hemorrhagic fever

dNTP: Deoxyribonucleotide triphosphate

Ec: Expected number of cases

E: East

Feb: February

Fw: Forward starter

G: Guanine

HI: House index

HPD: Highest probability density

IgG: Immunoglobulin G

IgM: Immunoglobulin M

I(): Function indicator

Jan: January

Jun: June

Jul: July

km: Kilometer

LLR: Log likelihood ratio

MAC-ELISA IgM: IgM antibody-capture enzyme-linked immunosorbent assay

Mar: March

$MgCl_2$: Magnesium chloride

M: Meter

mm: Millimeter

mM: Millimolar

n: Number of cases

N: North

Nov: November

NS: Nonstructural protein

Oct: October

OR: Odds ratio

PCR: Polymerase chain reaction

PD: Population density

Rv: Reverse starter

RNA: Ribonucleic acid

RR: Relative risk

RT-PCR: Reverse-transcriptase-polymerase chain reaction

S: South

Sep: September

T: Thymine

TTI: Time trend increase

U: Unit

v.: Version

W: West

µl: Microliter

°C: Degrees Celsius

1 Introduction

1.1 History of dengue

In the last decades, dengue has been a center of discussions in the field of global public health. According to the revision of the International Health Regulations in 2005, dengue was recognized as a concern for international health security (World Health Organization, 2012a). Therefore, strategies for prevention and control of dengue are deserved.

Occurrences of dengue have been recorded for over a millennium. The first register of a dengue-like illness was reported in the Chinese medical encyclopedia in 992 A.D. (Gubler, 1998). Some isolated outbreaks related to a syndrome that resemble dengue were registered in the 17th century in Asia and Central America (Gubler et al., 1997). In 1780, the United States suffered from a dengue outbreak. Concomitantly, the disease was occurring in the Caribbean islands, which maintained a close trade relationship with the United States. During that period, dengue was denominated as "break bone fever", due to painful and disabling symptoms (Morens et al., 2013). In the 18th century, sporadic outbreaks probably caused by dengue were registered, not only in the Americas, but also in Asia (Hayes and Gubler, 1992). By the end of this period, dengue was recognized as a disease characterized by specific signs and symptoms (Messina et al., 2014).

In 1906, the transmission of the etiological agent by mosquito bite was confirmed (Bancroft, 1906). Dengue virus was first isolated in 1943 in Japan, as a strain of dengue virus serotype 1 (DENV-1) (Messina et al., 2014). Until World War II, most dengue registrations occurred in coastal areas (Murray et al., 2013), including the Mediterranean region (Schaffner and Mathis, 2014). However, World War II enabled dengue to disperse inland, especially in Southeast Asia. Until the 1950's, dengue was known as a disease with benign presentations. However, in the post-war scenario, rapid urbanization and faster movements of humans over longer distances permitted different serotypes to be introduced into single regions concurrently. This lead to an endemic co-circulation of dengue virus serotypes, constituting a hyperendemic epidemiological scenario. This new situation gave rise to the emergence of the first cases of dengue hemorrhagic fever at the beginning of the 1950's (Holmes and Twiddy, 2003, Barreto and Teixeira, 2008; Snowden, 2008; Murray et al., 2013).

In the 1950's and in the 1960's, dengue virus circulation was restricted to some Asian countries (Gubler, 2012). In the Americas, a campaign was initiated in the 1940's aiming to eliminate the vector, which was already known to be also responsible for yellow fever transmission. This campaign was probably responsible for the practical absence of reports of dengue cases during the following decades on the continent. The Pan-American Health Organization, the American arm of the World Health Organization, coordinated this vertical campaign and had success in eliminating the vector in many American countries (Guzman and Kouri, 2003). Some of the key strategies applied in the campaign were the reduction of larval sources, the monitoring of larval density through different indexes, and the application of dichlorodiphenyltrichloroethane and malathion in sprays which were used at outdoor sites (Kyle and Harris, 2008). In the 1970's, however, the campaign was discontinued, permitting the reestablishment of the dengue vector in the Americas (Guzman and Kouri, 2003). Subsequently in this decade, dengue dispersed to other regions of the world, making it no longer a disease exclusively registered in Asia (Gubler, 2012).

According to the report of the different dengue serotypes, DENV-1 was first registered in Australasia (French Polynesia and Japan) in 1943, in the Americas (Barbados, Cuba, French Antilles, Granada, Paraguay and Puerto Rico) in 1977, in Africa (Sudan) in 1984, and in the Middle East (Saudi Arabia) in the 1990's. The first report of the dengue virus serotype 2 (DENV-2) occurred in Asia (Papua New Guinea and Indonesia) in 1944, followed by the Americas (Trinidad and Tobago) in 1953, and Africa (Nigeria) in 1964. Similarly to the other serotypes, the dengue virus serotype 3 (DENV-3) was reported initially in Asia (Philippines and Thailand) in 1953, then in the Americas (Puerto Rico) in 1963, in Africa (Mozambique) in 1984, and in the Middle East (Saudi Arabia) in 1994. Asia was also the first continent to report the dengue virus serotype 4 (DENV-4), in 1953 (Philippines and Thailand), followed by the Americas (Brazil, Cuba, Dominica, Puerto Rico and Virgin Islands) in 1981 (Messina et al., 2014).

The emergence of dengue virus occurred approximately 1,000 years ago, but the exact location where this event took place has not been established. It has been hypothesized that the Australasian region was the probable site of dengue emergence, due to the greater diversity of the sylvatic ancestors found there. The four serotypes of dengue virus evolved independently (Wang et al., 2000; Holmes and Twiddy, 2003), probably due to their different geographic locations or permanence in distinct populations. The first lineage of dengue virus serotype to emerge was the DENV-4, followed by DENV-2, DENV-1 and DENV-3 (Holmes and Twiddy, 2003). Initially, dengue occurred in a cycle involving non-human primates and the mosquito *Aedes (Stegomyia)*

albopictus (Wang et al., 2000; Messina et al., 2014), with humans being infected only sporadically. Dengue became more common over the past few centuries, with the establishment of the human-mosquito cycle, following the spread of the mosquito *Aedes (Stegomyia) aegypt*i to different regions in the world (Wang et al., 2000).

The mosquito *Aedes (Stegomyia) aegypti* is assumed to have originated in sub-Saharan Africa. The spread of this species of mosquito to other parts of the world is closely linked to the "Age of Discovery", from the 15th to 18th centuries, especially with the traffic of slaves from West Africa to the New World. Probably, at that period, ships to the Americas transported these mosquitoes. Australasian mosquitoes were hereafter derived from established American populations of this vector (Powell and Tabachnick, 2013).

The domestication of *Aedes (Stegomyia) aegypti* occurred originally in Africa, facilitated by the mosquitos' affinity for living close to human populations and using them as a source of blood feeding. In order to reach the human hosts, dengue viruses evolved under selective pressure adaptations to enable their transmission by the domesticated species *Aedes (Stegomyia) aegypti* (Powell and Tabachnick, 2013). The disease caused by dengue infection initially presented benign manifestations in humans. Due to the low fatality of the hosts, it was possible for the viruses to establish a transmission cycle involving humans. Even with few hosts, this physiological characteristic permitted a sustained viral spread (Holmes and Twiddy, 2003).

1.2 The dengue virus

The dengue virus is constituted by a positive single-stranded ribonucleic acid (RNA) involved by an icosahedral capsid enclosed in a lipoprotein envelope (Holmes and Twiddy, 2003; Whitehorn and Simmons, 2011; World Health Organization, 2011). The viral genome has approximately 11,000 nucleotides, responsible for encoding seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) and three structural proteins, which form the capsid, the membrane and the envelope. The envelope protein participates in the binding and fusion of the virus to the host-cell membrane, with its domain III interacting with cell receptors and neutralizing antibodies responsible for conferring the protective immune response (Weaver and Vasilakis, 2009).

There are four types of dengue virus, with cross-immunity between them being limited (Weaver and Vasilakis, 2009). Therefore, they compose a group of different serotypes (DENV-1, DENV-2, DENV-3, DENV-4), varying genetically and antigenically. Consequently, an infection by one serotype confers lifelong immunity against it (homologous protection), but only partial and temporary immune protection against the other three serotypes (heterologous protection), permitting the occurrence of sequential dengue infections (Sabin, 1952). These serotypes share approximately 62% to 67% of identical amino acid sequences (Westaway and Blok, 1997). Furthermore, each serotype is composed of genetic variants with less than 6% divergence in the envelope and NS1 junction (Rico-Hesse, 1990). These genotypes number four in DENV-1, six in DENV-2, four in DENV-3, and three in DENV-4 (Kyle and Harris, 2008), and vary in geographical distribution (Pinho et al., 2015), virulence and transmissibility (Rico-Hesse, 2010).

Taxonomically, dengue virus is classified into the family *Flaviviridae*, genus *Flavivirus*, as are some other human pathogens transmitted by vectors, such as yellow fever virus, Japanese Encephalitis virus, West Nile virus (Holmes and Twiddy, 2003), Zika virus (Zanluca et al., 2015), and other viruses. This taxonomic grouping is based on antigenic cross-reactivity, genomic organization and sequence similarity between the viruses of this genus (Weaver and Vasilakis, 2009).

1.3 The vector of dengue

Transmission of the virus from mosquitoes to humans occurs with its inoculation during blood feeding. Two species of the genus *Aedes (Stegomyia),* belonging to the family *Culicidae*, have confirmed vectorial competency, despite their distinct potential for triggering epidemics. The use of *Aedes* as a terminology of genus is maintained due to its classical and broad use. However, this genus of the mosquito was reclassified as *Stegomyia* in 2006. The mosquito *Aedes (Stegomyia) aegypti* is highly related to epidemics, especially in urban areas, due to its anthropophilic habitat and aggressive biting behavior. Contrary to *Aedes (Stegomyia) albopictus*, females of the species *Aedes (Stegomyia) aegypti* feed multiple times to complete a gonadotropic cycle, transmitting the virus across multiple human hosts during this process. Moreover, the vectorial capacity of *Aedes (Stegomyia) albopictus* is reduced, due to its non-urban habitat and less frequent contact with humans (World Health Organization, 2011).

The domestication of *Aedes (Stegomyia) aegypti* was the main event contributing to its development as an efficient vector for human diseases, transmitting also yellow fever, Chikungunya and Zika viruses. In addition to the mosquito's preference for humans as source of blood feeding, man-made water containers have proven to be ideal for egg deposition and larval development (Powell and Tabachnick, 2013). The proximity between sources of feeding and oviposition influences the flight distance of the mosquito, which normally is less than 150 m (Maciel-de-Freitas et al., 2010), but can reach 400 m (World Health Organization, 2011).

During warm periods, the vectorial capacity of *Aedes (Stegomyia)* mosquitoes is amplified, since an increase in 2°C in temperature is enough to reduce the period of viral incubation, prolonging the lifespan of the mosquito and accelerating its life cycle (Focks and Barrera, 2006; World Health Organization, 2011). In addition, temperature also influences the body size of female mosquitoes. A small body size in high temperature periods has an effect of forcing the ingestion of higher quantities of blood to obtain the amount of protein necessary to produce eggs. Blood ingestion in warm periods is also important to prevent dehydration of the vector (World Health Organization, 2011). Therefore, in warm seasons, *Aedes (Stegomyia)* mosquitoes are more active and more frequently infected, transmitting viral agents for a longer period due to the extended lifespan (Focks and Barrera, 2006; World Health Organization, 2011). The biting activity of these mosquitoes is higher in early morning and in late afternoon, but is not restricted to these periods, and can occur indoors and outdoors. In the latter case, it happens especially in peridomestic areas (Saifur et al., 2012).

Female mosquitoes lay about 50 to 120 eggs at one time. The eggs complete their embryonic development in 48 hours. After that, they can persist for more than one year in a dry environment, hatching once they get in contact with water. The larvae of *Aedes (Stegomyia) aegypti* present four stages of development. About 10 days are necessary for the emergence of the adult form of the mosquito; however, this time can be reduced to seven days in periods with higher environmental temperatures (World Health Organization, 2011).

In order to reach multiple human hosts, dengue viruses first need to infect vectors capable of transmitting them. This process occurs when the female mosquito bites a person infected by dengue virus in the viremic phase of the infection, which ranges from two days before until five days after the onset of disease symptoms. The viruses ingested with the blood meal replicate in the epithelial cells of the gut, later infecting the salivary glands and the genital tract of the mosquito.

Therewith the virus is able to infect the saliva and the eggs and be transmitted to humans as well as the vector's descendants, making dengue virus with the capacity of vertical transmission in the vector population. The period of incubation within the mosquito varies from eight to 12 days, and the vector remains infective for life (World Health Organization, 2011). The lifespan of the vector typically lasts from one to two weeks, but can reach more than 174 days (Pryor et al., 2001).

1.4 Pathogenesis of dengue

After the entry of dengue virus into the human body, it disseminates, probably through the capillary system. Then, the virus reaches and infects human cells in different tissues. Thus, high viral titers are achieved, resulting in a systemic infection. The first symptoms of dengue emerge with the host's antiviral response, consisting of interferon production for combating viral replication. A reduction in viral titers is observed after the second day of onset of symptoms (Simmons et al., 2015). Simultaneously, high levels of cytokines are released by macrophages, as representatives of the involved innate immune system, when interacting with T cells, inducing a discrete leukopenia and medullary depression (Figueiredo, 1999). Proinflammatory cytokines also have an indirect inflammatory effect on vascular endothelial cells (Whitehorn and Simmons, 2011). Therefore, in the course of dengue, a systemic inflammatory response induced by the infection is responsible for most of the presented symptoms (Simmons et al., 2015). In addition, B cell activation leads to a vigorous humoral response, with increasing levels of immunoglobulin G (IgG) and immunoglobulin M (IgM). These neutralizing antibodies are able to promote the lysis of the viral envelope and to block the viral receptors (Figueiredo, 1999).

The immunological response triggered by dengue infection is directly related to vascular alterations (Whitehorn and Simmons, 2011). Increased capillary permeability, coagulopathy and thrombocytopenia are some of the alterations that can be observed, especially in the period of decrease in body temperature (Simmons et al., 2015). An exacerbated immune response can lead to severe manifestations, due to three components that interact and reinforce each other. One is the uncontrolled cellular immunity, with activation of T cells by dendritic cells presenting viral components, producing elevated quantities of cytokines. Another is the stimulation of an excessive production of cytokines by the complement system and the associated anaphylatoxins. The third mechanism is speculated to primarily occur in sequential dengue infections, and is called antibody-dependent enhancement. In these cases, non-neutralizing heterologous antibodies recognize

dengue virus epitopes. Consequently, infectivity is enhanced as viral entry into cells through the corresponding receptors is facilitated. These antibodies are also associated with autoimmune processes, which can in turn also exacerbate the vascular leakage and the cytokine production (Nielsen, 2009).

Coagulopathy can also be mediated by cytokines, and thrombocytopenia can be induced by complement activation (Whitehorn and Simmons, 2011). Both factors are related to hemorrhages, one complication of dengue virus infection. In addition, platelet quality can be altered, and even in normal levels dysfunctional platelets can result in increased bleeding times (World Health Organization, 1997). Cases with atypical manifestations, such as encephalitis, myocarditis and hepatitis have also been documented (Gulati and Maheshwari, 2007). Even today, despite considerable knowledge of dengue pathogenesis and its relation to the immune response, a total understanding of the disease mechanisms has not been achieved (Whitehorn and Simmons, 2011).

Although some cases of dengue present with important pathophysiological alterations leading to complications such as dengue hemorrhagic fever and hypovolemic shock, which eventually may lead to death within 12 to 24 hours, most dengue cases are asymptomatic or present with a benign course (Brazilian Ministry of Health, 2016a).

1.5 Clinical management of dengue

The diagnosis of dengue in different phases of the disease may be challenging, due to other illness with similar signs and symptoms. Epidemiological knowledge of diseases prevalence in a certain location is an important aspect to consider in the differential diagnosis of dengue (World Health Organization, 2012b).

In the febrile phase, which can last up to seven days, high fever, facial flushing, ache in muscles and eyes, joint pain, photophobia, exanthema, headache, anorexia, nausea and vomiting are common manifestations (World Health Organization, 2012b). In this phase, febrile syndromes, such as enterovirosis, influenza, respiratory virosis, viral hepatitis, malaria, typhoid fever and arbovirosis such as Chikungunya, Oropouche and Zika, should be considered in the differential diagnosis. Similarly, febrile exanthematous syndromes could be confounded with dengue in their febrile phases, such as rubella, measles, scarlet fever, erythema infectiosum, sudden rash,

enterovirosis, infectious mononucleosis, parvovirosis, infection caused by cytomegalovirus, drug reactions, Kawasaki disease, Henoch-Schönlein disease and arboviroses such as Mayaro, Chikungunya and Zika (Brazilian Ministry of Health, 2016a). A positive tourniquet test and progressive leukopenia in this phase are indicative for dengue. In the febrile period, dengue cases can also present mild hemorrhagic manifestations in mucosa and skin (World Health Organization, 2012b).

The critical phase of dengue usually occurs in the period in which the fever decreases, which happens between three to eight days after the onset of symptoms. Most of the cases recover in this period; however, some of them present increased vascular permeability, triggering the emergence of warning signs. In this period, leukopenia and thrombocytopenia can precede the eventual plasma leakage, which is followed by an increase in hematocrit and changes in blood pressure and in pulse volume. Pleural effusions, ascites and hemorrhagic manifestations, such as prolonged bleeding in venipuncture sites and mucosal bleeding, can also occur. Warning signs, as persistent vomiting, abdominal pain and lethargy may be present (World Health Organization, 2012b). In this phase, hemorrhagic fever syndromes, such as hantavirosis, yellow fever, leptospirosis, severe malaria and rickettsiosis, can be confounded with dengue. Abdominal pain syndromes, such as appendicitis, bowel obstruction, liver abscess, pneumonia, urinary infection and acute cholecystitis, can also be confounded with dengue at this stage (Brazilian Ministry of Health, 2016a).

In cases that evolve to shock, low body temperature, metabolic acidosis, organ impairment, disseminated intravascular coagulation and severe hemorrhage can be observed (World Health Organization, 2012b). Other shock syndromes may be considered as differential diagnoses in this phase, such as meningococcemia, septicemia, meningitis, influenza type B, Brazilian purpuric fever, toxic shock syndrome and cardiogenic shock. Meningeal syndromes also have similar presentations to severe dengue, such as viral meningitis, bacterial meningitis and encephalitis (Brazilian Ministry of Health, 2016a).

The treatment of dengue is defined according to the clinical presentations, aiming at the control of the symptoms, and the prevention of deleterious clinical outcomes. In cases of dengue without warning signs, treatment usually comprises adequate fluid intake, bed rest, and administration of paracetamol. It is usually not necessary to manage cases without warning signs in hospital care. During the critical phase of the disease, follow-up of the patient without warning signs is necessary in order to detect evolution towards complications. By evaluating platelet counts, hematocrit and

the emergence of warning signs, the first signs of plasma leakage can be detected early, permitting a timely corresponding intervention (World Health Organization, 2012b).

In cases of dengue with warning signs or dengue with concomitant risk factors, such as pregnancy, infancy, old age or diabetes, admission to a reference health center is indicated for further treatment. Fluid intake, or when this is not possible, intravenous hydration, is essential. In cases with warning signs, intravenous hydration must be accompanied by monitoring of the hematocrit according to current protocols, in order to be able to evaluate the potential evolution of hemoconcentration. In addition, monitoring of vital signs, peripheral perfusion, urine output, glucose and organ functions must be conducted. For cases with concomitant conditions or risk factors, monitoring of the temperature pattern, fluid intake and losses, emergence of warning signs, hematocrit, leukocyte and platelet counts must be performed (World Health Organization, 2012b).

In cases of severe dengue, intravenous fluid resuscitation with isotonic crystalloid solutions without glucose is necessary to maintain circulation in order to combat plasma leakage. In cases that evolve to hypotensive shock, the use of colloid solutions is recommended. Normally, this process of resuscitation should be given in a time from 24 to 48 hours in order to avoid pulmonary edema and thrombophlebitis. In cases presenting with severe bleeding, blood transfusion can be considered as an ultimate measure (World Health Organization, 2012b).

1.6 Prognosis of dengue

Despite the absence of efficient antiviral medications against dengue virus, medical actions, such as controlled intravenous hydration, can substantially modify the clinical evolution of dengue if conducted cautiously, reducing the mortality rate to less than 1% of severe cases (Torres, 2006; World Health Organization, 2009).

It remains a challenge, however, to predict which patients will develop severe dengue or who will benefit from invasive medical treatment or hospitalization. This is especially true during epidemics, when provision of assistance for large numbers of dengue cases is needed. Efficient classification of patients aiming at a rational allocation of therapeutic resources is still not available. After all, the mortality rate in cases presenting dengue hemorrhagic fever remains high, since medical care is not always timely, or adequately provided (Tauil, 2007).

Therefore, it is necessary to keep the general population informed about severe and lethal forms of dengue and encourage the public to seek treatment already in the early stages of the disease, ideally on the first day after the emergence of fever and other clinical manifestations (Tauil, 2002). However, despite efforts in communications by press and other campaigns, a large number of patients consult health services relatively late, or even do not consult any medical care at all during their course of dengue (Montenegro et al., 2006).

The quality of the assistance and the organization of the health services are critical to the avoidance of deaths caused by dengue infection. Screening according to risk classifications may reduce the waiting times of the patients before reaching medical assistance, and the organization of the health services network may contribute to addressing epidemics effectively. The establishment of clinical protocols and reference systems based on risk classification enables a health care system to perform adequately in its function as primary health care provider in the light of a dengue outbreak, by allowing early detection and effective patient management (Brazilian Ministry of Health, 2009).

In face of the epidemiological and clinical picture of dengue, it is important to identify factors that support an assessment of the clinical evolution of the individual. These factors could contribute to creating a better screening protocol for the clinical management of dengue cases, enabling timely treatment of patients with potential to evolve to severe forms of dengue, and improving the disease prognosis.

Research projects investigating markers to support the prediction of severe forms of dengue at an early stage of the disease have been conducted. These studies provide evidence that patients who presented with dengue hemorrhagic fever had previously, in the febrile phase of illness, higher levels of creatine kinase, lactate dehydrogenase, and urea, and lower levels of triglycerides and albumin (Villar-Centeno et al., 2009). A decision tree targeting better clinical management of dengue cases has been developed previously, based on clinical and laboratory findings, such as levels of urea and protein. However, with high sensitivity and low specificity, it only permits detection of cases with dengue hemorrhagic fever about five days after the onset of symptoms (Lee et al., 2008a). Therefore, more investigations are necessary to identify patients with a potential to evolve to severe dengue at an early stage of the disease.

1.7 Global distribution of dengue

In any location, three factors are necessary to allow the occurrence of dengue infection: presence of the vector, circulation of dengue virus and availability of a susceptible human population. Climate plays an essential role in dengue distribution worldwide, since it mediates the presence of mosquitoes involved in dengue transmission. Therefore, dengue is endemic in tropical and sub-tropical areas, between the latitudes 35° North and 35° South, being uncommon in places above 1,000 m in altitude (World Health Organization, 2009). Dengue is favored particularly in locations where warm temperatures occur together with high humidity, with both climate factors influencing the observed seasonality of epidemics. Therefore, the peak of dengue incidence normally occurs at the beginning of the calendar year in the Southern hemisphere, and in the middle of the year in the Northern hemisphere, coinciding with the warmest periods in these regions (Schwartz et al., 2008).

Dengue is considered endemic in more than 120 countries. Consequently, more than the half of the world's population is at risk of dengue virus infection (Brady et al., 2012). The number of reports to the World Health Organization has increased over the last years, but these numbers are still far from the actual figures. Not only is this due to failure of surveillance processes, such as misdiagnosis or underreporting, but also because of a considerable number of asymptomatic cases that have to be expected wherever dengue virus infection occurs (World Health Organization, 2012a). According to a more recent estimate, 390 million new dengue infections occur annually, varying from 284 million to 528 million, with approximately 75% of these infections assumed to be asymptomatic. Therefore, the number of cases with clinical manifestations is about 96 million per year, with estimations varying from 67 million to 136 million (Bhatt et al., 2013). Among these cases, about half a million require hospitalization, and the number of deaths reaches 20,000 annually (Guzman et al., 2010). Worldwide, the annual number of cases presenting hemorrhage increased more than 500-fold from 1950 to 1998 (World Health Organization, 1999).

In fifty years, the global incidence of dengue increased 30-fold. Nowadays, dengue is still considered the most rapidly spread mosquito-borne viral disease in the world (World Health Organization, 2012a), despite the impressive progressions of other vector-borne infections, such as the Zika virus epidemic in 2015 and 2016. Additionally, dengue maintains a considerable potential for continuing its spread worldwide (Murray et al., 2013). Since the 1970’s, when looking

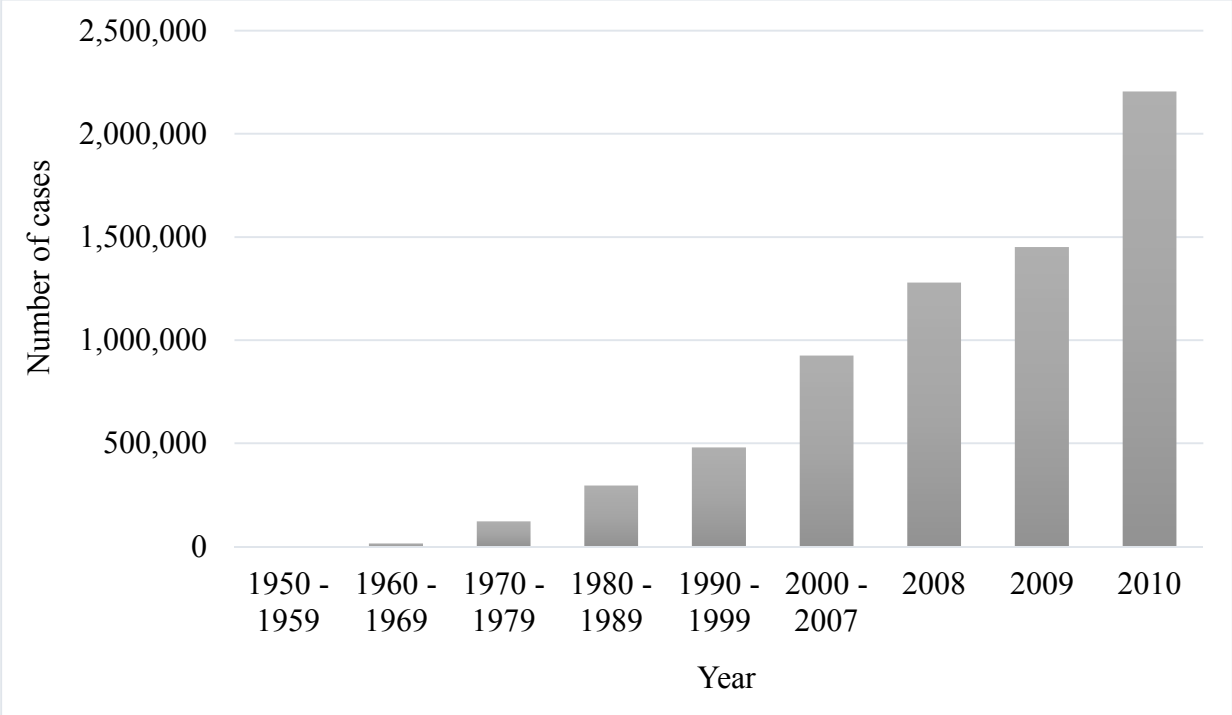
at the different serotypes of dengue virus, DENV-1 is the serotype most reported in the world, followed by DENV-2, DENV-3 and DENV-4 in decreasing order (Messina et al., 2014).

The largest share of new dengue infections in the world is concentrated in Asia, corresponding to about 70% of the globally apparent incident cases, or in total numbers corresponding to an average of 67 million annual infections, varying from 47 million to 94 million (Bhatt et al., 2013). In 2009, all countries in Southeast Asia reported autochthonous transmission of dengue. Southeast Asia is home to a population of 1.3 billion under risk for this disease. Eight countries in the region present hyperendemicity, with co-circulation of all four dengue virus serotypes (World Health Organization, 2012a). In Asia overall, reporting of DENV-2 has escalated rapidly, and registration of DENV-3 infections has been higher than DENV-1 records (Messina et al., 2014). The number of dengue cases reported has been increasing over time, especially in Indonesia, Thailand and Myanmar (World Health Organization, 2012a). However, India is the country in the region with highest estimations of dengue, accounting for roughly 34% of apparent incident cases in the world (Bhatt et al., 2013).

The Americas is the region with the second highest estimate of dengue, accounting for 14% of all new apparent dengue infections in the world. This corresponds to a mean of 13 million annual cases, ranging from 9 million to 18 million (Bhatt et al., 2013). Here, in all years since 2009, annual reports of dengue infections have been higher than one million. In 2013 and in 2015, these numbers surpassed two million incident cases (Pan American Health Organization, 2014; Pan American Health Organization, 2015). Almost all American countries have reported autochthonous transmission caused by all four dengue virus serotypes. This scenario of hyperendemicity has thereby increased the number of hemorrhagic cases (Dick et al., 2012). Before 1963, only DENV-2 was found in the Americas (Allicock et al., 2012). Afterwards, the American continent was responsible for the increasing global number of cases caused by DENV-3 in the 1990's, and for the persistent and ascending occurrence of DENV-4 (Messina et al., 2014).

Comparing the two most important world regions for dengue transmission, some differences can be observed. The prevalence of more severe clinical forms of dengue is higher in Asia than in the Americas. In Asia, most hemorrhagic forms affect children, while in the Americas these clinical forms affect both children and adults, with an increasing tendency in people under 15 years of age (Guzman and Kouri, 2003).

Figure 1.1. Annual average of dengue infections in the world reported to the World Health Organization from 1950 to 2010



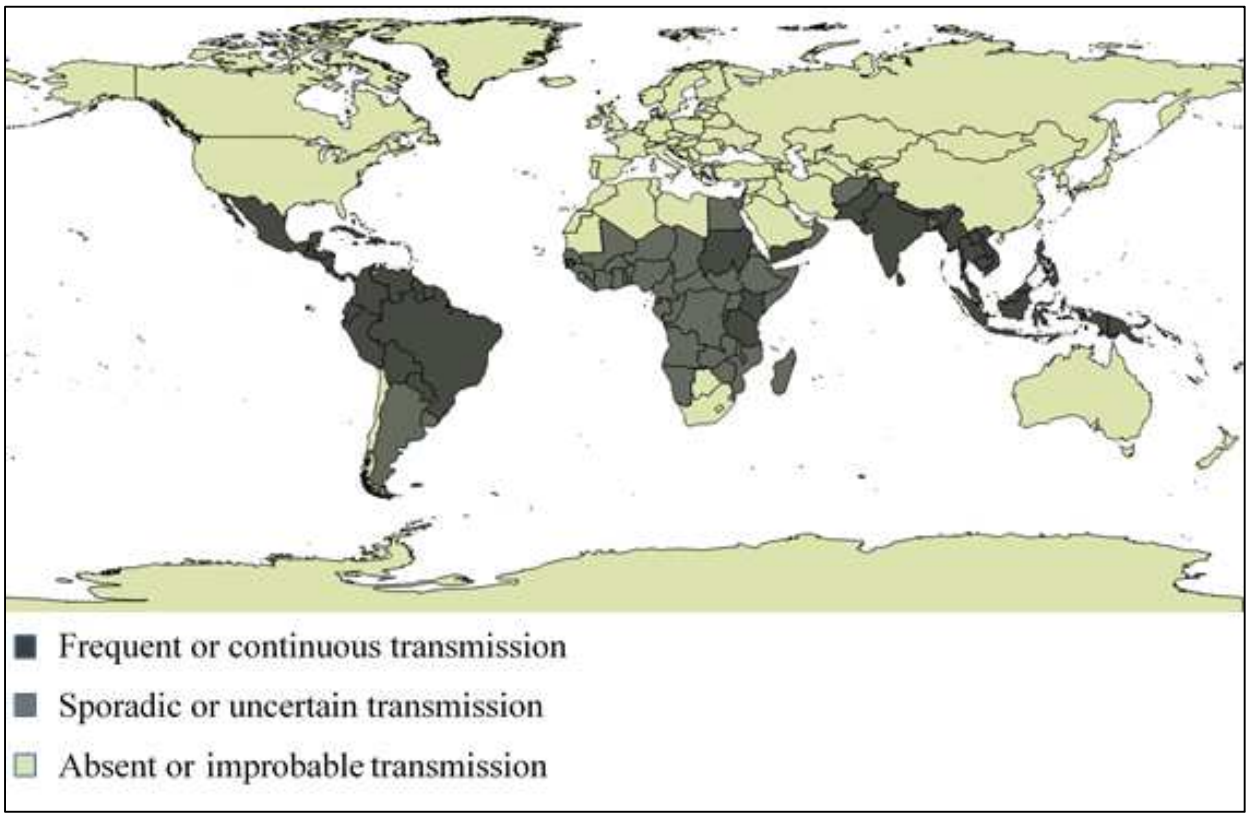
Data source: World Health Organization, 2012a.

Although dengue is not being considered as one of the main public health concerns in Africa, the estimated incidence of the disease is almost equivalent to that in the Americas. Africa accounts for 16% of estimated global incident dengue cases, which corresponds to an average of 16 million infections, ranging from 11 million to 22 million (Bhatt et al., 2013). However, in the African continent, deficient surveillance and low reporting impair the evaluation of dengue impact in the region (World Health Organization, 2009). The simultaneous occurrence of diseases with dengue-like signs and symptoms masks the perception of dengue infections, impairing the recognition of epidemics, such as that which occurred in Angola in 2013. There, more than 70% of dengue cases were diagnosed and treated erroneously as malaria (Sharp et al., 2015). In Africa, several areas present co-circulation of three dengue virus serotypes, and few areas reported all four serotypes (Messina et al., 2014). Apparently, dengue outbreaks have been increasing on the continent, both in frequency and in magnitude, mainly caused by DENV-2 circulation. Dengue transmission was considered endemic in 34 African countries in 2013 (Murray et al., 2013).

Another important area of dengue occurrence is the Western Pacific region, which presents a hyperendemicity caused by the co-circulation of all four dengue virus serotypes. In this region, the

numbers of reported cases have been increasing over the last decade. French Polynesia, New Caledonia, Vanuatu, and Australia are the countries that register most dengue cases in the Western Pacific (Murray et al., 2013). Countries of Oceania account for less than 0.2% of the estimated global number of infections, with an average of 0.18 million apparent cases and 0.55 million asymptomatic cases per year (Bhatt et al., 2013).

Figure 1.2. Dengue transmission in world by country in 2013



Data source: Center for Disease Control and Prevention (www.cdc.gov/dengue).

Other world regions are less prominent in the epidemiological scenario of dengue, but deserve attention due to recent evolutions. In the Eastern Mediterranean region, dengue is considered an emerging disease, and in the last decades Saudi Arabia, Sudan, Yemen and Pakistan registered outbreaks of this disease (World Health Organization, 2009; Murray et al., 2013). In Europe, the Madeira Island registered a dengue outbreak in 2012. Croatia and France reported autochthonous transmissions in 2010. In some European regions, the mosquito *Aedes (Stegomyia) albopictus* has been established as a potential vector for autochthonous transmissions since the 1990's (Murray et al., 2013).

1.8 Dengue in Brazil

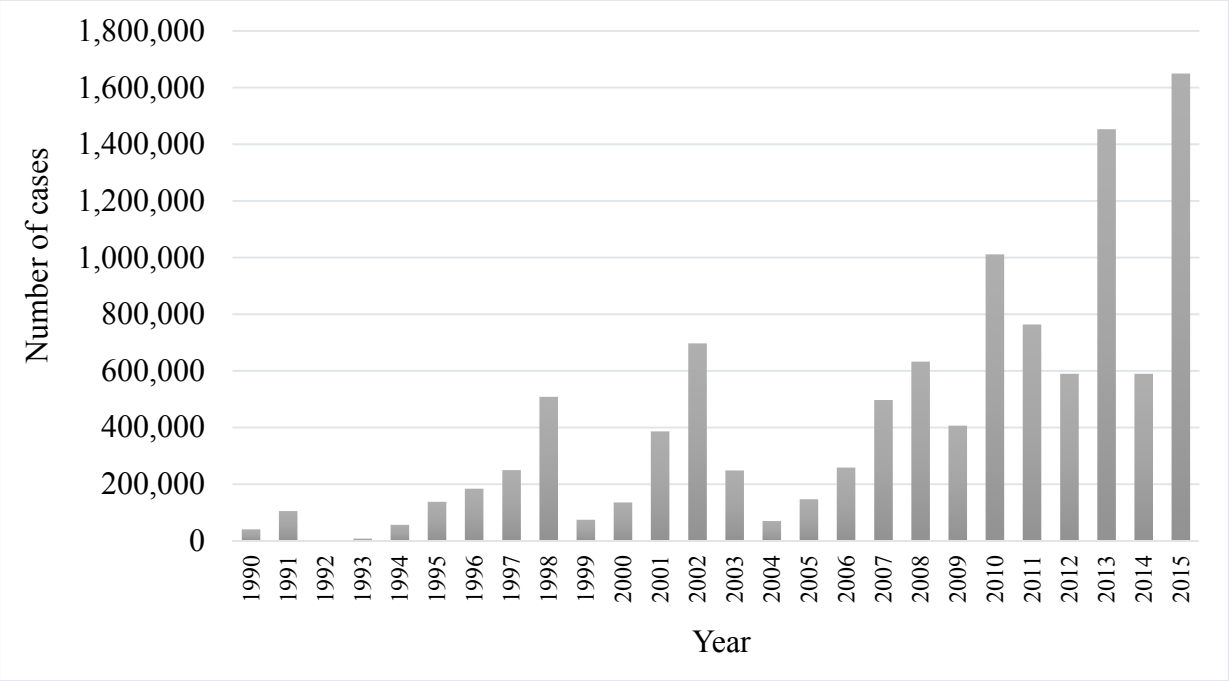
In the 1950's, a part of the population of the Brazilian Amazon region presented serological evidence of previous dengue infections (Barreto and Teixeira, 2008). In the following two decades, Brazil did not register any autochthonous dengue infections (Rodriguez-Barraquer et al., 2011). However, dengue has become an increasing public health concern in Brazil since the re-introduction of the virus in the country. In 1982, cases of DENV-1 and DENV-4 were detected in Roraima state, Northern region. In contrast to DENV-4, DENV-1 spread to other regions in Brazil in the following years. Hemorrhagic cases were not registered at that period due to the low endemicity of the disease, with circulation of only one serotype, but also because of the probable lack of its recognition (Barreto and Teixeira, 2008). However, in 1990, the first hemorrhagic cases and deaths related to dengue were reported in the country, mainly caused by the ongoing distribution of DENV-1 and the introduction of DENV-2 (Guzman and Kouri, 2003; Barreto and Teixeira, 2008). After the introduction of DENV-3 in 2001, the incidence of dengue in general and of hemorrhagic forms in particular increased (Barreto and Teixeira, 2008). In 2010, DENV-4 circulation was detected again in Brazil, triggering a national epidemic in 2013 (Brazilian Ministry of Health, 2015a).

The scenario observed in Brazil has characteristics of hyperendemicity, with co-circulation of four dengue virus serotypes. Dengue is largely distributed in the Brazilian territory, where almost 80% of the municipalities (n = 4,318) have the vector *Aedes (Stegomyia) aegypti* established. In Brazil, *Aedes (Stegomyia) aegypti* is the only vector with epidemiological importance, since so far *Aedes (Stegomyia) albopictus* has not been related to local dengue transmission, despite being present in more than 2,000 municipalities. As dengue is recognized as a disease of urban centers, it is favored in Brazil, where more than 80% of the population lives in urban areas. However, with the expansion of *Aedes (Stegomyia) aegypti* across its territory, an increasing incidence has been observed also in municipalities with lower population densities (Brazilian Ministry of Health, 2015a).

Dengue transmission in Brazil occurs in cycles of endemic and epidemic characteristics. Normally, epidemics occur in two consecutive years, being followed by a period of variable duration with lower incidence. Increasing incidence normally is a consequence of the introduction of a new dengue virus serotype or the substitution of the predominant serotype by a new one. This alternation or substitution of the predominant serotype is common and important to the

epidemiological scenario of dengue in an endemic area. The reduction in the incidence after an epidemic is related to the depletion of the susceptible population to the circulating serotype, or in other words, to the increase in herd immunity in a given population. The seasonal characteristic of dengue is evident in Brazil, where the evolution of epidemics usually occurs within a short period and the majority of cases are registered between October and May, the warmer and rainier periods in Brazil (Brazilian Ministry of Health, 2015a).

Figure 1.3. Total of dengue cases reported in Brazil, from 1990 to 2015

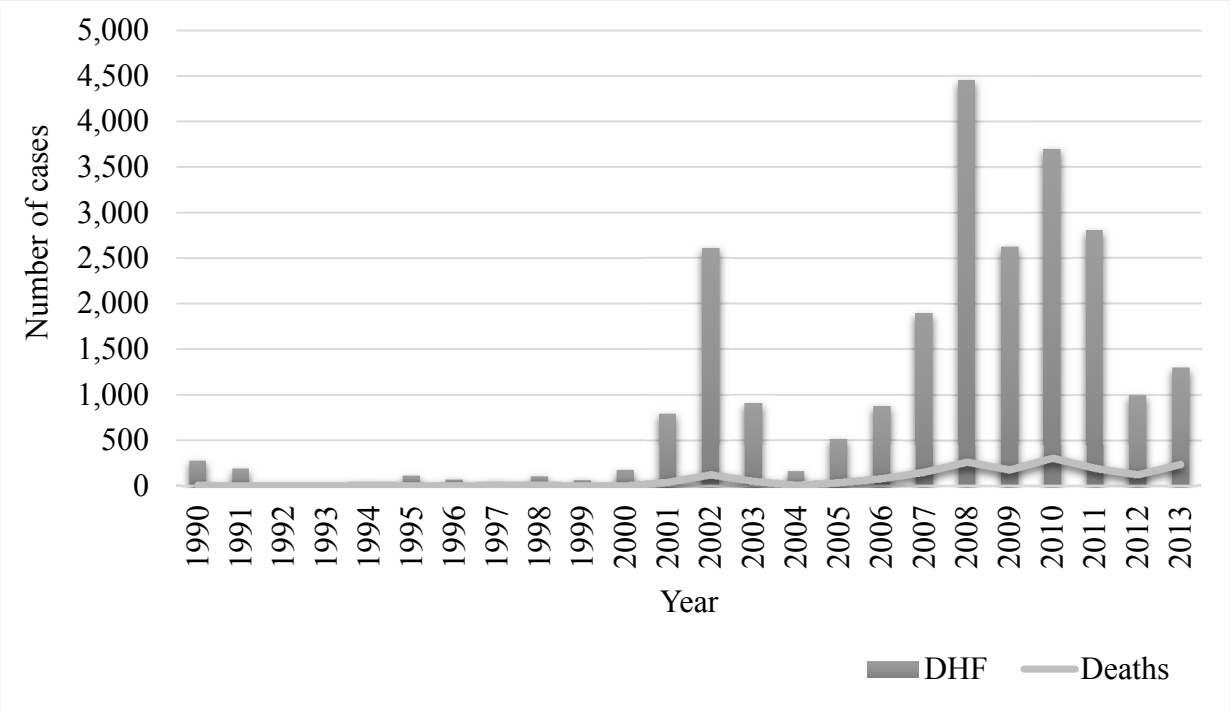


Data source: Brazilian Ministry of Health, 2015b.

In the beginning of the re-emergence of dengue in Brazil, the majority of the cases occurred in adults, which was the group more affected by the hemorrhagic forms of the disease. However, since 2006, a new trend has been observed, with increasing incidence of dengue, including hemorrhagic cases, in children. Nowadays, dengue in Brazil is a disease affecting all age groups (Brazilian Ministry of Health, 2015a). Because of the continuous presence of dengue transmission for more than three decades, the probability to be infected by dengue virus in Brazil is high and increases even more in older individuals, due to the longer time of exposure. This reality possibly will lead to a progressive reduction in the age for primary and sequential infections, increasing the proportion of severe dengue cases (Rodriguez-Barraquer et al., 2011). It may also increase the likelihood of critical epidemics, such as that which occurred in Rio de Janeiro state in 2009. In this state, in three months of the cited year, more than 9,000 hospitalizations and 230 deaths were

registered, mainly involving children. In Rio de Janeiro city, military support for health care and vector control was required in order to minimize the impact of the dengue epidemic (Barreto and Teixeira, 2008).

Figure 1.4. Total of cases of dengue hemorrhagic fever and deaths caused by dengue infection reported in Brazil, from 1990 to 2013



Data source: Brazilian Ministry of Health, 2015b.

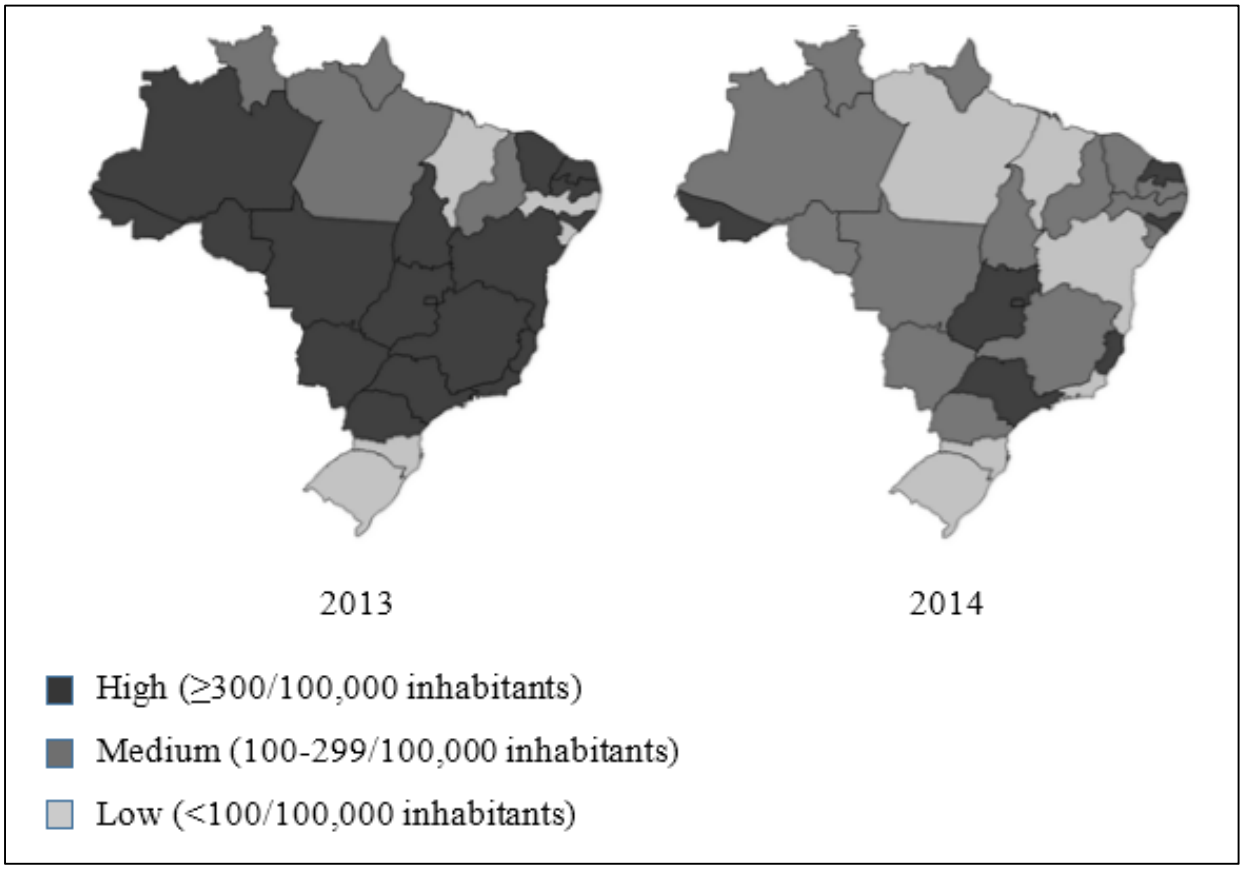
In recent decades, Brazil became the country with the highest numbers of reported dengue cases in the world, being recognized as the prime example of a worsening dengue situation across the globe (World Health Organization, 2012a). In 2015, the country registered the highest epidemic of its history, with more than 1.5 million cases reported, an incidence rate of 820 cases per 100,000 inhabitants, resulting in 863 deaths. In this year, more than 70% of dengue cases reported in the Americas were from Brazil (Pan American Health Organization, 2015). In the region, Brazil plays a role as a center of viral diversity of dengue virus, participating in gene flow with distant locations, and serving as a source of viral populations to other countries, especially those located along its borders (Allicock et al., 2012).

In a scenario of hyperendemicity, Brazil has been experiencing since the beginning of the 21st century an increasing occurrence of dengue, dengue hemorrhagic fever and deaths related to the

disease (Brazilian Ministry of Health, 2015a). This observation reinforces the importance of the country in the American region and in the world to the maintenance and amplification of dengue, making local public health efforts especially necessary for dengue control.

Figure 1.5 shows two maps comparing a year with high dengue incidence in Brazil followed by a year with medium incidence. In 2013, the incidence of dengue in Brazil was 722.4 cases per 100,000 inhabitants, while in 2014 the value of this indicator decreased to 290.5 cases per 100,000 inhabitants. The recent circulation of DENV-4 at that period contributed to the increase of dengue occurrence in Brazil in 2013.

Figure 1.5. Dengue incidence in Brazilian states in 2013 and 2014



Data source: Brazilian Ministry of Health, 2015b.

1.9 Dengue in non-endemic countries

Temperatures ranging lower than 10°C almost completely preclude the existence of dengue vectors in many territories in the world, but especially on the European continent (Schaffner and Marthis,

2014). However, expansion in the amount of international travel has been contributing to increased registrations of imported dengue cases in these non-endemic regions; and it seems that even with a higher detection of dengue in travelers, these registers are underestimated (Allwinn et al., 2008).

A study on dengue seroprevalence in travelers revealed a prevalence of 8.7% to 19.5%. Even considering the cross-reactivity observed in people vaccinated against yellow fever or other *Flaviviruses*, this proportion is considered high. The increasing numbers of dengue cases in returning travelers is corroborated by both the Robert Koch Institute in Berlin, Germany, and by the EuroTravNet in all of Europe (Allwinn et al., 2008; Schlagenhauf et al., 2015).

Southeast Asia is involved in approximately 60% of dengue infections acquired by European returning travelers (Allwinn et al., 2008). Travelling to Southeast Asia during the summer period in the Northern hemisphere increases the chances of dengue acquisition by travelers. Therefore, there are more registrations of imported dengue in Europe from August to October. This represents a risk for local dengue transmission, since the vector *Aedes (Stegomyia) albopictus* is present in at least 15 countries of the European region, and the majority of imported cases occur in the period with higher, therefore favorable, temperatures. Eggs of *Aedes (Stegomyia) albopictus* are able to resist sub-freezing temperatures, which could contribute to a future dengue emergence in temperate countries (World Health Organization, 2011).

International air travels have been increasing in Europe, including those to dengue endemic countries. From 2010 to 2013, 39% more European travelers visited areas under dengue transmission. Detection of viremic cases at airport borders is difficult due to the period of incubation, and due to the fact that most of the infected people have to be expected to be asymptomatic. Therefore, surveillance for rapid detection and response in order to avoid local dengue transmission is important yet challenging (Semenza et al., 2014). Travelers should be advised about protection against mosquito bites prior to trips to dengue endemic areas. Although rare, there are records of deaths caused by dengue in people returning from travels (Allwinn et al., 2008).

The importation of *Aedes (Stegomyia) aegypti*, especially in port areas, is also a concern for a future dengue re-emergence in Europe. Until 1950, this vector was established in many Mediterranean countries and was associated with epidemics in Greece and Turkey, which accounted for more than a million dengue infections and a thousand deaths (Schaffner and Mathis,

2014). In 2012, the spread of *Aedes (Stegomyia) aegypti* in Madeira Island, Portugal, which probably reached this European territory in tires transported in ships, caused an outbreak with more than 2,000 autochthonous cases, due to the introduction of DENV-1 from South America. This outbreak had repercussions in other European countries, which received a total of 82 travelers with dengue infections acquired on the island. The self-limited nature of this outbreak, which was decreasing in magnitude until its resolution in November, was probably more related to the lower environmental temperature than to human actions taken in that period for the disease control (Lourenço and Recker, 2014).

1.10 Economic and social impacts of dengue

Dengue has been imposing a high social and economic impact in regions where it is endemic. Investments are necessary in health care, in vector control programs and in other preventive actions. Absences in workplaces or in schools are other consequences of this disease, resulting in loss of productivity. In Southeast Asia, the region in the world that accounts for most incident cases, annual expenditure for dengue was approximately 950 million United States Dollars, with a burden of 214,000 disability-adjusted life years (Shepard et al., 2013). Although the Americas present lower dengue incidence and burden than Southeast Asia, the expenditures for dengue are higher in the American continent. Here, the annual costs induced by dengue illness from 2000 to 2007 were approximately 2.1 billion United States Dollars, and the estimated burden was 72,772 disability-adjusted life years. In the region, Brazil was the country with the highest economic and health burden impact, spending approximately 878 million United States Dollars annually, with a burden of 26,492 disability-adjusted life years (Shepard et al., 2011). In 2015, the budget for the National Program for Dengue Prevention and Control in Brazil was 3.5 million United States Dollars, without including costs for surveillance activities (Brazilian Ministry of Health, 2015c).

1.11 Future epidemiological perspectives for dengue

While dengue control continues to be limited by the management of its vector, it may be expected that the disease will maintain a territorial expansion, and may even increase in incidence around the world. In the era of globalization, fast and far-ranging human mobility favors the spread of dengue. A traveler in his viremic phase can carry the dengue virus to non-endemic areas or

introduce new serotypes and genotypes in endemic areas, contributing to the occurrence of outbreaks, since the local population may be highly susceptible to the newly introduced agent (Murray et al., 2013). Dengue is already considered a disease that can cause emergencies of international concern (World Health Organization, 2012a). Increasing international trade also contributes to the introduction of dengue vectors into new areas, enabling its autochthonous transmission (Lourenço and Recker, 2014).

Urbanization has been increasing in developing countries where dengue is an endemic disease, creating a propitious environment for mosquito breeding in close proximity to residential areas with high human population densities. In only three years, from 2011 to 2013, projections predicted an increase of 56% in rural-urban migration in dengue-endemic regions (World Health Organization, 2011). The disordered occupation of territory in these cities, with the creation of clusters without infrastructure and with poor socioeconomic conditions, has the potential to deteriorate even more the epidemiological situation of dengue in these locations. In some countries, an increasing dengue occurrence in rural areas has also been observed, due to the dispersion of the mosquito to these regions (Murray et al., 2013).

Climate change is another factor discussed in the future perspective of the dengue epidemiological scenario, having potential to influence the territorial expansion of this disease and to increase the viral transmission rates in current endemic areas. In 2100, the global temperature increase is estimated to be at least 2°C, but it may reach 4.5°C in some predictions, depending on continued use of fossil fuels and other greenhouse gas producing amenities. An increase of 2°C is sufficient to accelerate substantially the mosquito life cycle and to affect the feeding behavior, improving the vectorial capacity. If global warming will follow these predictions, in 2085 six billion people or 60% of the projected global population may live in areas under risk for dengue transmission. However, even without such an increase in temperature, 35% of the world's population is estimated to be living under this risk for the period (World Health Organization, 2011).

1.12 Public health policies on dengue

Efforts for dengue control have been prioritized by international and national health organizations. Dengue is listed among the 17 neglected tropical diseases defined by the World Health Organization. Since 2005, dengue is included in the International Health Regulations as a disease

that constitutes a health concern for the international community due to its capacity to trigger epidemics, inclusive of its potential for spreading to bordering countries (World Health Organization, 2012a).

The Global Strategy for Dengue Prevention and Control has established goals and actions on a global scale to be adopted in different countries of the world. This strategy intends to reduce dengue mortality by at least 50% and its morbidity by at least 25% by 2020, considering as references the situation in the years 2009 and 2010. In order to support these efforts, the World Health Organization has declared that in all dengue endemic regions, a dengue control plan and surveillance system should be instituted by 2020 (World Health Organization, 2012c).

In the Americas, the activities implementing the Integrated Management Strategy for Dengue Prevention and Control, and the Global Strategy for Dengue Prevention and Control have the objective to develop technical cooperation to deal with the disease. These actions aim to improve different areas of dengue management, such as surveillance, early diagnostics and therapy, and preparedness for and control of outbreaks. The American countries stated as one goal to reduce dengue mortality by least 30% by 2019, as compared to the 2014 rate (Pan American Health Organization, 2013).

1.13 Strategies for evaluating the epidemiology of dengue

In order to define effective policies for dengue control it is necessary to understand the determinants and the distribution of the disease in the population, which are factors investigated in epidemiology. The determinants are factors that influence the occurrence of dengue in its different clinical forms. The distribution of the disease is evaluated in terms of frequency and pattern. Classical study designs can be applied for these evaluations, whether character descriptive or analytical. Observational epidemiological studies are particularly important in order to understand the pattern of dengue occurrence in a population and the association of different factors with the outcome of the disease, by using different approaches, such as cross-sectional, case-control or cohort designs (Center for Disease Control and Prevention, 2012).

In addition to the classical studies on dengue occurrence in the population, other methods have been applied to better understand the epidemiology of dengue, such as spatial analysis, using

Geographical Information Systems, and molecular phylogenetic, using techniques of molecular biology.

Spatial analysis is a method used to measure characteristics and relations of a phenomenon with regard to its spatial location. The use of this method has become more frequent in different areas due to the availability of new technologies, such as Geographical Information Systems, which permit a computational treatment of geographic data. In Epidemiology, John Snow conducted a pioneer study applying spatial analysis in the 19th century, analyzing an outbreak of cholera that occurred in London in 1854. Despite the absence of knowledge about the etiological agent and pathways of cholera transmission, the mapping of deaths caused by this disease permitted detect a concentration of cases in proximity to a water pump. Therefore, the consumption of water from this pump was related to the occurrence of the disease, permitting the application of preventive actions. The use of spatial analysis in the field of health is especially important in the evaluation of environmental factors and the distribution of diseases, providing a better visualization of sources of exposure linked to the emergence of different health conditions. In Brazil, the use of spatial analysis in the health area is mainly applied in the evaluation of infectious diseases, including those transmitted by vectors (Rojas et al., 1999). Surveillance services use spatial analysis to evaluate the distribution of dengue cases and of mosquito breeding sites, showing that this method is important for defining preventive actions aimed at dengue control.

Since the 1950's, when the scientists Fred Sanger and Francis Crick noticed that it was possible to compare gene sequences in order to infer the relationship among specimens, a revolution started in the biological sciences. This observation founded the basis of what today is called molecular phylogenetics, the area of science aimed to infer historical relationships between individuals through comparison of macromolecular sequences (Pace et al., 2012). Gene mutations are the primary cause of evolution, and the results of nucleotides insertion, elimination, substitution, recombination, or conversion, can be inherited and become established in a population due to genetic drift or natural selection, with the new character generated by the mutations being fixed in the species (Forattini, 2005).

Molecular phylogenetics became crucial in order to clarify several aspects of human diseases, and has been playing an important role in the understanding of dengue, especially in the past 25 years. The classification of the different strains of dengue virus serotypes into subgroups of genotypes is supported by molecular phylogenetics (Castrillón, 2004). The idea that investigators can track back

relationships based on shared features of individual viral strains opened space for testing different hypotheses and scenarios. The molecular phylogenetic approach allows for the study of geographic, temporal and molecular characteristics of dengue along its evolution, given the opportunity of interpretation in a graphical scheme, such as a phylogenetic tree. The visualization of the relationship between strains of different geographical areas and the option to track dispersion routes permits, for example, a faster response through surveillance services. The possibility to measure the rate of molecular evolution using molecular clocks allows researchers to infer the speed of the emergence of local outbreaks and the relative influence of different epidemics in distinct parts of the globe, enabling prediction of the spread over time of new epidemics around the world, and setting the stage for an integrated global surveillance. In addition, the phylogenetic approach has improved the understanding of dynamics and sizes of dengue virus populations at different stages of infection and transmission. This methodology has also enhanced the capacity to understand viral pathogenesis (Weaver and Vasilakis, 2009). Knowledge about the strains circulating is also important for vaccine implementation and for the development of diagnostic tests (Nogueira et al., 2000).

Different strategies can be used to evaluate the epidemiology of dengue, providing valuable information for the health sector in order to better control the transmission of the disease and to provide adequate care in vulnerable scenarios for emergence of severe outcomes.

2 Rationale and objectives

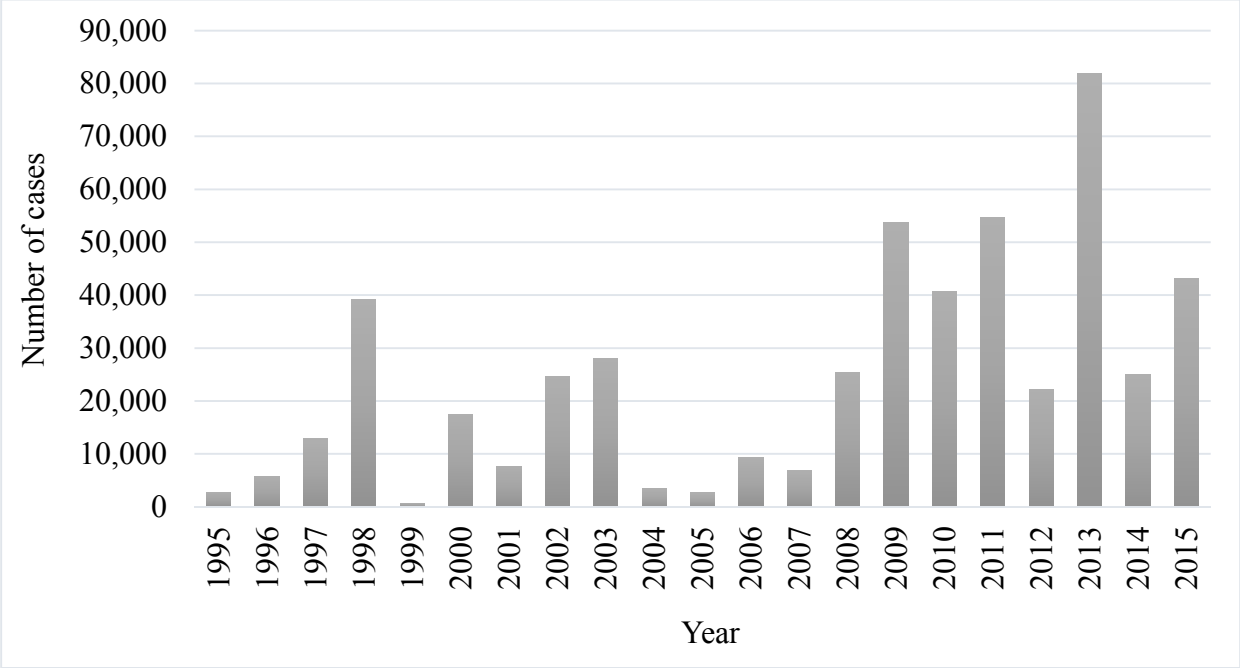
Dengue has been reported in Espírito Santo state, Brazil, since 1995, when the circulation of DENV-1 was detected. However, the mosquito *Aedes (Stegomyia) aegypti* has been present in this area since at least 1990, when it was found in 16 municipalities. Presently, this vector has infested almost all cities in the state, except one, resulting in 78 municipalities experiencing *Aedes (Stegomyia) aegypti* infestation (Health Department of Espírito Santo State, 2014). After DENV-1, all four dengue virus serotypes were introduced progressively in the state. In 1997, DENV-2 was detected in this territory; in 2002 the circulation of DENV-3 was determined, followed by the detection of DENV-4 in 2012.

The three main epidemics occurring in the state were in 2009, 2011 and 2013. In 2009, the recent circulation of the DENV-2 American/Asian genotype (Dettogni and Louro, 2012) triggered an increase in the incidence of dengue. In this year, 53,708 dengue cases were reported to the health department of the state (Health Department of Espírito Santo State, 2016). An association of the DENV-2 American/Asian genotype and dengue severity was found previously in the Americas (Rico-Hesse, 2003). Consequently, 2009 was the year that most accounted registrations of dengue hemorrhagic fever in Espírito Santo state, with 276 cases. In the period, 22 deaths were reported and 3,406 hospitalizations were registered (Brazilian Ministry of Health, 2016b).

In 2011, 54,648 dengue cases were reported in Espírito Santo state, with the new circulation of DENV-1 (Health Department of Espírito Santo State, 2016). In this year, 3,421 hospitalizations were registered (Brazilian Ministry of Health, 2016b). However, hemorrhagic cases in 2011 were less frequent than in 2009, totaling 192 registers, as well as five recorded deaths (Brazilian Ministry of Health, 2015b).

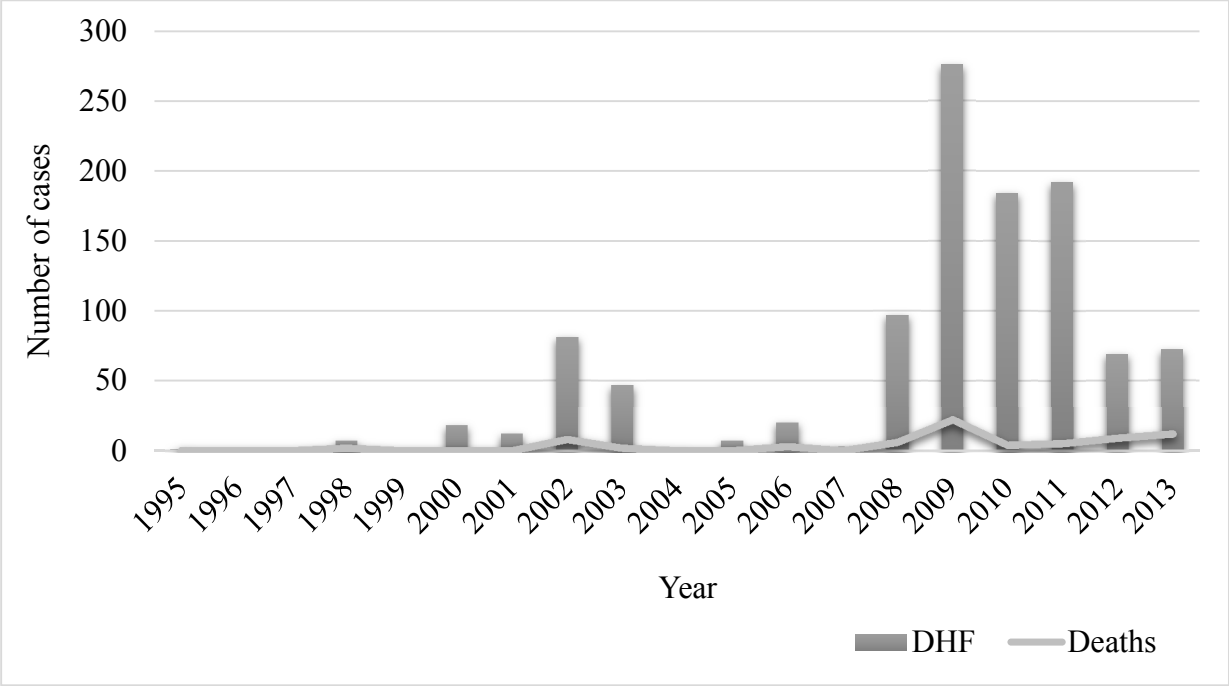
In 2013, Espírito Santo state had the highest registration of dengue in its history, with 81,892 cases recorded, due to the spread of DENV-4 after the introduction of this serotype in the previous year (Health Department of Espírito Santo State, 2016). However, the number of hospitalizations was lower than in 2009 and 2011, accounting for 1,595 registers (Brazilian Ministry of Health, 2016b), as well as the registration of dengue hemorrhagic fever, with a total of 74 cases. Deaths increased in 2013 in comparison to 2011, amounting to 12 cases (Brazilian Ministry of Health, 2015b).

Figure 2.1. Total number of dengue cases reported annually in Espírito Santo state, Brazil, from 1995 to 2015



Data source: Brazilian Ministry of Health, 2015b (1995-2008) and Health Department of Espírito Santo state, 2016 (2009-2015).

Figure 2.2. Total number of cases of dengue hemorrhagic fever and deaths reported annually in Espírito Santo state, Brazil, from 1995 to 2013

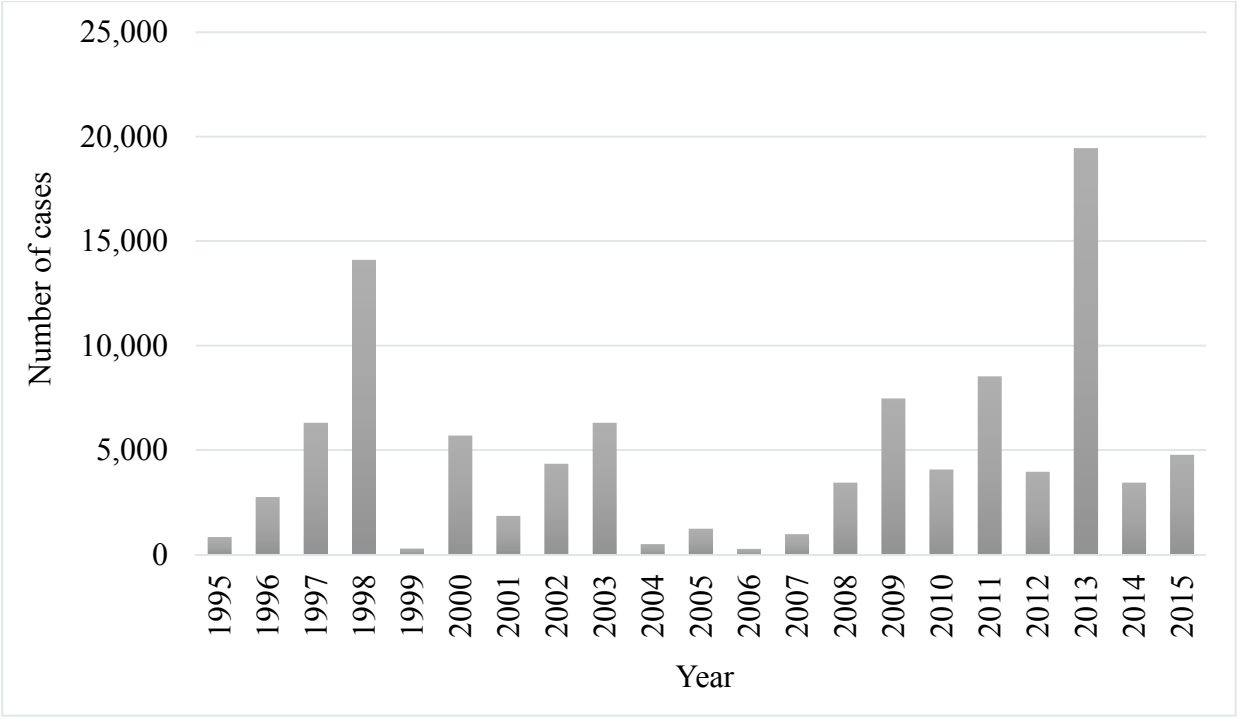


Data source: Brazilian Ministry of Health, 2015b.

Vitória, capital of Espírito Santo state, was the first city that reported dengue in the region, in 1995. Since then, dengue has become an important public health concern in the municipality. Among the diseases with mandatory reporting, dengue corresponds to more than 55% of these notifications every year (Health Department of Vitória Municipality, 2009).

The introduction and recirculation of dengue virus serotypes in Vitória is similar to that observed in Espírito Santo state. Accordingly, the number of reported cases in the city follows the pattern observed in the state. A record of reports was registered in 2013, accounting for 19,449 suspected dengue cases due to the recent introduction of the DENV-4. However, hospitalizations were more frequent in 2009, totaling 831, in a period of DENV-2 circulation (Brazilian Ministry of Health, 2016b). In this year, four dengue cases resulted in deaths (Brazilian Ministry of Health, 2015b).

Figure 2.3. Total number of dengue cases reported annually in Vitória, Espírito Santo state, Brazil, from 1995 to 2015



Data source: Health Department of Vitória Municipality.

The trend in the city shows an increasing incidence of dengue in children, with a corresponding increment in the number of severe cases in this age group (Cardoso et al., 2011), possibly leading to an alarming future of dengue epidemiological scenario in Vitória. Research on dengue in this setting is therefore important for the public health sector to take actions for the disease control.

2. Rationale and Objectives

Considering the absence of studies evaluating the dynamics of introduction and dispersion of dengue in the city, and the factors contributing to the emergence of severe cases in the recent epidemics occurring in Vitória, the present studies focus on the following objectives:

- Evaluate the origin of DENV-4 circulating in Vitória in 2013.

- Assess the patterns of dengue spread in Vitória in an epidemic after the first report of DENV-4.

- Generate hypotheses relating local factors to dengue dispersion during the epidemic of 2012 and 2013, such as House Index, population density and income.

- Evaluate the relation of the dengue virus serotypes circulating in the city and the emergence of severe cases.

- Evaluate the relation of gender and age with severe outcomes in the course of dengue.

3 Methods

3.1 Study area

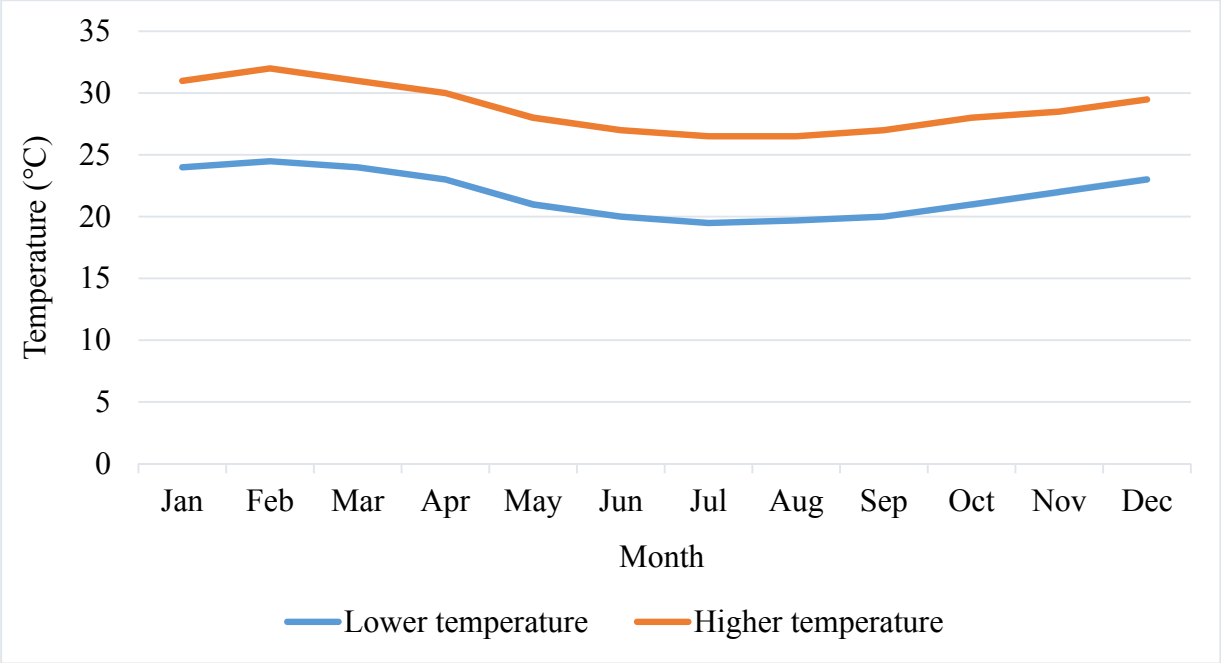
Vitória is the capital of Espírito Santo state, which is located in the coast of Southeast region in Brazil. The area of Espírito Santo state is approximately 46,097 km^2, bordering to the North the Bahia state, to the South the Rio de Janeiro state, to the West the Minas Gerais state and to the East the Atlantic Ocean. The population of the state was comprised of 3,514,952 individuals according to the Census conducted in 2010, which translates into a population density of 76 inhabitants/km^2. However, about 46.5% of the state population live concentrated in the metropolitan region of *Grande Vitória*, which comprises seven cities, with a much higher population density of 617 inhabitants/km^2 (Brazilian Institute of Geography and Statistics, 2010).

Vitória is located in the center of the metropolitan region, comprising approximately 98.2 km^2. Part of the city's territory is located on an island and the other part on the mainland. According to the Census conducted in 2010, its population comprised 319,163 inhabitants, with a demographic density of about 3,338 inhabitants/km^2 (Brazilian Institute of Geography and Statistics, 2010).

The climate in Vitória is characterized as tropical humid, with an average annual temperature of 24.2°C, higher average annual temperature of 28.5°C, and lower average annual temperature of 21.5°C. The average annual precipitation in the city is 1,153 mm (Brazilian National Institute of Meteorology, 2010; Institute of Research, Technical Assistance and Rural Extension of Espírito Santo State, 2015). In Vitória, the rainy and warm seasons of spring and summer are between October and March, when an increase in incidence of dengue can be noted.

Vitória is the Brazilian capital with the highest Gross Domestic Product per capita, corresponding to 85,794.00 *Reais* (21,448.5 Euros, 1.00 *Real* = 4.00 Euros) in 2011, and was in the fourth position of the Brazilian municipalities with highest Human Development Index in 2010, with a value of 0.845. However, the city presents a high level of inequity, with a Gini Index of 0.612 in 2010. The life expectancy in Vitória was 76 years in 2010 (Brazilian Institute of Geography and Statistics, 2010).

Figure 3.1. Higher and lower average temperatures registered in Vitória per month



Data source: Institute of Research, Technical Assistance and Rural Extension of Espírito Santo state, 2015.

3.2 Data collection

The secondary data used in the present studies was retrieved from the health services located in the city of Vitória, which are part of the municipal surveillance system with mandatory reporting of suspected dengue cases attending their facilities, both in the public and the private sector. Specific and standardized forms are used for this purpose, containing demographic, clinical and laboratory information. Physicians or other health professionals, such as nurses, fill in these forms. Dengue cases with severe manifestations must be reported within 24 hours. These reports are digitalized and stored in the System for Notifiable Diseases by the epidemiological surveillance service. At the same time, the epidemiological surveillance service performs the follow up on dengue cases, contacting them or the medical staff involved in the direct medical management of the cases. The System for Notifiable Diseases represents the data source of the evaluations on spatial variation in temporal trends of dengue cases that occurred in the epidemic of 2012 and 2013 in Vitória, and of the analysis on the relation of dengue virus serotypes (data available from 2009 to 2013), gender and age (data available from 2007 to 2013) with severe dengue outcomes. The

database was accessed through the epidemiological surveillance service at the Health Department of Vitória municipality.

The Brazilian Constitution that has been enacted in 1989 includes the access to health services as a fundamental right of all Brazilian citizens. Accordingly, in the federalized model of the National State organization, the decentralization of decision making and organization of the health services was adopted in Brazil. Therefore, despite most of the financing of public health services in Brazil being the responsibility of the central federal government, the municipalities are the main actors in the local health decisions. Within the *Sistema Único de Saúde*, instituted in 1992, the private health sector acts as a complement to the public health sector and is supervised by government agencies (Matta and Pontes, 2007). In Vitória, the *Sistema Único de Saúde* provides primary health care in 28 public health units and emergency care in two health centers. The laboratory detection of dengue virus serotypes is also a part of the epidemiological surveillance services as provided by the Health Department of Vitória. Thereby, laboratory tests are performed in local public laboratories. The laboratory confirmation by virological or immunological assays in suspected dengue cases in Vitória surpasses 10% of the total number of cases that are notified, in accordance with the recommendations of the Brazilian Ministry of Health. Vitória ranks number one in performance of the public health systems within Brazil.

Blood collection performed in order to conduct the phylogenetic evaluation of the DENV-4 circulation in Vitória was carried out in health units and emergency services of the public health sector of the municipality, from March 2013 to March 2014. The procedure of blood taking was performed by the local laboratory staff, after the researchers had explained the characteristics and objectives of the investigation to the patient, who then needed to voluntarily sign a consent form in order to be included into the study.

3.3 Definition of terms

Phylogenetic cluster is the term that defines a conglomerate of taxonomic units.

In the study on spatial variation in temporal trends of dengue cases in the epidemic of 2012 and 2013, a dengue case was defined as an individual with an acute febrile illness reported as a clinically suspected dengue case.

The localization of a dengue case was defined as the place of residence of the patient as described in the report form.

The average income in a given space-time cluster was defined as the average of monthly work payments received by all inhabitants of the corresponding space-time cluster, expressed in the Brazilian currency, *Reais*.

Subnormal agglomerate is the technical term used to define a place popularly known as *"favela"*. It contains a minimum of 51 houses grouped together, without title of property, with irregular provision of streets, unconventional form of the lots and inadequacy of services, such as garbage collection, water supply, electricity, sewage system and public illumination (Brazilian Institute of Geography and Statistics, 2010).

Verticalized areas are regions where most of the residential buildings have more than three floors, including ground floor.

Population density is the relation of the total number of residents living in a space-time cluster, divided by the area of the space-time cluster described in square kilometers (inhabitants/km^2).

House Index is an indicator used to assess the level of presence of *Aedes (Stegomyia) aegypti* in an area, evaluating houses randomly selected in order to search larvae presence. Therefore, it is calculated by dividing the number of buildings with larvae by the number of buildings surveyed. The House Index is expressed in percent (Focks, 2003; Brazilian Ministry of Health, 2012). In order to perform the survey, the districts are grouped in strata. Each stratum groups different districts with similarities in social and environmental characteristics. The present study assumed that the value of the House Index in a certain district was similar to the House Index in the strata that the district was included during the survey. The House Index of each space-time cluster was calculated as the median of the values of the House Indexes in the districts that formed the space-time cluster. The House Index is interpreted as a risk for dengue transmission, being low when the value is lower than 1%, medium when the value ranges from 1% to 3.9% and high when it reaches 4% or more (Brazilian Ministry of Health, 2012). The sampling for calculating the House Index is performed at least three times per year. In Vitória, during the period included in the study, the sampling was performed in October 2012 and March 2013.

Time Trend Increase is defined as the relative increase of dengue incidence inside and outside the space-time cluster over time (from September 2012 to June 2013), considering a log linear scale with a percent increase constant over time.

Space-time cluster is defined as a spatial conglomerate of dengue cases, where the Time Trend Increase inside is different from the Time Trend Increase outside the space-time cluster. The method to define a space-time cluster is described in the section 3.5.

Relative Risk is the estimated risk for acquiring dengue infection inside the space-time cluster divided by the estimated risk outside the space-time cluster, and represents the odds of acquiring dengue for people living in each space-time cluster as compared to the general population around the space-time cluster. Relative Risk is calculated according to the following formula, where n is the number of cases observed inside the space-time cluster, N is the total number of cases included in the dataset, and Ec is the expected number of cases (Kulldorff, 2015):

$$\mathrm{RR} = \frac{n/Ec(n)}{(N-n)/(Ec\,[N]-Ec[n])} = \frac{n/Ec[n]}{(N-n)/(N-Ec[n])}$$

In order to define the classification of dengue cases according to their outcome, the criteria of the Brazilian Ministry of Health used until 2013 was applied. In 2014, this classification was changed, taking into account the new approach adopted by the World Health Organization in 2009, which is based on identification of warning signs, and is therefore considered as easier to use in daily clinical practice (World Health Organization, 2009). However, the lack of information necessary to classify dengue cases that occurred before 2014 according to the new criteria ruled out the use of the new classification in the present studies. Nevertheless, the terms and criteria of the previous classification are still widely used by researchers, in turn allowing for better comparability of results.

According to the previous classification as used in the present studies, a case of dengue fever is characterized by fever with duration of up to seven days, and by presence of two or more signs and symptoms, such as headache, retro-orbital pain, myalgia, arthralgia, malaise, or rash (Brazilian Ministry of Health, 2013).

A case of severe dengue can be sub-classified into dengue hemorrhagic fever or dengue with complications (Brazilian Ministry of Health, 2013). In the classification of dengue with complications, the criteria used by the Brazilian Ministry of Health present particularities, including laboratory or clinical presentations that were not considered as severe uncommon clinical manifestations by the former classification of the World Health Organization (World Health Organization, 1997).

Cases of dengue with complications could present any severe clinical manifestation. Some of the neurological disorders that can occur are delirium, drowsiness, coma, depression, irritability, psychosis, dementia, amnesia, meningeal signs, paresis, paralysis, polyneuropathy, Reye syndrome, Guillain-Barré syndrome and encephalitis. Cardiac disorders, such as heart failure and myocarditis associated with myocardial dysfunction, reduction in ejection fraction and cardiogenic shock are also considered. Hepatic failure is another severe complication evidenced by hepatomegaly, elevated levels of hepatic enzymes and icterus. Manifestations related to plasma leakage, such as cavity effusion demonstrated by pleural effusion, pericardial effusion and ascites identified in ultrasound or radiography examinations, are also included in this definition. Other manifestations considered in these cases are thrombocytopenia equal to or less than 20,000/mm^3, gastrointestinal bleeding, total leukocyte count equal to or less than 1,000/mm^3 and death (Brazilian Ministry of Health, 2013).

Cases of dengue hemorrhagic fever need to present four characteristics simultaneously in order to be classified as such. First, all cases must have fever or recent febrile illness lasting up to seven days. Second, they must present thrombocytopenia demonstrated by platelet count equal to or less than 100,000/mm^3. Third, signs of hemorrhage must be demonstrated by at least one hemorrhagic manifestation, such as positive tourniquet test, hematuria, petechiae, menorrhagia, gingival bleeding, epistaxis, gastrointestinal bleeding or any other bleeding sites. Fourth, evidence of plasma leakage must be present, such as hemoconcentration demonstrated by at least a 20% increase in the hematocrit over the baseline at admission, or at least a 20% decrease in hematocrit after appropriate treatment, or cavity effusion or hypoproteinemia (Brazilian Ministry of Health, 2013).

Age distribution was categorized in four groups: children were defined as individuals aged one to nine years, adolescents as individuals aged 10 to 19 years, adults were people between 20 to 59 years of age and elderlies were people with an age of 60 years or above.

Death was the fatal event of which dengue infection was the basic cause, that is, dengue was the factor that initiated all pathological events that led directly to death.

3.4 Phylogenetic analysis

3.4.1 RNA extraction, cDNA amplification and sequencing

In the course of the present study, molecular analyses were conducted on 56 samples collected from patients with suspected dengue. In principle, a qualitative reverse-transcriptase-polymerase chain reaction (RT-PCR) assay has been used and was positive for dengue in 14 samples. The extraction of the viral RNA was performed by using the QIAamp RNA Blood Mini Kit™ produced by Qiagen©, following the indications of the manufacturer. Then, for complementary deoxyribonucleic acid (cDNA) synthesis, the High-Capacity cDNA Reverse Transcription Kit™ manufactured by Thermo Fisher Scientific© was used, according to the instructions provided by the manufacturer.

Two regions of the DENV-4 gene responsible for encoding the envelope protein and the NS1, which are the principal targets of the cellular immune responses as promoted by B cells against dengue virus, were amplified and sequenced (Simmons et al., 2015).

The primers used in the amplification were designed in house as follows:

For the NS1 gene: Fw-GCAATGGTTTTTGAATCTGCCTCTT, and Rv-TGTCCTGCAAACATGTGATTTCCAT, which generated an amplicon of around 1,600 base pairs.

For the envelope gene: Fw-CACGTATAAATGCCCCCTACTGGTC, and Rv-GCTGTGTTTCTGCCATCTCTTTGTC, which created a fragment of about 1,200 base pairs.

The polymerase chain reaction (PCR) had a final volume of 50 µl comprising 10 µl of template, 10X PCR Buffer, 1.5 mM of $MgCl_2$, 200 mM of dNTPs, 1 mM of each primer, and 1 U of Platinum Taq polymerase (Invitrogen©). The cycling process took seven minutes, with 45 cycles of 94ºC

for 30 seconds, 55ºC for 1 minute, and 72ºC for 1 minute. The fragments were purified using the GFX purification kit™ (GE Healthcare©) following the product instructions and sequenced using Sanger method (Sanger et al., 1977) with the same primers as of the detection reaction.

3.4.2 Model definition, tree reconstruction and molecular clock

The readings obtained in the molecular analysis were inspected for quality and for definition of consensus sequences. These consensus sequences were aligned with reference sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) in the software Clustal W©. A dataset with 93 sequences, including those obtained in the study and reference sequences of four dengue virus serotypes, was used to confirm the serotype.

A dataset with 46 sequences of the NS1 gene of different DENV-4 genotypes, including samples from Vitória and those from GenBank was created, as well as a dataset with 78 sequences of the envelope gene of distinct DENV-4 genotypes with samples from Vitória and reference sequences. Sequences from São Paulo state as a distinct geographic area were also included in these datasets, in order to evaluate the genetic variability of the DENV-4 circulating in Brazil.

Both datasets were used in the PhyML© package for the first reconstruction of the Maximum Likelihood phylogenetic trees. The envelope dataset was also used in the software BEAST v.1.5.3© in order to make phylogenetic estimates using a Bayesian Markov Chain Monte Carlo model (Drummond and Rambaut, 2007). Using the software MODELTEST©, it was determined that the best substitution model for the datasets was the GTR+I+G model (Posada and Crandall, 1998). An average rate of 7E-04 substitutions per site per year was used, based on previous estimated rates (Zanoto et al., 1996; Twiddy et al., 2003; Costa et al., 2012), to calculate the Time of the Most Recent Common Ancestor of the principal phylogenetic clusters of DENV-4 in Brazil. The Bayesian Skyline plot was performed under relaxed exponential and relaxed uncorrelated lognormal molecular clock, and the logistic, exponential, and constant models were assessed. Bayes Factor comparison was used to define the molecular clock and the model that best fit the data. Convergence of parameters in the Bayesian Markov Chain Monte Carlo was examined with Tracer v.1.4© (Drummond and Rambaut, 2007), with uncertainties of 95% Highest Probability Density intervals (95% HPD). Twenty million chains were enough for the convergence of all parameters (ESS.200). The trees were sampled at each 2,000 steps, resulting in a final file of

20,000 trees, summarized in a Maximum Clade Credibility tree using TreeAnotator (part of the BEAST© package), and visualized in FigTree v.1.2.2© (http://beast.bio.ed.ac.uk/FigTree).

3.5 Spatial variation in temporal trends analysis

In order to localize dengue cases that occurred between September 2012 and June 2013 in Vitória, the geocoding procedure was performed by using the plugin MMQGIS, which is part of the software Quantum GIS v.2.8.2-Wien™. This plugin searched the addresses of residence of dengue cases by using the website Google Maps©. The geocode was conferred in the shape map provided in the website Geoweb Vitória (www.geoweb.vitoria.es.gov.br), which was used to construct the maps presented in the study. This time period was used because it comprised the first epidemic after an unprecedented identification of DENV-4 in Vitória. Kernel maps constructed in Quantum GIS v.2.8.2-Wien™, using a circumference area of 1 km of diameter, enabled the visualization of hotspots of dengue occurrence in Vitória for each month.

The evaluation of spatial variation in temporal trends and the identification of space-time clusters were performed by using the software SaTScan™ (http://www.satscan.org/), which utilizes a scanning window. The period included in the analysis encompassed ten months. The latitude and the longitude where the cases were located was similar to the central position of the district of where they lived. The definition used in the program was that a space-time cluster had a circular shape with maximum 1 km of diameter, considering the area of the districts in Vitória. The Time Trend Increase was calculated based on the discrete Poisson probability model, and was presented as percent increase on a log linear scale along time.

In order to define a significant space-time cluster, the scan statistic uses the null hypothesis that the Time Trend Increase is equal in all areas of the scanning window, and presents the results as Log Likelihood Ratio and *P-value*. The value of the Log Likelihood Ratio increases according to the improbability of the difference found to occur due to chance. A Log Likelihood Ratio higher than 6.92 with a *P-value* lower than 0.05 indicates a significant space-time cluster, where the Time Trend Increase is significantly different from this value outside the space-time cluster. The Log Likelihood Ratio is calculated using the following formula, where N is the total number of cases, n is the number of cases inside the window, Ec[n] is the covariate adjusted expected number of

cases inside the window considering the null hypothesis, and I() is the function indicator (Kulldorff, 2015):

$$\text{LLR} = \left(\frac{N}{Ec/[n]}\right)^{n} \left(\frac{N-n}{N-Ec[n]}\right)^{N-n} I()$$

3.6 Cross-sectional analysis and data evaluated

3.6.1 Evaluation on dengue virus serotypes and dengue clinical outcomes

In order to evaluate the relation of the serotypes causing dengue infections and the clinical outcomes that were observed, a cross-sectional study was performed, which included 485 cases of dengue with information on dengue virus serotype responsible for the infections. These cases were reported in Vitória in the period from 2009 to 2013.

The 485 cases corresponded to 1.6% of all dengue cases registered in the System for Notifiable Diseases in the area during the same time period (n = 30,027). Viral isolation was used to determine the dengue virus serotype in 403 cases (83.1% of 485 cases), and was performed with the technique of inoculation in cell cultures of *Aedes (Stegomyia) albopictus* (C6/36), followed by a visualization by indirect immunofluorescence. In this method, the specific reaction of the antibodies against the four dengue virus serotypes is marked by fluorochrome (Gubler et al., 1984). The other 82 cases (16.9% of 485 cases) had the serotype determined by the RT-PCR, a technique that permits the amplification of the cDNA derived from the viral RNA using specific initiators of each dengue virus serotype (Lanciotti et al., 1992).

The laboratory tests for virus identification requires that samples be taken when the disease is in its viremic period, present in the first days of the onset of dengue clinical manifestations. Therefore, the blood collection for this analysis is indicated until three days after the emergence of febrile illness, since viral titers decrease after two days. In the course of dengue, the warning signs usually appear after three days of the onset of symptoms, with the decrease of fever. Thereby, these characteristics of dengue course minimize the chance of a selection bias due to the inclusion of

more severe cases. In addition, these tests are performed in sentinel sites and include patients systematically selected, aiming at results for surveillance purposes rather than for patient care.

The variables investigated in this analysis were gender, age, serotype, dengue outcome and clinical manifestations of severe dengue.

3.6.2 Evaluation on demographics and dengue clinical outcomes

The evaluation of the relation of gender and age with dengue clinical outcomes was performed using a cross-sectional approach, and included 6,703 dengue cases reported in Vitória from 2007 to 2013.

Two criteria were used to exclude cases: absence of specific laboratory confirmation of the dengue infection and age below 12 months. The exclusion of cases without laboratory confirmation occurred to avoid false positive dengue cases based merely on clinical suspicion, since the disease present unspecific clinical manifestations which makes it prone for confusion with other infectious febrile illnesses. The restriction regarding the age group excluded 82 cases, and was adopted because the registers in this age group presented inconsistencies, with age being reported in some cases in days, months or fractions of a year, raising doubts about correct and consistent data entry for this age group.

The cases included were confirmed by detection of the NS1 (n = 932, 13.9%), PCR (n = 107, 1.6%), IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA IgM) (n = 5,821, 86.8%), viral isolation (n = 397, 5.9%), histopathology (n = 8, 0.1%) or immunohistochemistry (n = 19, 0.3%).

For those cases with confirmation based on immunological testing, severe outcomes may have been already present at the time of sample taking, possibly favoring more severe cases here. The blood collection for performing MAC-ELISA IgM is carried out after six days of onset of symptoms, since the IgM can be detected from the third day up to 90 days in primary dengue infection, and from six to 40 days in sequential dengue infections (Brazilian Ministry of Health, 2013), and is therefore also detectable after the acute illness. Therefore, the blood collection for this test was performed during or after the emergence of warning signs. In addition, laboratory

confirmation is encouraged in cases with severe presentations and is compulsory in cases with a suspicion of dengue hemorrhagic fever (Brazilian Ministry of Health, 2013). This procedure increases the proportion of severe dengue in the study when compared to the proportion of severe dengue among all dengue cases that may have occurred in Vitória, influencing the magnitude of the effect measurement.

The variables included in the analysis were gender, age, clinical manifestations of severe dengue, dengue classification and death. Data on comorbidities and sequential infections were unavailable, impeding their evaluation.

3.7 Statistical analysis

Statistical analysis was conducted using the software R©. The confidence interval (CI) considered in the analysis was 95%, and a *P-value* lower than 0.05 was determined to define a significant difference.

3.7.1 Spatial variation in temporal trends analysis

The space-time clusters identified through the spatial variation in temporal trends analysis were divided in two groups: those with a higher Time Trend Increase than the overall population and those with a lower Time Trend Increase than the overall population. These two groups were compared in terms of population density, income, House Index in October, House Index in March, House Index difference and Relative Risk through the Mann-Whitney U-test.

3.7.2 Evaluation on dengue virus serotypes and dengue clinical outcomes

Two groups of dengue outcomes were defined: dengue fever and severe dengue. The comparison of dengue virus serotypes, gender and age group between the two dengue outcomes was performed by applying Pearson Chi-Square test or Fisher's Exact Chi-Square test. The comparison of clinical manifestations of severe dengue between the dengue virus serotypes was also performed by using Pearson Chi-Square test or Fisher's Exact Chi-Square test. Comparison regarding age as

continuous variable was performed by using Mann-Whitney U-test. Logistic regression was used to adjust for gender, age group, and dengue virus serotype, considering men, elderly and DENV-4 as reference categories. In the logistic regression, severe dengue was included as dependent variable. Descriptive analysis was used to define the distribution of dengue virus serotypes and severe dengue across the different years.

3.7.3 Evaluation on demographics and dengue clinical outcomes

The dependent dichotomous variable dengue outcome, defined as dengue fever and severe dengue, was used for comparison between gender and age groups using Pearson Chi-Square test. In the comparison of age as continuous variable, the Mann-Whitney U-test was used. Stratified analysis by gender was performed to compare differences in the age groups regarding the clinical manifestations presented in severe dengue courses, by performing Pearson Chi-Square test or Fisher's Exact Chi-Square test. Logistic regression was used to compare frequencies of severe dengue among gender and age groups, considering female and adults as reference groups. Descriptive analysis was used to define the distribution of severe dengue across the different age groups and years.

3.8 Ethical considerations

The study protocol was submitted to the Health Department of Vitória, to the Research Ethics Committee of the Federal University of Espírito Santo and to the Research Ethics Committee of the University Ludwig-Maximilians-Universität of Munich. It was approved in all institutions, in Brazil under the opinion number 881,909 and in Germany under the opinion number 231-15.

Blood collection was only performed after having clarified all characteristics of the study to the participants. All participants agreed with the terms and signed the consent form. The blood collection procedure was only executed when blood tests were necessary for other reasons, and the sample collected for this study consisted of a fraction of blood taken by the local staff of the public health units.

4 Results

4.1 Phylogenetic analysis

From 14 samples positive for dengue virus presence, amplification and sequencing were possible in eight samples for the envelope gene and in four samples for the NS1 gene. These sequences were obtained from ten samples: two with results of both envelope and NS1 genes, six with results of envelope gene, and two with results of NS1 gene. Sufficient quality for gene sequencing has been a challenge, therefore in some samples results could not be obtained for both gene loci.

The Maximum Likelihood phylogenetic tree of the envelope gene included the DENV-4 circulating in Vitória in the genotype II. These viruses from the city, from samples collected in 2013, were closely related to the DENV-4 identified in an epidemic in Roraima state in 2010 and in Mato Grosso state in 2012. The viruses from São Paulo state circulating in 2015 were also related to those from Vitória (Figure 4.1). A Bayes Factor of 4.96 demonstrated that the logistic model from the relaxed uncorrelated lognormal molecular clock was best fitting the data. However, the Time of the Most Recent Common Ancestor of all clocks and models were comparable (Table 4.1). The DENV-4 genotype II in Brazil is not monophyletic, clustering with viruses from Colombia and Venezuela. Two distinct introductions of DENV-4 genotype II occurred in Brazil. One introduction was limited to Pará state (phylogenetic cluster D) and occurred between 1.7 to 11.1 years before 2011 (mean of 5.8 years), having a Caribbean origin. The other introduction (phylogenetic cluster B) was originated from other South American countries, such as Venezuala and Colombia, spreading to several states in Brazil, which occurred between 4.7 to 12.1 years before 2015 (mean of 8.2 years).

The Maximum Likelihood phylogenetic tree of the NS1 gene showed a third DENV-4 introduction in Brazil, with the identification of viruses belonging to the DENV-4 genotype I circulating in Vitória in 2013. In the NS1 tree, two other isolates that were also present in the envelope tree clustered in the genotype II. The DENV-4 genotype I from Vitória was closely related to a strain detected in Bahia state in 2011 (Figure 4.2). The sequences obtained in the study were submitted to the GenBank, and can be accessed under the codes from KU745629 up to KU745648 (Table 4.2).

Figure 4.1. Maximum likelihood phylogenetic tree of dengue virus serotype 4 based on envelope gene sequences

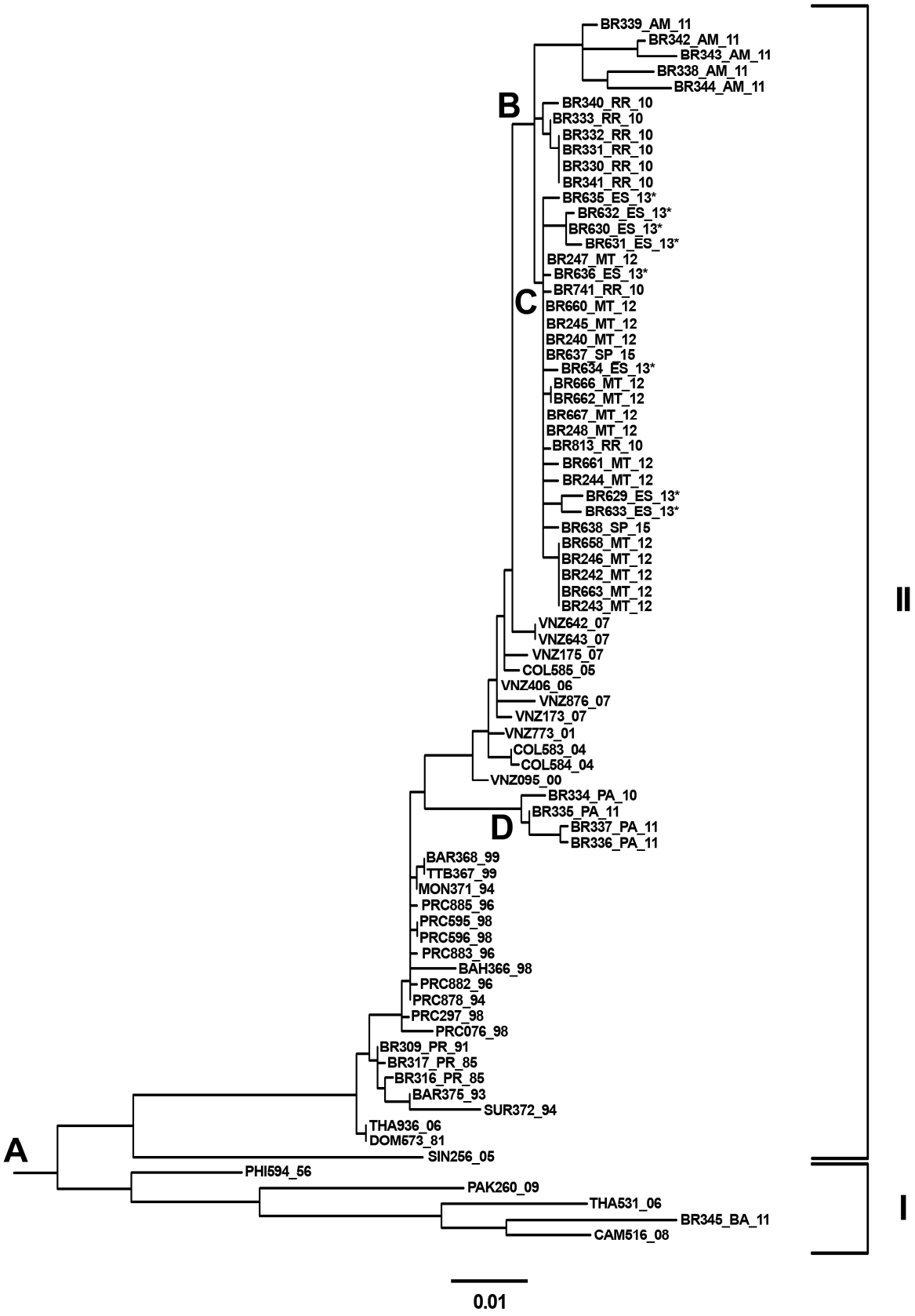


Figure 4.2. Maximum likelihood phylogenetic tree of dengue virus serotype 4 based on NS1 gene sequences

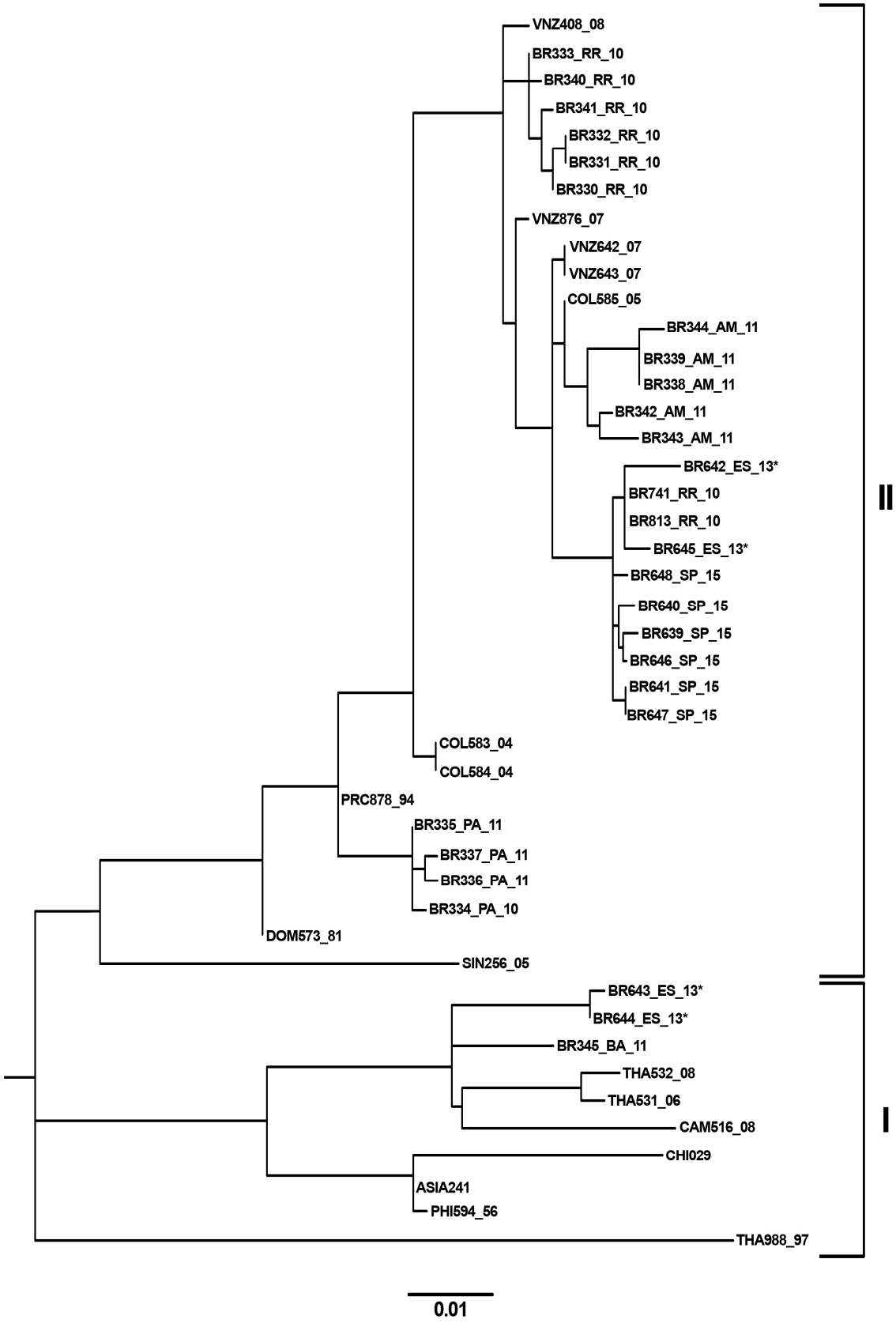


Table 4.1. Time of the Most Recent Common Ancestor for each node by using different molecular clocks

Node	Relaxed exponential molecular clock		Relaxed uncorrelated lognormal molecular clock	
	Bayesian Skyline plot	Logistic Model	Bayesian Skyline plot	Logistic Model
Phylogenetic Cluster A	114.9 (50.6-204)*	97.8 (44.1-171.4)*	114.9 (50.6-204)*	101.1 (47.4-167.2)*
Phylogenetic Cluster B	7.8 (4.4-11.6)*	8.5 (5.1-12.5)*	7.8 (4.4-11.6)*	8.2 (4.7-12.1)*
Phylogenetic Cluster C	5.5 (3-8.2)*	5.9 (3.3-8.7)*	5.5 (3-8.2)*	6 (3.4-8.9)*
Phylogenetic Cluster D	5.6 (1.7-10.6)*	5.7 (1.6-11.3)*	5.6 (1.7-10.6)*	5.8 (1.7-11.1)*

*Mean (Upper and Lower Values-95% HPD); Time of the Most Recent Common Ancestor corresponds to years before 2015 in the phylogenetic clusters A, B, and C, and to years before 2011 in the phylogenetic cluster D.

Table 4.2. Codification of the samples presented in the phylogenetic trees

Code	GenBank	Country	State	Year
BR339_AM_11	JQ513339	Brazil	Amazonas	2011
BR342_AM_11	JQ513342	Brazil	Amazonas	2011
BR343_AM_11	JQ513343	Brazil	Amazonas	2011
BR338_AM_11	JQ513338	Brazil	Amazonas	2011
BR344_AM_11	JQ513344	Brazil	Amazonas	2011
BR340_RR_10	JQ513340	Brazil	Roraima	2010
BR333_RR_10	JQ513333	Brazil	Roraima	2010
BR332_RR_10	JQ513332	Brazil	Roraima	2010
BR331_RR_10	JQ513331	Brazil	Roraima	2010
BR330_RR_10	JQ513330	Brazil	Roraima	2010
BR341_RR_10	JQ513341	Brazil	Roraima	2010
BR635_ES_13*	KU745635	Brazil	Espírito Santo	2013*
BR632_ES_13*	KU745632	Brazil	Espírito Santo	2013*
BR630_ES_13*	KU745630	Brazil	Espírito Santo	2013*
BR631_ES_13*	KU745631	Brazil	Espírito Santo	2013*
BR247_MT_12	KJ579247	Brazil	Mato Grosso	2012
BR636_ES_13*	KU745636	Brazil	Espírito Santo	2013*
BR741_RR_10	JN559741	Brazil	Roraima	2010
BR660_MT_12	JN596660	Brazil	Mato Grosso	2012
BR245_MT_12	KJ579245	Brazil	Mato Grosso	2012
BR240_MT_12	KJ579240	Brazil	Mato Grosso	2012
BR637_SP_15	KU745637	Brazil	São Paulo	2015
BR634_ES_13*	KU745634	Brazil	Espírito Santo	2013*
BR666_MT_12	KJ596666	Brazil	Mato Grosso	2012
BR662_MT_12	KJ596662	Brazil	Mato Grosso	2012
BR667_MT_12	KJ596667	Brazil	Mato Grosso	2012

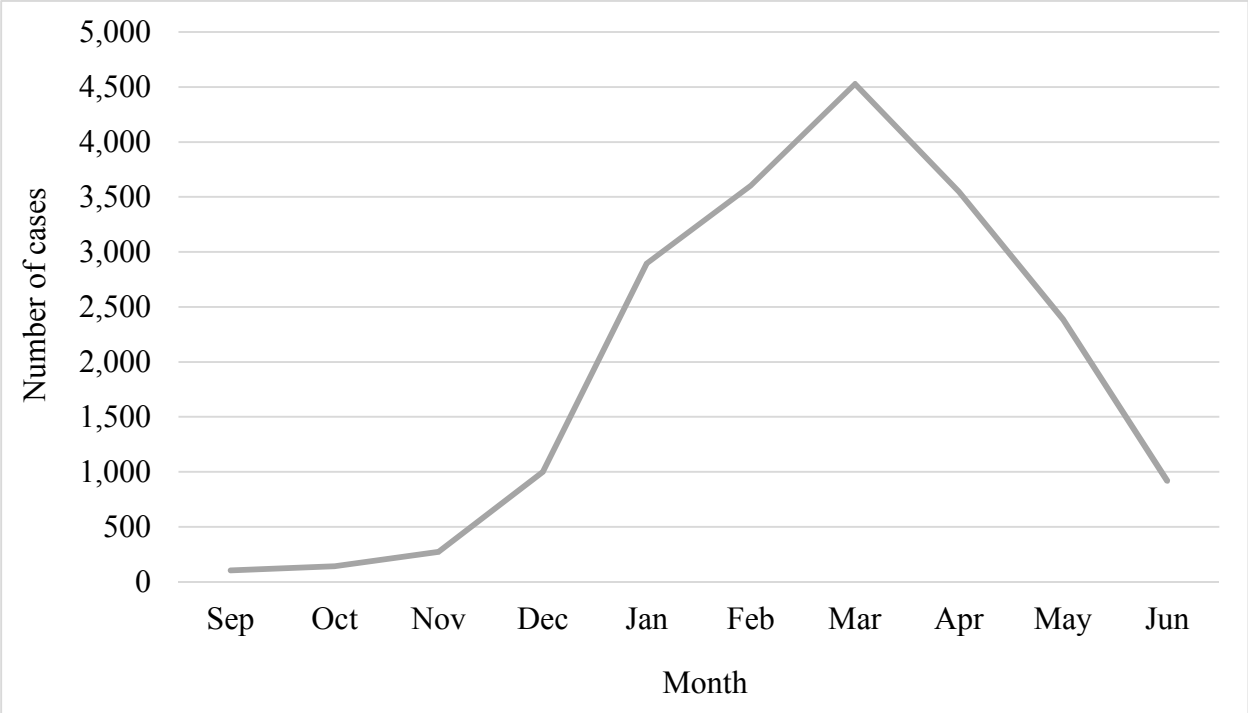
BR248_MT_12	KJ579248	Brazil	Mato Grosso	2012
BR813_RR_10	JN983813	Brazil	Roraima	2010
BR661_MT_12	KJ596661	Brazil	Mato Grosso	2012
BR244_MT_12	KJ579244	Brazil	Mato Grosso	2012
BR629_ES_13*	KU745629	Brazil	Espírito Santo	2013*
BR633_ES_13*	KU745633	Brazil	Espírito Santo	2013*
BR638_SP_15	KU745638	Brazil	São Paulo	2015
BR658_MT_12	KJ596658	Brazil	Mato Grosso	2012
BR246_MT_12	KJ579246	Brazil	Mato Grosso	2012
BR242_MT_12	KJ579242	Brazil	Mato Grosso	2012
BR663_MT_12	KJ596663	Brazil	Mato Grosso	2012
BR243_MT_12	KJ579243	Brazil	Mato Grosso	2012
VNZ642_07	GQ868642	Venezuela	-	2007
VNZ643_07	GQ868643	Venezuela	-	2007
VNZ175_07	HQ332175	Venezuela	-	2007
COL585_05	GQ868585	Colombia	-	2005
VNZ406_06	JN819406	Venezuela	-	2006
VNZ876_07	GQ199876	Venezuela	-	2007
VNZ173_07	HQ332173	Venezuela	-	2007
VNZ773_01	FJ639773	Venezuela	-	2001
COL583_04	GQ868583	Colombia	-	2004
COL584_04	GQ868584	Colombia	-	2004
VNZ095_00	FJ850095	Venezuela	-	2000
BR334_PA_10	JQ513334	Brazil	Pará	2010
BR335_PA_11	JQ513335	Brazil	Pará	2011
BR337_PA_11	JQ513337	Brazil	Pará	2011
BR336_PA_11	JQ513336	Brazil	Pará	2011
BAR368_99	AY152368	Barbados	-	1999
TTB367_99	AY152367	TrinTob	-	1999
MON371_94	AY152371	Montserrat	-	1994
PRC885_96	GQ199885	Puerto Rico	-	1996
PRC595_98	FJ882595	Puerto Rico	-	1998
PRC596_98	FJ882596	Puerto Rico	-	1998
PRC883_96	GQ199883	Puerto Rico	-	1996
BAH366_98	AY152366	Bahamas	-	1998
PRC882_96	GQ199882	Puerto Rico	-	1996
PRC878_94	GQ199878	Puerto Rico	-	1994
PRC297_98	EU854297	Puerto Rico	-	1998
PRC076_98	AY152076	Puerto Rico	-	1998
BR309_PR_91	GU318309	Brazil	Pará	1991
BR317_PR_85	GU318317	Brazil	Pará	1985
BR316_PR_85	GU318316	Brazil	Pará	1985
BAR375_93	AY152375	Barbados	-	1993
SUR372_94	AY152372	Suriname	-	1994
THA936_06	KM190936	Thailand	-	2006
DOM573_81	AF326573	Dominica	-	1981
SIN256_05	GQ398256	Singapore	-	2005
PHI594_56	GQ868594	Philippines	-	1956
PAK260_09	KF041260	Pakistan	-	2009
THA531_06	KF955531	Thailand	-	2006

BR345_BA_11	JQ513345	Brazil	Bahia	2011
CAM516_08	KF955516	Cambodia	-	2008

4.2 Spatial variation in temporal trends analysis

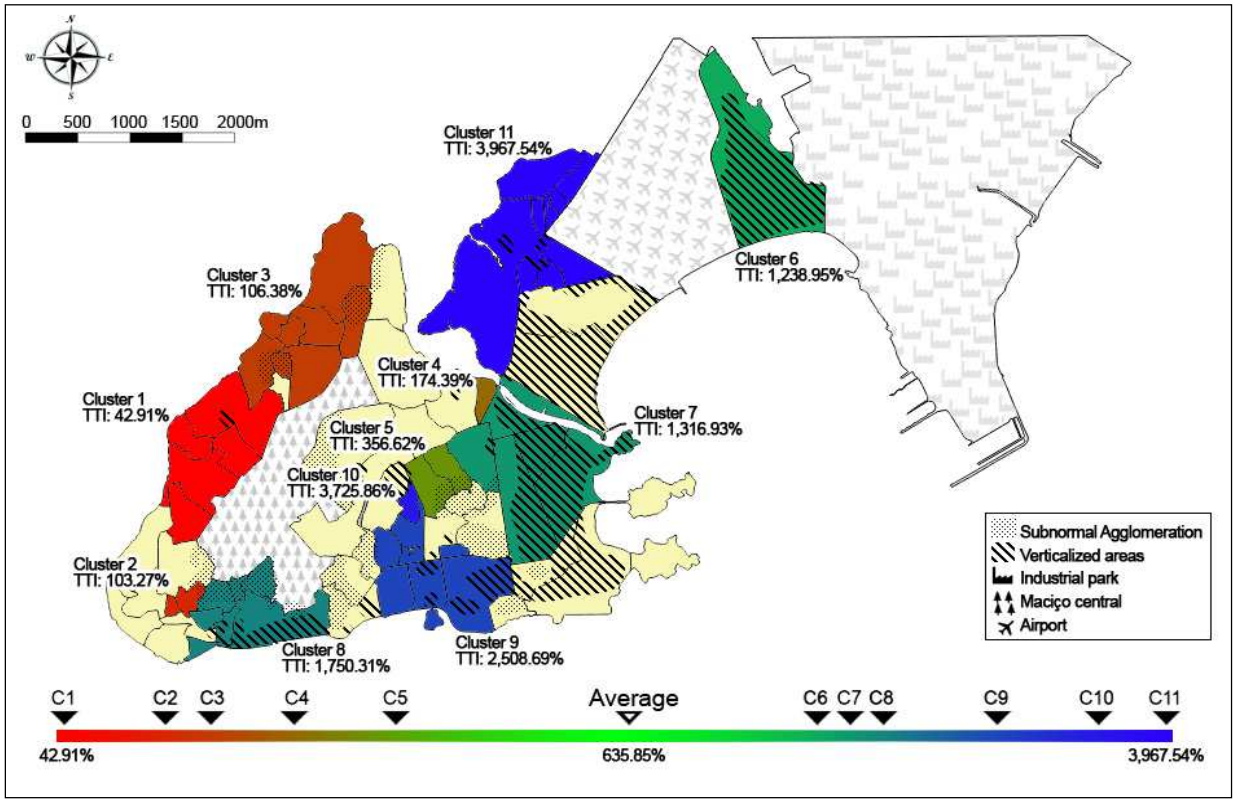
Overall, 19,397 suspected dengue cases were reported in Vitória from September 2012 to June 2013. However, 2.7% of them did not present data on address of residence. Therefore, only in 18,861 dengue cases (97.3%) it was possible to perform geocoding. The reporting of new dengue infections was lower in September 2012 (n = 105), reaching its peak in March 2013 (n = 4,529) (Figure 4.3).

Figure 4.3. Number of new cases of dengue reported in Vitória per month, from September 2012 to June 2013



Eleven space-time clusters were detected in the period. The Time Trend Increase in the overall territory of Vitória was 635.85%, and five space-time clusters (C1 to C5) presented a value lower than this, varying from 42.91% (C1) to 356.62% (C5), and six of them (C6 to C11) a value higher than this, varying from 1,238.95% (C6) to 3,967.54% (C11) (Table 4.3 and Figure 4.4).

Figure 4.4. Space-time clusters with higher or lower Time Trend Increase in Vitória and aspects of territorial occupation, from September 2012 to June 2013



The Relative Risk for dengue infection in the space-time clusters with lower Time Trend Increase varied from 1.26 (C3) to 3.05 (C4). The House Index in these space-time clusters in October 2012 ranged from 0.7 (C4) to 3.0 (C2 and C5), and in March 2013 it ranged from 0.9 (C3) to 3.0 (C5). A reduction in the House Index from October 2012 to March 2013 was observed in space-time clusters 2 and 3, while it increased in the space-time clusters 1 and 4, and remained the same in space-time cluster 5 (Table 4.3).

The Relative Risk for dengue infection in space-time clusters with higher Time Trend Increase varied from 0.52 (C6) to 1.58 (C10). The House Index in October 2012 ranged from 0.7 (C6) to 2.8 (C8), and in March 2013 it varied from 1.5 (C6) to 6.4 (C8). In all space-time clusters with higher Time Trend Increase, the House Index showed an increment over time (Table 4.3).

The Relative Risk to have a dengue infection was higher in space-time clusters with lower Time Trend Increase (Table 4.4). In the clusters with higher Time Trend Increase, solely the space-time cluster 10 presented a Relative Risk (RR = 1.58) higher than space-time cluster 1 (RR = 1.48) and

3 (RR = 1.26), which belonged to the space-time clusters with lower Time Trend Increase (Table 4.3).

The space-time clusters with lower Time Trend Increase presented lower income than space-time clusters with higher Time Trend Increase. However, population density and House Index were not different between space-time clusters with lower and higher Time Trend Increase (Table 4.4).

Dengue incidence was concentrated in the areas corresponding to the space-time clusters 1 and 3 in the beginning of the epidemic, but the disease spread in January 2013 to the space-time clusters 2, 4, and 5. In February 2013, dengue dispersed to larger areas, including the space-time clusters where in turn higher Time Trend increases could be identified. After April 2013, the cases increased considerably in the area of the space-time cluster 11 (Figure 4.5).

Figure 4.5. Dengue expansion during an epidemic in Vitória, from September 2012 to March 2013

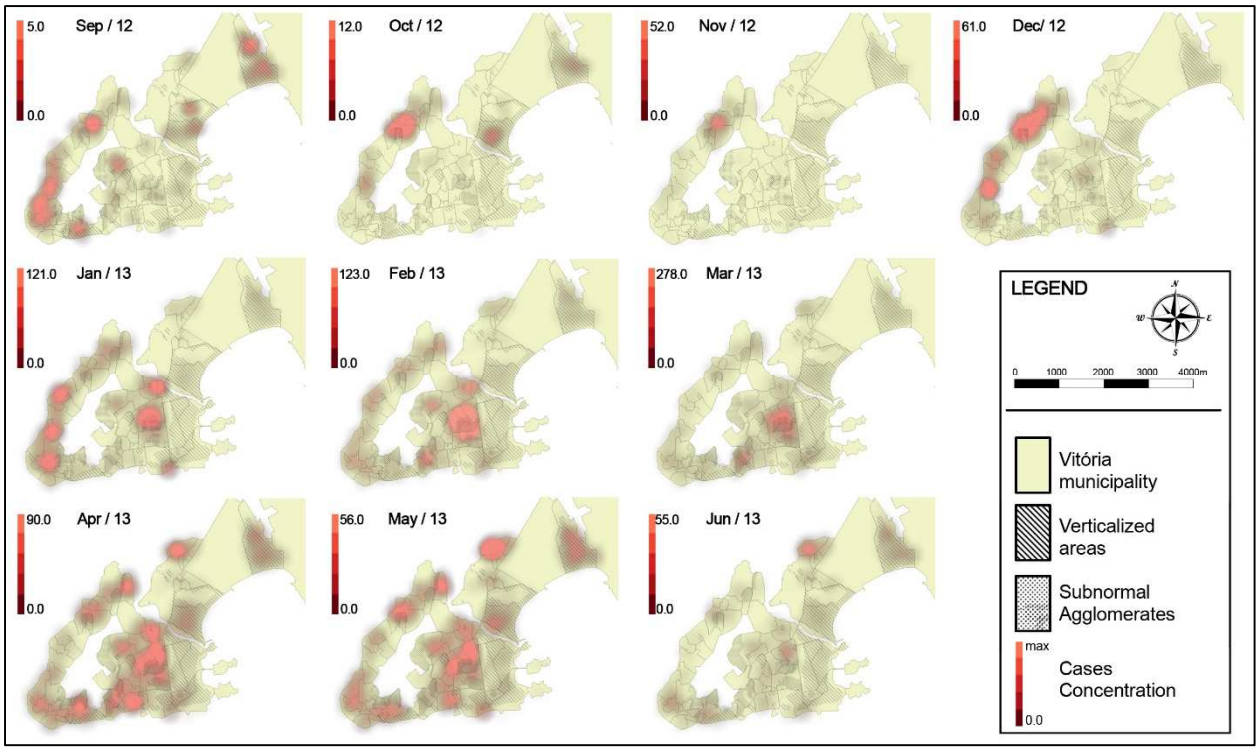


Table 4.3. Spatial variation in temporal trends for space-time clusters with lower (C1 to C5) and higher (C6 to C11) Time Trend Increase in Vitória, from September 2012 to June 2013

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	Vitória
Population	16,226	2,664	26,232	2,031	12,709	39,157	35,190	15,568	14,001	1,850	26,423	319,031
Area (km^2)	2.49	0.19	2.96	0.14	0.62	2.61	3.59	1.62	2.25	0.13	3.95	98.19*
PD (inhabitants/km^2)	6,516	14,021	8,862	14,507	20,498	15,003	9,802	9,610	6,223	14,231	6,689	3,338*
Income (reais)	662	538	514	620	652	2,260	2,607	1,136	1,506	683	1,394	
HI October (%)	1.4	3.0	1.6	0.7	3.0	0.7	1.8	2.8	2.2	2.2	1.2	-
HI March (%)	2.7	2.1	0.9	1.6	3.0	1.5	6.2	6.4	5.6	3.7	2.6	-
HI difference (%)	48	-43	-78	56	0	53	71	56	61	41	54	-
Cases observed	1,384	261	1,915	362	1,361	1,279	1,184	1,005	973	172	1,209	18,861
Cases expected	959.28	157.49	1,550.83	120.07	751.35	2,314.95	2,080.42	920.37	827.73	109.37	1,562.12	-
RR	1.48	1.67	1.26	3.05	1.87	0.52	0.54	1.10	1.19	1.58	0.76	-
TTI (%)	42.91	103.27	106.38	174.39	356.62	1,238.95	1,316.93	1,750.31	2,508.69	3,725.86	3,967.54	635.85
LLR	112.00	12.00	94.81	9.69	8.73	11.65	12.77	20.66	36.17	9.95	78.70	-
P-value	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	-

PD-Population density; HI-House Index: relation between the number of buildings with larvae of *Aedes (Stegomyia) aegypti* and the number of buildings evaluated; RR-Relative Risk: relation between cases expected and observed; TTI-Time Trend Increase: incidence increase inside the space-time cluster across the time; The *P-value* lower than 0.05, together with a LLR (Log Likelihood Ratio) higher than 6.92, indicates a significant space-time cluster where the TTI inside is significantly different from the TTI outside the space-time cluster;*Data before the State Law 9,972, in 2012.

Table 4.4. Comparison of space-time clusters with higher and lower Time Trend Increase

	Lower TTI (C1 to C5)	Higher TTI (C6 to C11)	*P-value**
PD (inhabitans/km^2)	14,021 (8,862-14,507)	9,706 (6,689-14,231)	0.66
Income (reais)	620 (538-652)	1,450 (1,136-2,260)	<0.01
HI October (%)	2.0 (1.0-3.0)	2.0 (1.0-2.0)	0.79
HI March (%)	2.1 (1.6-2.7)	4.65 (2.6-6.2)	0.13
HI difference (%)	0 (-43-48)	55 (53-61)	0.05
RR	1.67 (1.48-1.87)	0.93 (0.54-1.19)	0.02

TTI-Time Trend Increase: incidence increase inside the space-time cluster across the time; PD-Population density; HI-House Index: relation between the number of buildings with larvae of *Aedes (Stegomyia) aegypti* and the number of buildings evaluated; RR-Relative Risk: relation between cases expected and observed; * Mann-Whitney U-test; All values represented as median and interquartile range.

4.3 Evaluation on dengue virus serotypes and dengue clinical outcomes

Overall, 485 dengue cases detected between 2009 and 2013were included in the study, with 46.4% females and an overall median age of 26 years. Severe dengue affected 6.6% of the cases, with a lower proportion of females and higher age of people affected, although these mentioned differences were not significant between cases of severe dengue and those with dengue fever (Table 4.5).

Table 4.5. Demographic characteristics of the population included in the serotype evaluation

	Severe dengue	Dengue fever	Overall sample	*P-value*
Sample (%)	32 (100)	453 (100)	485 (100)	-
Proportion (%)	(6.6)	(93.4)	(100)	-
Female (%)	10 (31.3)	215 (47.5)	225 (46.4)	0.08[#]
Proportion (%)	(4.4)	(95.6)	(100)	-
Median Age	30	26	26	0.15[*]
(interquartile range)	(17-54)	(15-38)	(15-39)	

[#]Pearson Chi-Square test; [*]Mann-Whitney U-test.

In the study period, DENV-1 (77.3%) was the most frequently detected serotype, followed by DENV-4 (16.1%) and DENV-2 (6.4%). One case of DENV-3 was detected in a man with dengue fever, therefore no conclusive interpretations can be given for this serotype. Severe dengue

affected 4.5% of cases with DENV-1, 32.3% of cases with DENV-2, and 6.4% of cases with DENV-4. A significant correlation between DENV-2 and severe dengue was observed (*P-value* <0.01), while DENV-1 presented a lower proportion of this form of dengue presentation (*P-value* <0.01) (Table 4.6).

Table 4.6. Proportion of dengue fever and severe dengue cases for each dengue virus serotype

	Severe dengue	Dengue fever	Overall sample	*P-value*
Sample (%)	32 (100)	453 (100)	485 (100)	-
Proportion (%)	(6.6)	(93.4)	(100)	-
DENV-1 (%)	17 (53.1)	358 (79.0)	375 (77.3)	<0.01[#]
Proportion (%)	(4.5)	(95.5)	(100)	-
DENV-2 (%)	10 (31.3)	21 (4.6)	31 (6.4)	<0.01[#]
Proportion (%)	(32.3)	(67.7)	(100)	-
DENV-3 (%)	0	1 (0.2)	1 (0.2)	1.00[†]
DENV-4 (%)	5 (15.6)	73 (16.1)	78 (16.1)	0.94[#]
Proportion (%)	(6.4)	(93.6)	(100)	-

[#]Pearson Chi-Square test; [†]Fisher's Exact Chi-Square test.

In logistic regression, severe dengue was found more frequently in DENV-2 cases than in DENV-4 infections: adjusted odds ratio (OR) = 7.42; 95% CI = 2.21-24.93. A significant difference was not found between DENV-1 and DENV-2 infections regarding severe dengue occurrence (adjusted OR = 0.65; 95% CI = 0.23-1.88). The demographic variables included in logistic regression presented the following results: OR adjusted for females = 0.43 (95% CI = 0.19-1.01), OR adjusted for children = 0.19 (95% CI = 0.02-1.92), OR adjusted for adolescents = 0.48 (95% CI = 0.12-1.87), and OR adjusted for adults = 0.26 (95% CI = 0.07-0.96).

In DENV-1 and DENV-2 infections, the main signs of hemorrhage were positive tourniquet test and petechiae. Plasma leakage was mainly evidenced by hemoconcentration in DENV-2 cases, and hypoproteinemia in DENV-1 infections. In DENV-1 cases, the main signs of plasma leakage were hemoconcentration and cavity effusion, and for hemorrhage were hematuria and epistaxis. However, there was no significant difference of clinical manifestations between the dengue virus serotypes (Table 4.7).

Table 4.7. Clinical presentations of severe dengue according to different dengue virus serotypes

Clinical presentation	DENV-1		DENV-2		DENV-4		Overall serotypes
	n/N	*P-value*	n/N	*P-value*	n/N	*P-value*	n/N
Neurological disorder (%)	1/17 (5.9)	1.00†	1/10 (10.0)	0.53†	0/5 (0)	1.00†	2/32 (6.3)
Cardiac disorder (%)	1/17 (5.9)	1.00†	0/10 (0)	1.00†	0/5 (0)	1.00†	1/32 (3.1)
Plasma leakage (%)	6/14 (42.9)	0.90#	3/7 (42.9)	1.00†	2/4 (50.0)	1.00†	11/25 (44.0)
Hemoconcentration (%)	2/14 (14.3)	0.35†	3/7 (42.9)	0.30†	1/4 (25.0)	1.00†	6/25 (24.0)
Cavity effusion (%)	1/14 (7.1)	1.00†	0/7 (0)	1.00†	1/4 (25.0)	0.30†	2/25 (8.0)
Hypoproteinemia (%)	3/14 (21.4)	0.23†	0/7 (0)	0.53†	0/4 (0)	1.00†	3/25 (12.0)
Hemorrhage (%)	12/16 (75.0)	0.69†	6/8 (75.0)	1.00†	2/5 (40.0)	0.29†	20/29 (69.0)
Positive tourniquet test (%)	8/11 (72.7)	0.60†	3/4 (75.0)	1.00†	0/2 (0)	0.11†	11/17 (64.7)
Hematuria (%)	1/11 (9.1)	1.00†	0/4 (0)	1.00†	1/2 (50.0)	0.23†	2/17 (11.8)
Petechiae (%)	3/11 (27.3)	0.63†	3/5 (60.0)	0.27†	0/2 (0)	0.53†	6/18 (33.3)
Menorrhagia (%)	0/11 (0)	0.39†	1/5 (20.0)	0.28†	0/2 (0)	1.00†	1/18 (5.6)
Gingival bleeding (%)	2/12 (16.7)	1.00†	1/4 (25.0)	1.00†	0/2 (0)	1.00†	3/18 (16.7)
Epistaxis (%)	1/11 (9.1)	0.52†	1/4 (25.0)	1.00†	1/2 (50.0)	0.33†	3/17 (17.6)

n/N = number of patients with the clinical presentation/number of patients with data available; #Pearson Chi-Square test; †Fisher's Exact Chi-Square test.

4.4 Evaluation on demographics and dengue clinical outcomes

Overall, 6,703 confirmed dengue cases reported between 2007 and 2009 were included in this part of the study, with 43.4% males, 6.6% children, 22.5% adolescents, 61.8% adults and 9.1% elderlies, with a median age of 32 years. Severe dengue affected 11.3% of the overall cases, 13% of males and 10% of females (*P-value* <0.01). Among the age groups, 8% of children, 12.5% of adolescents, 10.5% of adults and 15.5% of elderlies presented severe dengue. The group of elderlies therefore had at large a higher proportion of severe dengue than other age groups (*P-*

value = 0.03) (Table 4.8). In the logistic regression, males and elderlies presented higher incidence of severe dengue when compared to females and adults. However, an increased occurrence of severe dengue was not demonstrated for children and adolescents in relation to adults (Table 4.9).

Table 4.8. Demographic characteristics of the population included, and dengue presentation by gender and age groups

Demographic characteristics	Severe dengue	Dengue fever	Overall sample	*P-value*
Sample (%)	759 (11.3)	5944 (88.7)	6703 (100)	-
Males (%)	379 (13.0)	2533 (87.0)	2912 (100)	<0.01[#]
Median Age (interquartile range)	34 (17-50)	31 (17-47)	32 (17-47)	0.03[*]
Children (%)	39 (8.8)	403 (91.2)	442 (100)	0.09[#]
Adolescents (%)	188 (12.5)	1318 (87.5)	1506 (100)	0.11[#]
Adults (%)	437 (10.5)	3707 (89.5)	4144 (100)	0.01[#]
Elderlies (%)	95 (15.5)	516 (84.5)	611 (100)	<0.01[#]

Children: 1-9 years old; Adolescents: 10-19 years old; Adults: 20-59 years old; Elderlies: 60-88 years old; [#]Pearson Chi-Square test; [*]Mann-Whitney U-test.

Table 4.9. Adjusted odds for severe dengue by gender and age group

Gender or age group	Adjusted OR (95% CI) [¥]
Males	1.34 (1.15-1.56)
Children	0.79 (0.56-1.12)
Adolescents	1.16 (0.96-1.39)
Elderlies	1.56 (1.23-1.99)

Children: 1-9 years old; Adolescents: 10-19 years old; Adults: 20-59 years old; Elderlies: 60-88 years old; [¥]Logistic regression: female and adults (20-59 years) were the reference groups.

The proportion of severe dengue in each age group varied depending on the respective calendar year. In 2010, a period of co-circulation of DENV-1, DENV-2, and DENV-3, children, adolescents and elderlies presented more frequently severe dengue. Children also presented more severe dengue in 2012, with the co-circulation of DENV-1 and DENV-4. Severe dengue affected more adolescents in years with circulation of DENV-2, such as 2009, or 2011, when DENV-2 and DENV-1 were circulating concomitantly. The same was observed for adults in 2009 and 2010. Elderlies presented higher proportions of severe dengue than other age groups in all years that

were evaluated, but especially during the period of DENV-2 circulation, such as 2009 and 2010 (Table 4.10).

Table 4.10. Dengue virus serotype identified per year from 2007 to 2013 and the annual proportion of severe dengue for each age group

	2007	2008	2009	2010	2011	2012	2013
Serotypes identified	-	2	2	1, 2, 3	1, 2	1, 4	4
Children (%)	0/0 (0)	0/1 (0)	0/1 (0)	4/26 (15.4)	28/273 (10.3)	2/19 (10.5)	5/112 (4.1)
Adolescents (%)	0/1 (0)	0/0 (0)	1/3 (33.3)	33/119 (27.7)	86/634 (13.6)	7/116 (6.0)	61/633 (9.6)
Adults (%)	0/0 (0)	0/11 (0)	2/15 (13.3)	69/344 (20.1)	118/1,345 (8.8)	25/339 (7.4)	223/2,090 (10.3)
Elderlies (%)	0/0 (0)	0/0 (0)	1/1 (100)	16/38 (42.1)	22/159 (13.8)	5/36 (13.9)	51/337 (13.5)

Children: 1-9 years old; Adolescents: 10-19 years old; Adults: 20-59 years old; Elderlies: 60-88 years old; n/N = number of patients with severe dengue/number of overall dengue cases in the respective age group.

Nine deaths were identified in the study group, five in adults and four in elderlies. The case fatality rate in cases of severe dengue in these groups was 11.4/1,000 in adults and 42/1,000 in elderlies.

Considering the proportion of clinical manifestations in severe dengue cases, children suffered more with hemorrhage and plasma leakage than other age groups. These differences varied according to gender, and children of female gender had more hemorrhage and epistaxis, whereas male children had more plasma leakage. Female adolescents presented a higher proportion of petechiae and male adolescents more hemorrhage, plasma leakage and cavity effusion. Adults presented less cavity effusion, and adult males had less plasma leakage, as compared to the overall sample. Elderlies presented less hemorrhage overall, despite the higher proportion of hematuria in male elderlies (Table 4.11 and Table 4.12).

Table 4.11. Clinical presentation of severe dengue in females by age group

Clinical presentation	Children		Adolescents		Adults		Elderlies		Overall sample
	n/N	*P-value*	n/N	*P-value*	n/N	*P-value*	n/N	*P-value*	n/N
Neurological disorder (%)	0/23 (0)	1.0	3/78 (3.8)	0.10	6/380 (1.6)	0.68	0/44 (0)	1.0	6/380 (1.6)
Cardiac disorder (%)	0/23 (0)	1.0	0/78 (0)	1.0	2/380 (0.5)	0.53	0/44 (0)	1.0	2/380 (0.5)
Plasma leakage (%)	11/21 (52.4)	0.08	29/72 (40.3)	0.26	115/332 (34.6)	0.21	10/36 (27.8)	0.36	115/332 (34.6)
Hemoconcentration (%)	4/21 (19.0)	1.0	12/72 (16.7)	0.62	62/332 (18.7)	0.24	4/36 (11.1)	0.26	62/332 (18.7)
Cavity effusion (%)	5/21 (23.8)	0.13	14/72 (19.4)	0.06	43/332 (13.0)	0.01*	5/36 (13.9)	0.86	43/332 (13.0)
Hypoproteinemia (%)	2/21 (9.5)	0.15	3/72 (4.2)	0.71	11/332 (3.3)	0.28	1/36 (2.8)	1.0	11/332 (3.3)
Hemorrhage (%)	19/22 (86.4)	0.04*	51/75 (68.0)	0.70	238/360 (66.1)	0.90	20/40 (50.0)	0.02*	238/360 (66.1)
Epistaxis (%)	6/18 (33.3)	<0.01*	4/47 (8.5)	0.79	24/232 (10.3)	0.15	2/20 (10.0)	1.00	24/232 (10.3)
Hematuria (%)	0/18 (0)	0.15	3/47 (6.4)	0.15	32/232 (13.8)	0.14	5/20 (25.0)	0.13	32/232 (13.8)
Gingival bleeding (%)	2/18 (11.1)	0.69	5/48 (10.4)	0.89	23/233 (9.9)	0.50	0/20 (0)	0.23	23/233 (9.9)
Gastrointestinal bleeding (%)	2/18 (11.1)	0.21	2/47 (4.3)	1.0	11/232 (4.7)	0.53	1/20 (5.0)	1.0	11/232 (4.7)
Petechiae (%)	5/18 (27.8)	0.62	24/48 (50.0)	<0.01*	77/233 (33.0)	0.06	6/20 (30.0)	0.76	77/233 (33.0)
Positive tourniquet test (%)	12/19 (63.2)	0.34	30/50 (60.0)	0.24	123/234 (52.6)	0.31	8/19 (42.1)	0.34	123/234 (52.6)

n/N = number of patients with the clinical presentation/number of patients with data available; * Significant *P-value* by using Pearson Chi-Square test.

Table 4.12. Clinical presentation of severe dengue in males by age group

Clinical presentation	Children		Adolescents		Adults		Elderlies		Overall sample
	n/N	*P-value*	n/N	*P-value*	n/N	*P-value*	n/N	*P-value*	n/N
Neurological disorder (%)	0/16 (0)	1.0	2/110 (1.8)	1.0	5/202 (2.5)	0.46	0/51 (0)	0.60	7/379 (1.8)
Cardiac disorder (%)	0/16 (0)	1.0	0/110 (0)	1.0	0/202 (0)	0.47	1/51 (2.0)	0.14	0/379 (0)
Plasma leakage (%)	10/16 (62.5)	0.04*	52/99 (52.5)	<0.01*	47/167 (28.1)	<0.01*	14/42 (33.3)	0.51	123/324 (38.0)
Hemoconcentration (%)	6/16 (37.5)	0.09	24/99 (24.2)	0.29	30/167 (18.0)	0.21	7/42 (16.7)	0.49	67/324 (20.7)
Cavity effusion (%)	4/16 (25.0)	0.25	22/99 (22.2)	<0.01*	13/167 (7.8)	<0.01*	5/42 (11.9)	0.73	44/324 (13.6)
Hypoproteinemia (%)	0/16 (0)	1.0	6/99 (6.1)	0.14	4/167 (2.4)	0.25	2/42 (4.8)	0.66	12/324 (3.7)
Hemorrhage (%)	11/16 (68.8)	0.16	64/105 (61.0)	0.02*	86/184 (46.7)	0.07	19/45 (42.2)	0.19	180/350 (51.4)
Epistaxis (%)	2/11 (18.2)	0.31	7/62 (11.3)	0.73	7/84 (8.3)	0.43	2/19 (10.5)	1.00	18/176 (10.2)
Hematuria (%)	0/11 (0)	0.22	9/61 (14.8)	0.72	12/83 (14.5)	0.58	7/19 (36.8)	<0.01*	28/174 (16.1)
Gingival bleeding (%)	1/11 (9.1)	1.0	9/62 (14.5)	0.07	6/83 (7.2)	0.40	0/19 (0)	0.23	16/175 (9.1)
Gastrointestinal bleeding (%)	2/11 (18.2)	0.12	3/62 (4.8)	1.0	4/83 (4.8)	0.75	1/19 (5.3)	1.0	10/175 (5.7)
Petechiae (%)	5/11 (45.5)	0.32	19/62 (30.6)	0.81	25/84 (29.8)	0.58	7/19 (36.8)	0.62	56/176 (31.8)
Positive tourniquet test (%)	7/11 (63.6)	0.76	38/61 (62.3)	0.22	48/84 (57.1)	0.77	5/19 (26.3)	<0.01*	98/175 (56.0)

n/N = number of patients with the clinical presentation/number of patients with data available; * Significant *P-value* by using Pearson Chi-Square test.

5 Discussion

Research is fundamental to the support of initiatives in the health sector focused on dengue control. The Global Strategy for Dengue Prevention and Control from 2012 to 2020 reinforces the necessity of basic and operational research, including investigations on early epidemic response, indicators for outbreak risks, transmission dynamics, and operational assessment (World Health Organization, 2012a). Investigations on risk factors related to dengue severity are also encouraged (Barreto and Teixeira, 2008).

The present study focused on understanding the dynamics involved in the spread of dengue and factors related to dengue clinical outcomes, aiming to better control the disease in terms of epidemiological surveillance actions.

The first approach that this study utilized to evaluate dengue spread dynamics was a phylogenetic evaluation of DENV-4 circulating in Vitória in 2013. This method is important in order to comprehend dengue epidemic patterns, and the effect of human movements and of virus evolution on the territorial expansion of dengue (Koo et al., 2013), by evaluating the relationship between different isolates (Carroll et al., 2014). It is important to consider the limitations of phylogenetic analysis in order to interpret correctly the results presented in phylogenetic trees. A phylogenetic tree represents the most-likely evolution of the viruses based on the comparison of their characters. However, as the gene sequence is the character of comparison, it is important to consider that extensive genetic recombination can occur between viruses, which can be depicted in a tree with relationships represented as a network of possible paths, instead a bifurcation. The results are also limited by sampling and can present evolutionary noise, such as when numerous mutations happen at the same nucleotide position, interfering in the obtainment of a phylogenetic tree that represents the real evolution (Holmes, 1998). In the health sector, the use of molecular and phylogenetic methods to investigate patterns of pathogen distribution and clustering is termed molecular epidemiology.

Before the present study, only one investigation was conducted which performed a phylogenetic analysis of dengue virus in Vitória. This study included only four samples of DENV-2, collected in 2009 (Dettogni and Louro, 2012). The present study is the first to evaluate the origin of DENV-4 circulating in Vitória, and was able to elucidate the genotypes circulating in the city and the probable origins of dengue virus strains detected during the largest epidemic registered in the city.

Two different genotypes of DENV-4 were detected in Vitória in the present study: the genotype I and the genotype II. The DENV-4 genotype I is of Asian origin, occurring in Sri Lanka, Philippines, Thailand and Japan (Weaver and Vasilakis, 2009; Pinho et al., 2013). Genotype I was absent from records in the Western hemisphere (Foster et al., 2003; Dussart et al., 2006) until the first report in Brazil in 2008 (Melo et al., 2009), probably due to a direct introduction of the virus from Southeast Asia (Nunes et al., 2012). The genotype II of DENV-4 is the most common in Brazil and the Americas (Nunes et al., 2012) and has been present in the Caribbean region for more than twenty years (Melo et al., 2009). This genotype originated in Asia, where it is frequent in Malaysia and Indonesia (Weaver and Vasilakis, 2009). It became established in the Caribbean at the end of the 1970's and beginning of the 1980's (Foster et al., 2003; Nunes et al., 2012). This genotype was introduced to South America on at least three separate occasions, originated from the Caribbean region, and has become widely established in these areas since then (Nunes et al., 2012).

The first detection of DENV-4 genotype I in Brazil and in the Americas occurred in the city of Manaus, the capital of the Amazon state, in 2008 (Melo et al., 2009, Souza et al., 2011), where local circulation was occurring, with the same genotype being identified in mosquitos collected in the city (Melo et al., 2009, Souza et al., 2011). This finding contradicts the usual route of dengue introduction to the Americas through the Caribbean (Melo et al., 2009), showing a new way of dengue introduction directly from Asian countries.

After the identification of the DENV-4 genotype I in Manaus, this genotype was found in 2010 in Salvador, the capital of Bahia state, a city that receives a high number of tourists every year (Pinho et al., 2015). Manaus and Salvador both have international harbors, through which they maintain intense commercial contact with Asian countries (Melo et al., 2009; Figueiredo et al., 2013), directly enabling the introduction of dengue viruses from the Asian continent.

The DENV-4 viruses belonging to the genotype I in Vitória were closely related to a strain identified in Bahia state in 2010. Their relation to the strains from Amazon state could not be evaluated, since only membrane and partial capsid gene sequences were available from the samples that had been collected in Manaus (Figueiredo et al., 2008). Bahia state borders Espírito Santo state in the North, and this geographical proximity could explain the facility of dengue dispersion involving both territories due to the intense human movements between them.

The DENV-4 genotype II from Vitória is closely related to the viruses circulating in Roraima state in 2010, in Mato Grosso state in 2012 and in São Paulo state in 2015. Countries that border the North of Brazil, such as Venezuela and Colombia, were the origin of the virus when it was introduced to the Brazilian territory. The first indications of DENV-4 infections in Brazil appeared in Roraima state in 2010. Roraima is bordered by Venezuela, a country to which the state maintains close connections, and which was at that time already a region endemic for dengue, with broad infestation by *Aedes (Stegomyia) aegypti*. Different mechanisms of cross-border dispersion from Venezuela into Brazil are thinkable, such as transportation of infected vectors or mobility of humans infected in Venezuela and then developing the viremic phase of dengue in Roraima state. The genotype II eventually reached almost all Brazilian states two years after its first detection in Brazil.

Despite the first report in Brazil of DENV-4 genotype II in 2010, its actual introduction is estimated to have taken place around the year 2007. A time gap between the serotype introduction and eventual identification has previously been reported for DENV-2 circulation in Brazil (Romano et al., 2010), and is probably influenced by the low level circulation of the new serotype in places where other serotypes are circulating concomitantly, stochastically delaying the identification of the new dengue virus serotype by the surveillance services.

The DENV-4 genotype II, probably introduced in this period, generated a more recent Brazilian lineage approximately in 2009 (phylogenetic cluster C), which also includes the strains collected in Vitória. Therefore, it is reasonable to assume that other Brazilian states were the source of the DENV-4 genotype II circulating in the capital of Espírito Santo state, rather than a direct introduction, for example, by international air or sea travel. Additional molecular data on DENV-4 from other Brazilian states and more local samples from Espírito Santo state would be necessary to evaluate the probable states involved in dengue introduction into Vitória. The states of Rio de Janeiro and São Paulo were inferred as possible origins of DENV-2 circulating in Vitória in 2009 (Dettogni and Louro, 2012), which is a plausible inference considering that both states are located in the Southeast region of Brazil, thus in geographic vicinity to Espírito Santo state.

The geographic location of Espírito Santo state in the Southeast, the richest region of Brazil, and its Northern border with Bahia state, facilitate the import and export of dengue virus by infected humans moving between the states. The presence of an international port and multinational companies in Vitória can also contribute to the dissemination of dengue to other countries.

However, according to this analysis, the city probably does not play a role in the international route of dengue introduction in Brazil.

The co-circulation of two different DENV-4 genotypes in Vitória is a concern, since this scenario can promote the emergence of severe cases (Añes et al., 2011) as suggested by some evidences showing that immunological protection can be partial for homologous infection (Forshey et al., 2016).

Therefore, the surveillance of dengue virus genotypes locally circulating and their relation to viruses from other places is important in order to understand the dynamic of dengue dispersion, routes of virus introduction, and probable epidemiological scenarios after the establishment of a certain genotype in the location, by considering the characteristics of the epidemics that they have caused in other places.

In Vitória, the circulation of both DENV-4 genotypes was related to an epidemic that accounted for 19,449 suspected dengue cases reported in 2013. In that year, the incidence of dengue in the city was 6,094 cases per 100,000 inhabitants, a number substantially higher than the threshold parameter of 300 cases per 100,000 that is used to declare an epidemic. In 2013, 153 cases were hospitalized and two deaths were registered. The main reason for the magnitude of this epidemic was the low immunity of the local population against DENV-4, since its circulation was unprecedented in the city.

However, after the identification of DENV-4 in Vitória in 2012, the pattern of dengue dispersion was not evaluated even during the epidemic that followed this event. The present study was the first performed with this aim, and has an advantage to be conducted in a period with high absence of immune protection against DENV-4 in the local population, preventing that the immunity status acts as a confounding factor on dengue distribution. Dengue occurrence is favored by certain social arrangements of human habitats and its spread is subject to influence of the environment. The visualization of these features in maps, displayed in computerized systems, is a valuable tool for the health sector for understanding the social and environmental characteristics correlated with dengue distribution (Fradelos et al., 2014).

Geographic Information Systems supports the construction of maps with geographically referenced information, and is an important instrument for surveillance, management and analysis

of different diseases. Methods to determine space-time clusters can help in evaluating the dynamics of dengue dispersion, permitting the identification of risk areas and thereby providing valuable information for disease control (Si et al., 2008). Considering this, the present study performed a spatial analysis of dengue cases, illustrated in maps, utilizing the geocode of 18,861 dengue cases occurring in Vitória between September 2012 and June 2013. The study also presented an analysis of spatial variation in temporal trends, which permitted detection of eleven space-time clusters of dengue in the city, providing information on the pattern of the disease dispersion along the ten months.

In the municipality, the dengue epidemic began with a progressive increase of incident cases in September 2012. Usually, this increase is not observed so early in the season, but it was precipitated by an unusual winter that presented constant rains, generating a hydric surplus higher than 180 mm in August 2012 (Institute of Research, Technical Assistance and Rural Extension of Espírito Santo State, 2015). The progressive increase in the number of dengue cases reported to the Health Department of Vitória per month reached its peak in March 2013, with more than 4,500 reports. High temperatures during this period favored the increase of dengue incidence in the city. In January 2013, the hottest month, had an average along the day of approximately 28°C and peaks of 36°C (Institute of Research, Technical Assistance and Rural Extension of Espírito Santo State, 2015). The reason behind the increase of the incidence is that temperature influences the mosquito's reproduction, life cycle and behavior, increasing the oviposition, the lifespan and the biting behavior, as has been highlighted in the introduction (Banu et al., 2011). The hydric surplus, registered in almost all months of the period studied, except February 2013 (Institute of Research, Technical Assistance and Rural Extension of Espírito Santo State, 2015), also favored the presence of breeding sites, increasing the presence of dengue vectors in the community. Therefore, the epidemic of 2012 and 2013 followed the pattern of dengue incidence observed in previous years, with the spring, and especially the summer (from December to March), accounting for more incident cases.

The lower Time Trend Increase in five space-time clusters demonstrates an explosive incidence in these sites at the beginning of the period evaluated, in comparison to space-time clusters with higher Time Trend Increase. The depletion of population without immunity against DENV-4 in space-time clusters with lower Time Trend Increase was probably the main reason for a lower increment of dengue incidence along the time within these sites. Similar observation was made for other Brazilian cities, where herd immunity was responsible for modulating dengue incidence

(Medronho, 2006; Chaade, 2007). The Relative Risk of having dengue infection was higher in space-time clusters with lower Time Trend Increase, demonstrating a more intense transmission of dengue within these territories.

Dengue transmission is characterized as highly locally restricted, since the vectors normally stay near to breeding sites. The flight range depends on the availability of feeding sources and reproduction sites, but usually does not exceed 150 m (Maciel-de-Freitas et al., 2010). Therefore, some local characteristics could be involved in the intensity of dengue transmission, such as higher presence of *Aedes (Stegomyia) aegypti* and high availability of hosts concentrated in small areas. These factors were explored in the present study, which evaluated House Index, income and population density, comparing these variables in space-time clusters with lower and higher Time Trend Increase.

Space-time clusters with lower Time Trend Increase were found to be areas of low-income. People living in low-income regions are particularly exposed to the mosquito *Aedes (Stegomyia) aegypti*, since the infrastructure in these places usually offers conditions for the establishment of breeding sites. The lack or intermittent supply of tap water is linked to the presence of water storage containers in houses, and the inefficient garbage collection encourages waste accumulation in inadequate places. In addition, low-educational levels, normally present in low-income populations, interferes in the involvement of the community in preventive actions (Braga et al., 2010). The relation of socioeconomic variables and dengue incidence was demonstrated in previous studies, which investigated low-income areas (Mondini et al., 2008; Braga et al., 2010), low socioeconomic status (Kikuti et al., 2015), poverty (Barreto et al., 2008), presence of subnormal agglomerates (Flauzino et al., 2009; Resendes et al., 2010), poor housing conditions (Stewart-Ibarra et al., 2014), low educational levels (Siqueira et al., 2004; Almeida et al., 2007; Siqueira et al., 2008; Costa et al., 2013) and inequity (Teixeira et al., 2011).

In Vitória, during the course of this study, the social reality imposed on a considerable part of its population became visible, especially in the region of São Pedro, where it is localized the space-time cluster 3, and Santo Antônio, where space-time clusters 1 and 2 are included. In these regions, it is not uncommon to observe garbage discarded in the streets or wastelands. In the district of Jesus de Nazareth, near to space-time cluster 9, sewage was exposed in alleyways and stairwells, even with its territory being located close to the richest districts of the city.

In the space-time clusters with lower Time Trend Increase, positive houses for larvae infestation had high proportions of breeding sites constituted by containers designed to store water for human consumption. In space-time cluster 1, 12.5% of breeding sites were detected in these containers, in space-time cluster 3, 28.6% to 60%, and in space-time cluster 5, 35.7%. This scenario demonstrates that the insufficient water provision in these low-income areas influences the presence of vectors.

The space-time clusters 2, 3 and 5 present areas of subnormal agglomerates, concentrating inadequate housing, unorganized occupation and absence of essential services (Brazilian Institute of Geography and Statistics, 2010). In other countries as well as in other Brazilian locations, areas of subnormal agglomerates have presented a high number of breeding sites (Thammapalo et al., 2005), leading to a persistent infestation by *Aedes (Stegomyia) aegypti* (Souza-Santos et al., 2000).

For many years, dengue control has been mainly focused on actions against the mosquito in its different life stages. However, the absence of actions for improving the infrastructure in endemic sites impairs the obtainment of effective results. Consequently, in the absence of improvements in infrastructure, it is not surprising to observe that *Aedes (Stegomyia) aegypti* infestation in four space-time clusters with lower Time Trend Increase (C1, C2, C3, and C4) remained at medium risk level for dengue transmission in March 2013. The information on House Index provided by the entomological surveillance and on dengue incidence provided by the epidemiological surveillance, have been used to define locations at risk of dengue transmission or in frank transmission of the disease, which are prioritized in control actions. However, these measures were not able to decrease the House Index to a low level in these prioritized areas until March 2013.

In addition, vector control programs have limitations. Nowadays, these actions are more focused on larval control than on adult mosquitos. Larvicide application and environmental management are two important actions taken, but their effectiveness is limited. Chemical control of the dengue vector is a method largely used in endemic areas. However, an increasing resistance over time with the use of these substances has been noted in vectors with different insecticides. In addition, activities related to this kind of control are resource consuming and rarely achieve elevated or continuous adequate coverage, impairing their results (Guzman et al., 2010), and moreover, they have potentially detrimental effects on the natural habitats of other species. The actions involving dengue control also need ample community participation (Teixeira et al., 2009), which is difficult to reach. The attenuation of dengue control efforts in interepidemic periods interferes with the

magnitude and capacity of responses during outbreaks. The activities for vector control, surveillance and community education should be consistent over time, and should not be discontinued according to dengue seasonality (Chang et al., 2011). The complete elimination of the dengue vector is almost impracticable, but it is clear that this should not be the sole focus of dengue control efforts. Even in well-structured control programs that reached low levels of *Aedes (Stegomyia) aegypti* infestation, the numbers of dengue infections in humans remained high (Teixeira et al., 2009). Other factors that impair vector control are the limited resources employed in its activities, the underestimation of its economic and social impacts and the local nature of dengue occurrence (Halstead, 2000).

Interestingly, despite the income difference between space-time clusters with lower and higher Time Trend Increase, the House Index was not significantly different between them in the studied periods. This shows the limitation of using this indicator as a parameter to define priority areas for dengue control. The entomological surveillance is based on the evaluation of different factors related to the vector, such as distribution, density, and habitats of the population, insecticide susceptibility and risk of dengue transmission. Larval survey is the most common approach of vector surveillance. In this method, larvae and pupae presence is evaluated, and they are collected in order to define the mosquito's species. There are three indexes used for monitoring the infestation by *Aedes (Stegomyia) aegypti*. The House Index consists of the percentage of houses infested with larvae or pupae, without considering the quantity and productivity of the positive containers found at these houses. The Container Index represents the percentage of containers with larvae or pupae, without evaluating their production. The Breteau Index evaluates the number of positive containers per 100 houses investigated, providing more information than the other indexes, but also without considering the productivity of the containers (World Health Organization, 2011). The three indexes are inaccurate measures of mosquito procreation, and do not present results on the adult form of the vector, which is responsible for the transmission of the virus (Teixeira et al., 2005).

The House Index, used in this study, is the parameter adopted by the Brazilian Ministry of Health to indicate the risk of dengue transmission as low, medium or high. However, no level of House Index is able to indicate whether dengue transmission will be impaired due to insufficient numbers of vectors (Almeida et al., 2007). A relationship between high larval densities and increased dengue incidence was observed previously (Barrera et al., 2011; Cordeiro et al., 2011; Padmanabha et al., 2012; Teixeira et al., 2012), but contrarily some studies did not find similar

results (Câmara et al., 2007; Chaade et al., 2007; Melo et al., 2007; Teixeira et al., 2011). Therefore, it is important to consider dengue transmission as a complex process, which is sustained by factors beyond the density of vectors (Barcellos et al., 2014).

Some other factors that contribute to an increased dengue transmission are the virus strain, which influences the magnitude and the duration of the viremia in humans, the susceptibility of the human population, which is impacted by genetic factors and the immunological profile and the introduction of a serotype in a community (Gubler, 1997).

Decision makers should evaluate different factors related to the increase of dengue incidence, considering the limitation of the House Index to determine priority areas for dengue control. Some of these factors to be considered are the dengue incidence in different districts in previous months, the immunity of the local population and the environmental characteristics that could favor the contact of the local population with the vector. In addition, problems emerging during the sampling process for determining the House Index, such as impossibility to access houses, should be discussed, as well as the effects of these difficulties in the House Index results.

The space-time clusters with higher Time Trend Increase suffered a slower progression of dengue incidence over time as compared to space-time clusters with lower Time Trend Increase. They presented an increment in the House Index between October 2012 and March 2013, and the higher presence of the vector probably contributed to increasing dengue transmission. In these sites, the House Index showed a high risk for dengue transmission for space-time clusters 7, 8, and 9, and medium risk for the space-time clusters 6, 10, and 11. However, the Relative Risk to have dengue infection in these space-time clusters was low, especially when compared with space-time clusters with lower Time Trend Increase. Within the space-time clusters with higher Time Trend Increase, those with lower income or located close to low-income areas presented a higher Relative Risk, such as the space-time cluster 10, located near to subnormal agglomerates of the space-time cluster 5, and the space-time cluster 8, where there is an area of subnormal agglomerate.

The space-time clusters with higher Time Trend Increase in Vitória present a high concentration of residential buildings with more than three floors, especially the space-time clusters 6, 7 and part of the space-time clusters 8 and 9, contrary to the space-time clusters with lower Time Trend Increase. At first sight, it is plausible to consider that areas with higher buildings present an increased human population density, permitting a close contact between vectors and the human

hosts, and thereby favoring dengue transmission. However, the space-time clusters with lower and higher Time Trend Increase did not differ significantly in population density. A notable difference between them is related to the housing conditions, which are poor in low-income areas, increasing the contact of population living in these sites with dengue vector, and consequently favoring the transmission of dengue virus.

In the map representing dengue dispersion, it is possible to observe the relation of low-income areas with the beginning of dengue spread, precipitating the disease occurrence in adjacent regions. The dispersion of dengue is characterized by centrifugal waves, influencing places near dengue hotspots (Barreto et al., 2008; Jefoo et al., 2011). Low-income areas and subnormal agglomerates seem to play a role as hotspots of dengue occurrence. For example, the space-time cluster 9 is located close to three subnormal agglomerates and was influenced by these areas, which triggered a posterior increment in the incidence of dengue inside this space-time cluster.

From September 2012 to December 2012, the occurrence of dengue was almost limited to the areas of space-time clusters 1 and 3, which are relatively separated from other regions of the city by the *Maciço Central*, a natural high altitude barrier located close to the center of Vitória Island. In January 2013, dengue incidence increased in other areas of the municipality, especially in low-income areas where subnormal agglomerates are located. After April 2013, areas adjacent to subnormal agglomerates presented an increased dengue transmission. A delayed increase in dengue occurrence observed in space-time cluster 11 could be possibly explained by its larger distance from low-income areas and subnormal agglomerates and relative isolation from other space-time clusters, which also probably influenced the low Relative Risk observed in this territory, despite the House Index indicating a medium risk for dengue transmission.

Preventive actions and the improvement of environment in low-income areas are important to minimize the risk of dengue transmission and disease spread inside them and in adjacent regions, especially after the identification of a new serotype or genotype circulating in the territory by the surveillance system. In Vitória, efficiency of control efforts can also influence the spread of dengue to other municipalities of the metropolitan region or other cities in the state, since its position as capital of Espírito Santo state generates an intense circulation of people from different municipalities in its territory.

Since aggregated data was used for the ecological approach in the study, definite conclusions about dengue dispersion in Vitória are limited. Other limitations come as a result of using secondary data, such as absence of address information in 2.8% of cases reported (n = 536), and the exclusion of asymptomatic or underreported cases that do not seek treatment from health services. These limitations impair capturing the real incidence in the city. The absence of laboratory confirmation in most reports permits the inclusion of cases diagnosed erroneously as dengue, but is important in order to assess the impact of the disease in the health system and in the community. The geocoding technique utilized in the study is not very precise, and in addition, the study could not evaluate the role of human movements in dengue virus acquisition, since it was not possible to determine the location where the individuals were when they were bitten by the infected mosquitoes.

Despite the limitations cited above, this study generates evidence for possible factors related to epidemiological patterns observed in the period, and was able to point out the characteristics of areas that need to be prioritized for dengue control when a new serotype is identified by the surveillance services. The absence of immunity against DENV-4 in most of the population at the beginning of the epidemic is an advantage for study purposes in order to allow for an analysis of the other factors related to dengue dispersion, while noting that it may be detrimental for the individuals being exposed to the infectious agent. Furthermore, the study demonstrated the relevance of the Geographic Information System as a tool for evaluation of disease distribution and for predicting dengue dispersion across the territory (Salje et al., 2012).

In addition to the introduction and spread of dengue serotypes in the population, their impact on the emergence of severe outcomes should be investigated. Factors related to the severity of dengue have not been completely elucidated. As a complex disease, both viral and host factors can influence dengue outcomes. The viral structure affects the pathogenesis of dengue virus (Leitmeyer et al., 1999). Studies have observed differences between dengue serotypes and genotypes related to their capacity to infect, virulence (Pongsiri et al., 2012) and magnitude of replication (Rosen, 1977; Gubler et al., 1978; Leitmeyer et al., 1999). However, all serotypes are able to induce hemorrhagic forms of dengue (Fried et al., 2010). It was hypothesized that viruses with higher replication are associated with increased dengue severity (Halstead, 1988; Gubler, 1997; Murgue et al., 2000; Vaughn et al., 2000; Libraty et al., 2002; Wang et al., 2003; Wang et al., 2006). It is thought that a high viral load could induce an accelerated production of antibodies,

which would influence the emergence of severe outcomes, such as those that occur in dengue hemorrhagic fever cases (Vaughn et al., 2000).

Besides the genetic background of the host, other factors could be related to dengue severity. Host gender could influence dengue outcome, since hormones possibly affect the immunological response. Thereby, females between 20 and 59 years old were more prone to develop severe dengue in a previous study (Guerra-Silveira and Abad-Franch, 2013). Age could also affects dengue severity, since children are considered the age group at higher risk of developing hypovolemic shock, due to their lower capacity in compensating for plasma leakage (World Health Organization, 2009). Presence of comorbidities, such as diabetes (Bravo et al., 1987; Cunha et al., 1999; Figueiredo et al., 2010; Pang et al., 2012), hypertension (Cunha et al., 1999; Figueiredo et al., 2010) and allergies (Figueiredo et al., 2010), were related to severe dengue outcomes, and individuals using salicylates had an increased risk of hemorrhages during the dengue course (Gorzoni et al., 2010). Ethnicity was another factor related to the host that could affect dengue outcome, since higher occurrence of hemorrhagic cases was observed in people with European origin in Brazil, when compared to those with African origin. Some genetic markers in African descendants were suggested to confer protective effects against dengue hemorrhagic fever (Teixeira, 2009).

Sequential dengue infection is seen as one of the main factors contributing to the emergence of hemorrhagic cases. In a previous study, about 90% of cases of dengue hemorrhagic fever and dengue shock syndrome were related to a second exposure to dengue virus (Mathew and Rothman, 2008). In cases of successive dengue infections, the risk of hemorrhagic forms increases 40 to 80-fold compared to primary dengue infection (Halstead, 2003). Around 2% to 4% of sequential dengue cases present severe forms of the disease (Malavige et al., 2004), hypothetically due to the antibody-dependent enhancement. Furthermore, the time between the two infections could influence the severity of the clinical manifestations, which are greater in intervals lower than five years. However, unfavorable effects are also possible to occur after intervals greater than 20 years. The sequence of serotypes responsible for infection was also suggested as favoring worse outcomes, as observed with infections of DENV-3 followed by DENV-2, by DENV-1 followed by DENV-3 and by DENV-2 followed by DENV-3 (Guzman and Kouri, 2003).

Firstly, the present study evaluated the association of different dengue virus serotypes and the emergence of severe outcomes, using a cross-sectional approach. This viral factor was evaluated

in 485 dengue cases confirmed by viral isolation or RT-PCR. These cases occurred in the capital of Espírito Santo state between 2009 and 2013, and were registered in the System for Notifiable Diseases. The analysis demonstrated an association between infections caused by DENV-2 and severe dengue. For the cases involved in this part of the study, age and gender did not influence the findings of the relationship between serotype and severe presentations of dengue.

The association of DENV-2 and severe outcomes of dengue was demonstrated in previous studies (Thomas et al., 2008), which showed an increased proportion of people presenting dengue hemorrhagic fever (Kalayanarooj and Nimmannitya, 2000; Vaughn et al., 2000; Nisalak et al., 2003; Fried et al., 2010) and dengue shock syndrome (Kalayanarooj and Nimmannitya, 2000; Huy et al., 2013) when the infections were caused by DENV-2. Different regions of the world experienced an increase in the number of severe dengue cases when this serotype was circulating in their territories (Dussart et al., 2012). Therefore, when DENV-2 was the predominant serotype circulating in these sites, the number of cases with dengue hemorrhagic fever emerging in the period was high, thereby characterizing the epidemic as severe (Guzmán et al., 1990, Balmaseda et al., 2006).

It is possible to observe the importance of DENV-2, in regards to the emergence of severe dengue, evaluating the epidemiological scenario in Vitória during years that registered local circulation of this serotype. Of the laboratory confirmed dengue cases in the municipality, 22.7% (n = 5) evolved to severe dengue in 2009, 23.4% (n = 125) in 2010, and 10.9% (n = 268) in 2011. These years were the only ones in the time series when DENV-2 was detected. In 2009, DENV-2 was the only serotype registered, and in 2010 and 2011 it was circulating together with other serotypes, such as DENV-1 and DENV-3. In 2010, DENV-2 was detected in 52.9% of the cases where serotype was determined, and was responsible for 88.9% (n = 8) of cases with severe dengue and laboratory determination of dengue virus serotype. This shows the importance of DENV-2 in the emergence of severe dengue in Vitória during the epidemic in 2010. The three years with DENV-2 circulation presented the highest number of hospitalizations in the period analyzed, corresponding to 831 in 2009, 260 in 2010, and 549 in 2011. This also shows the social and economic impact generated by DENV-2 circulation in the city.

The increased virulence of DENV-2 could be the result of different mechanisms, such as the stimulation of nitric oxide production by infected monocytes, which generates toxic and inflammatory effects in the organism, leading to the apoptosis of human cells (Valero et al., 2013).

Infections caused by DENV-2 also reach higher viral titer (Thomas et al., 2008), due to the efficient replication of this dengue virus serotype (Vaughn et al., 2000).

Despite the association of DENV-2 with severe dengue, milder hemorrhagic presentations were more frequent from a clinical standpoint, such as positive tourniquet test and petechiae. In a previous study, hemorrhagic manifestations, such as melena, hematuria, hematemesis, epistaxis and menorrhagia, were more common in DENV-2 infections (Balmaseda et al., 2006). Other symptoms indicative of severity were associated with this serotype in previous studies, such as signs of plasma leakage (Kalayanarooj and Nimmannitya, 2000; Thomas et al., 2014), demonstrated by cavity effusion (Fried et al., 2010), hemoconcentration (Thomas et al., 2014), and hypovolemic shock (Balmaseda et al., 2006), or thrombocytopenia (Thomas et al., 2014; Yung et al., 2015), internal hemorrhage (Balmaseda et al., 2006) and liver dysfunction (Kumaria, 2010). These clinical manifestations were not commonly registered in severe dengue linked with this serotype in Vitória, but the small sample size of severe dengue cases related to DENV-2, composed by ten registers, impaired the establishment of an association between clinical manifestations of severe dengue and DENV-2 infection.

Another serotype frequently related to severe presentations of dengue, such as dengue hemorrhagic fever (Nisalak et al., 2003) and dengue shock syndrome (Passos et al., 2004; Kumaria, 2010), is the DENV-3, which has also been linked with uncommon dengue manifestations, such as liver impairment (Kalayanarooj and Nimmannitya, 2000; Kumaria, 2010), in previous studies. Unfortunately, it was not possible to assess the influence of this serotype in the emergence of severe dengue in Vitória, since the evaluation was impaired by the limited sample, consisting of only a single case.

In the present study, DENV-1 infection was negatively associated with the emergence of severe dengue, similarly to previous studies that point to a lower proportion of severe cases in DENV-1 infections than in dengue caused by other serotypes (Corwin et al., 2001; Kumaria, 2010). The epidemiological scenario observed in Vitória in years with DENV-1 circulation, especially when DENV-1 was the predominant serotype, appears to indicate that this serotype is less likely to contribute to the emergence of severe outcomes. Of the cases with information on dengue virus serotype, 96.7% (n = 355/367) were related to DENV-1 in 2010, with 88.2% of all severe cases (n = 15/17) being caused by this serotype. Despite the proportion of severe cases linked with DENV-1 in 2011, compared to other serotypes, DENV-1 cases presented better clinical evolution,

especially considering that other factors related to severity were present in this year: DENV-1 and DENV-2 were co-circulating, and, after three years with a broad circulation of DENV-2, the proportion of sequential dengue infections was probably increased. The clinical manifestations in cases of severe dengue caused by DENV-1 were milder, such as hemorrhage demonstrated by positive tourniquet test and petechiae. A previous study also presented similar results (Balmaseda et al., 2006), and other investigations found that the plasma leakage was a rare manifestation in DENV-1 infections (Corwin et al., 2001; Kumaria, 2010). Contrary to previous reports (Thomas et al., 2014), gastrointestinal manifestations were not observed in the cases included in the present study. Nevertheless, the number of severe cases caused by DENV-1 was low (n = 17), impairing a better evaluation of the clinical manifestations of severe dengue in these cases.

Despite previous studies having corroborated the present results regarding the relation of DENV-1 and DENV-2 and the emergence of severe dengue, not all investigations are concordant with these findings. A study including solely hospitalized patients concluded that cases caused by DENV-1 and DENV-2 infections present the same chance to evolve to dengue hemorrhagic fever (Fox et al., 2011). However, restricting cases to only those requiring hospitalization introduces bias into the sampling, as hospitalized patients may be more likely than the general population to develop dengue hemorrhagic fever, potentially interfering with the results. Another study presented a result totally opposite to the findings of the present study. Conducted in Singapore, the research described a higher chance of developing dengue hemorrhagic fever in cases caused by DENV-1 infection when compared to those caused by DENV-2 infection. Nevertheless, it is important to note that the DENV-2 genotype circulating in this Asian country belonged to the Cosmopolitan group (Yung et al., 2015), which is considered to be different to the DENV-2 genotype circulating in Vitória, that clustered in the American/Asian group (Dettogni and Louro, 2012). As previously discussed, genotypes can vary in virulence and transmissibility (Rico-Hesse, 2010). Thereby, the circulation of DENV-2 Asian genotype in the Americas was accompanied by an emergence of cases presenting dengue hemorrhagic fever in this region. In addition, until 2013 all cases identified in the Americas that presented dengue hemorrhagic fever caused by DENV-2 were found to belong to the Asian genotype (Rico-Hesse, 2003).

Regarding infections caused by DENV-4, the present study did not find an increased or decreased proportion of evolution to severe dengue. In other populations, DENV-4 was related to less severe clinical presentations (Nisalak et al., 2003; Thomas et al., 2014), maybe due to the lower values of viral titers found in infections caused by this serotype (Gubler et al., 1981). This limitation of

DENV-4 in triggering severe dengue was illustrated by the epidemiological scenario observed in Vitória in periods when this serotype was predominant. In 2013, despite the large epidemic in the municipality, caused by the recent introduction of DENV-4, only 10.6% of laboratory confirmed cases evolved to severe dengue. In 2012, 7.6% of these cases presented this clinical outcome. Both years had a proportion of severe dengue inferior to that registered in 2009, 2010 and 2011. In the study sample, only four severe dengue cases caused by DENV-4 were present, impairing a reasonable evaluation of clinical manifestation of severe dengue in these cases. Previous studies, however, have shown an association between DENV-4 infection and cutaneous manifestations, such as petechiae and positive tourniquet test (Halsey et al., 2012), which was not possible to show with the data evaluated in this study.

Some limitations related to the use of secondary data are present in this study. These data were collected for surveillance purposes, and their precision was determined by the professional responsible for filling the forms and for digitalizing the information on the System for Notifiable Diseases. However, the use of standard forms to report and the checking of data carried out by the Surveillance Service, minimizes information bias due to misclassification of the cases. The determination of serotype was made in 485 cases systematically selected in sentinel sites, and was performed for surveillance purposes only, and therefore not related to the patient care, minimizing selection bias. In addition, physicians did not request these tests, making selection by clinical manifestations very unlikely. Only 1.6% patients randomly selected out of the 30,027 suspected dengue cases, reported from 2009 to 2013, were tested in order to define the serotype responsible for the infection. However, even including only 1.6% of dengue cases reported in the period, the sample size was large enough to find the associations with a sufficient power.

One limitation in conducting serotype identification for a large number of dengue cases is that there is a restricted time when blood collection permits the identification of the virus. The blood collection must be taken in the viremic phase of the disease, usually lasting only until the third day after the onset of symptoms. On the other hand, it prevents the inclusion of cases based on severity of manifestation of dengue, minimizing the selection bias, since the warning signs of dengue infection normally appear three days after the onset of symptoms, so after the blood samples have been taken.

Additional information regarding the genotypes of different virus serotypes circulating in Vitória would be valuable to discuss the relation of severe dengue with genotype, and to investigate

differences from results found in other sites. Molecular studies in Vitória are welcome in order to help to elucidate other epidemiological questions regarding the influence of viral factors and emergence of severe cases in the city. Considering the variables that could not be evaluated in the present study, due to absence of data in the report forms, prospective studies conducted in hyperendemic areas and in different centers are necessary in order to further elucidate the relation of severe dengue to different dengue virus serotypes, with larger sample sizes. Factors such as immunological status, serotype and genotype, presence of comorbidities and demographic characteristics could be considered at the same time, allowing for a broad comprehension of the complexity involved in the evolution to severe dengue.

The present study, even with limitations concerning the absence of data on sequential dengue infections and the presence of comorbidities, deepened its efforts to assess the role of demographic characteristics, such as gender and age, on the development of severe dengue. The present study investigated 6,703 dengue cases confirmed by viral identification or serological tests, which were reported in Vitória from 2007 to 2013.

Previous studies were not conclusive regarding the association of gender and severe dengue outcomes (Guha-Sapir and Schimmer, 2005), despite some evidences showing the influence of sexual hormones on physiological response against dengue virus infection (Guerra-Silveira and Abad-Franch, 2013). In the present study, males developed severe dengue in a higher proportion than females. However, the underlying mechanisms are unclear, and could not be evaluated with the data available. Additionally, gender-associated behavioral differences regarding when to seek treatment can also be suggested as a factor that influences the development of severe dengue, since a delayed intravenous hydration elevates the risk for severe outcomes. More studies are necessary in order to evaluate the association between genetic, hormonal and behavioral variables among males and females and the development of severe dengue.

Dengue presents distinct epidemiological patterns in terms of incidence in different age groups. Regions where dengue is endemic for more than 15 years present a higher proportion of infections among children than among older people, such as observed in Asia (Guha-Sapir and Schimmer, 2005; Hammond, 2005). In locations where dengue virus has been circulating for less than 15 years, dengue affects all age groups in similar proportions. In Vitória, dengue has been established for more than 20 years, and the proportion of people under 15 years old affected by the disease has increased throughout this period (Cardoso et al., 2010). Understanding the effect of age on the

emergence of severe outcomes of dengue is necessary, especially in light of these facts, in order to provide better assistance for the patients by preparing the health system to deal with this reality.

In the group of 6,703 dengue cases that were reported in Vitória, age was an important variable associated with the disease outcome. In the city, elderlies were more affected by severe dengue than younger people. Similar results were found in Singapore (Low et al., 2011). Additionally, elderlies presented a higher fatality rate, as shown by studies from different countries (Guzman et al., 2002; García-Rivera and Rigau-Pérez, 2003; Lee et al., 2008b; Liu et al., 2008; Lin et al., 2010; Leo et al., 2011). It seems that, in general, elderlies are at higher risk to develop severe dengue in any ethnicity or local epidemiological scenario.

Regardless of which serotype was dominantly circulating in Vitória, in every year from 2007 to 2013, elderlies were proportionally more affected by severe dengue than other age groups. The higher proportion of severe dengue in elderlies occurred in years with predominant circulation of DENV-2. Severe dengue occurred less in this age group in years with predominant circulation of DENV-1. Some factors associated with aging could have affected the incidence of severe dengue in elderlies. Sequential infections are more common in long periods of exposure while living in an endemic area. Therefore, the chance of having sequential dengue infections increases with increasing age. In Vitória, the presence of sequential dengue infections is plausible since over approximately two decades all four dengue serotypes have circulated in its territory. As physiological functions in the human body decline with age (Lee et al., 2008b), the immune system is affected (Opal et al., 2005), limiting the body's ability to combat oxidative stress induced by dengue infections (Rojas et al., 2007; Soundravally et al., 2008), since the monocytes present lower antioxidant response in elderlies (Valero et al., 2013).

Aging is also associated with decreased production of cytokines and, thereby, lower capacity to stimulate T cell production (Opal et al., 2005), contributing to the development of severe dengue and deaths, including cases influenced by bacterial co-infections in dengue course (Lee et al., 2008b; Rowe et al., 2014). Elderlies are also more likely to suffer from chronic diseases, including hypertension and diabetes (Low et al., 2011; Rowe et al., 2014). In order to prevent stroke, heart attack and other problems caused by blood coagulation, the regular use of salicylates is more common in elderlies, and this increases the risk of hemorrhages if a person acquires a hemorrhage prone infection such as the dengue (Gorzoni al., 2010). Unfortunately, it was not possible to characterize the presence of these variables in this age group due to the absence of related data.

Besides the association between severe dengue and old age, another factor that affects clinical management is that diagnosis in elderlies can be challenging, since symptoms typical for dengue may not appear in this age group (García-Rivera and Rigau-Pérez 2003; Lee et al., 2008b; Low et al., 2011; Rowe et al., 2014). In the present study, even presenting severe dengue, elderlies had less hemorrhagic manifestations than other age groups, except hematuria. Blood in urine was detected in seven of 12 cases presenting plasma leakage, and in two cases presenting concomitant platelet levels lower than 50,000/mm^3, which facilitated the diagnosis of severe dengue. However, independently of the presence of evident warning signs, hematuria should be checked in elderlies with dengue, as well as fecal occult blood, since hidden bleedings are more common in this age group (García-Rivera and Rigau-Pérez, 2003). Thus, monitoring elderly patients with complementary tests should be encouraged in order to provide a timely diagnosis of a potentially severe condition, permitting a fast implementation of the correct treatment.

In the present study, children did not show higher rates of severe dengue. Although they present a higher proportion of this clinical form in years with DENV-2 circulation, children had severe dengue with less frequency than other age groups, as what was observed in 2010. Nevertheless, children having severe dengue presented a higher proportion of hemorrhage, plasma leakage and epistaxis than other age groups, corroborating similar findings in other populations (Hammond et al., 2005; Hanafusa et al., 2008; The et al., 2012; Wichmann et al., 2004). The similarity of these results to those from other countries demonstrates the importance of physiological aspects inherent to children in the development of severe dengue, which are independent of ethnicity. The increased vascular permeability (Hammond et al., 2005; The et al., 2012), capillary fragility (Gamble et al., 2000) and underdeveloped homeostatic mechanisms (The et al., 2012) typical of childhood, influence the susceptibility to severe plasma leakage in this age group during the course of illness.

An additional factor to be considered in the clinical management of dengue in children is the difference between children and adults regarding the classical manifestations of dengue (Zambon et al., 2010). Headache, retro-orbital pain, nausea, vomiting, arthralgia, and myalgia are more frequent after the age of 11 years, while epistaxis, oliguria, and hepatomegaly are more common in children, especially in those younger than five years of age (Kumar et al., 2008). In addition, gingival bleeding, hematomas, running nose, and cough occur frequently in children younger than five years of age infected by dengue virus (Kittigul et al., 2007). Since the clinical manifestations of dengue are similar to that of other diseases typical in this age group, the diagnosis of dengue is

challenging in children, potentially leading to delayed treatment, which increases the occurrence of severe dengue (Zambon et al., 2010).

In adolescents, hemorrhage and plasma leakage, especially seen through cavity effusion, occurred in high proportion, but not as frequently as in children. Petechiae was the only manifestation more frequent in adolescents than in children, but only among females. Similarly to children, adolescents did not experience association with severe dengue, but in years with DENV-2 circulation, adolescents presented higher proportions of severe dengue than the general study population. Severe dengue occurred more frequently in adolescents than in children in all years, except 2012. This was probably due to a higher number of sequential dengue infections in adolescents, who were exposed for a longer time to an environment where dengue is endemic. In 2012, children proportionally evolved more frequently to severe dengue than adolescents, and this tendency should be confirmed in a future evaluation, since the incidence of dengue in people younger than 15 years of age is increasing in Vitória (Cardoso et al., 2011), raising the chance of children of presenting sequential dengue infections. Therefore, children should be considered a vulnerable group for severe dengue development, even without an association between childhood and severity being demonstrated in the present study, since their physiological characteristics and the new epidemiological scenario might have a potential to influence the severity in this age group.

Adults were the only age group showing a negative association with severe dengue, similar to what has been demonstrated by other studies from different countries (Rigau-Pérez et al., 2001; Ooi et al., 2003). Adults suffered less from severe dengue compared to overall study population in all years evaluated, independent of the serotype that has been circulating. The proportion of severe dengue in adults was higher in 2010, probably due to the simultaneous circulation of DENV-1, DENV-2 and DENV-3 in the city. Physiological aspects such as lower capillary permeability (Hammond et al., 2005) contributed to lower rates of plasma leakage and cavity effusion in adults and resulted in a protective effect against severe manifestations of dengue.

Besides the use of secondary data, other limitations were present in the evaluation of demographic characteristics and dengue outcomes. The exclusive inclusion of cases with laboratory confirmation increased the proportion of cases in the sample with severe dengue. The proportions of cases with severe dengue in the present study were therefore larger than that observed in Vitória. The order of sequential serotype infections and the time between the infections influences dengue severity (Guzman and Kouri, 2008), and could have affected the age groups differently. However,

it was not possible to consider these factors related to sequential dengue infections in the present study. Despite these limitations, the study succeeded in identifying which groups need special attention regarding clinical management, pointing to their need to be prioritized in a future vaccine implementation.

On December 9, 2015, Mexico has approved the application of the world's first vaccine against dengue. CYD-TDV, under the commercial name Dengvaxia™, is produced by Sanofi Pasteur©, a division of the company Sanofi©, and is a live recombinant tetravalent vaccine. The ability to generate an immune response for all four serotypes is essential in order to avoid the emergence of severe cases, as it is assumed to occur in sequential dengue infections (Singhasivanon and Jacobson, 2009). For the time being, Dengvaxia™ is the first and only licensed dengue vaccine, whilst some other candidates just started phase III trials. The vaccine efficacy against dengue was assessed to be 60.8% on average, but is highly dependent on the serotype: 42.3% against DENV-2, 50.3% against DENV-1, 74% against DENV-3, and 77.4% against DENV-4 (Villar et al., 2015). Additionally, the efficacy depended on the age of vaccinated individuals: 66% among those aged nine years or older, and 44% among children younger than nine years (Wilder-Smith and Gubler, 2015). Vaccine efficacy against severe dengue was 80.3% (Villar et al., 2015). As the license is limited for the use of individuals aged nine to 45 years, its benefit excludes exactly those populations with highest risk for severe dengue and worse clinical outcomes, as shown in the present study.

The World Health Organization recommends this vaccine for regions presenting high dengue transmission, with seroprevalence around 70% or higher in the age groups which are targeted for vaccine campaigns (World Health Organization, 2016), considering that the vaccine presented higher efficacy in individuals with previous dengue infections (Wilder-Smith and Gubler, 2015). As the vaccine is administered in a three dose series on months 0, 6 and 12, it cannot be effectively used for epidemic scenarios, but might be effective in terms of vaccination campaigns. The main goal in this case is reduce hospitalizations and costly demand in the health systems, since the vaccine could reduce in 81% the hospitalized cases (Wilder-Smith and Gubler, 2015). In February 2016, the first country that initiated a vaccination campaign against dengue was the Philippines. This country approved the vaccine on December 22, 2015. Brazil followed on December 28, 2015, and El Salvador on February 5, 2016. The limited efficacy of about 42.3% (varying from 14% to 61.1%) against DENV-2 (Villar et al., 2015) is a concern considering that this serotype causes a higher incidence of severe dengue than the other serotypes, as demonstrated by the present study.

Vitória is one of the centers where this vaccine has been tested since June 2011, but no additional cases of DENV-2 were identified in the city since then. Thereby, an evaluation regarding the effect of the vaccine in reducing severe outcomes and hospitalizations in a scenario of DENV-2 circulation was not possible. Hopefully, this aspect will be elucidated in the future, when the vaccine implementation will be accompanied by quality surveillance.

Development of other vaccines are in course and should be stimulated aiming to provide a better protection against dengue virus. An additional issue for the vaccine development is the possibility of a discovery of a probable new dengue virus serotype. The evidence of the existence of a dengue virus serotype five (DENV-5) circulating in Borneo Island, in the Malaysian territory, was presented in 2013, more than a half century after the last new dengue virus serotype had been described. However, the sample analyzed was collected from a human individual in this location in 2007. The entire genome of the virus was sequenced and used in a phylogenetic analysis. The phylogenetic tree obtained in this investigation showed that the strain from Borneo was located in an exclusive branch, not clustering with the other dengue virus serotypes already described. The immunological response induced by infection with the new serotype was also different from that generated by the other dengue virus serotypes (Normile, 2013). Apparently, DENV-5 occurs in a sylvatic cycle, but its detection in humans demonstrates the possibility of this serotype being involved in human transmission cycles in the future. The emergence of sylvatic strains affecting human populations must be considered, and more research should investigate this interaction, in order to provide successful development of safe and effective vaccines against dengue virus (Mustafa et al., 2015).

The control of dengue infections is challenging, and even with an effective vaccine being implemented in future, other efforts are necessary. Overcoming inequity is an essential element for dengue control, but in Brazil there is a long way to go before achieving this. According to the World Bank, the Gini Index in Brazil was 0.529 in 2013. Brazil was one of four countries with the highest inequity rating. Therefore, a large part of its population lives in inadequate conditions, despite Brazil being ranked as one of the world's ten strongest economies. Efforts from the health sector are not expected to be efficient without the involvement of other sectors and political will. Involvement of the community must be sought for preventive initiatives and for the identification of local residents presenting signs and symptoms of dengue. Community and religious leaders could be involved in this effort, as well as teachers at local schools.

Many investigators are dedicated to contributing to the improvement of the epidemiological scenario of dengue, with developments in control activities focused on the vector. One strategy is the use of bacteria of the genus *Wolbachia* to infect *Aedes (Stegomyia) aegypti*. The bacteria acts to prevent the installation of dengue virus in the midgut of the mosquito and also reduces the life duration of the vector. However, the reduction in the lifespan can also lead to the selection of viruses more efficient in infecting the mosquitoes in a brief timeframe (Christofferson and Mores, 2015). Transgenic sterile male mosquitoes has been tested for dengue control, reaching at least temporary success, reducing the number of mosquitoes captured in traps (Carvalho et al., 2015). These efforts are laudable and can help dengue control even if the current social scenario is maintained.

Regarding surveillance, attention to the laboratory component is necessary. A laboratory confirmation should be pursued for all suspect dengue cases, except during epidemics, when case numbers are quickly outnumbering laboratory capacities, and pre-test probability of clinical case definitions is high due to a largely increased incidence. In order to obtain good quality material for laboratory testing, it is necessary to collect and test the blood sample at the correct time. Tests conducted within the first three days after the onset of clinical manifestations should focus on virus identification, such as RT-PCR and viral isolation. After four days of onset of symptoms, serology is the method indicated to detect the antibodies against dengue virus (Beatty et al., 2010). However, serological detection is hampered by extensive cross-immunity across different *Flaviviruses*, such as antibodies developed in a previous dengue infection or after yellow fever vaccination, which is commonly encountered in regions where dengue virus is circulating.

The surveillance system should not be only passive, but also be based on active surveillance. In a scenario of exclusive passive surveillance, the detection of increased transmission normally occurs in a late stage, when dengue dispersion is already significant, losing the opportunity to start timely control actions. It occurs due to the low sensitivity of passive surveillance, which is influenced by the capacity to establish a correct diagnosis by clinicians and of the treatment seeking behavior of the population. Active surveillance helps to evaluate the epidemiological situation especially in interepidemic periods, assessing disease occurrence and the serotypes circulating, providing an early detection and prediction of dengue epidemics. This kind of surveillance requires laboratory capacity, as well as the establishment of sentinel sites and specifically trained staff. It is important to involve clinics as sentinel sites to provide monitoring of non-specific viral syndromes in non-

epidemic periods, while sentinel hospitals help to monitor dengue severity, especially in epidemic periods (World Health Organization, 2011).

The major objectives of dengue surveillance are the rapid detection of epidemics, which allows prompt and adequate responses. This includes the assessment of the disease burden with its social and economic impacts, the monitoring of dengue distribution and dispersion over time, the assessment of environmental risk factors and the evaluation of the effects of dengue control measures. The prediction of dengue outbreaks is another aim that must be pursued by surveillance services (Beatty et al., 2010). Finally, the activities might provide support for decision-makers to define resource allocation (World Health Organization, 2012a). In order to guarantee effective surveillance, the data collected must be of high quality, having precision and completeness. Therefore, the evaluation of the notification system is necessary on a periodic basis. The surveillance system should be a component of the national health information system, permitting monitoring of health indicators at different levels of the health administration, such as the progression of the morbidity and mortality in face of the health decisions taken (World Health Organization, 2012a). The reporting of dengue must be required in all health facilities, including the private sector and different levels of health care. During outbreaks, reporting of a suspected dengue case to the surveillance services should ideally occur within 48 hours. In the peak of transmission, at least a weekly report of aggregated information is necessary for dengue monitoring (Beatty et al., 2010).

As demonstrated by the present studies, surveillance service can also use tools, such as mapping through Geographic Information Systems, to evaluate disease distribution, to detect hotspots of dengue occurrence, to follow the spread of the disease, and to predict future patterns of dengue occurrence. Surveillance also can use phylogenetic evaluation of dengue virus strains and detection of dengue virus serotypes to predict the severity and magnitude of dengue occurrence. These information are valuable to decision makers in order to prepare the health system to provide a better assistance to the population. The present results reinforce the importance of training of medical staff to detect dengue and the warning signs of dengue severity in a timely manner, even in groups that represent a challenge for the diagnosis, such as children and elderly. In summary, investments in research, surveillance services, health care facilities, training of health professionals, vector control programs, sanitation, health education, and communication are necessary to overcome dengue after 30 years since its establishment in Brazil, and after more than 20 years of its installation in Vitória.

6 Conclusion

Two genotypes of DENV-4 were detected circulating in Vitória in 2013. The DENV-4 genotype I from Vitória was closely related to the dengue virus detected in Bahia state in 2011, which was probably imported directly from Asian countries. The DENV-4 genotype II detected in Vitória was closely related to strains collected in Roraima state in 2010, in Mato Grosso state in 2012 and São Paulo state in 2015. This virus was probably first introduced into Roraima state, coming from neighboring South American countries, such as Venezuela and Colombia and then spreading to other Brazilian states, where local evolution occurred. Apparently, Vitória is not an international route for dengue introduction in Brazil.

In the first epidemic triggered by the introduction of DENV-4 in the city, the five space-time clusters with higher incidence in the beginning of the epidemic period and higher risk for dengue transmission were characterized as low-income areas, with some presenting subnormal agglomerates. In comparison, the six space-time clusters with late increment in dengue incidence and lower risk for dengue transmission were located in high-income areas. The clusters with lower and higher Time Trend Increase did not differ regarding House Index or human population density, demonstrating the limitation of House Index to determine prioritized areas for dengue control. Evidence suggests that living in low-income areas, especially in proximity to subnormal agglomerates, makes a person more exposed to the vector, increasing the chance to be infected by dengue virus. Therefore, the transmission in space-time clusters with these characteristics was explosive, with a fast reduction in the population susceptible for dengue infection due to increased herd immunity. These space-time clusters play a role as hotspots of dengue occurrence, and the dispersion from them happened in centrifugal waves to near areas.

Considering the relationship between dengue virus serotypes circulating in Vitória and the emergence of severe outcomes, cases caused by DENV-2 infection presented a higher proportion of severe dengue than infections caused by DENV-1 or DENV-4. In years with DENV-2 circulation, the number of registrations of severe dengue in the city was higher than in other years, providing evidence to support the role of this serotype in triggering severe epidemics. Therefore, surveillance of circulating serotypes is important for predicting the severity of epidemics.

In Vitória, males, elderlies and children experienced worse outcomes in the course of dengue infection. Males and elderlies presented a higher proportion of severe dengue compared to other

groups, but children with severe dengue presented worse clinical manifestations, such as hemorrhage and plasma leakage. In elderlies, the clinical manifestations of severe dengue were less evident, with hematuria being more common in this age group than among young patients. The differences in clinical presentation of dengue with respect to the demographic characteristics of the people affected by the disease calls to attention the need for clinical protocols which consider the specificities of each age group in the course of dengue. Both elderlies and children should be prioritized in a future vaccine implementation.

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8 Annex

8.1 Curriculum vitae

Work experience

Position: Researcher.
Dates: From March 2010 to present.
Employer: Federal University of Espírito Santo.

Position: Intern.
Period: From October 2014 to December 2014.
Employer: Institut für Mikrobiologie der Bundeswehr.

Position: Lecturer.
Period: From August 2010 to June 2011.
Employer: Federal University of Espírito Santo.

Position: Intern.
Period: From August 2007 to July 2009.
Employer: Federal University of Espírito Santo.

Education

Period: From March 2010 to March 2012.
Title: Master in Public Health.
Institution: Federal University of Espírito Santo.

Period: From August 2005 to January 2010.
Title: Dental Surgeon.
Institution: Federal University of Espírito Santo.

8.2 List of publications

- "Factors related to severe dengue during an epidemic in Vitória, State of Espírito Santo, Brazil, 2011", Revista da Sociedade Brasileira de Medicina Tropical, v. 46, n. 5, p. 629-632, 2013.

- "Parental alcoholism and associated risk factors", SMAD Electronic Journal of Mental Health, Alcohol and Drugs, v. 7, p. 161-167, 2011.

8.3 Statement on pre-release and contribution

The thesis originated from four manuscripts, of which three were already submitted to international scientific journals.

The manuscript "Serotype influences on dengue severity: a cross-sectional study on 485 confirmed dengue cases in Vitória, Brazil" was submitted to the journal BMC Infectious Diseases on November 18, 2015 and the revised version was resubmitted on January 12, 2016.

The manuscript "Influence of demographics on clinical outcome of dengue: a cross-sectional study of 6,703 confirmed cases in Vitória, Espírito Santo State, Brazil" was submitted to the journal Epidemiology & Infection on February 24, 2016.

The manuscript "Evidence of co-circulation of dengue virus serotype 4 genotypes I and II during the epidemics in 2013 and 2014 in Vitória, Espírito Santo state, Brazil: A phylogenetic study" was submitted to the journal Molecular Biology Reports on February 24, 2016.

A fourth manuscript is in preparation, with the working title "Determination of space-time clusters and factors related to dengue dispersion during the first epidemic of dengue virus serotype 4 in a highly susceptible population: An ecologic study in Vitória, Brazil". This manuscript has not been submitted yet at the time of submission of the monography.

The PhD candidate participated in the study design, preparation and submission of documents to the ethic committees and the institutions involved in the research, organization of the study sites, data collection, database creation, mapping, statistical analysis, spatial variation in temporal trends analysis, thesis and manuscripts writing and revision.

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8.5 Affidavit

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Country

I hereby declare, that the submitted thesis entitled **"Phylogenetic, epidemiological and clinical studies on dengue and dengue virus in Vitória, Espírito Santo state, Brazil"** is the result of my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

The submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

I further declare that the electronic version of the submitted thesis is congruent with the printed version both in content and in format.

Munich, April 30, 2016

Place, Date

Signature of PhD Candidate