Detection of Dengue Virus in Department of Bolivar, Colombia, 2012-2013, Real-Time PCR

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ABSTRACT

Objective. To assess the presence of RNA from dengue virus by Real Time Polymerase Chain Reaction with Reverse Transcriptase (qRT-PCR), in anti-dengue IgM seronegative serum samples obtained from symptomatic patients with less than 5 days of fever, in the department of Bolivar, Colombia.

Materials and Methods. Cross sectional descriptive study, where 50 anti-dengue IgM seronegative serum samples were analyzed by qRT-PCR. In addition, we reviewed the epidemiological charts of each patient in order to collect sociodemographic and blood count (CBC) data.

Results. DENV-3 RNA was detected in seven (14 %) serum samples. The most common clinical manifestations were: fever (90 %; 45/50), headache (88 %; 44/50) and myalgia (74 %; 37/50). Regarding the CBC, the most significant findings were: thrombocytopenia (60 %; 30/50; 73.3% were men), and leukopenia (50 %; 25/50; 60% were men).

Conclusion. Detection and typing of DENV genome by qRT-PCR could be considered a diagnostic tool for Dengue in anti-dengue IgM seronegative serum samples, especially, within four days after the onset of the signs of the disease.

Indexing terms/Keywords

Dengue Virus; Dengue; Real-Time Polymerase Chain Reaction; RNA, Viral (source: MeSH, NLM).

Academic Discipline And Sub-Disciplines

Tropical Medicine, Public Health.

SUBJECT CLASSIFICATION

Virology.

TYPE (METHOD/APPROACH)

Cross sectional descriptive study.

INTRODUCTION

Dengue, the main arboviral infection in the world, is an acute viral disease epidemic in tropical and subtropical regions. It is transmitted to humans by the bite of infected mosquitoes from genus *Aedes*, being *Ae. Aegypti* and *Ae. albopictus* the main vectors in the Americas and Asia respectively [1,2]. An estimated 2.5 million people live at risk and the incidence of the disease is between 50 to 100 million cases/year, with an increasing trend due to the wide geographical spread of the virus and its vector [2-4].

Dengue virus (DENV) belongs to the *Flaviviridae* family as part of the *Flavivirus* genus, its viral genome is a single stranded RNA chain with positive polarity and has four serotypes antigenic and phylogenetically different (DENV-1 to DENV-4). This viral genome encodes three structural proteins: capsid protein (C), membrane (M) and envelope (E) and seven non-structural proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 [5, 6].

Since 2009, the World Health Organization defined a new classification for the severity of this arboviral infection based, essentially, on clinical data but with special emphasis on the presence of warning signs, which allows early identification of patients likely to evolve in serious cases. This classification defines three diagnostic categories: dengue without warning signs, dengue with warning signs and severe dengue [7].

According to evidence from epidemiologic studies, a few risk factors associated with severe forms of this viral disease have been identified, such as female sex, childhood, body mass index (BMI), presence of chronic diseases (diabetes, sickle cell anemia and asthma), serotype, ethnicity and certain genetic characteristics of the host [6-9].

In Colombia, a tropical country, this disease represents a priority public health problem, due to the re-emergence and intense transmission, as well as the behavior of epidemic cycles every two or three years, increased frequency of severe dengue outbreaks, the simultaneous circulation of different serotypes, *Ae. aegypti* infestation in over 90% of the national territory located below 2200 meters above sea level and the introduction of *Ae. albopictus* [10].

As in other endemic diseases, in the case of dengue, early reporting of cases is critical to identify epidemic outbreaks and initiating a timely medical response [11].

According to national reports of dengue (2008-2013), in the department of Bolívar cases of severe dengue have been documented, characteristic of the mesoendemic pattern in this region [12]. Additionally, during 2012, over 82% of dengue cases presented in this department were reported in three municipalities: Santa Rosa del Sur, El Carmen de Bolivar and Magangué [13].

Among the laboratory tests available for diagnosing Dengue, enzyme immunoassay, ELISA, to capture anti-dengue IgM is considered the method of choice due to its cost, low complexity and response time. However, these antibodies become detectable from the third to the fifth day of the disease in 50% of hospitalized patients and do not allow differentiation between different serotypes [9-14].

Therefore, the detection of nucleic acids by the technique of polymerase chain reaction with reverse transcriptase (RT-PCR), including the real-time variant (qRT-PCR), become early diagnostic confirmation possibilities, since they can detect viral RNA and serotype within the first 24 to 48 hours of a feverish state [5,15-17].

Consequently, the aim of this study was to evaluate the presence of dengue virus RNA by qRT-PCR in 50 serum samples, seronegative for anti-dengue IgM, obtained from patients with clinical symptoms compatible with dengue and less than five days of clinical evolution, in the department of Bolivar, Colombia.

MATERIALS AND METHODS

This is a descriptive cross-sectional study, in which we selected patients over five years of age with clinical symptoms compatible with dengue. Clinic-epidemiological records of each case were reviewed, documenting the results of blood count with differential leukocyte count and sociodemographic variables of patients.

Samples

Serum samples were provided by the Bolivar Department of Public Health Laboratory, reference center in collecting samples from municipal hospitals for diagnosis of dengue virus. Samples from selected patients originate in the municipalities: Santa Rosa del Sur, Carmen de Bolivar, San Pablo and Morales.

All samples were assayed for anti-Dengue IgM antibodies (UMELISA® Dengue IgM Plus), before being stored at -80 °C.

Viral Isolation

For the realization of the qRT-PCR controls were provided by the Biomedical Research Group from University of Sucre (DENV-1, strain 16007; DENV-2 strain 16681; DENV-3 strain 16562 and DENV-4 strain 1036).

RNA Extraction

To 800 µL of TRIzol reagent (TRI Reagent® - Sigma), we added 200 µL of serum, stirred for 15 seconds and allowed to stand at room temperature for 10 minutes; subsequently, 200 µL of chloroform were added, mixed by inversion for 15 seconds and allowed to stand at room temperature for three minutes; by centrifugation (refrigerated centrifuge Sigma 1-15PK) at 12000 rpm for 15 minutes, we obtained an aqueous phase which was separated and treated with 500 µL of cold isopropanol and allowed to stand at room temperature for 10 minutes; after that, it was centrifuged at 12000 rpm for 10 minutes and the supernatant was discarded; then, the obtained genomic material was washed with 1 mL of 75% cold ethanol, centrifuged later at 8700 rpm for five minutes and the supernatant was discarded; after drying the excess alcohol, it was resuspended in water treated with diethylpyrocarbonate (DEPC). All samples were stored at -80°C for 24 hours.

Oligonucleotides and probes

Specific TaqMan probes and primers for each serotype were targeting the genomic region encoding the envelope protein. Probes against DENV-1 and 4 were marked on 5' with 6-FAM and MGB-NFQ on 3', while those of DENV-2 and 3 were marked on 5' with VIC and MGB-NFQ on 3'.

All oligonucleotides and probes used for qRT-PCR assay were donated by the Biomedical Research Group from the University of Sucre.

qRT-PCR

For the reaction of molecular detection of DENV, we used the kit SuperScript® III Platinum® One–Step Quantitative RT-PCR System (Invitrogen Life Science Technologies). Two duplex reactions were conducted, each for simultaneous detection of DENV-1/DENV-2 and DENV-3/DENV-4.

The mixture contained 5 µl of RNA (approximately, 500 ng of total RNA), 12,5 µL of the 2X reaction mixture (0,4 mM of each dNTP and 6 mM of $MgSO_4$), 0,5 µL of each oligonucleotide (20 µM), 0,25 µL of each probe (20 µM), 0,5 µL of ROX as reference dye (25 µM), 0,5 µL of SuperScript^{TM} III RT/Platinum® *Taq Mix* and supplemented with water to a final reaction volume of 25 µL.

The thermal profile of the reaction was as follows: 50°C for 15 minutes for the cDNA synthesis step, 92°C for 2 minutes of initial denaturation and 50 cycles of 95°C for 15 seconds and 57°C for 1 minute for amplification. The StepOne^{TM} Real–Time PCR System (Applied Biosystems) equipment was used. In all trials we included positive controls (RNA extracted from control strains of DENV-1 to 4) and negative (ultrapure water). It was considered as positive all samples presenting amplification threshold cycle (Ct) <42.

RESULTS

Description of the population

This study was conducted in a population with a median age of 19 years (range: 6 to 81 years), which had a predominance of male sex and being cases in the age group corresponding to 15 - 45 years (table 1).

According to the clinic-epidemiological records, predominant notifications of dengue without warning signs were found (90%; 45/50), with ambulatory attention conduct (60%; 30/50) (table 1). There were few cases with a history of dengue (4%; 2/50), displacement in the last 15 days (10%; 5/50) or with a family member or partner with symptoms of dengue in the last 15 days (18%; 9/50).

As to the source, most samples corresponded to southern department municipalities (98%; 49/50), mainly Santa Rosa del Sur (95.9%; 47/49).

Regarding clinical manifestations, the most common were: fever (90%; 45/50), headache (88%; 44/50) and myalgia (74%; 37/50). The most relevant hematological findings were: thrombocytopenia (60%; 30/50), of which 73.3% of cases were male and leukopenia (50%; 25/50) with 60% of cases in men. 32% of cases presented simultaneously fever, headache and muscular pain, as thrombocytopenia and leukopenia.

Table 1: Epidemiological, clinical and laboratory characteristics of the studied population.

Characteristics	qRT-PCR		Total
	Cases (+) (n = 7)	Cases (-) (n = 43)	(n = 50)
Age			
Median	20	19	19
P25	17	10	12
P75	50	27	27
Age groups			
<15 años	1 (14,3 %)	16 (37,2 %)	17 (34 %)
15-45 años	4 (57,1 %)	23 (53,5 %)	27 (54 %)
46-60 años	1 (14,3 %)	3 (7,0 %)	4 (8 %)
>60 años	1 (14,3 %)	1 (2,3 %)	2 (4 %)
Sex			
Male	4 (57,1 %)	30 (69,8 %)	34 (68 %)
Female	3 (42,9 %)	13 (30,2 %)	16 (32 %)
Dengue			
Without warning signs	7 (100 %)	38 (88,4 %)	45 (90 %)
With warning signs	0	4 (9,3 %)	4 (8 %)
Severe	0	1 (2,3 %)	1 (2 %)
Hospitalized			
Yes	1 (14,3 %)	19 (44,2 %)	20 (40 %)
No	6 (85,7 %)	24 (55,8 %)	30 (60 %)
Leukocytes (cels/mm^3)			
Median	5350	4300	4300
P25	3400	2980	3300
P75	7543	5170	5515
Hematocrit (%)			
Median	40	41	40
P25	34	37	37
P75	45	46	45
Platelets (number/mm^3)			
Median	135000	125000	128000
P25	126250	104000	109000
P75	142000	163000	155000

Molecular detection of DENV

From the 50 serum samples tested, we determined the presence of dengue RNA virus in seven samples (14%), all of them corresponding to DENV-3 (figure 1) with Ct values from 36.0 to 40,5 (37,7 ± 1,5) (figure 2). The average Ct value for the positive control used for DENV-3 was 25,1 ± 1,0.

All DENV-3 positive samples came from Santa Rosa del Sur municipality collected between July and November 2012 (31st to 44th epidemiological week, 2012).
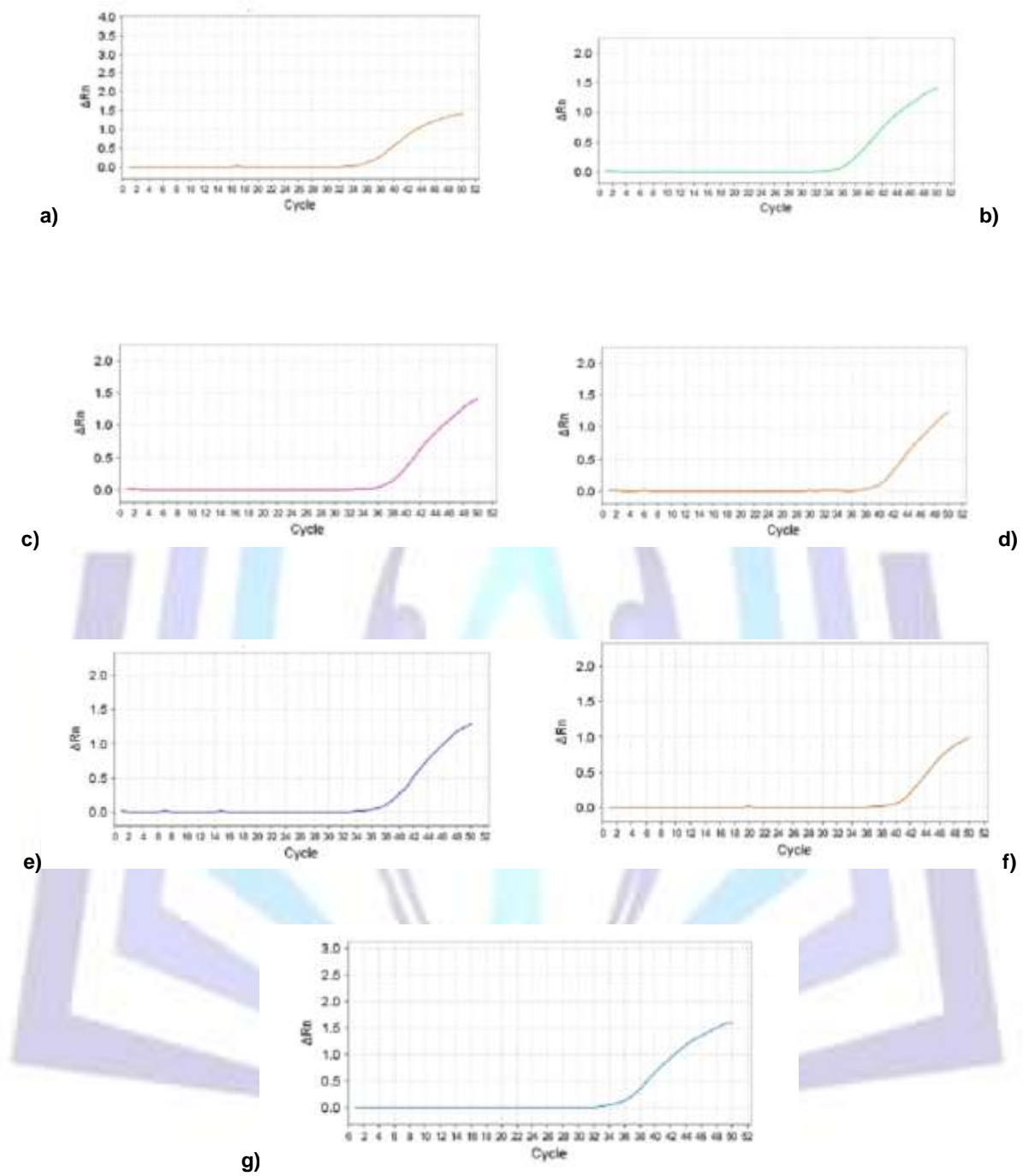
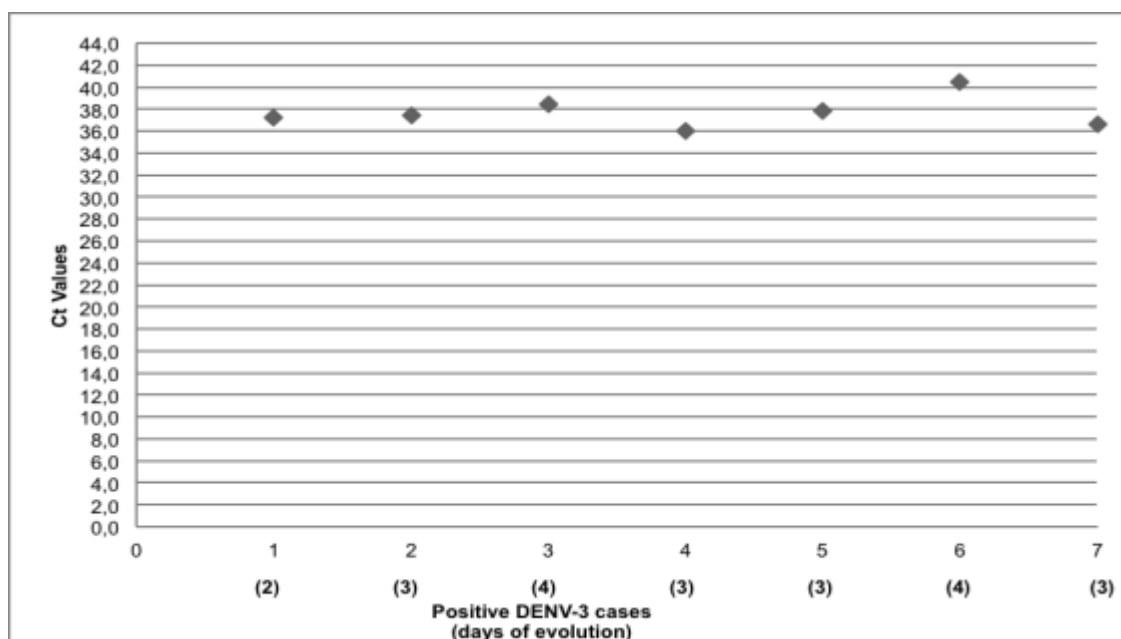Figure 1(a-g): DENV-3 positive serum samples by qRT-PCR.

Figure 2: Ct values of the DENV-3 positive cases according to the evolution time of the symptoms, expressed in days.

DISCUSSIÓN

The present study reports the detection and typing of dengue virus RNA by qRT-PCR technique in anti-dengue IgM negative serum samples, obtained from patients with less than five days evolution of fever.

The finding of seropositive cases in patients with clinical symptomatology in previously negative samples for titration of IgM antibodies, demonstrates the applicability of this molecular technique as an important diagnostic tool for early cases highly suspicious of dengue, in which the evolution of the disease obstructs early serological determination of IgM antibodies, similarly literature reiterates that the clinical diagnosis is not effective to differentiate dengue from other febrile conditions especially in its initial state, and that a technique such as qRT-PCR shows a high diagnostic sensitivity for this viral pathology [18-20].

Laue T et al. [21], without the application of real time technology, they reported that RT-PCR could detect dengue viral RNA in the great majority of patients, if serum samples were taken shortly after the onset of symptoms and before the detectable production of virus-specific antibodies; other authors have published similar findings with the application of RT-PCR [22-24] or qRT-PCR [25-27] in similar clinical situations.

In the case of DENV-3 seropositive samples, as in all qRT-PCR assay, the Ct value is considered inversely proportional to the concentration of initial genomic material [28,29]; Ct values obtained in this study suggest low viremia level. Although viremia in vertebrate hosts has been estimated for dengue between 10^5 and 10^9 RNA copies/mL [30], there are reports of situations with low plasma viremia during early stages of infection, for example, reinfection by DENV-3 with high levels of preexisting neutralizing antibodies [31,32], epidemics with endemic DENV-3 strains associated with low viremia in humans [30], primary infections with a duration of less than five days [33], newborn infected with DENV-2 through a contagious via associated with transfusion [34], and even infections of two genotypes of DENV-2 in a humanized mouse model [35].

In Colombia, the four serotypes of dengue virus have been associated with epidemics; however, DENV-1 and DENV-2 have the largest circulation [36]. In the case of DENV-3, it was absent for 20 years until its reintroduction in 2001 [37], to spread later, especially, in all endemic areas of the country, including the department of Bolivar [38].

Therefore, it is mandatory to analyze the behavior of results obtained in this study within the epidemiological situation of dengue in this Colombian department.

According to the reported dengue cases (2008-2013), the municipalities from Bolívar department present, mainly, two types of patterns: hypoendemic and mesoendemic. The first pattern indicates a risk for the rapidly appearance of outbreaks due to the large number of susceptible individuals and movement of a single viral serotype; the second indicates the presence of severe dengue cases and circulation of more than one viral serotype in the region [12].

In this study, all DENV-3 positive samples were geographically and temporally related, since they were obtained from patients treated at the Manuel Elkin Patarroyo Hospital from the municipality of Santa Rosa del Sur, during an epidemic outbreak that occurred from the 21st epidemiological week of 2012 (end of May) [39].

There are three important things to emphasize:

This study was able to identify DENV-3 in anti-dengue IgM seronegative samples; meanwhile serotyping of samples from the National Institute of Health (INS) was performed on anti-dengue IgM seropositive samples and the presence of DENV-2 was identified (40), the above would indicate that during this epidemic outbreak, at least these two serotypes were circulating, corresponding with the characteristics of the mesoendemic pattern from the region [12].

Another aspect to note is the correspondence during the outbreak in the most prevalent age group, patients aged 15 to 44 years (37) as well as the relationship between the origin locations of DENV-3 positive patients and localities that reported the highest number of dengue cases during the epidemic outbreak [40].

Beyond the methodological limitations of this study and the possible relationship of identified positive cases with the epidemiological characteristics of the reported outbreak, it is important to highlight that the detection and typing of the dengue virus genome by real-time RT-PCR in clinical samples is presented as a real and feasible possibility for early diagnosis of dengue cases, especially, during the first four days of fever evolution, acute period of the disease.

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CONFLICT OF INTERESTS

The author(s) declare that they have not conflit of interestes.

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