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Description of high rates of unapparent and simultaneous multiple dengue virus infection in a Colombian jungle settlement

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Abstract. Dengue disease statistics is mainly based on consulting patients with febrile illness, but misdiagnosed and asymptomatic cases are important to measure dengue epidemiology in endemic areas. The main objective of this work was to determine the prevalence of IgM and IgG antibodies or NS1 antigen and viral RNA in a group of healthy volunteers from an isolated village in Colombian Chocó rain forest. It found 51.7% of virologically PCR confirmed asymptomatic cases, despite low IgM seroprevalence. It was confirmed that all four serotypes are in the circulation and in 17.2% of individuals it detected natural co-infections of two or three different serotypes simultaneously. This is the first report in Colombia evaluating viremia in asymptomatic volunteers. These findings pose a big concern about the transmission of dengue virus by asymptomatic individuals because they can spread the virus without take appropriate control measures.

INTRODUCTION

Dengue virus (DENV) infection is the most important viral disease transmitted by mosquitos worldwide. More than 100 million cases with a wide spectrum of clinical manifestations are reported annually. The iceberg concept used to explain infectious diseases is well applicable to dengue disease and its characteristics because the majority of infections is asymptomatic or has mild signs, such as fever, arthralgia, myalgia, retro-orbital headache, maculopapular rash and leukopenia. Severe cases are the minority and are characterized by vascular leakage and/or hemorrhage, leading to hospitalization and fatality in hyper-endemic countries (Lam *et al.*, 2013).

In Colombia, dengue is an endemic-epidemic disease that represents a priority concern in public health because its transmission is rising in many Colombian provinces, increasing the disease burden. Epidemics occur every two or three years, and it has been confirmed that four serotypes are in circulation. In total, 72% of the 1 123 Colombian municipalities are less than 1 800 meters above sea level, and nearly all have *Aedes aegypti* in circulation. In a five-year period (2008-2012), there were 331 989 dengue cases, of which 7.2% (23 925) were severe dengue, with an approximate lethality of 2.5% (Ministry of Health, 2013). Presumably, the numbers may be greater because of case under-registration and misdiagnosis.

Dengue infection may occur with a sudden onset of fever, with mild symptoms, or as severe dengue, characterized by plasma leakage, hemorrhages or organ disease. However, the majority of infections are asymptomatic or unapparent and do not

deserve medical consultation (Guzman & Kourí, 2002). Asymptomatic cases indicate the existence of the vector mosquito and virus in a specific area, although because these individuals are not followed and interventions are not staged in their neighborhoods, these viremic subjects are responsible for continuous virus transmission. In a prospective study performed in Bangkok from 1980-1981, there were six more asymptomatic than symptomatic cases and all DENV-4 infections were asymptomatic (Burke *et al.*, 1988). Whereas Endy *et al.* (2002) reported the same number of symptomatic compared with asymptomatic cases, in Nicaragua (2001-2003) the ratio of asymptomatic/ symptomatic cases was 13:1 (Balmaseda *et al.*, 2006).

Recently, in an endemic area of Singapur, it was found that 78% of infections were unapparent, i.e., IgM-positive individuals who did not report fever or symptoms in the last 90 days (Yap *et al.*, 2013). Healthy but viremic people pose a major problem to health systems because they can spread the infection and may eventually act as blood donors, favoring endemicity in a particular region (Stramer *et al.*, 2013). Additionally, viremic individuals or individuals with asymptomatic infections may travel to non-endemic regions, introducing the virus and increasing the number of exposed people. In any event, these individuals are at risk of severe dengue because they have antibodies to one serotype, and these antibodies could act as enhancing antibodies during a secondary infection with another serotype.

In underdeveloped countries, and in Colombian particularly the reported numbers of dengue cases are mainly based on clinical diagnosis at primary health centers that report all suspected cases without laboratory confirmation. A small percentage of serum samples is sent to regional laboratories to undergo an IgM capture test, and a small proportion is shipped to the National Health Laboratory to try NS1 detection and PCR confirmation. For these reasons, case rates are presumably inaccurate.

MATERIALS

Study population and site

This descriptive cross-sectional study was conducted in the small indigenous village of Villa Nueva, which belongs to Quibdó, the capital of the Department of Chocó (Figure 1). The Villa Nueva village (with 188 Embera-Wounaan indigenous inhabitants) is located at 25 km from Quibdó and is accessible only by boat via travel through the Munguidó River. The village has 24 huts with wood walls, dirt floors and roofs made of tin and plastic.

This village was selected because a malaria outbreak had been reported in the previous months. Two visits were performed, with the first intended to count the houses and the number of residents in each home. In the second visit, the team visited nine selected houses to conduct a survey of the heads of household about knowledge, attitudes and practices (KAP) regarding malaria and dengue diseases and to collect blood samples. At this time, all of the subjects were healthy and did not report fever or any sign. The study protocol was reviewed and approved by the Ethics' Committee of Centro de Investigación Científica Caucaseco (Cali, Colombia) according to the national regulations of the Colombian Ministry of Health and the World Health Organization. All of the volunteers signed an informed consent form and authorized blood collection from them or from their children.

Serological and RT-PCR testing

Venous blood was drawn from 58 individuals, and the serum was separated and kept at 4ºC before shipping to the Virology Laboratory the next day. A second EDTA-anticoagulated blood sample was used to analyze red and white blood cells and other hematimetric indexes. The sample aliquots were then stored at -80ºC until processing for tests. Four different ELISAs for serology testing were performed following the instructions provided by the manufacturers: Capture UltraMicro-ELISA (Tecnosuma, La Havana) to detect IgM; Capture IgG ELISA (PanBio) to

Figure 1. Map of Americas showing in black the location of Colombia at the northwest corner of South America (asterisk). Inset: Map of Colombia Provinces. The arrow point out the Department of Chocó near to Pacific Coast where is located the Villa Nueva settlement.

detect elevated anti-dengue IgG antibody levels during secondary acute infections; IgG Indirect ELISA (PanBio, Australia) to detect memory antibodies and, finally NS1 viral antigen ELISA to detect the viral protein (PanBio).

For each sample, 140 µl of serum was used for RNA extraction using the QIAmp Viral RNA Mini Kit (Qiagen) and reverse transcribed with moloney murine leukemia virus transcriptase enzyme and random hexa-oligomers. The cDNA was amplified using the nested-PCR protocol reported by Chien *et al.* (2006). The first round was performed using the primers mD1- TCA ATA TGC TGA AAC GCG AGA AAC CG and D2- TTG CAC CAA CAG TCA ATG TCT TCA GGT TC, which amplify a fragment of 511 bp. This PCR product was 10 fold diluted and amplified using the primer mD1 and a primer set for the four dengue serotypes: rTS1- CCC GTA ACA CTT TGA TCG CT (DENV-1), mTS2- CGC CAC AAG GGC ATG AAC AGT TT (DENV-2), TS3- TAA CAT CAT CAT GAG ACA GAGC (DENV-3) and rTS4- TTC TCC CGT TCA GGA TGTC (DENV-4), which yielded different amplicon sizes that were visualized on ethidium bromide-stained agarose gels. In the cases in which two or more viral serotypes were detected, the second round was repeated using the primer pair specific to each serotype. These amplicons were sequenced to confirm their identity. Supernatants of C6/36 mosquito cells infected with each serotype or non infected were used as positive and negative controls respectively and distilled water as non-template control for each set of reactions.

To eliminate the risk of carry-over contamination of samples during PCR some procedures were adopted. The processing of blood samples and preparation of reagents were performed in a room different from that used for PCR amplification and amplicon analysis. Dedicated reagents, micropipettes, sterile tubes and filtered tips were used for RNA extraction and RT-PCR assembly.

DENV infection definition

Following the serological testing and PCR, current DENV infection was defined when a sample was Capture IgG ELISA positive or NS1 ELISA positive or RT-PCR positive. A second group was defined as non-current infection cases when IgG antibodies were detected in the Indirect IgG ELISA (infection history) or recent infection had been observed (capture IgM positive). A third group consisted of those who had negative results for all of the tests.

Data Analysis

Variables obtained from the KAP survey and serology or RT-PCR were tabulated in Microsoft Excel®, and analyses were performed using STATA. Data on seroprevalence and relationships with age or gender were analyzed by a Chi square test. Specificity, sensitivity, positive and negative predictive values and receiver operating characteristic (ROC) curves were estimated for each test.

RESULTS

A total of 58 serum samples (28 females) were collected from the indigenous people of Villa Nueva village, and nine KAP surveys were completed by the heads of household, describing their housing. No houses had piped water supply or piped sewage, then they have to use collected rainwater or the communal water fountain 20-100 meters from the house.

The blood samples were processed to evaluate hematimetric indexes (leukocyte count, lymphocytes, monocytes, erythrocyte count, blood cells, platelets, median corpuscular volume, hemoglobin concentration and hematocrit). The leukocyte count was significantly lower in the group with an infection history (n=28, WBC 7 790 ± 2 489) compared with the infected group (n=26, WBC 9 440 ± 3 591) (Kruskal-Wallis, $p<0.05$). The other parameters were not different between the groups.

We found a high rate of asymptomatic infections in this population (51.7%) because 30 subjects were RT-PCR positive (n=27) or IgM positive plus IgG Capture ELISA positive (n=3). Two subjects were febrile despite they did not report fever during the survey and one of them was PCR positive (Table 1). Eleven of the 27 viremic individuals had secondary infections (IgG Indirect ELISA positive). The mean age of the infected group was 13.4 years, and the rate of infection was not different among individuals below or above 15 years old (χ^2, $p>0.05$). This is the first report of confirmed viremia in healthy individuals who were recruited during a seroprevalence study in our country. The seroprevalence rate (IgM positive and/or IgG Indirect ELISA positive) in the study group was 43.1% (25/58), with the majority consisting of individuals over 15 years old, possibly due to long-term virus circulation. Strangely, none of the volunteers who had positive PCR results was positive by the NS1 antigen ELISA. All four DENV serotypes were detected in the volunteers. In 13 of 27 PCR-positive subjects (48.1%), DENV serotype 2 was detected, and in 10 individuals, 2 or 3 different serotypes were detected in the same serum sample, indicating natural co-infection (Table 2). The 32 sequenced amplicons shared between 87% (DENV-4) and a 98% (DENV-2) identity with reported dengue sequences in databases. They differ between them in 2-4 nucleotides, confirming that they are not a carry-over contamination of the samples or reagents.

DISCUSSION

Normally, due to *Aedes aegypti* invasion near towns, dengue disease is considered as an urban or semi-urban infection. However, in this work, we reported an outbreak of asymptomatic dengue cases in a small indigenous, jungle settlement, possibly due to circulation from hyper-endemic near cities

Table 1. Results of dengue diagnostic tests in different age groups*

Group	Age years	Positive diagnostic test No. (%)				
		Capture IgM (n=13)	Indirect IgG (n=29)	Capture IgG (n=6)	NS1 (n=0)	RT-PCR (n=27)
A	< 5 (n=9)	2 (22.2)	2 (22.2)	0 (0.0)	0 (0.0)	6 (66.7)
B	5 to 14 (n=21)	6 (28.6)	9 (42.9)	3 (14.3)	0 (0.0)	11 (52.4)
C	15 to 45 (n=25)	4 (16.0)	15 (60.0)	2 (8.0)	0 (0.0)	10 (40.0)
D	>45 (n=3)	1 (33.3)	3 (100)	1 (33.3)	0 (0.0)	0 (0.0)

* Number of positive tests is larger than patient number, because some samples were positive for two or three tests.

Table 2. Dengue serotypes distribution in different age groups

Detected virus	N	Age group
DENV-1	0	None
DENV-2	13	4 group A, 3 group B, 6 group C
DENV-3	2	1 group B, 1 group C
DENV-4	2	group A
DENV-3+4	8	1 group A, 7 group B
DENV-2+3+4	1	group C
DENV-1+3+4	1	group C

or virus sylvatic reservoirs and continuous transmission to naturally resistant humans.

The Colombian Department of Chocó is one of the most humid areas in the world and belongs to the tropical rainforest located near the South American Pacific coast, where most areas remain unexplored. There is little information about the Indian people Wounaan and their sanitary characteristics, and the vector-related factors associated with parasitic or viral infection transmission in this area are unknown. The Villa Nueva village is a poor and remote settlement of the Department of Chocó, with no public services except electricity. The absence of sanitary facilities forces the villagers to use water stored in open containers that turn into breeding grounds for mosquitoes. In addition, the dense crowding of people into each house (mean of 7 people) and the absence of adequate walls could increase mosquito spreading and therefore infection. These factors could partially explain the high rate of viremic subjects in this settlement. Although Chocó's small villages are isolated, they maintain contact and exchange with the main town of Quibdó, where mosquitos and virus circulate permanently.

Dengue infection has a wide range of signs and symptoms. It has been calculated that between 53 and 83% of cases in endemic regions are asymptomatic, and only a small proportion of cases are severe or fatal (Hoyos *et al*., 2012). In a recent research reported by Wang *et al.* (2015), it was found a high asymptomatic vs. symptomatic patient ratios, but different according with the outbreak virus serotype: higher during infections with DENV-1, while during the outbreak with DENV-3 the I: S ratio was lower.

Current dengue infection with no symptom or signs accounted for 51.7% of the study group in this work, indicating that this settlement had active virus transmission, which was enhanced by the poor sanitation conditions. Table 3 resumes the category and findings of each detected case. The individuals had been infected at least 3 days

Table 3. Serological and molecular findings in each dengue case (n=30)

Volunteer code	Diagnostic Tests			
	Capture IgM	Capture IgG	Indirect IgG	RT-PCR
02, 05, 10, 11, 16, 17, 18, 31, 32, 35, 36, 39, 40, 42, 43	Neg	Neg	Neg	Pos
8, 26, 27, 41	Neg	Neg	Pos	Pos
38	Pos	Neg	Neg	Pos
44, 48, 54	Pos	Pos	Pos	Neg
46, 49	Pos	Pos	Pos	Pos
47, 50, 51	Pos	Neg	Pos	Pos
53	Neg	Neg	Pos	Pos
56	Neg	Pos	Pos	Pos

before blood collection because they did not have viral antigen. DENV-2 was the most frequently detected serotype (13/27), but the most surprising finding was that in 10 viremic subjects, two or three different dengue serotypes were detected. This result was corroborated using both multiplex, uniplex PCR processing and sequencing. The main problem in this type of outbreak is that health systems cannot detect cases due to the absence of patients presenting signs; therefore, adequate measures are not taken.

The results reinforce the need for processing samples from suspected cases using multiple techniques because the infection case status and disease phase can only be determined using both serological and molecular tests simultaneously. In addition, the inclusion of the Capture IgG ELISA in the diagnostic panel allowed us to detect three secondary acute cases with no symptoms or viremia. The IgM prevalence of 22.4% observed here is higher than the rate reported previously in population studies (Yap *et al.*, 2013; Linnen *et al.*, 2011) but less than the rate reported by Sun *et al.* in asymptomatic subjects during an outbreak. As expected, 77.7% of viremic subjects were IgM negative, suggesting that infection had started few days earlier.

Interestingly, all the DENV RNA positive samples were negative in the NS1 antigen ELISA. This could be explained because 12 out of 27 PCR positive samples were secondary infections (IgG positive), antibodies which interfere with the test (Acosta *et al.*, 2014). The same report showed that 22% of NS1 negative febrile samples amplified viral RNA. Diagnostic NS1 sensitivity studies has been only performed in febrile patients demonstrating a lower sensitivity at more days of illness, even 11% of patients NS1 negative were PCR positive, a finding related with significant lower viral load in NS1 negatives (Ty-Hang *et al.*, 2009). Given that this was a cross-sectional study, we could not establish whether these subjects developed symptoms in the following days, but it is remarkable that all of the IgM-positive individuals did not report fever in the last 90 days.

Studies on asymptomatic viremic cases are scarce because most reports about unapparent dengue infection are obtained from individuals who change their immune status, reporting no symptoms from year to year. In the Villa Nueva population, the seroprevalence rate was less than the viremia rate, which indicated extensive transmission and spreading of the virus in this settlement. It has been proposed that concurrent infection with two or more serotypes may not necessarily lead to a more severe condition, and a low number of co-infections has been reported during dengue disease outbreaks in febrile patients (Bharaj *et al.*, 2008; Figueiredo *et al.*, 2011; Colombo *et al.*, 2013).

There is evidence of non-human primates' susceptibility to DENV, but the sylvatic transmission cycle in America is little known (Clark *et al*., 2013). Seroconversion of an isolated indigenous population in villages in Bolivia (Roberts *et al*., 1984), where *Aedes aegypti* was not present, has been reported, suggesting that sylvatic transmission could have occurred in these villages, even using reservoirs such as bats, rodents or marsupials (De Thoisy *et al*., 2009). Due to population movement, DENV presumably could move to primates and mosquitoes in forests near urban settings, establishing a reservoir for this infection (Vasilakis *et al*., 2011).

To our knowledge, this is the first report of an outbreak of asymptomatic viremia caused by multiple dengue serotypes. These unapparent infections could have been related to the co-evolution of indigenous inhabitants and the virus or to an abnormal immune response that does not lead to the classic secretion of immune mediators involved in the signs and symptoms. Recognize these asymptomatic cases could reshape the epidemic curves and give new approaches about managing the epidemic. Further investigation is necessary to characterize the genotypic properties of DENV in this area and the human genetic characteristics related to the observed clinical presentation. It is necessary to improve health, hygiene and economic conditions to reduce both mosquito circulation and virus spreading, given that sequential infections are the main risk factor for developing severe dengue.

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