

# Dengue virus serotype 3 (genotype III) from Colombia: A perspective of its pathogenic potential

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## Abstract

The introduction of DENV-3 genotype III in Latin American countries has been associated with dengue outbreaks, and the role of the virus with respect to the occurrence of dengue haemorrhagic fever (DHF) has been different depending on the country. We have conducted research on the relative abundance of DENV-3 in relation to the incidence of DHF in a Colombian endemic area. Additionally, it was explored using phylogenetic analyses whether or not viruses are genetically distinct in relation to the severity of dengue. Viral isolation was made from serum samples collected during the period from January 2007 to October 2008. Sequences from the envelope gene of viruses from Colombia and Latin American countries isolated from DF and DHF patients and submitted to GenBank were compared. We found that in 2007–2008 the predominance of DENV-3 declined as compared to 2002–2004 (28.3% versus 87.8%), whereas the DENV-1 and DENV-2 predominance increased (54.7% versus 2.7% and 16.9% versus 5.4%, respectively). This relative abundance of serotypes coincided with an increase of DHF compared with the period of the highest DENV-3 dominance (25.9% versus 4.6%). Phylogenetic analyses showed that: (i) there is no relationship between DENV-3 clades and the severity of the disease; and (ii) Colombian viruses clustered apart from those coming from countries where DENV-3 has caused severe dengue. The results suggest that DENV-3 could not play any important role in the occurrence of DHF in Colombia, and that local viruses are genetically distinct from Latin American viruses associated with epidemics of DHF.

**Keywords:** Dengue serotypes; Dengue haemorrhagic fever; DENV-3 genotype III; Colombia.

## Introduction

Dengue virus exists as four antigenically distinct viruses designated as serotypes (DENV-1, -2,

-3, and -4), belonging to genus *Flavivirus* of family *Flaviviridae*<sup>[1]</sup>. Infection with any one of these serotypes generally leads to a mild, self-limiting febrile illness called dengue fever (DF).

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Nonetheless, in a few cases, the viral infection leads to severe, sometimes fatal, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)<sup>[2]</sup>. Epidemiological studies have identified sequential infection with different serotypes as a risk factor for DHF/DSS<sup>[3,4]</sup>. Despite the much higher frequency of secondary infections in areas where two or more DENV serotypes are present, only a small percentage of patients develop DHF<sup>[5,6]</sup>. The infecting viral strain is hypothesized to influence the severity of dengue. It has been demonstrated that dengue virus serotypes and strains within a serotype may vary in their ability to cause DHF<sup>[7-9]</sup>.

DENV-3 viruses are phylogenetically grouped into four genotypes by Lanciotti et al.<sup>[10]</sup> or five genotypes by Wittke et al.<sup>[11]</sup>. In Latin America, this serotype was present between 1963 and 1977, and reappeared in Nicaragua and Panama in 1994<sup>[12]</sup>. It then dispersed to Central and Caribbean American countries<sup>[13-15]</sup>. In South America, DENV-3 appeared first in Brazil<sup>[16]</sup> and Venezuela<sup>[17]</sup> in 2000, and then dispersed to neighbouring countries in the following years<sup>[18-21]</sup>. Viruses isolated before 1994 were DENV-3 genotype IV, and those isolated after 1994 were DENV-3 genotype III<sup>[10,17,18-20]</sup>. Recently, Brazilian isolates in 2002 and 2004 were grouped into genotype I<sup>[22]</sup>, but the precise classification has been controversial, considering that this genotype was classified as genotype V by Nogueira et al<sup>[23]</sup>.

In Colombia, the presence of DENV-3 genotype III was detected for the first time in 2001 in the Departamento de Santander, the region where the present study was conducted. The reappearance of the virus coincided with an extended epidemic but increase in the number of DHF cases was not observed<sup>[21,24]</sup>. The same was seen in Mexico<sup>[13]</sup>, Puerto Rico<sup>[14]</sup>, Venezuela<sup>[17]</sup> and Peru<sup>[20]</sup>, where a great number of dengue cases occurred after the

re-introduction of the virus, but DHF cases were rare. In contrast, in Brazil<sup>[25]</sup>, Paraguay<sup>[26]</sup> and Cuba<sup>[15]</sup>, DHF/DSS in DENV-3-infected patients was frequent and some of them died. Likewise, the predominance of this dengue serotype in India and Sri Lanka has been associated with an increased incidence of DHF/DSS<sup>[27,28]</sup>.

It is difficult to determine the causes for different clinical outcomes in dengue patients infected with DENV-3 of genotype III. This is due to the limitations of our knowledge about the role of host factors and the virus-specific determinants of virulence. In this study we investigated the predominance of the virus circulating during 2007 and 2008, five years after its occurrence in the Departamento de Santander, with relation to the occurrence of DHF. We have also studied the genetic relationships of Colombian DENV-3 isolates to determine if viruses in DF patients diverge or have distinct geographical origin from those in DHF patients.

## Methods

### Study area

Santander is one of the 32 states (departments) of Colombia with a total number of 87 municipalities. Located in the north-central part of the country close to Venezuelan border, it covers an area of 30 537 km<sup>2</sup>. Its Capital is Bucaramanga, which together with three nearby municipalities, constitutes the seventh largest metropolitan area of Colombia with one million inhabitants (2005). It has a population density of 1012/km<sup>2</sup>, and an annual average temperature of 22 °C. At least 94% of dengue cases occurring in Santander originated in Bucaramanga and the metropolitan area. The numbers of DHF cases out of total dengue cases in Santander during the period 1998–2008, as reported by the state Secretariat of Health<sup>[29,30]</sup>, are shown in Table 1.



**Table 1:** Annual DHF cases reported by the state Secretariat of Health, Departamento de Santander, Colombia, 1998–2008

Year	Dengue cases	DHF	
		Total	%
1998	23 826	881	3.7
1999	4956	25	0.5
2000	1525	130	8.5
2001	10 530	779	7.4
2002	10 356	523	5.0
2003	6638	288	4.3
2004	1669	50	2.9
2005	1586	419	26.4
2006	2341	496	21.2
2007	5167	1 331	25.7
2008*	2579	676	26.2

**Source**<sup>[29,30]</sup>: \*From January to October, information supplied by Luis Gualdrón, División de Epidemiología, Secretaría de Salud, Santander, Colombia.

## Serosurveillance

A total of 680 serum samples from clinically suspected dengue patients, reported to the dengue surveillance programme set up by the state Health Secretariat, were included in the study. Between 18 January 2007 and 11 October 2008, acute serum samples for virus isolation were selected every week from total samples sent to the Public Health Laboratory in Bucaramanga. Only sera from patients with fever of unknown origin (not from respiratory, diarrhoea or other apparent causes) were included, and a reporting form was completed with clinical and laboratory data collected from each patient.

## Virus isolation

The isolation of viruses from the acute phase samples was attempted in C6/36 cells as previously described<sup>[21]</sup>. Briefly, 100 µl of serum

was added onto cell monolayers and, after centrifugation, 1 ml of culture medium was added. Cells were incubated at 32 °C, and analysed for the presence of virus on the 12<sup>th</sup> post-infection day by using a polyclonal anti-dengue antibody (Instituto Evandro Chagas, Brazil) in a direct immunofluorescence assay.

## Typing of viruses

Serotype identification of the virus isolates was carried out by a seminested reverse transcription-PCR (RT-PCR) protocol on the basis of that described by Lanciotti et al<sup>[31]</sup>. Briefly, viral RNA was extracted from 140 µl of cell-infected culture supernatant by using Trizol<sup>®</sup> (GIBCO BRL, Grand Island, NY), followed by reverse transcription with forward primer D2. cDNA was subjected to PCR amplification with D1 and D2 primers for 42 cycles, and a second round of amplification was conducted with a mixture of type-specific reverse primers (TS1-TS4). PCR reaction product was electrophoresed through a 2.5% agarose gel, stained with ethidium bromide, and photographed.

## Anti-dengue IgG antibodies

IgG antibodies were screened in the sera collected 0–4 days after the onset of symptoms from dengue virus isolation-positive cases by using the PanBio IgG ELISA kit (PanBio Inc., Brisbane, Australia). Primary or secondary infection status was determined by the absence or presence of IgG antibody in an acute-phase sample.

## Sequence analysis

The envelope gene sequences of Colombian DENV-3 genotype III isolates were used along with some representative global isolates. Sequences of the remainder genotypes were included as outgroup. All sequences used in the



**Table 2:** Isolates of DENV-3 analysed

Location*	Strain	Year	Clinical status	Accession no.
Brazil	68784	2000	DF	AY038605
Brazil	BR74886/02	2002	DSS	AY679147
Brazil/Porto Velho	D3BR/PV5/02	2002	DF	DQ118875
Brazil/Porto Velho	D3BR/PV2/03	2003	DF	DQ118872
Brazil/Porto Velho	D3BR/PV4/03	2003	DF	DQ118874
Brazil/Ribeirão Preto	D3BR/RP1/03	2003	DF	DQ118877
Brazil/Ribeirão Preto	D3BR/RP2/03	2003	DF	DQ118879
Colombia/Norte de Santander	COD3_OC092	2005	DF	FJ189462
Colombia/Santander	COD3_01072	2001	DF	FJ189450
Colombia/Santander	COD3_02200	2002	DF	FJ204475
Colombia/Santander	COD3_LV073	2003	DF	FJ189458
Colombia/Santander	COD3_LV016	2003	DHF	FJ189454
Colombia/Santander	COD3_LV038	2003	DHF	FJ189455
Colombia/Santander	COD3_LV058	2003	DHF	FJ189457
Colombia/Santander	COD3_LV057	2004	DF	FJ189456
Colombia/Santander	COD3_LV433	2004	DHF	FJ189461
Colombia/Santander	COD3_LV428	2004	DHF	FJ189460
Cuba	Cuba116/00	2000	DF	AY702032
Cuba	Cuba580/01	2001	DSS	AY702030
Cuba	Cuba21/02	2002	DF	AY702031
Mexico	MEX6097	1995	DF	AY146763
Mexico/Oaxaca	OAXACA-MX/00	2000	DF	DQ341207
Mexico/Quintana Roo	6889/QUINTANA ROO-MX/97	1997	DF	DQ341205
Mexico/Quintana Roo	6896/QUINTANA ROO-MX/97	1997	DF	DQ341206
Mexico/Yucatan	4841/YUCATAN-MX/95	1995	DF	DQ341202
Mexico/Yucatan	6584/YUCATAN-MX/96	1996	DF	DQ341203
Mexico/Yucatan	6883/YUCATAN-MX/97	1997	DF	DQ341204
Nicaragua	Nicaragua24/94	1994	DHF	AY702033
Paraguay/Asunción	D3PY/AS10/03	2003	DF	DQ118883
Paraguay/Asunción	D3PY/AS9/03	2003	DF	DQ118885
Paraguay/Fernando de la Mora	D3PY/FM11/03	2003	DF	DQ118886
Paraguay/Pedro Juan Caballero	D3PY/PJ4/03	2003	DF	DQ118887
Venezuela/Aragua	LARD6007	2000	DF	AY146765
Venezuela/Aragua	LARD6315	2000	DF	AY146767
Venezuela/Aragua	LARD6318	2000	DF	AY146768
Venezuela/Aragua	LARD6397	2000	DF	AY146769
Venezuela/Aragua	LARD6411	2000	DF	AY146770



Location*	Strain	Year	Clinical status	Accession no.
Venezuela/Aragua	LARD5990	2000	DF	AY146771
Venezuela/Aragua	LARD6218	2000	DHF	AY146766
Venezuela/Aragua	LARD7110	2001	DHF	AY146776
Venezuela/Aragua	LARD7812	2001	DHF	AY146777
Venezuela/Aragua	LARD7984	2001	DHF	AY146778

**Isolates used as outgroup in this study**

Location*	Strain	Year	Accession no.
Malaysia	LN6083	1994	AF147460
China	80-2	1980	AF317645
Fiji	29472	1992	L11422
India	1416	1984	L11424
Indonesia	1280	1978	L11426
Malaysia		1981	L11427
Philippines	H87	1956	L11423
Puerto Rico	PR6	1963	L11433
Puerto Rico	1339	1977	AY146761
Samoa	1696	1986	L11435
Sri Lanka	1326	1981	L11431
Sri Lanka	1594	1985	L11436
Sri Lanka	260698	1989	L11437
Sri Lanka	2783	1991	L11438
Thailand	D86-007	1986	L11441
Thailand	D88-303	1988	AY145714
Thailand	D95-0014	1995	AY145724
Thailand	D97-0106	1997	AY145728

\* Country and/or state or city

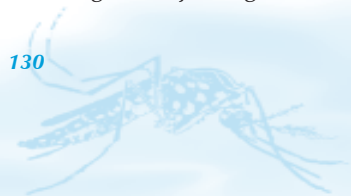
study were deposited in GenBank (Table 2). Colombian viruses were isolated in our laboratory from patients suffering from either DF or DHF in previous studies<sup>[21,24]</sup>. The sequences were aligned in the Muscle software v. 3.7<sup>[32]</sup> using the default parameters and the model of nucleotide substitution that best fits the data set was determined using a hierarchical likelihood ratio test<sup>[33]</sup> using the Modeltest software<sup>[34]</sup>. A maximum likelihood phylogenetic tree was reconstructed in the phyML software v. 3.0<sup>[35]</sup> where the starting tree was found using the neighbour-joining method. A Bootstrap

analysis with 10 000 pseudo-replicates was conducted to place confidence values on grouping within the tree.

## Results

### Dengue serotypes and infection pattern

A total of 53 dengue viruses were isolated from 426 and 254 serum samples collected from febrile cases enrolled in 2007 and 2008



respectively. The serotypes detected were DENV-1 (n=29), DENV-2 (n=9) and DENV-3 (n=15), and no isolates of DENV-4 was obtained. Primary infection was more frequent in DENV-1 (86.9%) and DENV-3 (73.3%) than DENV-2 (55.6%)-infected patients.

### DENV-3 predominance and DHF

In 2007, DENV-3 (42.2%) was the most prevalent serotype followed by DENV-1 (36.3%) and DENV-2 (21.2%). In 2008, in contrast, DENV-1 (85%) became the dominant serotype followed by DENV-2 (10%), while DENV-3 (5%) was detected to a much lesser extent. This temporal relative abundance of dengue serotypes coincided with an increase in the frequency of DHF cases with respect to the period of the highest DENV-3 dominance. This is, from 4.6% (861/18 663; DENV-3=87.8%) between 2002 and 2004 to 25.9% (2922/11 673; DENV-3=28.3%) between 2007 and 2008 (Tables 1 and 3).

### DENV-3 phylogenetic diversity

The aligned final data set comprised 60 sequences. The ingroup included 42 sequences

of the entire E gene (1479 bp in length) from patients suffering from either DF or DHF/DSS (Figure). The Tamura and Nei plus Gamma (TrN +  $\Gamma$ ) model was the best fit to the data with an  $\alpha$  value (shape parameter) of 0.24. The single phylogenetic tree obtained revealed five different groups of DENV-3 viruses that could be assigned to genotypes. The analysis clearly distinguished the two different genotypes (III and IV) detected in Latin America. All the 2001–2004 Colombian DENV-3 isolates grouped into genotype III, along with viruses from Latin American countries that were isolated after 1994. Viruses isolated from DHF patients did not cluster apart with isolates from DF patients. Consequently, there were no phylogenetically distinct groups related with disease severity when using the envelope gene. Nonetheless, Latin American viruses could be grouped in two clades. One clade grouped strains from Mexico, Venezuela, Colombia and Nicaragua, where the disease outcome has been benign in the majority of DENV-3-infected patients<sup>[13,17,24,36]</sup>. The second clade grouped the isolates from Cuba, Brazil and Paraguay, where infections resulted in either fatalities or serious visceral and nervous system involvement<sup>[15,25,26]</sup>.

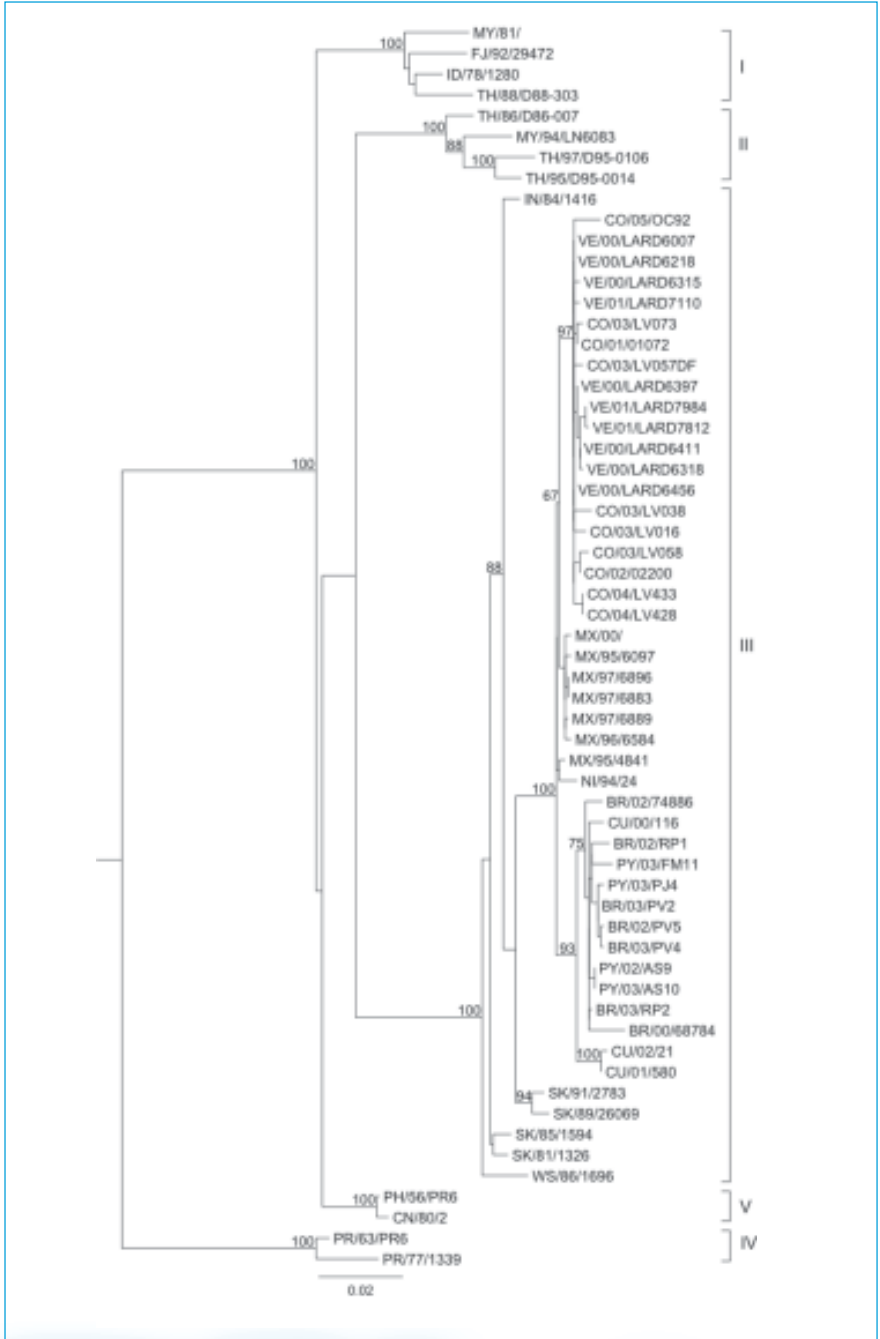
**Table 3:** Annual temporal predominance of dengue virus serotype, Departamento de Santander, Colombia

Year	Serotype: count (%)				Source
	DENV-1	DENV-2	DENV-3	DENV-4	
1998	8 (57.1)	6 (42.9)	0	0	*
1999	2 (50)	2 (50)	0	0	*
2000	1 (10)	7 (70)	0	2 (20)	*
2001	1 (4)	10 (40)	9 (36)	5 (20)	*
2002	1 (6.7)	1 (6.7)	13 (86.6)	0	*
2003	0	1 (2.7)	37 (97.3)	0	*
2004	1 (4.8)	2 (9.5)	15 (71.4)	3 (14.2)	*
2007	12 (36.3)	7 (21.2)	14 (42.2)	0	§
2008	17 (85)	2 (10)	1(5)	0	§

\*: Ocazonez et al.<sup>[21]</sup>. §: this study.



**Figure:** Maximum likelihood phylogenetic tree of 60 DENV-3 envelope gene sequences. Viruses are listed by abbreviation for country, year and strain (Table 2). Bootstrap values above 50 are shown above the branches.



## Discussion

Our results show that at least three dengue serotypes have been simultaneously present in the Departamento de Santander between 2007 and 2008, and that the relative abundance of serotypes had a distinct pattern each year. To our knowledge, dengue virological surveillance in 2005 and 2006 was not carried out in Santander. Under these circumstances, we used data from previous studies in the same region of the country for the period 1998–2006 to identify the relationship between the occurrence of DHF and the abundance of DENV-3.

DENV-3 was the predominant serotype in Santander in the period 2002–2004, while DENV-1, DENV-2 and DENV-4 were found in considerably lower frequency. This temporal serotypes distribution coincided with a decrease in the frequency of DHF with respect to the previous year<sup>[21,24]</sup>. Between 2007 and 2008, in contrast, DENV-3 declined and DENV-1 and DENV-2 increased, and the frequency of DHF was six-fold higher with respect to 2002–2004 (Tables 1 and 3). Although the severity of dengue in accordance with WHO parameters<sup>[37]</sup> in patients enrolled in the present study was not determined, however, in previous studies<sup>[21,24]</sup> conducted in Santander, DHF was less frequent in DENV-3 patients compared with DENV-2-infected patients (10.9% versus 27.5%), and that there was no DSS or fatal case caused by DENV-3. Moreover, the period of the highest predominance of DENV-3 coincided with a decrease of DHF, compared with the period of DENV-2 dominance.

Phylogenetic analyses of isolates of DENV from severe cases or DHF epidemics suggest that viral factors can have an influence on the outcome of viral infection<sup>[8]</sup>. In this study, segregation of DF- versus DHF-associated viruses on the basis of E gene sequences was not observed. This finding is in agreement with a study of DENV-3 viruses from Venezuela<sup>[17]</sup>.

Likewise, Miagostovich et al.<sup>[38]</sup> did not find any differences among the untranslated region (UTR) sequences of viruses isolated from fatal or DF patients in Brazil. Additional studies investigating other genes within the DENV-3 viruses are necessary to infer with more certainty the genetic basis of virulence.

It seems that the strains of DENV-3 genotype III circulating in the Americas exhibit different pathogenic potential. During the 1998 epidemic in Nicaragua, 11.8% out of DENV-3-infected patients developed DHF and fatalities were not registered<sup>[36]</sup>. In Venezuela, even though the virus caused the largest epidemic seen after 1989, during the period of its highest predominance only 8% of dengue cases were severe and death in DENV-3-infected patients was not reported (Dirección de Epidemiología y Análisis Estratégico, 2001). Although, in Mexico, the severity of dengue increased in the mid-1990s after the introduction of DENV-3, the continuous presence of Asian genotype of DENV-2 seems to have played a more important role in DHF outbreaks<sup>[13]</sup>. On the contrary, the introduction of DENV-3 into Brazil, Paraguay and Cuba was associated with severe disease. The virus caused an epidemic in the state of Rio de Janeiro with 1831 DHF cases, and a total of 91 deaths in DENV-3-infected patients<sup>[16]</sup>. In Paraguay, the predominance of the virus resulted in 28 129 dengue cases, out of which 55 were haemorrhagic and 14 ended fatally. The fatality rate was 25.4%<sup>[26]</sup>. In Cuba, 12 886 dengue cases occurred during the 2001 DENV-3 epidemic, of which 70 suffered from DHF and 3 of them ended fatally<sup>[15]</sup>.

Our phylogenetic analyses clearly revealed that isolates of DENV-3 from Colombia belong to the same genotype as isolates from Brazil, Paraguay and Cuba. Why did the predominance of the virus result in epidemics with a distinct severity of dengue? One explanation involves genetic diversity within the isolates of the virus. It has been suggested that DENV-3 viruses



might have gone through a period of *in situ* evolution within Latin American countries after its introduction, diversifying into distinct phylogenetic groups<sup>[17]</sup>. A genetic shift in DENV-3 of genotype III has been suggested as the cause for the emergence of an invasive strain responsible for the increased frequency of DHF in Sri Lanka<sup>[9,28]</sup>. We found that isolates coming from countries where DENV-3 has not been associated with severe disease (Colombia, Venezuela and Mexico) grouped apart from isolates coming from countries where the virus has caused deaths (Brazil, Paraguay and Cuba)<sup>[19,39,40]</sup>. Additional studies are necessary to evaluate whether or not a genetic shift in viruses from Latin America is occurring.

Other aspects might influence the severity of dengue during the DENV-3 outbreaks. Virus-specified determinants of virulence at the level of susceptibility to cross-neutralization, more prone to enhancement by dengue antibodies, and variation in the ability to infect and be transmitted by their mosquito vector, have been proposed<sup>[27]</sup>. On the other hand, human genetic resistance to infection caused by viral strains more virulent, and herd immunity of the respective populations, should also be considered.

An observation that caught our attention in the present study was the increased incidence of DHF during the period 2005–2008 (25%) compared with the period 1998–2004 (4.5%). We did not investigate the temporal distribution of dengue serotypes in Santander in 2005–2006. Nonetheless, we conducted a study in the municipality of Ocaña, located 299 km from Bucaramanga in the north-eastern part of Santander (data not published). In Ocaña, in 2005, DENV-2 (77.5%) was the dominant serotype followed by DENV-3 (12.5%), and DENV-1 (5%) was isolated to a much lesser extent. In 2006, DENV-1 (57.5%) became dominant, followed by DENV-3 (27.5%) and DENV-2 (22.5%). We can speculate that in Bucaramanga the predominance of DENV-2

and DENV-1 could have also increased in 2005 and 2006, respectively, despite DENV-3 continuing as the prevalent serotype; and, in this context, the frequency of DHF cases could have increased. One explanation could be that the co-dominance of DENV-2 and DENV-3 in 2001 coincided with increased DHF cases in Bucaramanga during the period 1998–2004 (Tables 1 and 3), and that DENV-1 and DENV-2-infections were more associated with DHF than DENV-3-infections<sup>[21]</sup>. In Nicaragua (1999–2003), the predominance of DENV-2 was associated with infections with shock to a greater extent, and the predominance of DENV-1 with an increased number of infections with severe manifestations<sup>[41]</sup>. In addition, the marked increase of DHF in Latin America has been largely attributed to the increased frequency of DENV-2 infections<sup>[4,42]</sup>.

We cannot exclude that the increase of DHF cases in Santander between 2005 and 2008 could be due to the failure of physicians in clinics to collect sufficient data to fulfil the requirements for the WHO case definition. In the case of some patients, serial haematocrit tests required to estimate the degree of haemoconcentration were not available; and as such, patients with platelet counts below 100 000/mm<sup>3</sup> and/or with haemorrhages might have been classified as a DHF case.

Taken together, our results in this study and previous studies suggest that the presence of DENV-3 in the north-central part of Colombia since 2001 has had a minor role in the occurrence of DHF. More studies need to be conducted to clarify the pathogenic potential of the virus in Colombia. The phylogenetic analysis suggests that DENV-3 Colombian viruses could be genetically distinct from the viruses of the same serotype coming from neighbour countries with potential to cause DHF. Thus, continuous virological surveillance should be a priority in the Colombian dengue endemic areas.



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