VOLUME 00 NO 00

Secondary dengue virus infections during the 2009 outbreak in Buenos Aires

E. Tittarelli[1,2], P. R. Barrero[1,2,†], A. S. Mistchenko[1,3] and L. E. Valinotto[1,2]

1 *Laboratorio de Virología Hospital de Niños 'Ricardo Gutiérrez', Buenos Aires, Argentina*
2 *Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina*
3 *Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Buenos Aires, Argentina*

Abstract

OBJECTIVES To evaluate the occurrence of secondary dengue virus (DENV) infections during the 2009 outbreak in a non-endemic area. Viral loads were evaluated in serum from acute-phase patients, comparing primary and secondary infection.

METHODS Serum samples from patients with clinical diagnosis of suspected dengue were referred to the Virology Laboratory at 'Ricardo Gutiérrez' Children's Hospital. Dengue-positive samples were classified as primary or secondary DENV infections through serological methods (anti-DENV IgM and IgG). Viral loads were measured by quantitative real-time PCR (qRT-PCR) in samples obtained in the first 5 days of infection. Statistical analyses were performed to evaluate factors that might correlate with differences in the viral load of primary or secondary infection.

RESULTS A total of 229 DENV cases were confirmed; among them, 22.7% were secondary infections. No significant differences were found between the viral load of primary and secondary infections.

CONCLUSION We detected a high percentage of secondary DENV infections in a non-endemic area; this finding might correspond to socio-demographic characteristics of the group under study or indicate a previous cryptic DENV circulation causing inapparent infections.

keywords dengue virus, secondary infections, viral load

Introduction

Dengue virus (DENV) is a small single-stranded RNA virus that belongs to the genus *Flavivirus*, family Flaviviridae. DENV is primarily transmitted among humans by female *Aedes aegypti* mosquito. Dengue is endemic in over 100 countries, where more than 2.5 billion people are at risk of infection. Almost 390 million people are infected annually, of whom 96 million manifest clinical symptoms [1].

Dengue virus is grouped into four serotypes (DENV-1–4) which do not confer cross-protective immunity. Moreover, evidence indicates that immunity to one serotype increases the risk of more severe disease upon infection with a different serotype. DENV infections cause a wide spectrum of clinical manifestations and the severity of the disease results from the interaction between viral factors and the host immune response [2].

Symptoms due to the infection appear after an incubation period of 3–10 days, with a median of 5.9 days [3]. In most primary infections, levels of anti-DENV IgM antibodies are detectable after the fifth day from fever onset, whereas levels of anti-DENV IgG are detectable around 5–15 days after fever onset [4]. In contrast, in secondary infections, anti-DENV IgG is detectable in the first 5 days of symptoms, and IgG titres are significantly higher than IgM titres due to pre-existing plasma cells that rapidly produce antibodies against the previous DENV serotype [2]. Antibodies generated during the primary infection bind to the current DENV serotype in a non-neutralising manner, thus favouring the secondary infection by increasing the number of infected cells and enhancing the viral production, a phenomenon known as antibody-dependent enhancement (ADE) [5].

Sometimes DENV infections are not diagnosed because they are confounded with symptomatically similar illnesses or do not cause symptoms strong enough to be detected. Both primary and secondary infections can be inapparent raising the possibility of secondary DENV infections being far more common than expected [6]. Nowadays, DENV transmission is growing dramatically

[†]Deceased.

© 2015 John Wiley & Sons Ltd

in urban and semi-urban locations worldwide, becoming a public health problem. A significant increase in the incidence has been also observed in the last two decades in the Americas, where Argentina's bordering countries, excepting Uruguay, have reported dengue cases with cocirculation of more than one serotype [7, 8].

Until 2009, the Emergent Pathogens Monitoring Program in Buenos Aires city detected only cases acquired in the northern provinces of Argentina or in foreign countries. However, in 2009, a DENV-1 outbreak occurred in Argentina and by February reached the metropolitan area of Buenos Aires, where local transmission was detected for the first time and a total of 624 cases were reported, the highest number of DENV cases in the area to date [9, 10].

The aim of this work was to investigate the occurrence of secondary DENV infections during the 2009 outbreak in a non-endemic area such as the metropolitan area of Buenos Aires, considering the possibility of inapparent DENV infections and to evaluate viral loads in patients with primary and secondary DENV infection.

Methods

Samples

Under the Emergent Pathogen Surveillance Program of the Government of the City of Buenos Aires, a total of 229 DENV cases were confirmed at the Virology Laboratory at 'Ricardo Gutiérrez' Children's Hospital. Serum samples from these patients were tested to distinguish primary from secondary infections. Viral loads were assessed on a subset of 64 DENV-confirmed acute-phase samples. All samples analysed in this study were submitted with complete epidemiological data including vaccination status and recent travel history.

Primary and secondary infections

Dengue virus-confirmed samples were tested by three methods to discriminate primary from secondary infections. The results of at least two of the three methods had to be consistent to define the type of infection. The first method calculates the ratio of anti-DENV IgM to anti-DENV IgG antibodies detected by DxSelect capture ELISA (DxSelectTM; Focus Diagnostic, Cypress, CA, USA) for samples obtained during the first 15 days of illness. As a result, primary infections are characterised by an excess of IgM (IgM/IgG > 1.7), whereas secondary infections are characterised by an excess of IgG (IgM/IgG < 1.7) [11]. The second method is a commercially available kit that detects an elevated level of anti-DENV IgG antibodies (samples during days 6–15 after the fever onset), which is indicative of active secondary infection (Dengue IgG Capture ELISA kit; PanBio Diagnostics, Alere, Brisbane, Australia). The third method considers that an infection is secondary when anti-DENV IgG antibodies are detected during the first 4 days of illness (DxSelectTM; Focus Diagnostic).

Molecular studies

Viral RNA was extracted from sera by standard EZ1 BioRobot protocol (EZ1 Virus Mini Kit v2.0; QIAgen, Valencia, CA, USA) and stored at −20 °C. Multiplex reverse transcription followed by polymerase chain reaction was performed to determine DENV serotypes [12]. To compare the viral loads of primary and secondary DENV infections, a representative subgroup of 64 DENV-confirmed acute-phase samples with complete epidemiological data from the outbreak of 2009 was further studied by real-time one-step reverse transcription PCR (qRT-PCR, adapted from ref. [13]). Briefly, the capsid region was amplified using IScript™ One-Step RT-PCR Kit for Probes (Bio-Rad, Hercules, CA, USA), the primer pair 5′-nt255-ATACCYCCAACAGCAG GAATT-nt275-3′ and 5′-nt403-AGCATRAGGAG CATGGTCAC-nt384-3′, and the probe 5′-nt276-6FAM TTGGCTAGATGGRGCTCATTCAAGAAGAAT TAMRA-nt305-3′. Cycling conditions were 50 °C for 30 min followed by 5 min at 95 °C, 45 cycles of 95 °C for 30 s, and 30 s at 55 °C. The qRT-PCRs were performed in duplicate on a CFX-96 real-time PCR detection system (Bio-Rad). Positive and negative controls were included in each run. Synthetic positive controls were obtained by cloning DENV-1 amplicon of Hawaii strain (kindly provided by Institut Pasteur, Paris, France) into pGEM-T® vector (Promega Inc, Madison, WI, USA). A relative quantification was performed by means of a standard curve built from serial dilutions of a cloned quantified plasmid. The detection limit of qRT-PCR was 1800 copy/ml. Viral loads are expressed as the logarithms of the calculated number of copies standardised per millilitre of plasma (log copy/ml).

Data were collected and analysed with Bio-Rad CFX Manager Software (version 2.1.1022.0523.) and reported following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [14].

Statistical methods

Viral loads of primary and secondary infections were compared using *t*-test and one-way analysis of variance complemented by Fisher's least significant difference test

 © 2015 John Wiley & Sons Ltd

Table 1 DENV-1-confirmed cases

	Primary dengue	Secondary dengue	Not classified	Total
Dengue cases	159 (69.43%)	52 (22.71%)	18 (7.86%)	229
Age	36	39	36	36
Sex	F: 76, M: 83	F: 23, M: 29	F: 10, M: 8	F: 109, M: 120
Yellow fever vaccine: yes	6	6	1	13
Yellow fever vaccine: no	114	31	9	154
Yellow fever vaccine: unknown	39	15	8	62
Local transmission	137 (75.28%)	33 (18.13%)	12 (6.59%)	182
Travel to endemic zones	22 (46.81%)	19 (40.42%)	6 (12.77%)	47

F: female; M: male.

with the InfoStat software package (v. 2012) [15]. Statistical significance was defined as $P < 0.05$.

Ethics

The study was approved by the Medical Ethics and Research Committees of 'Ricardo Gutiérrez' Children's Hospital, Buenos Aires, Argentina.

Results

Epidemiologic characteristics

During the 2009, 229 DENV cases were confirmed in our laboratory, 182 of which had no recent travel history to endemic zones. DENV-1 was the only serotype detected during the outbreak. Most of the cases (71.6%) occurred in April 2009. Of the total confirmed cases, 109 were female and 120 male, with ages ranging from 2 years to 80 years and a median of 36 years.

As shown in Table 1, according to the results obtained by the three methods employed to discriminate primary from secondary infections (Table S1), more than one-fifth of the cases were secondary infections. Cases that could not be classified corresponded to inconclusive results or inappropriate timing of the sample. Of the secondary infections, 23 were female and 29 were male, with a median age of 39 years. The youngest secondary case was a 9-year-old boy.

Viral load differences between primary and secondary infections

A total of 64 DENV-1-confirmed acute-phase serum samples with complete epidemiological data were suitable for viral load determination (48 of primary and 16 of secondary infections, median age of 34 years; 34.37% females and 65.63% males; 85.94% reported no recent travel history to endemic zones).

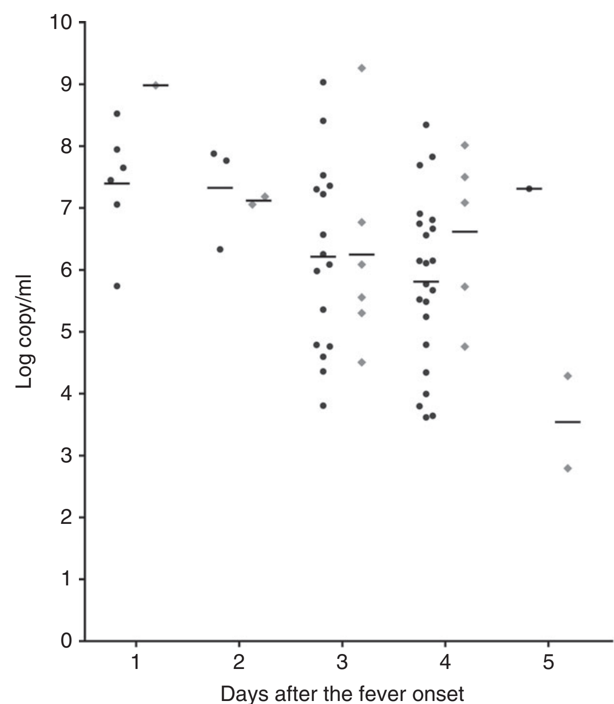

Figure 1 Viral load determined by qRT-PCR in serum samples from patients with primary (●) and secondary (◆) dengue virus infections.

In primary infections, the mean viral loads decreased from 7.40 ± 0.95 to 5.81 ± 1.37 log copy/ml in the first and fourth day after the fever onset, respectively. As to the secondary infections, values varied from 8.98 ± 0.00 to 6.62 ± 1.34 log copy/ml in the first and fourth day after the fever onset, respectively (Figure 1).

Viral loads were evaluated each day after the fever onset; no statistical differences were found between both types of infections during the days 3 and 4. No statistical analyses were performed for days 1, 2 and 5 due to too few available samples.

© 2015 John Wiley & Sons Ltd

Additional statistical analyses were performed to take into account other factors that might affect the viral load of DENV infection. No significant differences were found between primary and secondary infections when age and gender were considered.

Discussion

In this study, we show the occurrence of secondary DENV infections in a non-endemic area. During the first DENV outbreak in the metropolitan area of Buenos Aires, a high percentage of the DENV cases (22.71%) were secondary infections. Considering that metropolitan Buenos Aires is a non-endemic area where DENV local transmission was detected for the first time during the 2009 outbreak, the finding of more than 20% of secondary cases is rather striking and highlights the need of vector control interventions. Several studies report that a high percentage of dengue infections are inapparent, in both endemic and non-endemic areas [16, 17]. Inapparent DENV infections do not cause symptoms or might be misdiagnosed as symptomatically similar illnesses, especially in non-endemic areas where healthcare workers might not be aware of the circulation of the virus. Also, as the case forms attached to each sample report only recent travel history, but not previous travel to endemic zones, the possibility of a first infection being contracted in an endemic zone must be considered. Further studies should be promoted to elucidate the prevalence of anti-DENV antibodies among residents of the metropolitan area of Buenos Aires. These data will be of major importance in planning the management of patients at risk of suffering secondary DENV infections in a future outbreak.

The acute-phase serum samples collected during this outbreak allowed us to evaluate and compare viral loads from primary and secondary DENV-infected patients by qRT-PCR. We showed that regardless of the type of infection, viral load decreases with the progress of the disease. It is noteworthy to mention that for days 3 and 4 after fever onset, we found no statistical differences between viral loads of primary and secondary infections. Laue *et al.* [18] reported high viral load ($>10^6$ RNA molecules/ml) in early serum samples (day 1 and 2 after the fever onset) from two patients with suspected secondary infection. In agreement with those findings, our results also show viral loads >6 log copy/ml on days 1–2 after fever onset. However, we found these high values not only in secondary, but also in primary infections. The most dominant hypothesis usually used to explain the disease severity upon secondary infections is the ADE phenomenon [19, 20]. This hypothesis suggests that the antibodies generated after a primary infection with a certain serotype can bind to virions of a different serotype with low affinity, thus favouring cell infection [21, 22]. *In vitro* studies demonstrate that this enhanced viral infectivity turns into a higher viral titre when measured in culture supernatants [20]. However, this result might not be easily extrapolated to the clinical progress of DENV infections, as they are highly dependent on the complex interactions between viral replication and the host immune response [23, 24].

There is a lack of studies comparing viral loads in primary and secondary dengue virus infections in serum samples, and we hope that our results will contribute to elucidate the factors implied in dengue severity and offer a more accurate prognosis to patients with dengue.

Acknowledgements

We would like to thank M. Viegas and M. Natale for critical discussion of the manuscript.

References

1. Bhatt S, Gething PW, Brady OJ *et al.* The global distribution and burden of dengue. *Nature* 2013: **496**: 504–507.
2. Martina BEE, Koraka P, Osterhaus ADME. Dengue virus pathogenesis: an integrated view. *Clin Microbiol Rev* 2009: **22**: 564–581.
3. Chan M & Johansson MA. The incubation periods of dengue viruses. *PLoS One* [Internet]. 2012: **7**. (Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3511440/) [1 August 2014].
4. Hu D, Di B, Ding X *et al.* Kinetics of non-structural protein 1, IgM and IgG antibodies in dengue type 1 primary infection. *Virol J* 2011: **8**: 47.
5. Flipse J, Wilschut J, Smit JM. Molecular mechanisms involved in antibody-dependent enhancement of dengue virus infection in humans. *Traffic* 2013: **14**: 25–35.
6. Grange L, Simon-Loriere E, Sakuntabhai A, Gresh L, Paul R, Harris E. Epidemiological risk factors associated with high global frequency of inapparent dengue virus infections. *Front Immunol* 2014: **5**: 280.
7. Dick OB, Martín JLS, Montoya RH, del Diego J, Zambrano B, Dayan GH. The history of dengue outbreaks in the Americas. *Am J Trop Med Hyg* 2012: **87**: 584–593.
8. Five-fold increase in dengue cases in the Americas over the past decade [Internet]. 2014. (Available from: http://www.paho.org/hq/index.php?option=com_content&view=article&id=9657&Itemid=1926&lang=en) [7 August 2014]
9. Seijo A, Romer Y, Espinosa M *et al.* [Outbreak of indigenous dengue in the Buenos Aires Metropolitan Area. Experience of the F. J. Muñiz Hospital]. *Medicina (B Aires)* 2009;**69**:593–600.

© 2015 John Wiley & Sons Ltd

10. Tittarelli E, Mistchenko AS, Barrero PR. Dengue virus 1 in Buenos Aires from 1999 to 2010: towards local spread. *PLoS One* 2014: **9**: e111017.
11. Kuno G, Gómez I, Gubler DJ. An ELISA procedure for the diagnosis of dengue infections. *J Virol Methods* 1991: **33**: 101–113.
12. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992: **30**: 545–551.
13. Leparc-Goffart I, Baragatti M, Temmam S *et al.* Development and validation of real-time one-step reverse transcription-PCR for the detection and typing of dengue viruses. *J Clin Virol* 2009: **45**: 61–66.
14. Bustin SA, Benes V, Garson JA *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009: **55**: 611–622.
15. Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M & Robledo CW. *InfoStat [Internet]*. FCA, Universidad Nacional de Córdoba, Argentina: Grupo InfoStat. (Available from: http://www.infostat.com.ar) [10 September 2015].
16. Halstead SB. Is there an inapparent dengue explosion? *Lancet* 1999: **353**: 1100–1101.
17. Sun J, Luo S, Lin J *et al.* Inapparent infection during an outbreak of dengue fever in Southeastern China. *Viral Immunol* 2012: **25**: 456–460.
18. Laue T, Emmerich P, Schmitz H. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. *J Clin Microbiol* 1999: **37**: 2543–2547.
19. Boonnak K, Slike BM, Burgess TH *et al.* Role of dendritic cells in antibody-dependent enhancement of dengue virus infection. *J Virol* 2008: **82**: 3939–3951.
20. Kou Z, Lim JYH, Beltramello M *et al.* Human antibodies against dengue enhance dengue viral infectivity without suppressing type I interferon secretion in primary human monocytes. *Virology* 2011: **410**: 240–247.
21. Murphy BR, Whitehead SS. Immune response to dengue virus and prospects for a vaccine. *Annu Rev Immunol* 2011: **29**: 587–619.
22. Gujarati TP, Ambika G. Virus antibody dynamics in primary and secondary dengue infections. *J Math Biol* 2014: **69**: 1773–1800.
23. Silveira GF, Meyer F, Delfraro A *et al.* Dengue virus type 3 isolated from a fatal case with visceral complications induces enhanced proinflammatory responses and apoptosis of human dendritic cells. *J Virol* 2011: **85**: 5374–5383.
24. Boonnak K, Dambach KM, Donofrio GC, Tassaneetrithep B, Marovich MA. Cell type specificity and host genetic polymorphisms influence antibody-dependent enhancement of dengue virus infection. *J Virol* 2011: **85**: 1671–1683.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Values of serological assays used to discriminate primary from secondary infections.

Corresponding Author E. Tittarelli, Laboratorio de Virología Hospital de Niños ‘Ricardo Gutiérrez’, Gallo 1330, (1425), Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 49643118; Fax: +54 11 49644320; E-mail: estefaniatittarelli@conicet.gov.ar

© 2015 John Wiley & Sons Ltd