Detection of dengue virus in sera of Brazilian blood donors

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BACKGROUND: Dengue is the most important arboviral disease in the world. Dengue viruses (DENVs) have produced huge outbreaks in Brazil in the past 25 years with more than 5 million reported cases. During these epidemics, asymptomatic individuals infected with DENV could donate blood and serve as a source of virus dissemination in the community. Here, we studied the circulation of DENV in healthy individuals during an epidemic outbreak.

STUDY DESIGN AND METHODS: The study included 500 serum samples from healthy blood donors collected at the Hemotherapy Center of Ribeirão Preto, Brazil, during a dengue outbreak. The presence of DENV RNA in the serum samples was screened by real-time reverse transcription–polymerase chain reaction (PCR). The virus serotype was determined by a heminested PCR procedure. A partial fragment of the NS5 gene sequence was used for phylogenetic analysis.

RESULTS: DENV RNA was detected in the serum sample of 2 of 500 (0.4%) individuals. Both of them were infected with DENV-3 Genotype III, a virus that has been circulating in Brazil in the past decade.

CONCLUSION: Individuals with asymptomatic DENV infection can be blood donors and serve as a source of virus dissemination in the community. Further studies are needed to determine the risk of recipient infection by DENV as a result of transfusion in Brazil, especially during epidemic periods.

Dengue virus (DENV), which belongs to the genus *Flavivirus*, family *Flaviviridae*, comprises four antigenically related, but genetically distinct, viruses named DENV Types 1, 2, 3, and 4 (DENV-1, -2, -3, and -4). DENV has a single-stranded, positive-sense RNA genome of fewer than 10,800 nucleotides, surrounded by a nucleocapsid and covered by a lipid envelope that contains the viral glycoproteins. The RNA genome contains a single open reading frame flanked by two untranslated regions (5′ and 3′ UTRs). The single open reading frame encodes a precursor polyprotein, which is co- and posttranslationally cleavaged resulting in the formation of three structural proteins, capsid (C), membrane (M), and envelope (E), and seven nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5.[1,2] Dengue is an arboviral disease transmitted by *Aedes* mosquitoes.[3] Annually, 50 million dengue infections occur annually and approximately 2.5 billion people live in dengue endemic countries.[4] DENVs have produced large outbreaks in Brazil in the past 25

ABBREVIATIONS: DENV(s) = dengue virus(-es); PFUs = plaque-forming units; RCHRP = Regional Center of Hemotherapy of Ribeirão Preto; WNV = West Nile virus.

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years with more than 5 million reported cases.[5] Considering that approximately 50% of DENV-infected individuals remain asymptomatic, and that the duration of viremia prior to the onset of symptoms is estimated in 1 to 2 days, it is possible that viremic individuals donate blood and serve as a source of virus dissemination in the community.[6-10] The objective of the present study was to detect blood donors infected with DENV in Ribeirão Preto city, São Paulo state, Brazil, during a period of dengue outbreak.

MATERIALS AND METHODS

Locale of the study

This study was carried out in Ribeirão Preto, a city with a total population of 547,417 inhabitants according to IBGE-BRAZIL.[11] Ribeirão Preto is situated 313 km northeast of São Paulo city, the capital of São Paulo state; it has a tropical climate with rainy summers (mean temperature, 25°C) and dry winters (mean temperature, 19°C).

Study samples

Serum samples of 500 blood donors collected randomly between February and May 2010 in the Regional Center of Hemotherapy of Ribeirão Preto (RCHRP) were included in this study. The RCHRP received 8223 donations in this period, which coincided with an epidemic of dengue in the city. The serum samples were separated by centrifugation after collection in the RCHRP and immediately sent to the Virology Research Center of the School of Medicine of Ribeirão Preto, São Paulo University, packed in a cooler with ice packs. The serum samples were aliquoted and rapidly stored at –70°C until use. The subsequent tests were carried out anonymously with approbation in the ethical committee of the Clinics Hospital of Ribeirão Preto, São Paulo University (Process 764/2009).

Extraction of RNA

The viral RNA was extracted from 140 mL of serum samples or controls (supernatant of DENV-3–infected C6/36 cells) using the viral RNA mini kit (QIAamp, Qiagen, Hilden, Germany), following the manufacturer's recommendations.

Detection of DENV RNA in serum samples

The detection of DENV genome was performed using a one-step real-time reverse transcription–polymerase chain reaction (RT-PCR), as previously described.[12] Briefly, a total of 25 µL of reaction mixture contained 0.5 µL of SuperScript III RT Platinum *Taq* mix (reverse transcriptase, *Taq* DNA polymerase, and RNase inhibitor; Invitrogen, Carlsbad, CA), 0.2 mmol/L of each primer, 12.5 µL of 2× SYBR Green buffer, and 10 µL of purified RNA. The amplification cycle was as follows: 50°C for 20 minutes for reverse transcription, 95°C for 5 minutes for reverse transcriptase inhibition, and *Taq* DNA polymerase activation, followed by 45 cycles of PCR amplification with denaturation at 95°C for 15 seconds, annealing at 54°C for 40 seconds, and extension at 72°C for 30 seconds. Finally, to verify the specificity of the PCR amplification products, a melting curve was constructed incubating the amplification products from 60 to 90°C with an increase of 0.2°C/sec. The melting temperature values of the specific amplicons were in the range of 80.57 to 81.73°C. A standard curve was constructed using decimal serial dilutions of RNA obtained form a viral seed (4.8×10^8 plaque-forming units [PFUs]/mL). The viral title in serum samples was expressed as PFUs/mL. The sensitivity of the test was 10 PFUs/mL.

DENV serotype determination

The virus serotype was determined by a multiplex heminested RT-PCR, which amplifies a part of the NS5 gene of flaviviruses.[13] Briefly, the RT reaction mixture contained 8 µL of RNA, 200 U of reverse transcriptase (Superscript; Invitrogen), 4 µL of 5× first-strand buffer (250 mmol/L Tris-HCl [pH 8.3], 375 mmol/L KCl, 15 mmol/L $MgCl_2$), 1.5 µL of dithiothreitol (0.1 mol/L), 20 U of RNase inhibitor (RNaseOUT; Invitrogen), 0.6 µmol/L FG2 reverse primer (5′-GTGTCCCATCCTGCTGTGTCATACAGCATACA-3′), 10 mmol/L deoxynucleoside triphosphate (dNTP) mixture, in a final volume of 20 µL. The mixture was incubated at 42°C for 50 minutes for RT and at 95°C for 5 minutes to inactivate the reverse transcriptase. The PCR mixture contained 4 µL of cDNA, 1 U of *Taq* Platinum DNA polymerase (Invitrogen), 5 µL of 10× PCR buffer (200 mmol/L Tris-HCl [pH 8.4], 500 mmol/L KCl), 2 mmol/L $MgCl_2$, 0.3 µmol/L forward FG1 primer (5′-TCAAGGAACTCCACACATGAGATGTACT-3′), and 10 mmol/L dNTP mixture, in a total volume of 50 µL. The mixture was subjected to 30 cycles of 94°C for 1 minute, 53°C for 1 minute, and 72°C for 2 minutes, followed by a final extension step at 72°C for 5 minutes. The expected size of this amplification product is 958 bp. The second step of the nested PCR was carried out in the same conditions as mentioned including 1 µL of the first reaction and 0.3 µmol/L of each internal DENV-1, -2, -3, and -4 primers. Ten microliters of the second step of the nested PCR products was electrophoresed on a 2% (wt/vol) agarose gel, stained with ethidium bromide, and visualized with an ultraviolet light. The expected sizes for DENV-1, -2, -3, and -4 are 472, 316, 659, and 253 bp, respectively.

Nucleotide sequencing of the PCR products

The fragments of 958 bp obtained in the first step of the heminested RT-PCR were directly sequenced. The fragments were purified from 2.0% agarose gel using a gel extraction kit (QUIAquick, Qiagen, Chatsworth, CA) following the manufacturer's recommendations. The puri-

fied products were sequenced using primers FG1 sense and FG2 antisense. The products were sequenced using a genetic analyzer (ABI PRISM 3500, Applied Biosystems, Foster City, CA). The sequences obtained in this study were submitted to GenBank and registered with the following accession numbers: JN997393 and JN997394. The sequences were aligned with NS5 gene sequences of other DENV-3 retrieved from GenBank using computer software (BioEdit v7.0.0, http://www.mbio.ncsu.edu/bioedit/bioedit.html; and MEGA 5.0, http://www.megasoftware.net/).[14,15] Aligned sequences were analyzed with the software to identify the best-fit model of nucleotide substitution for phylogenetic reconstruction (Modeltest, http://darwin.uvigo.es/software/modeltest.html).[16] The phylogenetic relationships among strains were reconstructed by the neighbor joining and Tamura & Nei model using MEGA 5.0. The analyses were statistically supported by bootstrap using 1000 replicates.

RESULTS

The presence of DENV RNA was investigated by real-time RT-PCR in serum samples of 500 healthy blood donors. The test was positive for 2 of 500 (0.4%) of the individuals, which showed an amplification product with melting temperature between 80.57 and 81.73°C, indicating a specific DENV RNA detection.[17] These two serum samples were subjected to a multiplex heminested RT-PCR for flaviviruses, which produced 659-bp amplicons compatible with the size expected for DENV-3 (Fig. 1). To confirm and better characterize the DENV-3 detected in the serum samples, the amplicons of approximately 958 bp obtained in the first step of the heminested RT-PCR were directly sequenced. These two sequences were aligned with other 69 NS5 gene sequences of DENV-3 obtained from GenBank. Based on this alignment, a phylogenetic tree was constructed, showing clearly that both viruses detected in the serum of blood donors belonged to DENV-3 Genotype III (Fig. 2).

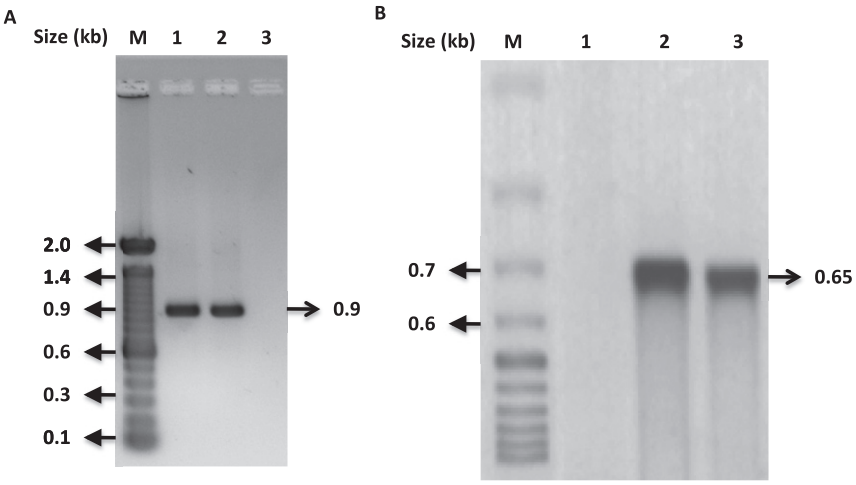

Fig. 1. Agarose gel electrophoresis showing amplicons obtained by the RT–heminested PCR for flavivirus. (A) Amplification reaction products (958 bp) from two blood donors (Lanes 1 and 2). These amplicons were obtained in the first phase for detection of virus genera of the multiplex heminested RT-PCR for flavivirus (Primers FG1 and FG2). (B) Amplicons of 659 bp from two blood donors (Lanes 2 and 3). These amplicons were obtained in the second phase, for detection of virus species, of the multiplex heminested RT-PCR for flavivirus (Primers FG1 and nD3) and are suggestive of dengue Serotype 3.

DISCUSSION

The risk of DENV transmission by blood transfusion was clearly demonstrated in Singapore and Hong Kong.[8,9] In addition, several blood donors have been found to be infected with DENV in retrospective studies.[6,7] Therefore, blood transfusion might have an important role for dissemination of DENV in the community.

Brazil has suffered several epidemics of dengue in the past years, and Ribeirão Preto is one of the cities with the highest number of reported cases per 100,000 people.[18] In this study, we have investigated the presence of blood donors in Ribeirão Preto infected with DENV using a highly sensitive real-time RT-PCR[12] and found a positivity of 0.4% (2/500). The detection of the virus was further confirmed by a heminested RT-PCR[13] and by nucleotide sequencing, which indicated that both viruses belong to the Serotype 3, Genotype III, a virus that has been circulating in the country in the past decade.

Previous studies have shown that the incidences of blood donors infected with DENV were consistent with the magnitude of the outbreaks during the studies.[6,7] Those studies found a higher incidence of infected blood donors in Honduras (0.3%, 2004-2005), which suffered an epidemic with 2.8 cases per 1000, and lower incidence in a population in the vicinity of São Paulo city (0.03%, 2003) and Puerto Rico (0.07%, 2005), which suffered epidemics with 0.7 cases per 1000 and 0.73 cases per 1000, respectively.[6,7] In Ribeirão Preto, a great epidemic of dengue occurred during the study period (February-May 2010) with 26,625 cases in a population of 547,417 (48.6 cases/1000). In this period, we have found an incidence of 0.4% blood donors infected with DENV, which is a bit higher than the incidence observed in Honduras, corroborating the importance of the epidemic magnitude in the number of infected blood donors.

Other studies carried out in Mexico and Brazil fail to find blood donors

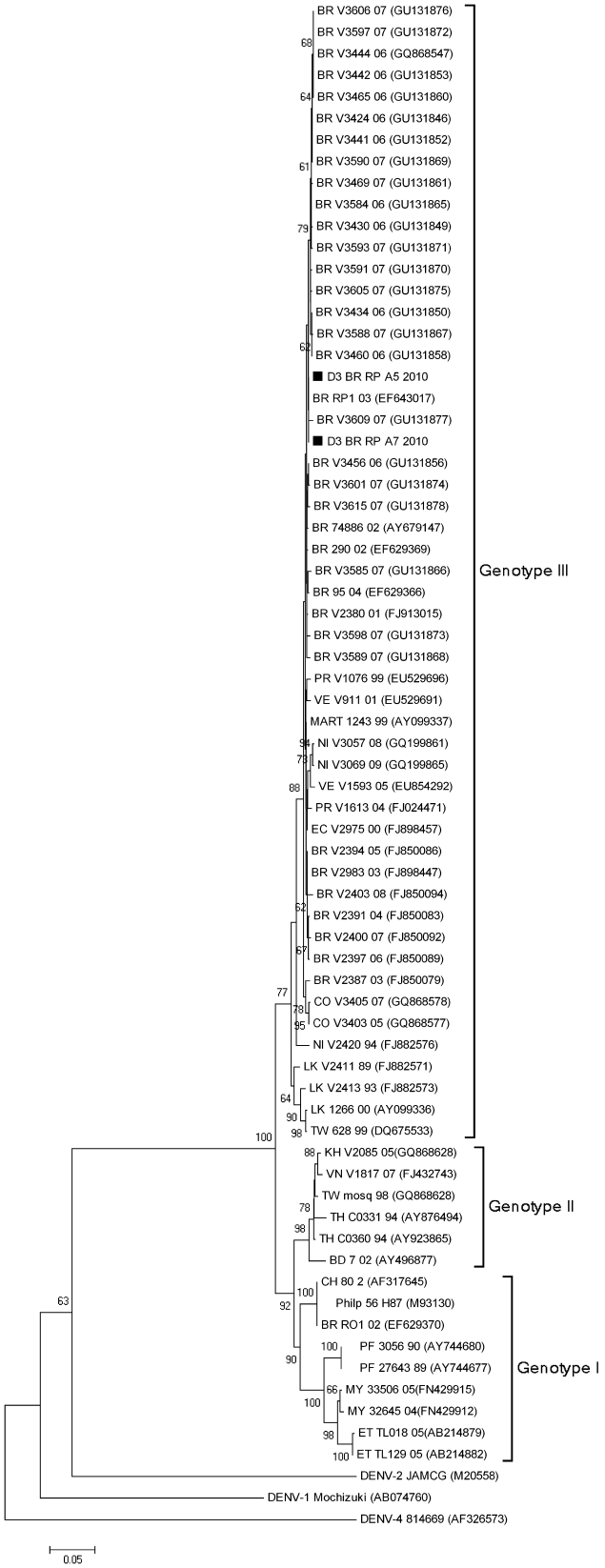

Fig. 2. DENV-3 phylogenetic tree based on the NS5 partial gene sequences. The tree was constructed using the method of neighbor joining with 1000 bootstrap replications. DENV-1, DENV-2, and DENV-4 were used as outgroup. Branch lengths are proportional to percentage of divergence. Bootstrap support values are shown for key nodes only (values <60% not shown). (■) Strains D3/BR/RP/A5 2010 and D3/BR/RP/A7 2010. The country of origin, years of isolation, and GenBank accession numbers are shown under the tree.

infected with DENV and this failure might be related to the method used for virus detection.[19,20] Both studies used a conventional RT-PCR for the DENV RNA detection, which is less sensitive than the real-time RT-PCR used in our study and the commercial transcription-mediated amplification method used in previous studies.[6,7,12]

Transmission by blood transfusion is well recognized for another flavivirus, the West Nile virus (WNV), which represents a serious problem for blood banks in North America. The first outbreak of meningoencephalitis caused by WNV in the Americas was reported in New York City.[21] The virus quickly spread throughout North America becoming a serious public health problem and causing epidemics that strike man and horses. By 2007, in the United States, 27,379 WNV cases were reported including 8885 cases of encephalitis and 1060 fatalities.[22] It was observed that WNV, even producing an acute and transient infection, was transmitted through blood transfusions. It was discovered in 2002 when there were 23 cases of WNV infection transmitted by blood transfusion.[23] As a consequence, measures were taken by the system connected to all blood products in North America. Rapid commercial methods available for the diagnostic test in all donated blood were created. Therefore, other standard rules were implemented to control WNV in blood products. These measures virtually eliminated the problem of transmission of WNV through blood transfusions, but increased the price of blood products.[24] Dengue transmission by blood transfusion highlighted in this study could become an emerging problem and must be reported to public health and transfusion medicine authorities in Brazil. It would be necessary to monitor the donated blood to check the presence of DENV contamination. It would be also very important to actively seek for dengue cases that may be associated with transfusion of blood products.

In summary, we showed here a relevant epidemiologic problem related to dengue. During an epidemic, 0.4% of blood donors were infected with DENV. These findings raise concern and directly threaten the safety of blood products that could transmit DENV through transfusion. Further studies monitoring the virus presence in blood products are necessary in Brazil where dengue outbreaks are constantly occurring.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION**.

REFERENCES

1. Henchal E, Putnak J. The dengue viruses. Clin Microbiol Rev 1990;3:376-96.
2. Lindenbach B, Thiel H, Rice C. Flaviviridae: the viruses and their replication. In: Knipe D, Howley P, editors. Fields virology. Philadelphia (PA): Lippincot Williams & Wilkins; 2007. p. 1101-51.
3. Mackenzie J, Gubler D, Petersen L. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 2004;10:S98-109.
4. WHO. World Health Organization. Dengue and dengue haemorrhagic fever. 2002; Fact Sheet No. 117. Geneva.
5. Figueiredo L. Dengue in Brazil: 1999-2009. Dengue Bulletin (World Health Organization) 2010;34:6-12.
6. Mohammed H, Linnen JM, Muñoz-Jordán JL, Tomashek K, Foster G, Broulik AS, Petersen L, Stramer SL. Dengue virus in blood donations, Puerto Rico, 2005. Transfusion 2008;48: 1348-54.
7. Linnen JM, Vinelli E, Sabino EC, Tobler LH, Hyland C, Lee TH, Kolk DP, Broulik AS, Collins CS, Lanciotti RS, Busch MP. Dengue viremia in blood donors from Honduras, Brazil, and Australia. Transfusion 2008;48:1355-62.
8. Tambyah PA, Koay ES, Poon ML, Lin RV, Ong BK; Group T-TDIS. Dengue hemorrhagic fever transmitted by blood transfusion. N Engl J Med 2008;359:1526-7.
9. Chuang VW, Wong TY, Leung YH, Ma ES, Law YL, Tsang OT, Chan KM, Tsang IH, Que TL, Yung RW, Liu SH. Review of dengue fever cases in Hong Kong during 1998 to 2005. Hong Kong Med J 2008;14:170-7.
10. Nishiura H, Halstead SB. Natural history of dengue virus (DENV)-1 and DENV-4 infections: reanalysis of classic studies. J Infect Dis 2007;195:1007-13.
11. Instituto Brasileiro de Geografia e Estatistica I. Censo 2010. 2010.
12. Dos Santos HWG, Poloni T, Souza KP, Muller VDM, Tremeschin F, Nali LC, Fantinatti LR, Amarilla AA, Castro HLA, Nunes MR, Casseb SM, Vasconcelos PF, Badra SJ, Figueiredo LTM, Aquino VH. A simple one-step real-time RT-PCR for diagnosis of dengue virus infection. J Med Virol 2008;80:1426-33.
13. de Morais Bronzoni R, Baleotti F, Ribeiro Nogueira R, Nunes M, Moraes Figueiredo L. Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. J Clin Microbiol 2005;43:696-702.
14. Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999;41:95-8.
15. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28: 2731-9.
16. Posada D. ModelTest Server: a web-based tool for the statistical selection of models of nucleotide substitution online. Nucleic Acids Res 2006;34:W700-3.
17. Dos Santos HW, Poloni TR, Souza KP, Muller VD, Tremeschin F, Nali LC, Fantinatti LR, Amarilla AA, Castro HL, Nunes MR, Casseb SM, Vasconcelos PF, Badra SJ, Figueiredo LT, Aquino VH. A simple one-step real-time RT-PCR for diagnosis of dengue virus infection. J Med Virol 2008;80:1426-33.
18. Secretaria de Estado da Saúde de São Paulo. Programa de Vigilância e Controle da Dengue Secretaria de Estado da Saúde de São Paulo. Doenças CDCD, editor. São Paulo, 2010; 65.
19. Rodríguez Rodríguez D, Garza Rodríguez M, Chavarria AM, Ramos-Jiménez J, Rivera MA, Taméz RC, Farfan-Ale J, Rivas-Estilla AM. Dengue virus antibodies in blood donors from an endemic area. Transfus Med 2009;19:125-31.
20. Lavezzo LC, Dos Santos Santana V, Terzian AC, Mondini A, de Moraes Bronzoni RV, Sperança M, Dias SM, Rossit AR, Machado RL, Nogueira ML. Arboviruses in blood donors: a study in the Amazon region and in a small city with a dengue outbreak. Transfus Med 2010;20:278-9.
21. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, Murray K, Huang A, Rosenberg A, Greenberg A, Sherman M, Wong S, Layton M; 1999 West Nile Outbreak Response Working Group. The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med 2001;344:1807-14.
22. Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases. West Nile virus: statistics, surveillance and control. Atlanta (GA): CDC; 2007.
23. Harrington T, Kuehnert MJ, Kamel H, Lanciotti RS, Hand S, Currier M, Chamberland ME, Petersen LR, Marfin AA. West Nile virus infection transmitted by blood transfusion. Transfusion 2003;43:1018-22.
24. Dodd RY. Emerging pathogens in transfusion medicine. Clin Lab Med 2010;30:499-509.