Dengue viremia in blood donors from Honduras, Brazil, and Australia

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BACKGROUND: Dengue fever and hemorrhagic disease are caused by four dengue virus (DENV) serotypes (DENV-1 to -4), mosquito-borne flaviviruses with increasing incidence, and expanding global distributions. Documented transfusion transmission of West Nile virus raised concern regarding transfusion-transmitted DENV.

METHODS: A DENV RNA assay was developed based on transcription-mediated amplification (TMA) blood screening assays routinely used by blood centers worldwide. Sensitivity was established by endpoint dilution analyses of DENV-1 RNA transcript and pedigreed tissue culture standards for all four DENV-serotypes. Frozen plasma samples were tested from 2994 donations from Honduras (September 2004-January 2005), 4858 donations from Brazil (February-April 2003), and 5879 donations from Australia (March-September 2003). Type-specific polymerase chain reaction (PCR) assays were used to quantify and genotype TMA repeat-reactive samples; viral cultures, type-specific antibody, and antigen assays were also performed.

RESULTS: The TMA assay detected 14.9 copies per mL DENV-1 transcript (95% detection limit), with comparable sensitivity for all four serotypes. Honduran donors yielded 9 TMA repeat-reactive samples (0.30%); 8 were confirmed by PCR, with 3 DENV serotypes detected and viral loads from fewer than 3×10^4 to 4.2×10^4 copies per mL; and 4 samples yielded infectious virus. Three (0.06%) Brazilian samples tested repeat-reactive; 2 (0.04%) were PCR-positive (serotypes DENV-1 and -3; 12 and 294 copies/mL). No Australian donor samples tested repeat-reactive.

CONCLUSION: Dengue viremia rates among asymptomatic blood donors ranged from 0.30 percent in Honduras to 0.04 percent in Brazil. Future studies are needed to establish rates of transfusion transmission by viremic donations and clinical consequences in recipients.

Dengue fever (DF) is a mosquito-transmitted disease caused by four closely related dengue virus serotypes (DENV-1 to -4) of the genus *Flavivirus*.[1,2] Infection with one serotype provides lifelong immunity to the infecting serotype only. Persons can acquire a second serotype, and secondary infections place them at greater risk for dengue hemorrhagic fever (DHF), the more severe form of the disease.[3,4] DHF is characterized by bleeding manifestations, thrombocytopenia, and increased vascular permeability that can lead to life-threatening shock.

ABBREVIATIONS: DENV = dengue virus; DF = dengue fever; DHF = dengue hemorrhagic fever; PFU(s) = plaque-forming unit(s); PRNT(s) = plaque reduction neutralization test(s); S/CO = signal-to-cutoff; TMA = transcription-mediated amplification; TT = transfusion-transmitted; WNV = West Nile virus.

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There has been marked global reemergence of dengue infections and clinical disease in the past 25 years, with frequent, larger epidemics associated with more severe disease.[1,2,5-7] The World Health Organization (WHO) estimates that in 2004, there were 50 million to 100 million cases of DF, 500,000 cases of DHF, and 20,000 consequent deaths, making dengue the most important arboviral disease of humans.[7] Dengue incidence has increased substantially in the Americas due to expansion of its primary urban mosquito vector, *Aedes aegypti*, after yellow fever control efforts were curtailed in the 1970s.[6-14]

Concern over potential transmission of DENV and other arboviruses by blood transfusions increased after documentation of transfusion-transmitted (TT) West Nile virus (WNV) during recent epidemics in the US.[15-18] As may be the case with DENV, TT-WNV was not appreciated over decades of spread in Africa, the Middle East, and Europe, due to failure to launch specific studies to implicate transfusions and difficulty differentiating mosquito-borne from TT cases. DENV and WNV are similar in important respects supporting the hypothesis that dengue can be TT: 1) both flaviviruses are efficiently transmitted to humans via the bite of infected mosquitoes; 2) a large proportion (53%-87%) of infections are asymptomatic, and symptomatic infections are preceded by a 2- to 5-day viremic phase;[3,4,19-22] 3) levels of viremia during the incubation phase can exceed 10^6 virions per mL;[3,4,22,23] and 4) transmission of both viruses has been documented after organ transplantation and health care worker accidents.[24-27]

Despite these facts suggesting that TT-dengue may frequently occur, there is only one case of reported TT-DENV.[28] Determination of the prevalence of DENV in blood donations would serve as the first step in assessing the risk of TT. We describe development of a DENV nucleic acid amplification technology (NAT) assay and results obtained using this assay to screen 13,731 blood donor samples collected during epidemic outbreaks in Brazil and Honduras and a seasonal outbreak in Queensland, Australia.

MATERIALS AND METHODS

Blood donations

Residual samples from asymptomatic volunteer blood donors in Honduras, Brazil, and Australia were obtained during periods of outbreaks of clinical dengue disease in each country[14,29] (Informe, Departamento de Virología, Secretaría de Salud de Honduras). Plasma from donors in Honduras and Brazil, derived from routine ethylenediaminetetraacetate pilot tubes, was aliquoted and frozen within 24 hours of phlebotomy; samples were subsequently recoded after linkage to donor demographic data (sex, age, region of collection), such that subsequent testing was performed anonymously. The 2994 plasma samples from Honduras were collected during dengue outbreaks in Tegucigalpa and San Pedro Sula regions from August 2004 to January 2005. The 4858 plasma specimens from Brazil were archived during a DENV outbreak in the vicinity of São Paulo city from February to April 2003. The 5879 Australian samples were collected in plasma preparation tubes (PPT, Becton Dickinson, Franklin Lakes, NJ), centrifuged, and frozen within 24 hours of collection. These donations were obtained during small regional outbreaks from March to September 2003 in Townsville (n = 1457) and Cairns (n = 3240), with additional samples included from Brisbane as controls (n = 1182). The Australian samples remained linked to donor identifiers. The study protocols were approved by ethics committees in each country and by the Committee for Human Research at University of California, San Francisco.

Dengue transcription-mediated amplification assay

The qualitative DENV RNA assay uses the same technology as a particular FDA-licensed WNV assay (Procleix, Chiron Corp., Emeryville, CA).[16,18,30] The assay targets genomic sequences of DENV that are highly conserved across all four serotypes.[1] Testing was performed using an automated system (Procleix Tigris, Chiron Corp.), which can complete up to 1000 tests in 14 hours.[30] Assay results were reported in relative light unit values, which were used to derive signal-to-cutoff (S/CO) values. An internal control RNA was added to each sample to determine individual reaction validity. Cutoff values for the Dengue transcription-mediated amplification (TMA) assay internal control and analyte signals were calculated with the same formulae used for the Procleix WNV assay.[30] Donor samples were screened in singlet and reactive samples (S/CO > 1.0) were retested. Initial-reactive samples that retested negative were classified as false-reactive and used to calculate specificity. Samples that tested repeat-reactive were subjected to supplemental testing.

Determination of analytical sensitivity of dengue TMA assay

An analytical sensitivity panel was prepared using an in vitro synthesized transcript from a cloned genomic fragment created from a DENV-1 specimen. The analytical sensitivity panel, composed of 6 members at 0, 1.2, 3.7, 11.1, 33.3, and 100 copies per mL, was tested in 80 replicates, with results (proportion of replicates testing reactive) subjected to probit analysis (SAS Institute, Inc., Cary, NC). To establish comparable sensitivity to all four DENV serotypes, tissue culture stocks of DENV-1, -2, -3, and -4 were obtained from the Centers for Disease Control and Prevention (CDC) that had been quantified by endpoint titration inoculation onto Vero cells to define plaque-forming units per mL (PFUs/mL). These stocks were

diluted to 1 PFU per mL and then subjected to serial threefold dilutions using difibrinated human serum. Ten replicate assays were performed on each dilution for each subtype, and results are expressed as proportion of replicates that tested reactive and mean S/CO of reactive samples.

Supplemental dengue assays

Real-time polymerase chain reaction assays for confirmation, genotyping, and quantitation

RNA from 200 µL of plasma was extracted, reverse-transcribed, and polymerase chain reaction (PCR)-amplified using allele-specific primers for each serotype. Each assay run included duplicate 10-fold dilutions of the CDC tissue culture stocks corresponding to DENV-1, -2, -3, and -4 (see above), such that specific DENV concentrations could be estimated by regression analysis. A value of 300 copies per PFU was used to assign viral copy levels.

Dengue virus cell culture and plaque reduction neutralization testing

Recovery of live virus using cell culture (C6/36 cell lines) and serotype-specific plaque reduction neutralization tests (PRNTs) were performed at the CDC (Ft. Collins, CO) as previously described.[31]

Dengue immunoglobulin M and immunoglobulin G and NS1 antigen enzyme-linked immunosorbent assays

For further characterization of the reactive samples, dengue-specific immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies were measured using commercially available IgM- and IgG-capture microwell assay kits (Dengue Duo, PanBio Diagnostics, Brisbane, Australia). The pan-E dengue early antigen enzyme-linked immunosorbent assay (ELISA; PanBio Diagnostics) was also used to detect free dengue NS-1 antigen in confirmed viremic samples from Honduras; this assay employs monoclonal anti-NS1 bound to microwell test strips to capture NS-1 antigens, which are detected by ELISA.[32,33]

RESULTS

Analytical sensitivity of the dengue blood screening assay

To determine the sensitivity of the dengue TMA assay, an in vitro transcript of DENV-1 RNA was prepared, quantified, and used to establish an analytical sensitivity panel (Fig. 1). Probit analysis indicated a 95 percent detection limit of 14.9 copies per mL (95% confidence interval [CI], 11.5-20.9 copies/mL) and a 50 percent detection limit of 3.5 copies per mL (95% CI, 3.0-4.1 copies/mL). Table 1 presents results from testing serial dilutions of tissue culture virus standards of DENV-1, -2, -3, and -4 and demonstrates comparable detection of the four DENV serotypes. At 0.11 PFU per mL, 10 of 10 replicates of DENV-1, -2, and -4 were detected, while DENV-3 virus was detected in 9 of 10 replicates. For DENV-2 and DENV-4, 100 percent detection was seen down to 0.037 PFU per mL. Comparing these analytical results to those generated with the DENV-1 transcript panel, we estimate that 1 PFU of the DENV-1 corresponds to approximately 300 RNA copies.

Prevalence and characteristics of dengue viremia in blood donor specimens

Plasma samples from 2994 blood donors were collected in two geographic regions in Honduras in late 2004 and early 2005 (Table 2), which spanned the typical dengue season in Honduras, as well as the peak period of the epidemic during the 2004 to 2005 rainy season. The DENV TMA assay yielded 12 initial-reactive samples, 9 of which were repeat-reactive; S/CO values of the repeat-reactive samples ranged from 21.48 to 38.09, while the 3 initial-reactive-only samples had S/CO values of 1.01 to 17.64. Because confirmatory testing with type-specific PCR was limited to TMA repeat-reactive samples, prevalence rates may be underestimated. For example, an initial-reactive sample with a relatively high S/CO of 17.64 may have been a positive sample with a false-negative result in repeat TMA testing due to low viral titer. Type-specific PCR confirmed the DENV RNA in 8 of 9 TMA repeat-reactive samples and documented three dengue serotypes (Table 3), consistent with circulating serotypes in

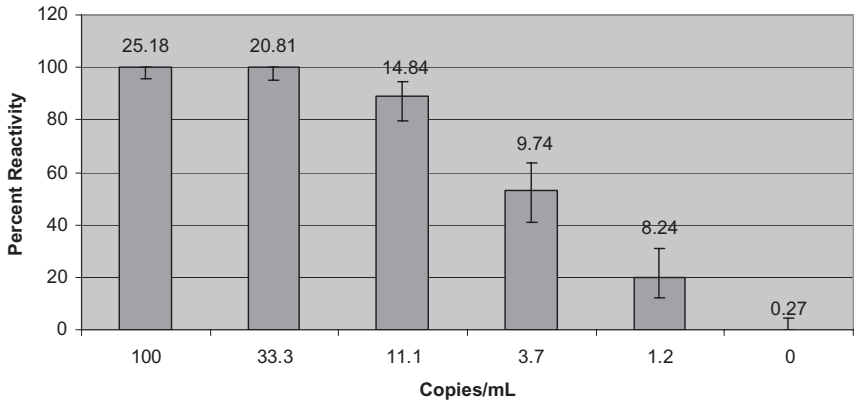

Fig. 1. Analytical sensitivity of the dengue TMA assay based on detection of in vitro synthesized DENV-1 transcript. Approximately 80 replicate assays were tested for each copy level. Error bars represent 95 percent exact CIs for the percentage reactive at each RNA copy level. The mean S/CO values are shown above each bar. For the virus-containing panel members (100-1.2 copies/mL), the mean S/CO results were calculated from the reactive results.

TABLE 1. Detection of DENV serotypes using tissue culture–derived virus*

	DENV-1		DENV-2		DENV-3		DENV-4	
PFUs/mL	Number reactive/number tested (% reactive)	Mean S/CO	Number reactive/number tested (% reactive)	Mean S/CO	Number reactive/number tested (% reactive)	Mean S/CO	Number reactive/number tested (% reactive)	Mean S/CO
1	10/10 (100)	24.91	10/10 (100)	20.85	10/10 (100)	15.35	10/10 (100)	25.46
0.33	10/10 (100)	21.95	10/10 (100)	20.97	10/10 (100)	7.49	10/10 (100)	25.89
0.11	10/10 (100)	8.62	10/10 (100)	19.28	9/10 (90)	3.20	10/10 (100)	25.18
0.037	9/10 (90)	7.79	10/10 (100)	19.58	NT	NT	10/10 (100)	24.54
0	0/10 (0)	0.21	0/10 (0)	0.43	0/10 (0)	0.13	0/10 (0)	0.42

* Serial dilutions of CDC standards for each DENV serotype were tested in 10 replicates.
NT = not tested.

TABLE 2. Summary of dengue TMA initial-reactive and repeat-reactive donor samples by country and region

Country	Country region	Collection period	Number tested by region	Total tested per country	Number of TMA initial-reactive donations	Number of TMA repeat-reactive donations	Detection rate*
Honduras	Tegucigalpa	August 2004-January 2005	1178	2994	12	9	1:333
	San Pedro Sula	September 2004-January 2005	1816				
Brazil	São Paulo City	February-April 2003	4858	4858	8	3	1:1619
Australia	Townsville	2003	1457	5879	5	0	NA
	Cairns	2003	3240				
	Brisbane	2003	1182				

* Detection rate based on number of TMA repeat-reactive donations per number of donations tested.
NA = not applicable.

TABLE 3. Demographic characteristics and supplemental laboratory test results for donations that tested repeat-reactive by dengue TMA assay

Specimen ID	Draw date	Initial/repeat TMA results (S/CO)*	Genotype	PCR viral load (Copies/mL)	Growth in culture	NS-I antigen EIA	IgM ELISA (S/CO)†	IgG ELISA (S/CO)†	PRNT antibody titers				Interpretation (primary vs. secondary infection)
									DENV-1	DENV-2	DENV-3	DENV-4	
Honduras													
DV-00164	Sep 14, 2004	28.44/33.88	DENV-4	4.4×10^2	DENV-4	NR	0.56	0.22	<10	<10	<10	<10	Primary
DV-00220	Sep 17, 2004	33.83/38.02	DENV-4	3.9×10^3	DENV-4	NR	0.67	1.85	40	320	80	80	Secondary
DV-00477	Jan 10, 2005	32.91/38.09	DENV-4	4.2×10^4	DENV-4	NR	0.73	1.16	320	80	80	40	Secondary
DV-00545	Oct 7, 2004	28.61/32.21	DENV-1	4.7×10^2	DENV-1	NR	0.53	0.36	10	80	10	10	Primary
DV-00802	Oct 29, 2004	28.68/21.48	DENV-4	78	Negative	NR	0.88	1.07	640	80	160	80	Secondary
DV-01110	Nov 19, 2004	27.18/33.15	DENV-1	2.3×10^2	Negative	NR	0.60	0.25	<10	<10	<10	<10	Primary
DV-01328	Dec 3, 2004	28.55/33.07	DENV-1	7.4×10^3	Negative	NR	0.47	0.94	80	320	80	40	Secondary
DV-02837	Nov 9, 2004	25.39/24.52	DENV-2	3	Negative	NR	1.02	0.58	80	80	40	80	Primary
DV-02921	Nov 16, 2004	3.43/24.77	ND	Undetected	Negative	NR	4.04	6.73	>20,480	>20,480	>20,480	>20,480	Secondary
Brazil													
SPDV-260	Mar 11, 2003	30.31/30.09	DENV-1	12	NA	NA	NA	NA	<10	<10	<10	<10	Primary
SPDV-3304	Mar 6, 2003	22.91/28.05	DENV-3	294	NA	NA	NA	NA	10	20	20	10	Primary
SPDV-4665	Mar 19, 2003	12.19/15.82	ND	Undetected	NA	NA	NA	NA	40	320	180	40	Secondary

* TMA S/CO values ≥ 1.0 are considered reactive.
† IgM and IgG ELISA S/CO values ≥ 1.0 are considered reactive.
NA = not applicable; ND = not detected; NR = not reactive.

Honduras and neighboring countries in 2004 and 2005 (Informe, Departamento de Virología, Secretaría de Salud de Honduras). Viral loads of confirmed positive samples ranged from fewer than 3 to 42,000 copies per mL. Four of the 9 TMA repeat-reactive samples yielded DENV on culture with serotypes consistent with the results from PCR; the culture-positive specimens all had viral loads above 300 copies per mL, whereas 4 of the 5 culture-negative specimens had viral loads of fewer than 300 copies per mL. All 8 viremic donation specimens tested negative for the presence of NS1 antigen. The dengue antibody status of the 8 confirmed viremic samples was determined using IgM, IgG, and serotype-specific PRNT assays (Table 3). Five of 9 TMA repeat-reactive donations tested IgG-reactive and had PRNT titers of greater than 320 to one or more DENV serotypes, consistent with secondary infections; in 4 of these cases the antibody specificity by PRNT was to a different serotype than the contemporary virus, while in 1 case PRNT titers of at least 1280 were detected to all 4 serotypes—this case had IgM reactivity and very low viral load precluding serotyping by PCR. The final four RNA-positive donations tested negative for dengue IgG and had low (<80) or absent (<10) PRNT titers, indicating probable primary DENV infections.

We screened 4858 archived plasma specimens from blood donations collected during a 2003 dengue outbreak in the São Paulo region of Brazil (Table 2). This screening resulted in 8 initial-reactive and 3 repeat-reactive samples (Table 3). Four of the 5 initial-reactive samples that were negative on repeat testing had initial S/CO values of less than 2.0, consistent with nonspecific TMA reactivity. The two repeat-reactive samples with high S/CO values (22.91 and 30.08) were identified as DENV-1 (12 copies/mL) and DENV-3 (294 copies/mL) by serotype-specific kinetic PCR; these samples were negative by PRNT analysis and interpreted as primary infections. The final repeat-reactive sample was negative by type-specific PCR assays, but had PRNT reactivity to DENV-2 and -3, suggesting a secondary infection with very low viremia at the time of the TMA-reactive donation. Viral culture and IgM, IgG, and NS1 serology were not possible due to insufficient volume of the Brazilian specimens.

Plasma from 5879 donations from Queensland, Australia, collected in 2003, was screened by dengue TMA assay and yielded 5 initial-reactive samples, all of which had low S/CO values ranging from 1.09 to 2.45 (Table 2). All 5 samples were nonreactive on repeat TMA testing and classified as false-positive samples.

Specificity of the dengue RNA blood screening assay

Of the 13,731 samples tested from all three countries, 13 test results were considered false-positive based on initial reactivity that could not be confirmed by repeat TMA or

supplemental testing, yielding a specificity for the prototype dengue TMA blood screening assay of 99.91 percent.

DISCUSSION

The DENV TMA assay developed for this study had comparable analytical sensitivity (approx. 15 copies/mL, 95% limit of detection) and clinical specificity (99.95%) to TMA-based assays currently employed worldwide to screen blood donors for human immunodeficiency virus (HIV)-1 and hepatitis C virus (HCV) RNA and to screen donors in high-risk regions of the world for hepatitis B virus DNA or WNV RNA.[16,18,23,30,34] For HIV and HCV, the rapid replication kinetics and high viral loads of infected donations during the acute preseroconversion phase have allowed blood donor screening to effectively employ so-called "minipool NAT" screening strategies.[35] For WNV, the viremia after mosquito transmission is transient and relatively low titer, such that minipool NAT screening failed to interdict a proportion of RNA-positive and infectious donations.[16,18] Consequently, a strategy of targeted individual donation NAT screening was developed, which involves real-time monitoring of minipool NAT yield, with conversion to individual-donation NAT in epidemic regions and time periods, thus interdicting low-titer viremic units that might be infectious.[16,18,36]

This study, which used individual-donation NAT for DENV, identified viremic units with relatively low-level RNA based on viral loads by quantitative PCR and in some cases low S/CO values in the TMA assay. These results are consistent with animal and human studies demonstrating low-titer viremia during the asymptomatic or presymptomatic 3- to 5-day acute viremic phase.[20-22] The combined results from our study suggest that individual sample NAT may be required for efficient interdiction of potentially infectious donations, despite the excellent analytical sensitivity of the TMA assay. Finally, the failure of an NS1 antigen enzyme immunoassay (EIA) to detect viremia in the nine infected donations from Honduras is consistent with delayed detection of antigenemia relative to RNA in acute dengue infection.[21,32]

Given expanding global spread of the four dengue serotypes, it was important to establish that the prototype dengue TMA assay has similar analytical and clinical sensitivity to all four serotypes. This is particularly critical since reinfections by a heterologous serotype are more pathogenic than primary infections.[3,4] If an RNA-positive transfusion transmits DENV to recipients previously infected with a heterologous dengue serotype(s), these patients would be at high risk for DHF. Consequently, in areas such as Asia and Latin America where all four serotypes are now circulating, a blood-screening assay that lacks sensitivity to one or more serotypes could allow breakthrough transmission of that serotype to recipients with antibody to heterologous serotypes, precipitating enhancement of infection and anamnestic responses manifesting as severe clinical disease.

The yields of confirmed viremic donations observed among the three countries represented in this study are consistent with the magnitude of dengue outbreaks within each country during the study accrual periods. In Honduras, there were 19,971 cases of DF and 351 cases of DHF in 2004 in a population of 7.2 million (2.8 cases/1000). In Brazil, there were almost 300,000 reported DF and DHF cases in 2003. Although the city of São Paulo (population 11 million) was not severely impacted by the epidemic (753 cases), surrounding cities from which study donations were accrued were severely affected, with 27,876 cases notified in the State of São Paulo, which has 38 million residents (0.7 cases/1000). In Australia between March and September 2003, there were 449 dengue cases in Cairns (population of 122,192) and 20 cases in Townsville (population 151,725) for a combined rate of 1.7 per 1000, with the source for the outbreak tracked to travelers with rare secondary autochtonous transmissions.[29] The subtypes detected by blood donor TMA testing, based on type-specific PCR and serotyping of culture isolates, were consistent with serotypes documented in corresponding regional epidemics: Serotypes 1, 2, and 4 were detected in Honduras with a predominance of Serotype 4 and Serotypes 1 and 3 were detected in Brazil.

Based on serologic markers (IgM, IgA, and PRNT) in the viremic specimens, we were able to classify infected donors with respect to whether they were experiencing their initial dengue infection or a second or even third DENV infection. Given the pathogenesis implications of reinfection with a heterotypic subtype, it would be of great interest to follow viremic individuals identified by RNA screening to study viral dynamics and host genetic, innate, and adaptive immunologic factors that correlate with clinical outcome, in the context of primary infection versus reinfection and relative to titer and specificity of preexisting antibodies. Similar follow-up studies of acutely infected blood donors identified by NAT screening for HIV, HCV, and WNV have contributed significantly to our understanding of viral dynamics and disease pathogenesis.

In conclusion, we have developed a donor screening assay with excellent analytical sensitivity (15 copies/mL) and clinical specificity (>99.9%). Confirmed prevalence rates among asymptomatic blood donors ranged from 0.30 percent in Honduras to 0.04 percent in São Paulo, Brazil, with no viremic donations detected in Australia.

Despite the high prevalence rates observed in this study, which suggest that DENV viremic donations may occur frequently in endemic regions, only a single DENV TT case has been documented worldwide. The ability to recognize DENV TT may be complicated by the high overall prevalence rates in the general populations of

DENV-affected regions, making it difficult to distinguish mosquito-borne and TT infections. Additionally, the presence of potentially protective antibodies in either DENV viremic donations or in recipients may play a role in reducing TT cases in such populations. Although this study raises many questions about blood safety in DENV endemic regions, it would be premature to recommend DENV NAT donor blood screening without additional studies.

Future studies are needed to establish rates and correlates of TT by viremic donations and clinical consequences to recipient health. We have recently launched such a study in Honduras, which experienced a very large epidemic in 2007. Application of the dengue NAT assay to donors or other populations, with follow-up of viremic individuals, could also contribute to understanding determinants of pathogenesis and play a role in evaluation of dengue vaccines, which are in active development and entering clinical trials.[37]

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