virus was quickly inactivated by the heat treatment, and infectious virus became undetectable within 5 minutes (Fig. 1). Total viral reduction for both the bulk pasteurization and the terminal pasteurization is shown in Table 2.

FIII precipitation

After the 15 percent ethanol precipitation, dengue virus was detectable with the $TCID_{50}$ assay in both the supernatant III and the FIII, with a majority of infectious virus in the FIII. Viral reduction from the FII + III to the supernatant III was calculated and is shown in Table 3.

Virus filtration

The immunoglobulin spiked with dengue virus was processed at 24 to 25°C through the 35N virus filter within 7 hours. No infectious virus was detectable by the $TCID_{50}$ assay in the immunoglobulin filtrate, the sample obtained when the virus filter was reversely flushed with purified water, or the virus-spiked immunoglobulin control standing along the whole virus filtration process. To differentiate physical separation from the chemical inactivation by the low pH, the samples were further quantified for dengue virus with the quantitative RT-PCR assay. The RT-PCR data show that dengue virus was much more concentrated in the back-flush fraction than in the immunoglobulin filtrate. Viral reduction by the virus filtration was calculated and shown in Tables 3 and 4. These results indicate that dengue virus is effectively removed by the 35N virus filtration.

TABLE 2. Clearance of dengue virus in the albumin process*

Process step	Reduction of virus (log)
FIV precipitation	≥5.18†/≥5.35†
Bulk pasteurization	≥4.61
Terminal pasteurization	≥4.94/≥5.44
Cumulative	≥10.12‡/≥10.79‡

* Data are shown from duplicate experiments, except bulk pasteurization, which was conducted once.
† The data from the $TCID_{50}$ assay, but not the RT-PCR assay, are included.
‡ "Bulk pasteurization" is not included in the "cumulative," because it is similar mechanistically to "terminal pasteurization."

TABLE 3. Clearance of dengue virus in the immunoglobulin process*

Process step	Reduction of virus (log)
FIII precipitation	2.16/2.65
Virus filtration	3.37†
S/D treatment	≥5.05/≥5.38
Chromatography	3.66‡/4.18‡
Cumulative	≥14.24/≥15.58

* Data of single virus filtration experiment and duplicate experiments of other processing steps are shown.
† Only RT-PCR data are included.
‡ Virus reduction caused by the presence of S/D is not included.

S/D treatment

The presence of S/D was cytotoxic to the virus detector Vero E6 cells; when diluted 1000-fold, S/D did not affect the determination of virus titer. The kinetics of inactivation of dengue virus in the immunoglobulin during the 16-hour S/D treatment at 28 to 30°C is shown in Fig. 2. Dengue virus was quickly inactivated by the S/D treatment, and infectious virus became undetectable within 1 minute. Total viral reduction for the S/D treatment was shown in Table 3.

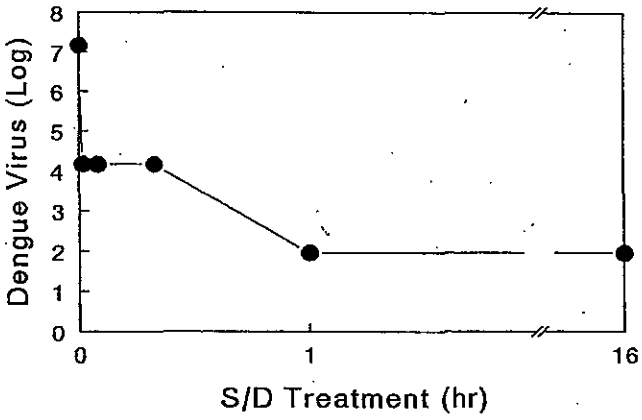

Fig. 2. Inactivation of dengue virus in immunoglobulins by S/D treatment over time. Stock dengue viruses (7.16 log/mL) was spiked at a ratio (vol/vol) of 1:9 in the S/D treatment step; when it was spiked to the immunoglobulin sample neutralized to pH 7.0 a viral titer of 7.00 log was obtained.

TABLE 4. Clearance of dengue virus in the virus filtration*

Assay	Load	Immunoglobulin filtrate	Back-flush	Log reduction, load → filtrate
$TCID_{50}$	8.37	≤2.58†	≤2.51†	≥5.79
Quantitative RT-PCR	8.47	5.10	7.08	3.37

* The numbers shown are total viral titers (log number multiplied by volume), and the stock virus spiked at a ratio of 1:49 had a titer of 7.67 log per mL.
† No dengue virus was detected, and the number was calculated according to the Poisson distribution.

TABLE 5. Clearance of dengue virus in the chromatography*

Assay	Resin	Load	Flow through	Eluate	Log reduction, load → eluate
$TCID_{50}$	New	8.20	≤6.19†	≤1.16†	≥7.04
	Used	8.04	≤6.19†	≤1.11†	≥6.93
Quantitative RT-PCR	New	9.24	8.31	5.58	3.66
	Used	8.99	8.14	4.81	4.18

* The numbers shown are total viral titers (log number multiplied by volume), and the stock virus spiked at a ratio of 1:20 had a titer of 7.67 and 7.83 log per mL for the new and used resins, respectively.
† No dengue virus was detected, and the number was calculated according to the Poisson distribution.

Cation-exchange chromatography

After the chromatography step of the immunoglobulin process, no infectious virus was detectable by the $TCID_{50}$ assay in the column load, the flow-through fraction, the eluate fraction, or the virus-spiked load control standing along the whole chromatography process. Total viral reduction from the column load to the eluate fraction was at least 7.04 and at least 6.93 log for the new resin and the 476-cycled used resin, respectively (Table 5). Because S/D was present in the starting material, the elimination of dengue virus could be a result of inactivation by the chemicals. To differentiate physical removal from chemical inactivation, the samples were further quantified for dengue virus with the quantitative RT-PCR assay. The RT-PCR data show that a majority of dengue virus was observed in the flow-through fraction. Total viral removal by the chromatography process was calculated to be 3.66 and 4.18 log for the new resin and the 476-cycled used resin, respectively (Tables 3 and 5).

DISCUSSION

Viral safety is of paramount importance for human plasma–derived therapeutic proteins such as albumin, α1-proteinase inhibitor, clotting factors, and immunoglobulins. Recent documentation of blood-associated transmission[1-3] and continuous regional outbreaks of dengue fever have prompted a validation of clearance of dengue virus in the manufacture processes of the plasma-derived products. It was the intention of this study to investigate clearance of dengue virus in individual steps of manufacture processes of plasma-derived albumin and immunoglobulins. The results shown in Tables 2 and 3 clearly demonstrate for the first time that specific virus removal and inactivation procedures reduce the risk of dengue virus transmission by more than 10 log cumulatively in plasma-derived albumin and immunoglobulins.

In this study, cold ethanol precipitation is very effective in inactivating dengue virus in the albumin process, but mildly effective in removing dengue virus in the immunoglobulin process. This difference in effectiveness is probably due to the fact that higher concentrations of ethanol were used in the albumin process. It is fairly reasonable to speculate that other therapeutic proteins prepared from plasma by similarly high concentrations of ethanol, for instance, α1-proteinase inhibitor and transferrin purified from the Cohn FIV, would have a good safety margin with regard to transmission of dengue virus.

Pasteurization inactivated dengue virus very quickly and effectively in the albumin process. The presence of a high concentration of albumin or the albumin stabilizing agent sodium caprylate did not seem to protect dengue virus from the heat inactivation. Caprylate has been shown to be an effective virus-inactivating agent at millimolar concentrations under acidic conditions;[9-12] however, caprylate appears unlikely to contribute much to the viral inactivation capacity of the pasteurization step as in the albumin formulation it is used under neutral pH, which do not favor the formation of the active component—the nonionized form of caprylate. As shown by albumin's long history of viral safety in clinical applications, the dedicated viral inactivation step in albumin manufacture processes has been very robust in the inactivation of many different viruses including West Nile virus and bovine viral diarrhea virus (BVDV), both from the same Flaviviridae family as dengue virus.[13,14]

Virus filtration was very effective in separating dengue virus from the immunoglobulin filtrate (Table 4). The data suggest that chemical inactivation by the low pH condition can probably contribute to the viral clearance capacity of this process step. In a separate study with BVDV, which is of similar size but not sensitive to low pH treatment, a majority of the spiked BVDV was trapped in the Planova 35N filter, which was recovered in the back-flush sample (unpublished observation).

Like pasteurization, S/D treatment very quickly and effectively inactivated dengue virus in the immunoglobulin process. This dedicated viral inactivation step in the immunoglobulin manufacture processes has been very robust in the inactivation of many different viruses including West Nile virus and BVDV.[13,14]

The cation-exchange chromatography was originally intended to remove S/D from the immunoglobulin process; however, it was also observed in this study to effectively remove dengue virus by affinity adsorption. In addition, this purification step was mildly effective in the physical removal of BVDV (unpublished observation). Although the chromatographic process may not be a

robust viral removal step in general, it is indeed effective in the clearance of dengue virus.

In summary, this study has shown that effective clearance of dengue virus is achieved in the manufacture processes of albumin and immunoglobulins, providing additional evidence supporting the viral safety of plasma-derived products.

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一般的名称	①乾燥抗HBs人免疫グロブリン ②ポリエチレングリコール処理抗HBs人免疫グロブリン		研究報告の公表状況	TRANSFUSION 2008; 48: 1348-1354	公表国 プエルトリコ	
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研究報告の概要

＜背景＞
輸血を介したデングウイルス感染が1例報告されている。しかし、流行地域でのデング熱の発生率が高いこと、無症候感染の割合が高いこと、およびウイルス血症の中央値が5日であることから、輸血に関連したデング感染は、報告されているよりもさらに広範囲に拡がっている可能性がある。
＜研究デザインおよび方法＞
2005年9月20日から12月4日までにプエルトリコにある米国赤十字へ提供された全ての血液ドネーションのデングウイルス(DENA) RNAの陽性率を、transcription mediated amplification (TMA) 法と呼ばれる特異タイプの核酸増幅検査を使って測定した。TMAの測定結果が2回くり返して陽性であったものを、TMA法陽性のドネーションと定めた。TMA陽性のドネーションについて、IgM抗体のELISA、RT-PCRおよびウイルス培養による検査を行った。
＜結果＞
検査を行った血液ドネーション16,521検体のうちの12検体（0.07%）が、TMA陽性であった。4検体がRT-PCR陽性（DENVセロタイプ2、3）であった。4つのRT-PCR陽性のうちの3つでウイルスが培養できた。TMA陽性であった12のドネーションのうちの1つがIgM陽性であった。他の輸血ウイルス感染（C型肝炎、WNV）のルーチンのミニプールスクリーニングでされているのと同じように1:16に希釈すると、5つのドネーションだけがTMA陽性であった。
＜結論＞
1,000のドネーション中のほぼ1つがDENV RNAを含み、TMA陽性ドネーションからウイルスが培養できたが、このことは輸血感染のリスクが、WNVについて供血者全員へのスクリーニングがされる前の米国に存在していたリスクに類似していることを示している。WNVと同じく、IgM抗体のスクリーニングは有効でない可能性があり、感染性を有しているドネーションのいくつかはミニプールによって見逃されるであろう。輸血後のデング感染の患者において、輸血感染を考慮すべきである。

報告企業の意見	今後の対応
デングウイルス感染症の流行地域における献血中からデングウイルスが約0.1%の確率で検出されたとの報告である。 血漿分画製剤からのデングウイルス伝播の事例は報告されていない。万一、原料血漿にデングウイルスが混入しても、BVDをモデルウイルスとしたウイルスバリデーション試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

使用上の注意記載状況・その他参考事項等

代表として静注用ヘブスブリンーIHの記載を示す。
2. 重要な基本的注意
(1)本剤の原材料となる血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体陰性で、かつALT（GPT）値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の抗HBs抗体を含有する血漿を原料として、Cohnの低温エタノール分画で得た画分からポリエチレングリコール4000処理、DEAEセファデックス処理等により抗HBs人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びろ過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。

TRANSFUSION COMPLICATIONS

Dengue virus in blood donations, Puerto Rico, 2005

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BACKGROUND: A single instance of transfusion-transmitted dengue infection has been reported. The high incidence of dengue in endemic countries, the high proportion of asymptomatic infection, and the median 5-day viremia, however, suggest that transfusion-associated dengue transmission may be more widespread than documented.

STUDY DESIGN AND METHODS: The prevalence of dengue virus (DENV) RNA was determined in all blood donations to the American Red Cross in Puerto Rico from September 20 to December 4, 2005, using a specific type of nucleic acid amplification test called transcription-mediated amplification (TMA). TMA-positive donations were defined as those having two repeatedly reactive TMA results. TMA-positive donations were tested by enzyme-linked immunosorbent assay for immunoglobulin M (IgM) antibodies, by reverse transcription–polymerase chain reaction (RT-PCR), and by viral culture.

RESULTS: Twelve (0.07%) of 16,521 blood donations tested were TMA-positive. Four were positive by RT-PCR (DENV serotypes 2 and 3). Virus was cultured from 3 of 4 RT-PCR–positive donations. One of the 12 TMA-positive donations was IgM-positive. Only 5 donations remained TMA-positive when diluted 1:16, as is done for routine minipool screening for other transfusion-transmissible viral infections (hepatitis C, human immunodeficiency, West Nile viruses [WNVs]).

CONCLUSION: Nearly 1 in 1000 blood donations contained DENV RNA, and virus could be cultured from TMA-positive donations, suggesting a transfusion transmission risk similar to that which existed in the United States for WNV before universal donation screening. Similar to WNV, IgM antibody screening is likely to be ineffective, and some potentially infectious donations will be missed by minipool screening. Transfusion transmission should be considered in patients with dengue after blood transfusion.

Dengue virus (DENV) is a mosquito-borne flavivirus transmitted by the bite of an infected *Aedes* spp. mosquito. Infection by each of the antigenically distinct serotypes (DENV-1, -2, -3, and -4) confers lifelong serotype-specific immunity. Subsequent infection with another serotype is possible because immunity to heterologous serotypes is short-lived. Most (53%-87%) dengue infections are asymptomatic or mildly symptomatic.[1-3] Dengue infection is characterized by a median 5-day viremia, and in clinically apparent infections, symptom onset occurs 1 day after onset of viremia.[4,5] The clinical spectrum of dengue infection ranges from dengue fever to dengue hemorrhagic fever, dengue shock syndrome, and death. Primary dengue infections often present with features of classic dengue fever including acute onset of fever, arthralgia, myalgia, retroorbital pain, headache, and rash. Subsequent infection with a second dengue serotype increases the risk of developing dengue hemorrhagic fever, which is characterized by fever, thrombocytopenia (platelet count $\leq 100 \times 10^9$/L), hemorrhagic manifestations, and evidence

ABBREVIATIONS: ARC = American Red Cross; DENV = dengue virus; IC = internal control; IR = initially reactive; S/CO ratio = signal-to-cutoff ratio; TMA = transcription-mediated amplification; WNV = West Nile virus.

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Conflict of interest: JML and ASB are employees of Gen-Probe, Inc. and are owners of equity stock options in Gen-Probe.

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of increased vascular permeability and plasma leakage.[6,7] With timely supportive care, dengue hemorrhagic fever case-fatality rates can be reduced to less than 1 percent.[8,9]

The principal dengue vector is *Aedes aegypti*. It is found throughout the tropics and subtropics and in limited areas of some states in the southeastern United States. *Aedes albopictus* is also a competent vector for dengue and has been implicated previously in dengue outbreaks.[10] Although it has not been detected in Puerto Rico, *A. albopictus* exists in some parts of the Americas including more than 20 states in the eastern half of the United States.[11] Autochthonous dengue transmission does sporadically occur in southern Texas along the United States-Mexico border, with the most recent outbreak occurring in the contiguous border towns of Brownsville, Texas, and Matamoros, Tamaulipas (Mexico).[12,13] This suggests the endemicity of dengue in South Texas and the risk of reemergence of dengue in states that border Mexico as well as in southeastern states with competent vector(s) and subtropical climates. Research, however, has found that differences in housing (e.g., use of air conditioning and screens) and lifestyle may prevent this from happening.[12,13]

Although few reports document DENV transmission through receipt of infected blood,[14] tissues,[15] or organs,[16] transfusion-associated dengue transmission may be more common than previously recognized. The high proportion of asymptomatic infections, the median 5-day period of detectable viremia, and the high incidence, especially during outbreaks, suggest that a substantial number of donors could be viremic at the time of donation. In addition, nosocomial transmission of DENV via needle-stick injury[17-21] further indicates the transmissibility of DENV by infected blood. Viremic individuals may unknowingly donate blood before symptom onset or if they remain asymptomatic. West Nile virus (WNV), a related mosquito-borne flavivirus, may provide a useful model for assessing transfusion-associated DENV transmission. Transfusion transmission of WNV is well documented, and all blood donations in the United States are screened using WNV-specific nucleic acid amplification tests (NATs).[22,23]

Dengue was first identified in Puerto Rico in 1963 and is now endemic year-round with occasional islandwide outbreaks. A mean of 5446 (range, 2416-10,048) suspected cases were reported annually during the nonoutbreak years from 1990 to 2004, whereas 6039 cases were reported in 2005 (incidences of 151 versus 159 per 100,000 population/year). Approximately 77,000 blood donations are collected annually by the American Red Cross (ARC) collection centers and blood donation drive sites in Puerto Rico. These donations are used in the continental United States, Puerto Rico, and elsewhere in the Caribbean. To assess the potential for transfusion-associated dengue infection in Puerto Rico, we tested all blood donations to the ARC for dengue viral nucleic acid using a recently developed dengue-specific NAT during an 11-week period of seasonally heightened dengue activity in 2005.

MATERIALS AND METHODS

We analyzed demographic data collected from blood donors and plasma specimens from all blood donations to ARC blood collection centers and blood drives in Puerto Rico from September 20 to December 4, 2005. This study period commenced 2 weeks after the peak of seasonally heightened dengue activity in Puerto Rico (Fig. 1). Plasma specimens containing ethylenediaminetetraacetate as an anticoagulant (BD Vacutainer PPT plasma preparation tubes, BD, Franklin Lakes, NJ) from all blood donations during this study period were retained in a repository at the ARC facility in Gaithersburg, Maryland.

All specimens were first screened for the presence of DENV RNA using a DENV-specific NAT developed by Gen-Probe, Inc. (San Diego, CA) that uses transcription-mediated amplification (TMA). Specimens were tested by TMA at Gen-Probe by trained ARC staff. All initially reactive (IR) specimens were retested and TMA-positive

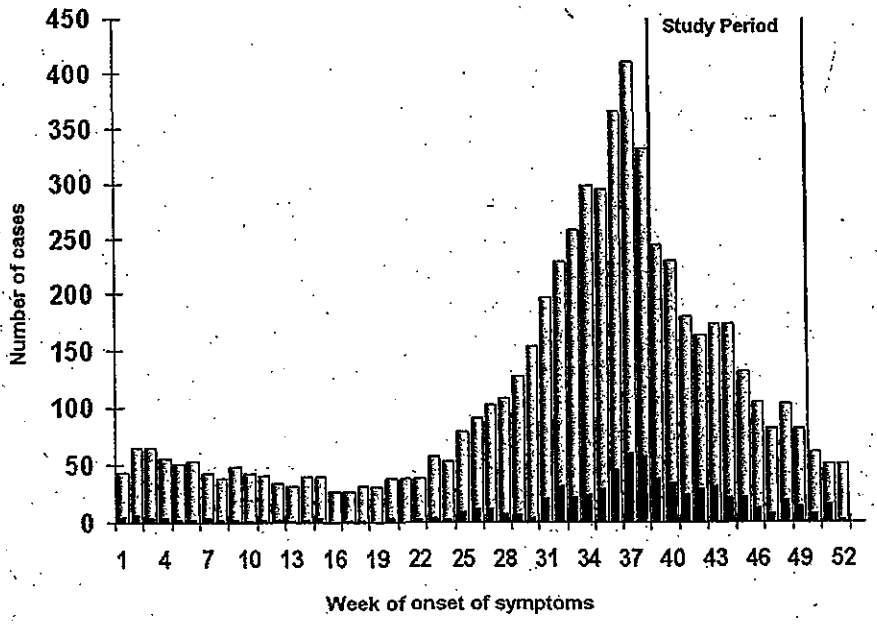

Fig. 1. Number of suspected* (□) and confirmed† (■) dengue cases by week of symptom onset, Puerto Rico, 2005. *Suspected = reported case of dengue with a clinical suspicion of dengue. †Confirmed = laboratory-confirmed (by serology or virology) case of dengue.

specimens were those that were repeatedly reactive; all others were considered to be TMA-negative. Both initial and repeat TMA screening were performed using individual specimens. All IR specimens were sent to the CDC's Dengue Branch Laboratory in San Juan, Puerto Rico, for supplemental testing by reverse transcription–polymerase chain reaction (RT-PCR).[24] Testing of donations was unlinked to donor personal identifiers; thus, subsequent contact with donors or recipients was not possible. Deidentified data from blood donation records were used in the statistical analysis (described below). The data were stored on a single password-protected terminal at the CDC, and no attempt was made to trace the donors. The study protocol was approved by the Institutional Review Board of the ARC.

TMA

Testing was performed using a prototype dengue TMA assay on a fully automated system for NAT blood screen (Procleix Tigris system, Chiron Corp., Emeryville, CA). The assay uses the same chemistry as other human immunodeficiency virus-1/hepatitis C virus and WNV assays (Procleix and Ultrio, respectively, Chiron Corp.)[25-27] and targets sequences that are conserved across all four serotypes. Thus the assay used is capable of detecting all four dengue serotypes. TMA is an isothermal RNA transcription amplification system using bacteriophage T7 RNA polymerase and Moloney murine leukemia virus reverse transcriptase (MMLV RT) to produce RNA amplicons via DNA intermediates. Viral lysis and magnetic-based target capture of viral RNA are followed by amplification and detection with the use of chemiluminescent probes.[26] This technique is able to detect 3.4 West Nile viral copies per mL at a 50 percent detection rate.[28] The analytical sensitivity of the DENV TMA assay used in this study is very similar, with 50 percent detection at 3.5 viral RNA copies per mL and a sample volume of 0.50 mL.[29] Assay results were reported in relative light units, which were used to derive signal-to-cutoff (S/CO) ratios. Cutoff values for the Dengue TMA assay internal control (IC) and analyte signals were calculated using the same formulae used for the Procleix WNV Assay.[30] A sample was considered reactive if the analyte S/CO ratio was at least 1.0, nonreactive if the analyte S/CO ratio was less than 1.0 and the IC signal was above the IC cutoff, and invalid if the analyte S/CO ratio value was less than 1.0 and the IC signal was below the IC cutoff.

Supplemental testing

All TMA-positive specimens were retested at a 1:16 dilution in plasma screened negative for all infectious disease markers including dengue RNA at Gen-Probe to determine the efficacy of testing blood donations by minipooled methods. The TMA-positive and IR specimens were tested using a real-time RT-PCR assay for the detection of NS5 gene sequence (TaqMan, Applied Biosystems, Foster City, CA).[24] This RT-PCR test is multiplexed and detects the four dengue serotypes in one reaction. It can also be used to quantitatively measure viral RNA in blood specimens with a sensitivity of approximately 1×10^3 to 5×10^3 viral RNA copies per mL. The sample volume is 20 µL derived from a 100-µL RNA extract obtained from a 0.24-mL serum specimen. All TMA-positive and IR specimens were also tested for the presence of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody using IgM MAC–enzyme-linked immunosorbent assay (ELISA) and IgG ELISA, respectively.[31,32] Virus was isolated on C6/36 cells and by mosquito isolation.[33,34]

Statistical analysis

A trend analysis using simple linear regression was performed to determine if there was a change in the number of blood donations collected during the study period. The prevalence of DENV RNA was determined by dividing the number of TMA-positive donations by the number of blood donations collected during the study period. Information about donor characteristics (see Table 1) was obtained from the ARC's electronic donor database and included date of collection, gender, date of birth, zip code of residence, zip code of donation site, donation status (first-time donor or repeat donor), phlebotomy procedure (whole blood, plateletpheresis, or leukapheresis), and donation type (allogeneic, directed, or autologous). Both donor residence and donation site were recoded into the three regions of Puerto Rico set by the United States Postal Service (San Juan Metropolitan Area, west, and east) and then treated as binary variables in the analyses (i.e., San Juan metropolitan area versus other). Age was stratified by its median and considered as a binary categorical variable in analyses.

Differences in TMA positivity by donor characteristics were assessed by the Fisher's exact test and exact logistic regression. Potential covariates identified for inclusion in the final multivariable model included covariates with a p value less than 0.20 on bivariate analysis. Age was added to the model a priori given its association with dengue infection.[2] All comparisons were made with the use of a two-tailed test, and a Type I error rate of 0.05 was used to assess significance.

RESULTS

A total of 16,521 blood donations were collected during the 11-week study period (mean, 1502 donations per week [range, 281-1864] without a significant trend in donation frequency). Twelve donations (0.73 per 1000 donations) were TMA-positive, with two or less identified per week. Eleven of these 12 donations were whole