

## ORIGINAL ARTICLE

# Screening the Blood Supply for West Nile Virus RNA by Nucleic Acid Amplification Testing

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## ABSTRACT

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N Engl J Med 2005;353:460-7.

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**BACKGROUND**

The use of nucleic acid amplification tests of “minipools” of 16 samples to screen blood donors for West Nile virus RNA began in July 2003. We report the yield and characteristics of positive donations and the incremental yield and safety of nucleic acid amplification tests of individual donations.

**METHODS**

Reactive minipools were analyzed to identify the individual reactive donations. For the regions with the highest yield on minipool testing, retrospective nucleic acid amplification testing was performed on individual donations that were negative on minipool testing. Reactive donations were confirmed by alternative nucleic acid amplification tests and IgM and IgG tests, and donors were followed to document seroconversion.

**RESULTS**

From July 1 through October 31, 2003, 677,603 donations were prospectively screened for West Nile virus by minipool testing, yielding 183 confirmed viremic donations (0.027 percent, or 1 in 3703 donations). Retrospective individual testing of 23,088 donations from high-prevalence regions that were negative on minipool testing yielded 30 additional units with a low level of viremia, with 14 additional viremic units detected by prospective testing of individual donations late in the 2003 transmission season. Of all the viremic units detected, 5 percent were detected only by individual testing and were negative for IgM antibody, 29 percent were detected by individual testing after IgM seroconversion, and 66 percent were detected by minipool testing. West Nile virus infection was confirmed in both recipients of IgM-negative units that were reactive on individual testing, whereas neither recipient of antibody-positive blood components that were reactive on individual testing was infected. In 2004, prospective testing of individual donations in regions that yielded donations that were reactive on minipool testing resulted in a 32 percent incremental yield of units with a low level of viremia that would have been missed by minipool testing.

**CONCLUSIONS**

Although nucleic acid amplification testing of minipools of blood donations prevented hundreds of cases of West Nile virus infection in 2003, it failed to detect units with a low level of viremia, some of which were antibody-negative and infectious. These data support the use of targeted nucleic acid amplification testing of individual donations in high-prevalence regions, a strategy that was implemented successfully in 2004.

**W**EST NILE VIRUS, A MOSQUITO-borne flavivirus, emerged as a cause of meningoencephalitis in the United States in 1999, and infections reached epidemic proportions in 2002.<sup>1,2</sup> In 2002, West Nile virus was shown to be transmissible by transfusion, when 23 cases were documented.<sup>3,4</sup> In late 2002, the Food and Drug Administration (FDA), U.S. blood-collecting organizations, and test-kit manufacturers accelerated programs to develop nucleic acid amplification tests to screen blood donors for West Nile viremia in an effort to implement such programs before the 2003 transmission season.<sup>4-7</sup> The resulting assays involved the testing of pools of 6 to 24 samples, or "minipools," an approach that is now also routinely used to screen blood donors for human immunodeficiency virus (HIV) and hepatitis C virus (HCV).<sup>8</sup>

We report the results of a large, multicenter testing program conducted during the summer and fall of 2003 and of studies that compared minipool results with those obtained by nucleic acid amplification tests of individual (undiluted) donations. Our results allow an estimation of the number of infections averted in 2003 by the implementation of screening with minipool nucleic acid amplification tests and an estimation of the additional benefit of screening individual donations in regions with seasonal epidemics of West Nile virus infections. We also summarize experience with implementation of a targeted screening strategy involving nucleic acid amplification testing of individual donations in 2004, a strategy that successfully identified units with a low level of viremia that would have been missed by minipool testing.

## METHODS

Blood Systems Laboratories screens blood donations in two FDA-licensed laboratories in Tempe, Arizona, and Bedford, Texas. Clients include 18 blood-collection facilities owned by Blood Systems (Scottsdale, Ariz.) and 59 other community-based and hospital-based blood-collection programs. The geographic areas covered by this client base include much of the Southwest, the South, the Central Plains, and parts of California. The annual testing volume is approximately 2.2 million donations, which represents approximately 20 percent of the U.S. blood supply.

The West Nile virus Transcription-Mediated Amplification system (Procleix WNV Assay, Gen-

Probe and Chiron) was used for nucleic acid amplification tests. This technique involves lysis of viral particles in plasma, either from individual donations or from a minipool of plasma specimens from 16 donations and the isolation of West Nile virus RNA with the use of probes bound to magnetic beads, amplification with the use of RNA transcription, and subsequent detection by a chemiluminescent probe.<sup>9,10</sup> All samples within a reactive minipool are then tested individually. The assay has an analytical sensitivity of approximately 4 RNA copies per milliliter when used for individual donations (50 percent limit of detection by probit analysis of dilutions of West Nile virus standards) and a sensitivity of approximately 45 copies per milliliter when used for minipool testing.<sup>10</sup>

Individual specimens identified as reactive were evaluated by means of a confirmatory algorithm with the use of multiple assays for West Nile virus.<sup>10-12</sup> These tests included an alternative nucleic acid amplification test (either a modification of a TaqMan polymerase-chain-reaction [PCR] assay or another primer-based transcription-mediated amplification assay, performed by Bayer Reference Laboratory) and assays of plasma for viral IgM and IgG antibody (Focus Diagnostics).<sup>10,13</sup> All donors with reactive tests were promptly asked to enroll in a follow-up study, involving return visits approximately every week. Follow-up specimens were tested for West Nile virus RNA and for IgM and IgG antibodies against West Nile virus. A confirmed positive result was defined by the detection of viral IgM in either the index specimen or a follow-up specimen, the detection of viral RNA in the index specimen by means of the alternative nucleic acid amplification test, or the detection of viral RNA in a follow-up specimen by means of a transcription-mediated amplification assay.<sup>11</sup> Since the actual times at which donors returned for follow-up varied (resulting in intermittent blood collection) and since seroconversion would have occurred in the interval between the last seronegative and the first seropositive result, median times (and interquartile ranges) to IgM and IgG seroconversion were estimated with the use of an analysis in which data were censored in the intervals between visits.<sup>14</sup> The viral load in confirmed positive index donations for which frozen plasma components were available for analysis was evaluated by a kinetic PCR assay based on target-capture TaqMan techniques (Chiron).<sup>10</sup>

Blood Systems Laboratories also conducted geo-

graphically and temporally targeted nucleic acid amplification testing of individual donations in 2003 according to two protocols. The first protocol involved testing of individual frozen specimens from donations previously found to be negative on nucleic acid amplification testing of minipools of specimens from regions with a high prevalence of West Nile virus infections. Donors whose specimens were retrospectively determined to be reactive on individual testing were asked to enroll in the follow-up study. In addition, in-stock blood products from these donors were retrieved, and in collaboration with the Centers for Disease Control and Prevention (CDC), recipients of blood components from donors confirmed to be positive for West Nile virus on nucleic acid amplification testing of individual donations were evaluated to ascertain whether transmission of West Nile virus had occurred.<sup>15</sup> The second protocol was conducted in selected blood-collection regions that had had a high number of reactive minipools on nucleic acid amplification testing in the previous weeks. Individual donations were prospectively screened exclusively by nucleic acid amplification testing (i.e., minipool testing was not performed). Reactive units were subsequently diluted 1:16 and retested individually to determine what the results of minipool testing would have been. No blood components that were reactive on nucleic acid amplification testing of individual donations were transfused during this prospective study.

In June 2004 Blood Systems Laboratories implemented a targeted screening strategy involving nucleic acid amplification testing of individual donations (described elsewhere in detail<sup>16</sup>) and real-time tracking of the results of minipool testing. Prospective nucleic acid amplification testing of individual donations was implemented in geographically defined zones if there were two or more reactive donations on minipool testing and a rate of more than 1 reactive minipool per 1000 tested. Testing reverted to the minipool format when regions had had no individual donations with reactive tests for at least seven consecutive days and had a weekly rate of reactivity of fewer than 1 per 1000 donations.

All studies were approved by the FDA and the relevant institutional review boards. All donors and recipients gave written informed consent to undergo screening and follow-up testing for West Nile virus. Drs. Busch, Tomasulo, and Kleinman and Ms. Caglioti designed the studies; supervised data

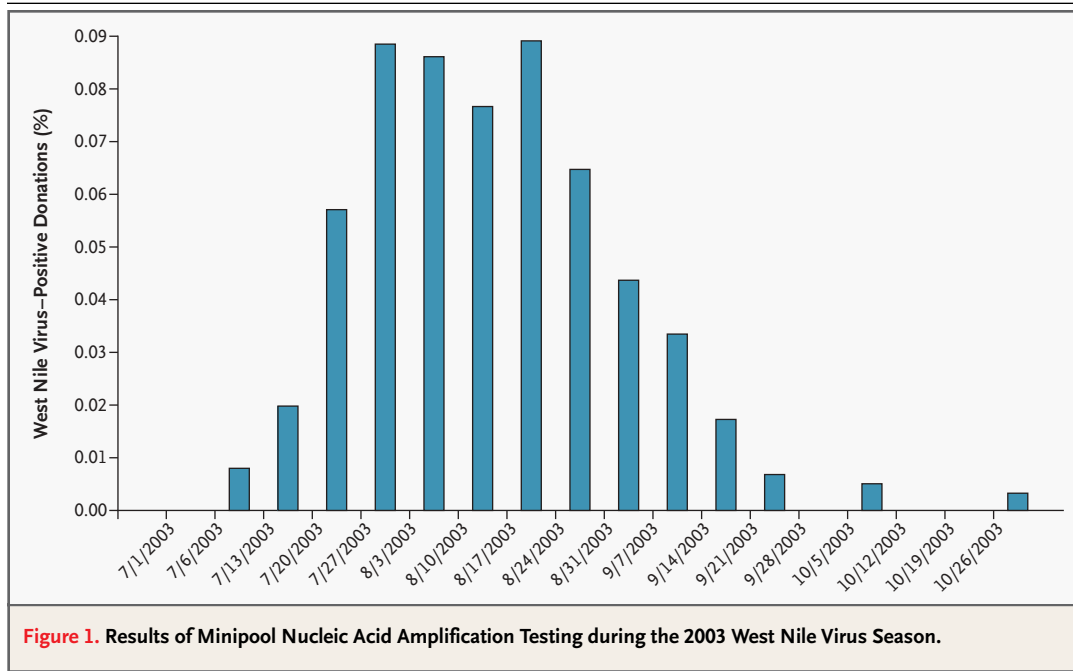
collection, management, and analyses; and drafted and revised the manuscript. Drs. Robertson, Tobler, Linnen, and Shyamala and Ms. McAuley supervised testing. Dr. Kamel supervised donor follow-up and participated in “look-back” activities involving recipients of blood components. All authors approved the manuscript, which was written primarily by Drs. Busch and Kleinman.

## RESULTS

The results of minipool nucleic acid amplification testing during the active West Nile virus season in 2003 are shown in Figure 1. Of 677,603 donations tested between July 1 and October 31, 2003, 183 were confirmed to be positive, for an aggregate rate of 0.027 percent (1 in 3703 donations). The highest rates occurred during a six-week period from mid-July through mid-August.

Forty-seven additional viremic units were detected on nucleic acid amplification testing of individual donations. Retrospective testing of 23,088 individual donations that had been negative on minipool testing, collected from donor centers in Texas, North Dakota, and South Dakota (i.e., centers with high rates of reactivity on minipool testing during the summer of 2003), identified 30 additional confirmed positive specimens. Prospective testing of individual donations was subsequently performed on 3964 donations collected in North Dakota and South Dakota in September 2003 and identified 17 confirmed positive donations, of which 14 tested negative for West Nile virus with the use of the transcription-mediated amplification system at a 1:16 dilution, indicating they would have been missed by minipool testing. Thus, 186 units were detectable by minipool testing and 44 units were detectable only by nucleic acid amplification testing of individual donations.

West Nile virus antibody status was determined for 41 of the 44 donations that were negative on minipool testing and confirmed positive by individual testing (3 samples had insufficient volume for testing). Thirty-one specimens (76 percent) had detectable West Nile virus antibody: 10 were IgM-positive, and 21 were positive for both IgM and IgG. In contrast, only 16 of the 183 confirmed positive donations detected by minipool testing (9 percent) were positive for IgM antibody at the time of donation ( $P < 0.001$  by the chi-square test). Among 145 viremic donors who were initially seronegative and who enrolled in the follow-up assessment, West



Nile virus–specific IgM antibody appeared a median of four days after donation (interquartile range, one to six) and virus-specific IgG antibody appeared a median of two days later (interquartile range, one to five).

The median viral load for 143 of the 183 specimens confirmed to be positive on minipool testing with sufficient volume for quantitative PCR analysis was 3519 copies per milliliter (range, less than 50 to 690,159). Twelve IgM-positive donations identified by minipool testing had significantly lower viral loads than 131 IgM-negative donations identified by minipool testing (median, less than 50 and 5325 copies per milliliter, respectively;  $P < 0.001$  by the two-sample Wilcoxon rank test). As expected, viral loads were very low in donations detected only by nucleic acid amplification testing of individual donations: 21 of these 44 samples (48 percent) were reactive on only one of two replicate tests, indicating that the viral load was near the limit of detection of the transcription-mediated amplification assay, and 16 of 22 evaluated by TaqMan PCR (73 percent) had RNA levels that were below the limit of quantitation (i.e., fewer than 50 copies per milliliter).

We performed a subanalysis of 113 confirmed viremic donations identified from July 1 through September 30, 2003, in North Dakota and South Dakota from a donor population that had been

screened with the use of both minipool and individual nucleic acid amplification testing. Table 1 shows the overall rates of detection of units confirmed positive by minipool testing and by individual testing alone, as well as the incremental rate of detection of viremia by nucleic acid amplification testing of individual donations throughout the epidemic, with adjustment to account for the proportion of units tested individually. Minipool testing detected 66 percent of viremic units detected by nucleic acid amplification testing of individual donations. Five percent of viremic donations were detectable by individual testing alone and were negative for West Nile virus antibody; 7 percent were reactive on individual testing alone and were positive for IgM but negative for IgG; and 22 percent were reactive on individual testing alone and were positive for both IgM and IgG (Table 1).

On the basis of previous studies of inoculation of West Nile virus in humans<sup>17</sup> and animals,<sup>18,19</sup> acute-phase infection is thought to be characterized by a brief period of very-low-level viremia shortly after inoculation (as reflected by the viral-load data presented above and the finding of IgM-negative specimens that were reactive on nucleic acid amplification testing of individual donations). This period is followed by a longer interval (approximately seven days) with an increasing and then decreasing viral load, which makes the viremia de-

**Table 1.** Yield of Minipool and Individual Nucleic Acid Amplification Testing of Donations from North Dakota and South Dakota, July 1 through September 30, 2003.

Variable	Total No. of Donations	Minipool Testing	Individual Nucleic Acid Amplification Testing Alone			
			Total No.	IgM–	IgM+, IgG–	IgM+, IgG+
No. of donations tested	27,009	27,009	22,641	22,641	22,641	22,641
No. of confirmed positive units	113	79	34*	5	6	21
Rate of detection (no. of confirmed positive units/100 donations)	0.443	0.292	0.150	0.024†	0.028†	0.099‡
Adjusted no. of confirmed positive units‡	119	79	40	6	8	26
Proportional yield (%)	100	66	34	5	7	22

\* Two units had insufficient volume for serologic testing.

† The value was adjusted to account for serologic classification of 32 of 34 units that were identified by individual testing alone.

‡ The value was adjusted to account for individual testing of 22,641 of the 27,009 units that underwent minipool testing.

tectable by minipool testing. Finally, as IgM and IgG seroconversion evolves, the viral load decreases to a level detectable only by testing of individual donations. Given this natural history, Figure 2 presents the biweekly yield data for North Dakota and South Dakota according to the results of individual and minipool tests and antibody tests. The figure shows that although testing of individual donations identified additional donations with a low level of viremia throughout the epidemic, the characteristics of these units shifted from primarily antibody-negative early in the epidemic to predominantly IgM-positive and IgG-positive late in the season ( $P < 0.001$  by Fisher's exact test).

Case investigations were initiated for 17 recipients of blood components from 14 donations that were negative on minipool testing but identified as having a low level of viremia on retrospective nucleic acid amplification testing of individual donations. On the basis of clinical symptoms and serologic analysis for West Nile virus, two recipients of seronegative donations with low-level viremia were infected with West Nile virus, probably as a result of transfusion, whereas two recipients of components from one donation that was reactive on individual testing alone and was positive for IgM and IgG were not infected.<sup>12</sup> The evaluation of the remaining 13 recipients was deemed inconclusive owing to a lack of follow-up laboratory data to support or rule out West Nile virus infection (Montgomery S and Brown J, CDC: personal communication).

During the 2004 epidemic (from May 1 to October 23, 2004), analysis of 1,065,212 donations by

minipool testing yielded 71 confirmed viremic donations (Table 2). An additional 58,679 donations (5 percent of all donations tested) were prospectively tested individually with the use of previously described triggers,<sup>16</sup> and 54 donations were confirmed to be viremic. Sufficient volume was available to test 48 of these 54 donations at a 1:16 dilution: 27 were negative (and thus classified as reactive on individual testing alone), and 21 were positive (and thus classified as detectable by minipool testing). Of the 27 donations identifiable by individual testing alone, 23 were IgM-positive and 4 were IgM-negative. Thus, as seen in Table 2, targeted testing of individual donations in the regions of the 2004 epidemic yielded percentages of units detectable by minipool testing (76 percent) and by individual testing alone (24 percent) and a serologic profile for units detectable by individual testing alone that were similar to the percentages and profile observed in North Dakota and South Dakota during the 2003 epidemic (66 percent and 34 percent, respectively).

## DISCUSSION

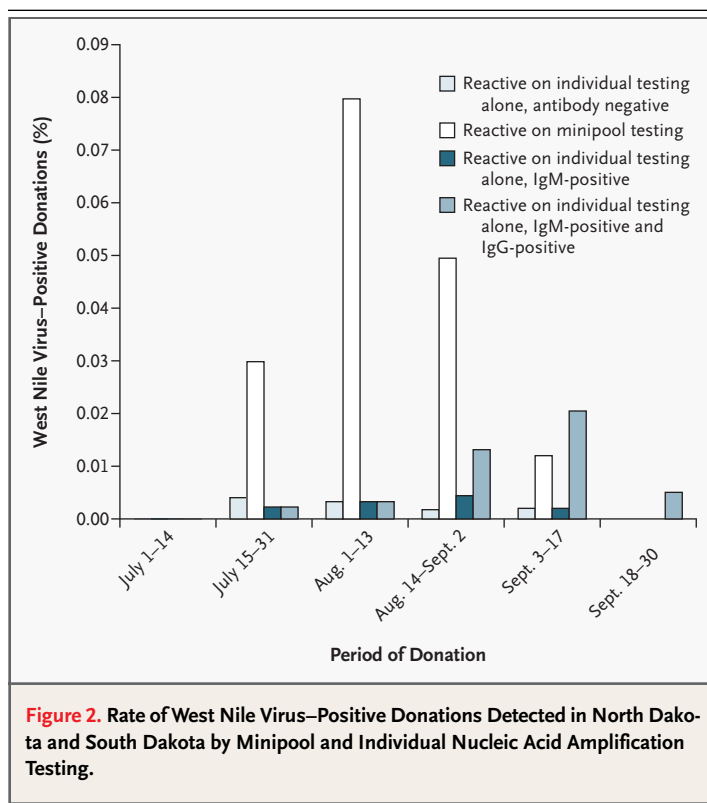
The implementation of nucleic acid amplification testing for West Nile virus RNA in 2003 resulted in the identification of 183 confirmed viremic units, with 47 additional infected units detected by targeted testing of individual donations. Nationally, the combination of minipool testing and targeted testing of individual donations resulted in the identification of approximately 1000 viremic donations.<sup>11,15</sup> Since, on average, each unit is made into 1.45 transfusable components,<sup>20</sup> the transfusion



of almost 1500 viremic components (most of which lacked antibody and would be expected to be infectious) was averted in 2003.

Our study confirms previous data from clinical cases and experimental inoculation studies indicating that infected persons would probably have low titers of West Nile virus.<sup>2,3,12,15,17-19</sup> Unlike seronegative donors with HIV and HCV infection and positive results on minipool testing, who usually have viral titers of  $10^5$  to  $10^7$  copies per milliliter,<sup>6</sup> in our study, the median number of copies of West Nile virus RNA was only about 3500. This observation of a low viral load combined with data indicating that proven transfusion-transmitted cases of West Nile virus infection occurred from donors with low viral titers<sup>3,15</sup> prompted us to study retrospectively the incremental value of individual nucleic acid amplification testing as compared with minipool testing and to implement individual testing prospectively in selected high-prevalence regions in late 2003 and 2004. We found that individual testing identified up to 50 percent more viremic donors than were detected by minipool testing. Five percent of all viremic donations were identifiable by individual testing alone and were antibody-negative and thus were donations that have been shown to be infectious. (Both recipients of units that were antibody-negative and reactive on individual nucleic acid amplification testing alone in our study were infected.) These donations were detected at a fairly constant rate throughout the epidemic. In contrast, the additional yield of antibody-positive donations identified by means of individual testing alone was minimal when tests were performed during the early weeks of the epidemic, but toward the end of the epidemic it increased to levels greater than those observed with minipool testing.

The incremental safety to be achieved by the use of individual testing over minipool testing is difficult to quantify because of the unknown risk of transmission by donations with low-level viremia that contain West Nile virus antibody. The absence of transmission of West Nile virus from two antibody-positive components identified by individual testing alone in this study is consistent with the observation that no documented case of post-transfusion infection has been attributed to a seroreactive donation, despite the relatively high frequency of such donations during the later stages of West Nile virus epidemics.<sup>5,10,15</sup> This is also consistent with in vitro and animal infectivity experiments suggest-



ing that IgM and IgG antibodies neutralize infectivity.<sup>19</sup> Similar experience with hepatitis A virus, an acute infection that also leads to the production of neutralizing IgM antibody, has indicated that the concurrence of viremia and IgM production does not result in infectivity.<sup>7,21</sup> Thus, one working hypothesis is that viremic units that contain IgM (and particularly those that also contain IgG) are not infectious when transfused.

The pattern of observed viremia (i.e., the percentage of West Nile virus RNA–positive donations detected by minipool testing as compared with the percentage detected by individual testing) and seroreactivity (presence of IgM and IgG) of viremic units changed dramatically through the 10 weeks of the epidemic, strongly suggesting that the greatest benefit of individual testing can be obtained by implementing such screening early in the epidemic when new infections are on the rise. This observation led Blood Systems Laboratories to adopt a new nucleic acid amplification testing strategy during the 2004 epidemic. A designated level of reactive minipool tests in a defined geographic region was used to determine when sufficient risk existed to implement individual testing.<sup>16</sup> This strategy

**Table 2.** Actual and Adjusted Systemwide Yield of Minipool and Individual Nucleic Acid Amplification Testing of Donations Screened from May 1 through October 23, 2004.

Type of Nucleic Acid Amplification Test	Total Tested	Confirmed Positive Results		Adjusted Confirmed Positive Results*	
	no. (%)	no. (%)	no./100 donations	no. (%)	no./100 donations
Minipool	1,065,212 (95)	71 (57)	0.007	95 (76)	0.009
Individual	58,679 (5)	54 (43)*	0.092	30 (24)	0.051
Total	1,123,891 (100)	125	0.012	125	0.012

\* Of the 54 samples confirmed to be positive by nucleic acid amplification testing of individual donations, 48 were available for retesting at a 1:16 dilution; 27 of the latter (56 percent) were found to be negative and therefore classified as reactive on individual testing alone. This percentage was applied to the 54 positive units detected by individual testing to project that 30 of these donations would be positive with the use of this method alone and that 24 would have been detected by minipool testing. The reclassification is reflected in the column that provides adjusted confirmed positive results.

was designed to balance the residual risk of transfusion-transmitted West Nile virus infection accompanying the use of minipool testing against the limited capacity for individual testing, given the current limitations of automation.<sup>5</sup> This strategy proved highly effective, with the identification and removal of at least 27 units that would have been missed by minipool testing in regions with West Nile virus epidemic activity. They included at least four units that were IgM-negative and hence likely to transmit West Nile virus to recipients. Moreover, the similarity of the systemwide distribution of the yields of the two tests in 2004 (an increase in the rate of detection by approximately 32 percent with the use of individual over minipool testing) with that observed in a region of epidemic infection in 2003 indicates that our targeting of individual testing was appropriately directed to regions with an increased yield.

Before the initiation of blood-donor screening, information from experimental studies of the inoculation of West Nile virus conducted in the early 1950s in patients with advanced cancer indicated that the duration of viremia (as assayed by intracerebral injection of virus into mice) was approximately six or seven days.<sup>17</sup> Contemporary data from primate and murine models of West Nile virus infection are consistent with this estimate.<sup>7,18,19</sup> On the basis of the proportional rates of detection of viremia in the early phases of infection in asymptomatic viremic blood donors, we estimate that the duration of antibody-negative viremia detectable only by nucleic acid amplification testing of individual donations is shorter (one or two days). Our data cannot be used to estimate the length of the phase in which specimens are reactive on individual testing and antibody-positive, since a cross-sectional analysis would be biased: the frequency of

donation in this convalescent phase is reduced as a result of the signs and symptoms of West Nile virus infection.<sup>2</sup> As compared with other transfusion-transmissible infections (e.g., HIV and HCV), West Nile virus has a similar interval in which it is detectable only by nucleic acid amplification testing of individual donations.<sup>22</sup> In contrast, the duration of viremia detectable by minipool testing is much shorter for West Nile virus infections than it is for HIV and HCV infections, in which high-titer viremia is detected for weeks or months before seroconversion and usually persists for many years after seroconversion.

In conclusion, although the use of minipool screening in 2003 prevented hundreds of West Nile virus infections, it failed to detect donations with a low level of viremia, some of which were antibody-negative and infectious. Our 2003 data supported the use of targeted nucleic acid amplification testing of individual donations in high-prevalence regions, a strategy that was successfully implemented in 2004. On the basis of the price of reagents for previously licensed nucleic acid amplification tests, the costs of performing minipool and individual screening at Blood Systems Laboratories, and the observed yields of minipool testing, the cost of minipool screening was \$120,000 per unit intercepted in 2003 and \$232,000 per unit intercepted in 2004. The reduced cost utility in 2004 reflects the decreased rate of viremic donations detected, illustrating the close inverse relationship between yield and cost-effectiveness. The targeted individual screening of approximately 60,000 donations in the summer of 2004 resulted in a 33 percent increment in yield and cost only \$32,000 per incremen-

tal case detected. In contrast, had individual testing been performed for West Nile virus during all of 2004, the cost per viremic donation detected would have been \$281,000. This approach of performing targeted testing of individual donations on the basis of real-time monitoring of the yield of minipool testing may prove to be a rational and cost-effective donor-screening paradigm for other agents similar to West Nile virus that cause seasonal and regional epidemics.

Supported in part by a grant (R01-CI-000214) from the Centers for Disease Control and Prevention (CDC) and the Blood Systems Foundation.

Dr. Busch is an employee of Blood Systems, a not-for-profit company that collects and tests donated blood for West Nile virus and other infections as described in this article; is a member of Scientific Advisory Boards of Chiron and Gen-Probe, the manufacturers of the West Nile virus assays that were evaluated in this study; and reports having received an unrestricted research grant from Chiron and speaking honoraria from Chiron and Gen-Probe. Ms. Caglioti, Dr. Robertson, Ms. McAuley, Dr. Tobler, Dr. Kamel, and Dr. Tomasulo

are employees of Blood Systems. Dr. Linnen is an employee of Gen-Probe and reports owning equity stock and stock options in Gen-Probe. He is named on patents filed by Gen-Probe for the West Nile virus nucleic acid amplification assay described in this article. Dr. Shyamala is an employee of Chiron and is named on patents filed by Chiron for the West Nile virus nucleic acid amplification assay described in this article.

Reagents for performing the retrospective and prospective West Nile virus transcription-mediated amplification testing on collections in 2003 were supplied to Blood Systems at no charge by Gen-Probe. The costs for reagents to perform minipool screening in 2003 and 2004 and individual screening in 2005 were paid to Chiron and Gen-Probe on a cost-reimbursement basis, as detailed in an FDA Investigational New Drug (IND) application. All other sample-collection, shipping, and labor costs related to West Nile virus screening and acquisition of follow-up specimens were funded by Blood Systems, with reimbursement from hospitals or other blood centers. The cost of follow-up serologic and PCR testing was covered by Gen-Probe and Chiron as part of the IND application.

We are indebted to the staff at Blood Systems Laboratories and the United Blood Services and other donor centers for their effort in support of this study and to Susan Montgomery and Jennifer Brown at the CDC for coordination of look-back investigations of recipients exposed to West Nile virus.

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