Analytical and clinical sensitivity of West Nile virus RNA screening and supplemental assays available in 2003

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BACKGROUND: Transfusion-transmitted West Nile virus (WNV) infections were first reported in 2002, which led to rapid development of investigational nucleic acid amplification tests (NAT). A study was conducted to evaluate sensitivities of WNV screening and supplemental NAT assays first employed in 2003.

STUDY DESIGN AND METHODS: Twenty-five member-coded panels were distributed to NAT assay manufacturers. Panels included five pedigreed WNV standards (1, 3, 10, 30, and 100 copies/mL), 15 or 16 donor units with very-low-level viremia identified through 2003 screening, and four or five negative control samples. Samples were tested neat in 10 replicates by all assays; for NAT screening assays, 10 replicates were also performed on dilutions consistent with minipool (MP)-NAT. The viral load distribution for 142 MP-NAT yield donations was characterized, relative to the analytical sensitivity of MP-NAT systems.

RESULTS: Analytical sensitivities (50% limits of detection [LoD] based on Poisson model of detection of WNV standards) for screening NAT assays ranged from 3.4 to 29 copies per mL; when diluted consistent with MP pool sizes, the 50 percent LoD of screening NAT assays was reduced to 43 to 309 copies per mL. Analytical sensitivity of supplemental assays ranged from 1.5 to 7.7 copies per mL (50% LoD). Detection of RNA in donor units varied consistent with analytical LoD of assays. Detection of low-level viremia after MP dilutions was particularly compromised for seropositive units, probably reflecting lower viral loads in the postseroconversion phase. Based on the viral load distribution of MP-NAT yield donations (median, 3519 copies/mL; range, <50-690,000), 13 to 24 percent of units had viral loads below the 50 percent LoD of screening NAT assays run in MP-NAT format.

CONCLUSION: WNV screening and supplemental assays had generally excellent analytical sensitivity, comparable to human immunodeficiency virus-1 and hepatitis C virus NAT assays. The presence of low-level viremic units during epidemic periods and the impact of MP dilutions on sensitivity, however, suggest the need for further improvements in sensitivity as well as a role for targeted individual-donation NAT in epidemic regions.

West Nile virus (WNV), a mosquito-borne flavivirus infection, emerged as a cause of meningoencephalitis in the United States in 1999 and reached epidemic proportions in 2002.[1,2] Evidence began accumulating in the summer of 2002 that WNV could be transfusion-transmitted, culminating in 23 documented cases during that year.[3,4] In late 2002, the Food and Drug Administration (FDA), US blood collecting organizations, and test kit manufacturers began an accelerated program to develop nucleic acid amplification tests (NAT) for detection of WNV for implementation before the 2003 mosquito transmission season. Assays were developed for implementation in minipool (MP) formats (i.e., specimens from multiple [6 or 16] donations are pooled and the pool is tested), similar to the procedures routinely used for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) NAT blood donor screening.[5,6] Investigational supplemental assays were also developed for confirming viremia in the NAT-reactive individual-donation (ID) samples identified from reactive MPs, as

ABBREVIATIONS: BSL = Blood Systems Laboratory; IC = internal control; ID = individual donation; LoD = limits of detection; MP(s) = minipool(s); TMA = transcription-mediated amplification; WNV = West Nile virus.

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well as for testing follow-up samples from donors of reactive units. From July through November 2003, approximately 1000 confirmed viremic donations were detected by US blood centers with these NAT assays, resulting in prevention of approximately 1500 transfusion-transmitted WNV infections.[7,8]

Although a dramatic success, WNV MP-NAT screening did not completely eliminate transfusion-transmitted WNV infections. Studies conducted during the 2003 transmission season confirmed at least six "MP-NAT breakthrough" infections, which were attributed to units that had levels of viremia below the sensitivity of MP-NAT screening systems.[8] Furthermore, large-scale retrospective studies of specimens from donors in epidemic areas and periods established that a substantial fraction (20%-30%) of WNV RNA–positive blood donations have very-low-level viremia that is detectable by ID-NAT but not by MP-NAT.[8] Moreover, analyses of follow-up specimens from donors detected as WNV RNA–positive by MP-NAT screening have documented persistent very-low-level viremia, detectable only by ID-NAT, for weeks following immunoglobulin M (IgM) seroconversion. Studies are under way to further characterize the viral and antibody (IgM, immunoglobulin G [IgG], and plaque neutralization capacity) status of such low-level viremic donations and follow-up specimens, as well as to investigate the infectivity of units with different viral load and antibody profiles.

These findings have led to significant concern over the residual risk of WNV transmission from MP-NAT–screened blood transfusions. Several different strategies have been considered to reduce this risk, including targeted conversion from MP-NAT to ID-NAT and reduced blood collections in epidemic regions. A better understanding of the analytical and clinical sensitivity of WNV NAT assays, in the context of MP-NAT and ID-NAT, is critical to these considerations. We report results of a comparative study of the sensitivity of WNV screening and supplemental NAT assays employed by US and Canadian blood centers in 2003.

MATERIALS AND METHODS

Study design

A protocol was developed and commitments to participate obtained from participating NAT assay manufacturers and testing laboratories. A coded panel was developed composed of frozen plasma specimens from donations that had been identified to contain very-low-level viremia and various profiles of IgM and IgG reactivity and for which large volumes of plasma were available (>200 mL). This coded panel also included positive control (a dilution series of a WNV reference reagent) and negative control specimens. The panel was distributed to manufacturers of WNV RNA screening and supplemental assays that were used for blood donor testing in the United States in 2003 (i.e., Roche Molecular Systems, Pleasanton, CA; Gen-Probe Inc., San Diego, CA; National Genetics Institute [NGI], Los Angeles, CA; Chiron Corporation, Emeryville, CA; and Bayer Reference and Testing Laboratories (BRTL), Berkeley, CA). Screening NAT assays were performed on all panel members both neat and at dilutions consistent with relevant MP-NAT screening practices (Roche, 1:6; Gen-Probe, 1:16 and 1:4 [an intermediate dilution under consideration for future WNV MP-NAT screening]). Supplemental NAT assays were run neat. All testing was performed in replicates of 10 at each input level (neat and mock MP-NAT dilutions). Testing laboratories reported back results of the coded panel members as the number of reactive results divided by the number of replicates tested for each panel member. The results were compiled and analyzed with categorical and Probit analyses.

Panel composition

The panel consisted of 25 members as summarized in Table 1. Plasma components (fresh frozen plasma or recovered plasma) corresponding to 15 donations with low-level viremia were identified by Blood Systems Laboratory (BSL, Tempe, AZ) either by MP-NAT (16-member MPs) or retrospective ID-NAT WNV screening studies with the Procleix WNV Assay (Gen-Probe screening assay; n = 13)[9] or through the America's Blood Centers (ABC)-Retrovirus Epidemiology Donor Study (REDS) plasma recall study (n = 2).[10] These 15 plasma units included: 1) 3 units with WNV RNA that had been missed by MP-NAT but detected by retrospective ID-NAT and that lack detectable WNV antibodies based on Focus IgM and IgG assays; 2) 3 units lacking WNV antibodies that were detected by MP-NAT but have very-low-level viremia based on borderline reactive signal-to-cutoff values by MP-NAT and viral loads of less than 50 copies per mL; 3) 3 units detected by MP-NAT that have detectable WNV IgM antibodies and very-low-level viremia based on borderline reactive MP-NAT signal-to-cutoff values and viral loads of less than 50 copies per mL; 4) 3 units missed by MP-NAT but which tested repeat reactive by retrospective ID-NAT and which contain detectable IgM and weak IgG reactivity; and 5) 3 units that have high-level IgM and IgG reactivity and very-low-level viremia based on inconsistent detection of RNA by replicate ID-NAT. These 15 selected units were carefully thawed, mixed, and subaliquoted at BSL into a repository containing small (1-10 mL) and large (25-50 mL) volumes of frozen plasma (–70°C). Two 50-mL aliquots from each unit were subsequently thawed to room temperature to allow cryoprecipitate to fully dissolve, followed by mixing and low-speed centrifugation to clear nonsoluble particulate matter. Supernatant plasma was then distributed into coded tubes with appropriate volume for each company's planned testing (13-25 mL) and

TABLE 1. Characteristics of specimens represented in the WNV low-level viremia panel*

Panel code	Source	Baseline data: Original NAT screening results	Baseline data: Viral load	Baseline data: IgM	Baseline data: IgG	IgM and IgG SC on follow-up
Analytical sensitivity standards						
22	CBS† reference reagent	NA	1			
25	CBS reference reagent	NA	3			
24	CBS reference reagent	NA	10			
21	CBS reference reagent	NA	30			
23	CBS reference reagent	NA	100			
Negative controls						
1, 2, 6, 10, 15A	BSRI NC plasma	NA	0	Negative	Negative	
Before seroconversion (IgM- and IgG-negative)						
13	BSL donor unit	MP-positive	<50	Negative	Negative	Yes
14	BSL donor unit	MP-positive	<50	Negative	Negative	Yes
17	BSL donor unit	MP-positive	<50	Negative	Negative	Yes
8	BSL donor unit	MP-negative/IDT‡-positive	78	Negative	Negative	Yes
20	BSL donor unit	IDT-positive (end of run)	141	Negative	Negative	Yes
7	BSL donor unit	MP-negative/IDT-positive	470	Negative	Negative	Yes
15B	Nebraska donor unit	MP-negative/IDT-positive	<50	Negative	Negative	Yes
After seroconversion (IgM only)						
3	BSL donor unit	MP-negative/IDT-positive	<50	Positive	Negative	Yes
5	BSL donor unit	MP-negative/IDT-positive	<50	Positive	Negative	Yes
9	BSL donor unit	MP-negative/IDT-positive	<50	Positive	Negative	Yes
11	BSL donor unit	MP-positive	<50	Positive	Negative	Yes
After seroconversion (IgM- and IgG-positive)						
4	BSL donor unit	MP-negative/IDT-positive	<50	Positive	Positive	Yes
12	BSL donor unit	MP-positive	<50	Positive	Positive	Yes
18	ABC study donor unit	IDT 7/10 reps.	<50	Positive	Positive	NA
19	ABC study donor unit	IDT 2/10 reps.	<50	Positive	Positive	NA
16	BSL donor unit	MP-positive	89	Positive	Positive	Yes

* The original panel member 15, derived from a negative control unit (15A), was replaced by an ID-NAT-positive, MP-NAT-negative unit implicated in WNV transmission (15B), in the panels distributed to the manufacturers or screening assays.
† CBS = Canadian Blood Services.
‡ IDT = individual-donation testing.
SC = seroconversion; reps = replicates; NA = not available.

immediately refrozen to –80°C. Five WNV RNA– and antibody-negative plasma units were similarly processed as negative controls. A proposed WNV reference reagent was obtained from Health Canada and used to construct positive control specimens; this reference reagent was characterized in detail in a multicenter study reported recently in **TRANSFUSION**.[11] The reference reagent was prepared by diluting heat-inactivated, tissue culture WNV (concentration approx. 2×10^9 copies/mL) in pooled human plasma negative for WNV, HCV, HIV-1, and hepatitis B virus (HBV) to a final concentration of approximately 1000 copies per mL. The titer of the stock was determined by the endpoint dilution method (method of maximum likelihood with the statistical package GLIM[12]). The reference material was serially diluted in WNV-negative plasma (confirmed by each manufacturer) to levels of approximately 1, 3, 10, 30, and 100 RNA copies per mL.

After initial preparation of the 25 member study panels, we gained access to a volume of plasma from a unit that had been implicated in a MP-NAT breakthrough transfusion transmission case in 2003.[13] Owing to the limited volume of specimen available from this donation, it was represented only in the panels submitted to the two manufacturers of screening NAT assays by substituting coded aliquots from this infectious low-level viremic unit for one of the negative control aliquots in the original panel. Therefore, Roche and Gen-Probe received 16 low-viremic clinical samples, 5 positive control samples, and 4 negative control samples, whereas the supplemental assay manufacturers received panels consisting of 15 clinical samples, 5 positive control samples, and 5 negative control samples.

The panels were shipped on dry ice to each participating site. The screening NAT companies were responsible for performing dilutions of all specimens with WNV-negative plasma as diluent to mimic MP sizes currently used or under consideration. All testing was performed according to the manufacturers' standard testing protocols.

Qualitative screening and supplemental WNV NAT assays

Gen-Probe. Two transcription-mediated amplification (TMA) assays were used for testing at Gen-Probe: the Procleix WNV assay (Gen-Probe screening assay) and the

Gen-Probe alternative TMA assay (a supplemental assay that targets a different WNV genomic region). Both assays use the same assay protocols and semiautomated platform as currently in use with the FDA-licensed Procleix HIV-1 and HCV assay and are completely compatible with the fully automated TIGRIS system.[6] Both assays employ a magnetic particle–based target capture method and use a sample input volume of 0.5 mL. The two assays use different oligonucleotide primers for TMA and different acridinium ester labeled oligonucleotide probes for chemiluminescent detection of amplicon.

Roche. The TaqScreen WNV Test uses generic sample preparation and real-time reverse transcription-PCR (RT-PCR) to detect WNV RNA in plasma samples and uses the Roche Diagnostics' COBAS AmpliPrep and COBAS TaqMan Instruments to achieve full automation of the test. Sample preparation on the COBAS AmpliPrep Instrument uses a chaotropic lysis reagent and magnetic glass particles to capture total nucleic acid in the sample. The instrument adds an internal control (IC) to each sample along with the lysis reagent to serve as a control for target recovery and amplification-detection and prepares the RT-PCR after sample processing. The prepared reaction tubes are transferred to the COBAS TaqMan instrument for RT-PCR amplification and real-time fluorescence detection. The IC and WNV RNA are amplified and detected simultaneously with probes labeled with different fluorescent reporter dyes. One negative control and one WNV-specific positive control were included as controls in each run.

Bayer. This qualitative target-capture PCR WNV assay was developed at Chiron Corporation and validated at Chiron and Bayer for use as a supplemental assay. The assay involves target-specific magnetic particle–based isolation of nucleic acid from 0.5 mL of sample followed by PCR amplified detection with fluorescently labeled oligonucleotide probes. Each sample also included a WNV-specific IC RNA modified for the probe-binding sequence that served as a control for target isolation and amplification. One-step RT-PCR reagent (ABI, Foster City, CA) was added to the isolated target-IC and amplification-detection was performed during 50 cycles of PCR in ABI Prism 7900. Fluorophores distinct for target (6-FAM) and IC (TET) were used to simultaneously detect the target amplicons. Each run included high, medium, and low WNV-positive and -negative calibrators tested in triplicate. The samples were scored as detectable, not detectable, and invalid depending on the status of target and IC detection at 45 cycles of amplification.

NGI. NGI's supplemental qualitative WNV PCR involved extraction of 1 mL of plasma, followed by four separate amplification reactions (540 µL total plasma input). Each amplification reaction was analyzed by Southern blot for both WNV-specific sequences and IC-specific sequences. Other than use of WNV-specific primers and probes, the assay is identical to NGI's HCV UltraQual assay that is approved by FDA for plasma donor screening.

Quantitative WNV RNA assay

A quantitative target-capture PCR WNV viral load assay was developed by Chiron Corporation and consists of two steps: target capture and amplification-detection. A total of 400 µL of capture reagent (WNV-specific and linker probes and magnetic particle beads) and 500 µL of each specimen were used per test. This suspension was incubated in a 60°C water bath for 20 minutes to lyse the virus, solubilize the proteins, and release viral RNA. The samples were then cooled and the beads with the hybridized target washed three times in a HEPES buffer (0.3 mol/L NaCl, 0.5% Nonidet P-40, pH 7.5). After removal of the wash buffer, 100 µL of one-step RT-PCR master mix (ABI) containing both amplification and detection primers corresponding to the capsid region of WNV was added to the beads. The WNV target and IC-specific signals were monitored with the ABI Prism 7900 system. Results were quantified with a nine-member standard curve (40-160,000 copies/mL) generated from serial dilutions of WNV cultured in Vero cells, with results analyzed with computer software (SDS, ABI). WNV RNA qualification panel-QWN702 (BBI, Boston, MA) served as the primary standard to quantitate the Vero cell cultured WNV stock used for preparing the nine-member secondary standard panel. Based on replicate analyses of analytical standards across the dynamic range of the assay, the lower limit of accurate quantitation is 50 copies per mL, with coefficients of variation of 0.22 to 2.6 percent; samples yielding results above the 160,000 copies per mL standard are diluted 1:10 and retested to yield values within the assay's dynamic range.

Compilation and analysis of study panel results

After each company reported results on the coded panels, a summary database was compiled that included predicate (previously determined RNA and antibody) and study results. The analytical sensitivities (50 and 95% limits of detection [LoD]) were determined by the Poisson model of detection based on the 10 replicate results for each of the five-member dilution series of the reference reagent. For each assay method, data from all replicates were pooled to give the number of positive samples of the number tested at each dilution step. Fifty and 95 percent LoD were calculated based on results from each such dilution series with the method of maximum likelihood with the statistical package GLIM.[13] For clinical samples, the detection rates (number reactive results/number replicate tests performed) of each assay, neat and at mock MP dilutions, were calculated. Results were summarized by stage of infection as defined by serostatus of donor units.

Viral load distribution of MP-NAT yield donations

To understand how the differential sensitivities of the qualitative WNV NAT assays, as determined from the results of the low-level viremia sensitivity panel, would correlate with detection of viremic units currently identified by MP-NAT screening, we characterized WNV viral loads for 142 donations that had been detected at BSL in 2003 with the Gen-Probe WNV TMA assay and 16-member MPs. The distribution of viral loads of these MP-NAT–positive units was determined, and the proportion of these units falling below the 50 percent LoD of each NAT assay was calculated.

RESULTS

Analytical sensitivity and specificity

Table 2 summarizes the analytical sensitivity for all assays, as determined by the Poisson model of detection of 10 replicate results on the 5 half-log serial dilutions of the tissue culture–derived virus standard. For neat samples, the 50 percent LoD were 3.4 and 29 copies per mL and 95 percent LoD 15 and 125 copies per mL for the Gen-Probe and Roche screening assays, respectively ($p < 0.05$). Similar assay sensitivity differences were noted at the operating MP dilutions, with a 50 percent LoD for the Gen-Probe screening assay of 43 copies per mL when performed on 1:16 dilutions, and a 50 percent LoD for the Roche assay of 309 copies per mL on 1:6 dilutions ($p < 0.05$).

The supplemental WNV assays had similar analytical sensitivities to one another, with 50 percent LoD in the 1.5 to 7.7 copies per mL range and 95 percent LoD between 6.4 and 33 copies per mL. Results of all assays were negative on the negative control samples represented in the study panel, except for one false-reactive result (of 50 replicates) reported for the Bayer supplemental assay.

Clinical sensitivity for low-level viremic specimens

To summarize data on the ability of each assay to detect WNV RNA in the low-level viremic units represented in the panel, results were grouped based on the antibody profile of each donation represented in the study panel. For the seven RNA-positive and IgM-negative (preseroconversion) units evaluated by the two screening assays, WNV RNA was detected in 40 and 91 percent of 70 replicates tested neat by the Roche and Gen-Probe assays, respectively (Table 2). The detection of viremia was substantially lower when these assays were performed on dilutions of these specimens consistent with the pool size in operational MP testing, with detection of 9 and 33 percent of replicates as RNA-positive by the Roche and Gen-Probe assays, respectively. For the supplemental NAT assays, reactive results were reported for 77 to 97 percent of replicates performed neat on the viremic seronegative donor specimens represented in the panel.

The detection rates for the six samples containing low-level RNA and IgM antibody but lacking IgG antibody were consistently lower for each assay than was observed on the low-level RNA-positive, antibody-negative units, with detection rates ranging from 3 to 75 percent for neat specimens and 0 to 15 percent for mock MP-diluted specimens. The rates of detection of WNV RNA in the panel samples containing IgG as well as IgM were similar to the rates seen with the IgM-positive and IgG-negative speci-

TABLE 2. Detection of WNV RNA by screening and supplemental NAT assays in serial dilutions of WNV reference reagent and in low-level viremic blood donor units, represented in the study panel*

	Screening NAT assays, neat		Supplemental NAT assays, neat			Screening NAT assays, MP dilutions		
Assay (assay code): / Measure	Gen-Probe (A)	Roche (B)	NGI (C)	Bayer (D)	Gen-Probe (E)	Gen-Probe 1:4 (G)	Gen-Probe 1:16 (H)	Roche 1:6 (I)
Analytical sensitivity†								
50% LoD	3.4	29	6.1	7.7	1.5	13	43	309
95% LoD	15	125	26	33	6.4	55	184	1,336
Clinical sensitivity								
No IgM or IgG (6 or 7 donor units)‡	64/70 (91)	28/70 (40)	55/60 (92)	46/60 (77)	63/65 (97)	41/70 (59)	23/70 (33)	6/70 (9)
IgM only (4 donor units)	30/40 (75)	1/40 (3)	23/40 (57)	10/40 (25)	28/40 (70)	11/40 (27)	6/40 (15)	0/40 (0)
IgM and IgG (5 donor units)	29/50 (58)	5/45 (11)	27/49 (56)	7/50 (14)	29/48 (60)	13/50 (26)	7/50 (14)	1/50 (2)
Negative controls	0/40	0/40	0/50	1/50 (2)	0/40	NT	NT	0/40

* Results for analytical sensitivity are presented as 50 and 95 percent LoD, defined as the copy number at which an assay detects WNV RNA on 50 or 95 percent of replicate determinations based on Probit analysis. Clinical sensitivity and results on negative controls are presented as number (%) of reactive over number of replicates determinations reported. Donor units were subcategorized based on presence of IgM and IgG antibody. NT = not tested.

† 50 and 95 percent LoD were derived from probit analysis of results on serial dilutions of a WNV reference reagent.[11]

‡ For the two screening assays, seven RNA-positive, IgM-negative specimens were evaluated, whereas only six of these had sufficient volume for evaluation by the supplemental assays (see Materials and methods).

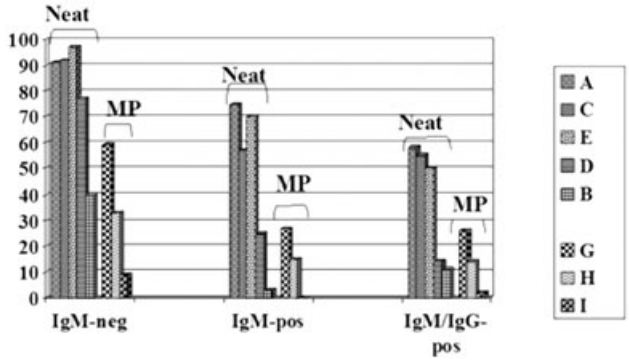

Fig. 1. Comparative performance of WNV NAT assays on low-viremic units, sorted by antibody profile of donation. Assay codes are given in Table 1. neg = negative; pos = positive.

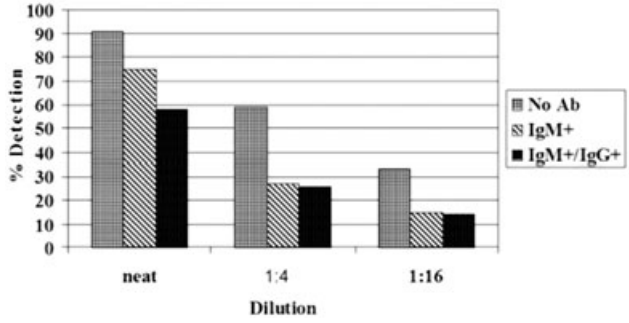

Fig. 2. Impact of dilution on detection of WNV RNA in donor units lacking WNV antibody (No Ab) versus those containing WNV IgM (IgM+) or IgM and IgG (IgM+/IgG+). Results are based on 10 replicates of the Gen-Probe screening NAT assay performed in parallel on neat and 1:4 and 1:16 dilutions.

mens. Figure 1 contrasts the rates of detection for all seven assays represented in the study, according to the stage of infection of the clinical samples defined by serologic pattern. The observation of lower rates of RNA detection in samples containing IgM and IgG, compared to those lacking WNV antibody, reflects progressively lower-level viremia in units collected after seroconversion.

By use of the Gen-Probe screening assay, we analyzed data from testing the 16 low-viremic clinical specimens neat, as well as at 1:4 and 1:16 dilutions. This allowed us to examine the impact of progressive dilution on detection of viremia in units from different stages of primary infection. As seen in Fig. 2, viremic samples lacking WNV antibody had detection rates of approximately 90 percent when tested neat, 60 percent when tested at a 1:4 dilution, and 30 percent when tested at a 1:16 dilution. In contrast, samples with IgM and IgG were more difficult to detect when diluted, with 60 to 70 percent rates of detection neat,

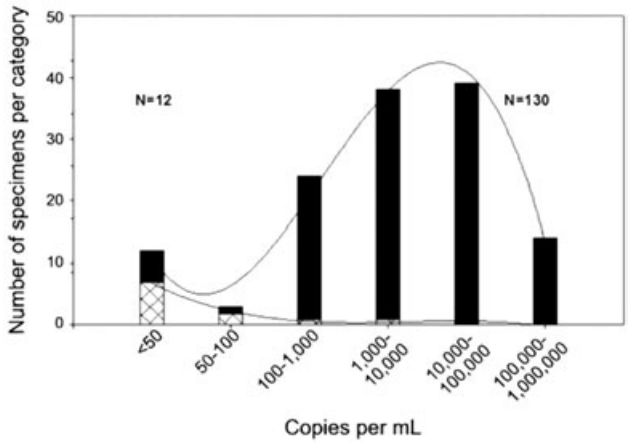

Fig. 3. Histogram of viral loads for 142 confirmed viremic units identified by MP-NAT screening with the Gen-Probe NAT assay. Results for samples lacking IgM (n = 130) are shown as solid bars whereas results for samples containing IgM (n = 12) are shown as hatched bars.

but only approximately 25 percent rates of detection at 1:4 dilution and 15 percent rates of detection at 1:16 dilution.

Viral load distribution of units detected by MP-NAT in routine screening

The panel results above focused on detection of WNV RNA in selected, low-level viremic units. To place these findings in context, it is important to consider the capacity of each assay to detect WNV RNA in representative units identified by routine MP-NAT. We therefore characterized viral loads and determined the distribution of viremia in all 142 WNV MP-NAT–positive units that had been detected by BSL in 2003 (with the Gen-Probe WNV TMA screening assay and 16-member MPs) and for which plasma components were retrieved enabling determination of viral loads. Figure 3 shows this distribution, both for the total group and for subgroups of 12 MP-NAT yield donations containing WNV IgM and the 130 units lacking detectable IgM. The median viral load for the 142 MP-NAT yield donations was 3519 copies per mL and the range of less than 50 to 690,000 copies per mL. The proportion of units falling below the 50 percent LoD of the two screening MP-NAT assay systems were calculated based on the analytical sensitivity data presented above. Based on the lower limit of detection of the quantitative assay, 13 percent (19/142) of the units had viral loads that were inferred to be below the 50 percent LoD of Gen-Probe screening NAT assay performed in MP format (43 copies/ mL at 16-fold dilution), whereas 24 percent (34/142) had viral loads below the Roche assay 50 percent LoD (309 copies/ mL at 6-fold dilutions).

DISCUSSION

The purpose of this study was to evaluate the low-end sensitivity of investigational WNV assays first available in 2003 in a head-to-head comparison. This was important in light of the generally lower levels of viremia observed with donations from WNV-infected donors, compared to the viral loads seen with HIV and HCV window-period units detected by MP-NAT screening.[5] The issue of low-level sensitivity became particularly critical when retrospective ID-NAT studies conducted in the last half of 2003 identified moderate rates of low-level viremic donations identifiable only by ID-NAT[8,9] and particularly after breakthrough transmissions were documented that were traced to low-level viremic units that had been missed by MP-NAT.[8,12]

The results of our study document generally good analytical sensitivities of these investigational WNV NAT assays when applied to undiluted specimens. Indeed, despite the rapid development timeline, the sensitivities of these WNV assays were comparable to the sensitivities of NAT assays for donor screening for HIV-1, HCV, and HBV.[5,6,14] As expected, the analytical and clinical sensitivities of the two screening platforms were significantly better in the context of individual donation compared to MP testing. When tested neat these assays 50 percent LoD ranged from 1.5 to 29 copies per mL, whereas when diluted consistent with current MP-NAT screening, the 50 percent LoD was reduced to 43 to 309 copies per mL. The rates of detection of WNV RNA were more variable in the donor units represented in the clinical sensitivity component of the study, with one screening assay (Roche) demonstrating significantly lesser sensitivity compared to other assays, in the context of both ID-NAT and MP-NAT screening.

Caution should be exercised in extrapolating our results from this panel that overrepresented low-level viremic units to the sensitivity of screening assays to detect viremic donations during an epidemic. Based on results from Blood System Research Institute's retrospective and prospective studies comparing MP- and ID-NAT, MP-NAT would have detected 71 percent of viremic units detectable by ID-NAT and, most important, 95 percent of viremic units lacking IgM and IgG antibodies.[9] Of note, all units implicated to date in MP-NAT breakthrough transmission cases have lacked IgM antibodies, and IgM is known to neutralize WNV infectivity in animal model studies.[9,15] Based on our analysis of the viral load distribution of units detected by MP-NAT in 2003, approximately 11 percent of units that were detected by Gen-Probe's screening MP-NAT assay might have been missed by the less sensitive Roche MP-NAT assay (the difference between the 24% of units with viral loads below the 50% LoD for Roche and the 13% with viral loads below the 50% LoD for Gen-Probe). This estimate of the impact on WNV transmission risk attributable to the differential sensitivities of the two MP-NAT assays is less than the differential sensitivities of ID-NAT versus MP-NAT for each assay documented in our panel study.

One approach to increase detection of units with low-level viremia missed by MP-NAT would be to reduce the pool size. Our analysis of the sensitivity of Gen-Probe's screening assay on serial dilutions of clinical samples showed that for viremic donor units lacking WNV antibodies RNA was detected in inverse proportion to the dilution level (pool size). This suggests that there is a fairly even distribution of viral loads in units from donors in the early "ramp-up" phase of viremia. In contrast, samples with IgM and IgG were more difficult to detect when diluted, with 60 to 70 percent rates of detection when tested neat, approximately 25 percent rates of detection at 1:4 dilution, and approximately 15 percent rates of detection at 1:16 dilution. These results likely reflect a lower level distribution of viremia in the postseroconversion stages of infection, resulting in relatively poor detection irrespective of the MP dilution level. These findings indicate that to significantly increase detection of low-level viremic units (and particularly those collected during the convalescent phase of infection) it will be necessary to perform ID-NAT rather than create smaller sized MPs.

The performance of the supplemental WNV NAT assays was uniformly good. These assays were approximately equal in sensitivity to one another and to the more sensitive NAT screening test. Importantly, based on these data, the supplemental assays are more sensitive than both screening assays performed in the context of MP-NAT, and therefore they can serve as effective supplemental tests to resolve the true infectious status for units detected through MP-NAT. When samples are screened by ID-NAT with the most sensitive screening test, however, there is substantial overlap in the probit sensitivity curves for screening and supplemental assays. Consequently, index units that screen as reactive by ID-NAT but are negative by a supplemental NAT assay require additional testing to confirm unit-donor status, including a combination of replicate NAT, serologic testing of the index donation specimen, and follow-up evaluation.

This study has several important limitations. First, all but one of the low-viremia clinical specimens were selected based on detection by the Gen-Probe screening assay. This could have biased the findings in favor of that assay. The relative clinical sensitivity of the assays based on these potentially biased samples, however, correlates well with the analytic sensitivity differences of the assays. Furthermore, the results for the one sample that had been originally screened with the Roche system were consistent with the findings based on the Gen-Probe selected specimens. Second, the study panel was small, and additional studies with larger numbers of specimens are warranted to confirm these findings and to evaluate enhanced ver-

sions of the assays as they are developed. Third, the assessment of the viral load distribution of MP-yield units and the proportions of these units that would be detectable by different NAT assays is dependent on the accuracy of the viral load assay employed, as well as the comparability of results from this assay with the 50 percent LoD of the MP-NAT assays determined based on the results from testing serial dilutions of the WNV reference reagent. We believe, however, that the 11 percent differential detection capacity of the two screening MP-NAT assays is a robust finding, because any consequence of imprecision in quantitation of the viral load distribution of MP-yield units would be comparable for both assays.

It is important to recognize that WNV NAT assays and screening strategies are evolving rapidly.[16] To improve sensitivity for the 2004 WNV season, Roche modified the assay evaluated in the current study by lowering the cycle-threshold used to define the assay cutoff. Roche has also developed an assay with further sensitivity enhancements that should be available before the 2005 season (J. Gallarda, personal communication). Gen-Probe has also modified its screening NAT assay to improve specificity (J. Linnen, personal communication). Replicate panels of the clinical specimens used in this study have been made available to both manufacturers to help assess the impact of these changes on detection of low-level viremic units.

REFERENCES

1. Petersen LR, Marfin AA, Gubler DJ. West Nile virus. JAMA 2003;290:524-8.
2. Centers for Disease Control and Prevention. Provisional surveillance summary of the West Nile virus epidemic—United States, January-November 2002. JAMA 2003;289:293-4.
3. Pealer LN, Marfin AA, Petersen LR, et al. Transmission of West Nile virus through blood transfusion in the United States in 2002. N Engl J Med 2003;349:1236-45.
4. Iwamoto M, Jernigan DB, Guasch A, et al. Transmission of West Nile virus from an organ donor to four transplant recipients. N Engl J Med 2003;348:2196-203.
5. Stramer SL, Glynn SA, Kleinman SH, et al. Detection of HIV-1 and HCV infections among antibody-negative blood donors by nucleic acid amplification testing. N Engl J Med 2004;351:760-8.
6. Grant PR, Busch MP. Nucleic acid amplification technology methods used in blood donor screening [review]. Transfus Med 2002;12:229-42.
7. Dodd RY. Emerging infections, transfusion safety, and epidemiology. N Engl J Med 2003;349:1205-6.
8. Centers for Disease Control and Prevention (CDC). Update. West Nile virus screening of blood donations and transfusion-associated transmission—United States, 2003. MMWR Morb Mortal Wkly Rep 2004;53:281-4.
9. Caglioti S, McAuley J, Robertson GF, et al. Yield of West Nile virus RNA screening of U.S. blood donors [11-abstract book part 1]. Blood 2003;102:57a.
10. Tobler LH, Bianco C, Glynn SA, et al. Detection of West Nile virus RNA and antibody in frozen plasma components from a voluntary market withdrawal during the 2002 peak epidemic. Transfusion 2005;45:480-6.
11. Saldanha J, Shead S, Heath A, Drebot M, West Nile Virus Collaborative Study Group. Collaborative study to evaluate a working reagent for West Nile Virus (WNV) RNA detection by NAT. Transfusion 2005;45:97-102.
12. Macedo de Oliveira A, Beecham B, Lanciotti R, et al. West Nile virus blood transfusion-related infection despite nucleic acid testing. Transfusion 2004;44:1695-9.
13. Francis B, Green M, Payne C, eds. The GLIM system: release 4 manual. Oxford: Oxford Science Publications, Clarendon Press; 1993.
14. Biswas R, Tabor E, Hsia CC, et al. Comparative sensitivity of HBV NATs and HBsAg assays for detection of acute HBV infection. Transfusion 2003;43:788-98.
15. Diamond MS, Sitati EM, Friend LD, et al. A critical role for induced IgM in the protection against West Nile virus infection. J Exp Med 2003;198:1853-62.
16. Custer BS, Tomasulo PA, Murphy EL, et al. Triggers for switching from minipool testing by nucleic acid technology to individual donation nucleic acid testing for West Nile virus: analysis of 2003 data to inform 2004 decision making. Transfusion 2004;44:1547-54.