Detection of emergent strains of West Nile virus with a blood screening assay

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BACKGROUND: West Nile virus (WNV) is a threat to transfusion safety. WNV Kunjin strain (WNV_{KUN}) is endemic across parts of Australia; however, human infection is believed to be infrequent and is often associated with relatively minor symptoms. A virulent strain, closely related to WNV_{KUN} (termed $WNV_{NSW2011}$) was recently identified as the etiologic agent of encephalitis in Australian horses. The aim of this project was to investigate whether a commercially available WNV blood screening assay can detect different strains of WNV_{KUN}, including the virulent $WNV_{NSW2011}$, in human blood donor samples.

STUDY DESIGN AND METHODS: Plasma samples were spiked with four different strains of WNV_{KUN}, as well as a prototype WNV strain, at high, medium, and low viral loads. Spiking was confirmed with real-time reverse transcription–polymerase chain reaction (RT-PCR), before testing with the Procleix WNV transcription-mediated amplification (TMA) blood screening assay (Grifols).

RESULTS: All WNV strains used were detectable by RT-PCR after being spiked into plasma. Additionally, all viral spiked samples were reactive by WNV TMA.

CONCLUSION: We experimentally demonstrate that a commercially available WNV blood screening assay can detect different strains of WNV_{KUN}. Given that WNV can be transfusion transmissible, it is essential to confirm that emergent strains are detectable by existing blood screening methods.

Arthropod-borne viruses (arboviruses) can be transmitted through blood transfusion because infection results in a period of asymptomatic viremia, in populations with a variable and occasionally high infection incidence.[1] West Nile virus (WNV) was first documented to be transfusion transmissible in 2002; since this time, other arboviruses including dengue, chikungunya, Zika, and Ross River viruses have posed a risk to blood supply safety.[1,2] To help maintain transfusion safety with respect to WNV, blood donations in North America are screened for WNV RNA by nucleic

ABBREVIATIONS: JEV = Japanese encephalitis virus; TMA = transcription-mediated amplification; WNV = West Nile virus; WNV_{KUN} = WNV Kunjin strain.

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acid amplification technology (NAT)-based blood screening assays.

WNV is a single-stranded, positive-sense, RNA virus in the Japanese encephalitis virus (JEV) serogroup of the *Flavivirus* genus (family *Flaviviridae*). The virus is maintained in an enzootic transmission cycle, with mosquitoes (primarily *Culex* spp.) as the vector and passerine birds as the reservoir host. Humans and horses are incidental dead-end hosts. Being an RNA virus, WNV exists within hosts as an assemblage of related, but nonidentical viral variants.[3] Indeed, this high level of genetic diversity is related to increased fitness in mosquitoes, where the increased diversity is maintained.[4] To date, viral genetic changes in WNV have not been associated with a modified disease incidence in humans.[5]

A virulent strain of WNV (termed $WNV_{NSW2011}$), with enhanced transmission by mosquitoes, emerged in horses in the state of New South Wales (NSW) in eastern Australia in 2011.[6,7] This novel virus was shown to be closely related to the WNV variant, WNV Kunjin strain (WNV_{KUN}), which is enzootic in northern Australia. WNV_{KUN}, as well as $WNV_{NSW2011}$, cluster together with strains circulating in the United States, Africa, southern and eastern Europe, and the Middle East, however, to form their own, distinct clade, Clade 1b.[8] This virulent strain possesses two known markers of WNV virulence, a phenylalanine residue at Amino Acid 653 in NS5 as well as glycosylation of the E protein at Residue 154, which are not seen in WNV_{KUN}.[6] There are only 42 amino acids different between $WNV_{NSW2011}$ and WNV_{KUN}, resulting in 18 nonconserved changes, while there are 89 amino acid disparities between this strain and WNV_{NY99}, with 38 nonconserved changes.[6] Although WNV_{KUN} may cause encephalitic disease in humans, this is reported infrequently, and the majority of infections are asymptomatic.[9] WNV_{KUN} is frequently detected in mosquito and sentinel bird surveillance in the Northern Territory and Western Australia.[9] The clinical course of infection in humans is poorly understood; however, it appears to be milder than for WNV in the United States.

Exposure to WNV_{KUN} is likely to offer protection against WNV infection; however, given the low number of notifications each year[10] and low seroprevalence in NSW blood donor populations,[11] much of the Australian population is likely to be potentially susceptible to WNV were it to become introduced and/or established in Australia. Endemic Australian mosquitos are capable of transmitting WNV, and a number of Australian bird species have the potential to be infected with WNV.[12] Moreover, the potential WNV transmission cycle would likely not be impacted by endemic flaviviruses, given these viruses are not common in urban areas.[12]

It is known that WNV can be transmitted by transfusion,[1] WNV_{KUN} is circulating in Australia,[10,11] Australian blood donors have been exposed to WNV_{KUN},[11] there is high genetic diversity in the WNV genome,[3] and virulent forms of the virus are known to emerge.[6] Given this, knowledge of whether commercially available WNV NAT blood screening assays can detect different strains of WNV_{KUN}, including the more virulent $WNV_{NSW2011}$, is needed. Based on sequence similarity between WNV and WNV_{KUN} it is likely that commercially available blood screening assays, such as the WNV transcription-mediated amplification (TMA) assay, will detect WNV_{KUN}; however, this is yet to be demonstrated experimentally for a range of viral strains. Therefore, we aimed to determine if the WNV TMA blood screening assay can detect different strains of WNV_{KUN} spiked into plasma. This project is not only relevant for transfusion safety in Australia, but also elsewhere in the world where new and virulent strains of WNV emerge.

MATERIALS AND METHODS

Viral strains

Four different strains of WNV_{KUN}, as well as a prototype WNV strain (used as a control to ensure the spiking method produces a sample that is capable of being detected by the WNV TMA assay), were used. These include WNV_{MRM16}, $WNV_{NSW2011}$, WNV_{K68967}, WNV_{Gu0631}, and WNV_{NY99} (Table 1).

Cell culture

Aedes albopictus salivary gland cells (C6/36; ATCC# CRL-1660) were cultured in RPMI 1640 (Thermofisher, Scoresby, Victoria, Australia) supplemented with 10% fetal bovine serum (FBS; Sigma, Castle Hill, New South Wales, Australia), penicillin (50 U/mL), streptomycin (50 μg/mL), and L-glutamine (2 mmol/L; Gibco, Mulgrave, Victoria, Australia) at 28°C and 5% CO_2. African green monkey kidney (Vero) cells (ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermofisher) supplemented with 2% FBS, penicillin (50 U/mL), streptomycin (50 μg/mL), and L-glutamine (2 mmol/L; Gibco) at 37°C and 5% CO_2.

Viral production and determination of stock concentration

Viral strains (WNV_{MRM16}, $WNV_{NSW2011}$, WNV_{K68967}, WNV_{Gu0631}, WNV_{NY99}) were grown to 10^6 to 10^7 PFUs/mL by propagation in subconfluent mosquito C6/36 cells. At 5 days postinfection the supernatant was collected, centrifuged, aliquoted, and stored at −80°C until required. The concentration of virus in the aliquots was determined by plaque assay as previously described.[6]

Fresh-frozen plasma

This study had approval from the Australian Red Cross Blood Service Human Research Ethics Committee. All donations were from eligible, voluntary donors. Fresh-frozen

TABLE 1. Characteristics of the WNV strains used in the study

Strain	Source	Place of isolation	Year
WNV_{MRM16} (reference strain)	Mosquitoes	Mitchell River Mission, Queensland (Northeastern Australia)	1960
WNV_{K68967}	Mosquitoes	Western Australia	2009
WNV_{GU0631}	Mosquitoes	Eastern Australia	2000
WNV_{NY99} 4132 (reference strain)	Humans/ mosquitoes	New York	1999
$WNV_{NSW2011}$	Horse brain	New South Wales (Eastern Australia)	2011

plasma (FFP) was sourced from eligible, voluntary blood donors. Briefly, plasma was prepared by plasmapheresis using an apheresis system (Haemonetics PCS 2, Haemonetics, Braintree, MA).

Detection of neutralizing antibodies in FFP

FFP samples were heat-inactivated and titrated in serial twofold dilutions from 1:20 to 1:2560 in DMEM without FBS in wells of a 96-well microtiter plate as previously described.[11] Titers of not more than 20 were classified as being negative for neutralizing antibodies against either virus, whereas titers of at least 40 were classified as being positive.

Viral spiking

Only FFP that was negative for WNV-neutralizing antibodies were included in this study. Viral strains (see Table 1) were spiked at three different concentrations to reflect differing levels of viremia: high (final concentration, approx. 10^5 PFU/mL), medium (final concentration, approx. 10^3 PFU/mL), and low (final concentration, approx. 10^1 PFU/mL). These concentrations were chosen as they reflect levels seen in blood donor samples.[13] Viruses were first diluted in sterile phosphate-buffered saline (PBS) to a concentration of 1×10^6 and then diluted in plasma to the desired concentration. The control plasma contained PBS only. Separate aliquots were prepared in cryogenic vials (Corning Ltd., Clayton, Victoria, Australia) for each viral strain at each concentration. Samples were stored at −80°C.

Real-time reverse transcription–polymerase chain reaction

Published viral strain-specific real-time reverse transcription–polymerase chain reaction (RT-PCR) assays were used for the detection of viral nucleic acid.[6] One spiked aliquot was tested for each viral strain. First, viral RNA was extracted using a RNA virus kit (NucleoSpin, Macherey-Nagel GmbH & Co., Düren, Germany) and then converted to cDNA using a cDNA supermix kit (qScript, Quanta Biosciences, Gaithersburg, MD). The quantitative PCR method was performed using SYBR Green PCR master mix (Life Technologies, Mulgrave, Victoria, Australia) on the real-time PCR system (ViiA 7, Life Technologies) using the qWN_F (TTGAGTGTGATGACCATGGGAG) and qWN_R (TAGCTGGTTGTCTGTCTGCG) primers that recognize a conserved sequence in the NS4A gene.

TABLE 2. Detection of WNV strains by WNV quantitative RT-PCR and WNV TMA

			WNV TMA		
			Analyte S/CO		
Strain	Dilution	WNV quantitative RT-PCR	Mean	SD	Status
MRM16	High	D	32.64	0.219317	R
	Med		32.53	1.434724	R
	Low		32.50	2.360953	R
K68967	High	D	34.33	0.545558	R
	Med		34.04	0.126623	R
	Low		33.65	0.459601	R
GU0631	High	D	33.66	0.603352	R
	Med		33.04	0.10116	R
	Low		33.26	1.23557	R
NY99	High	D	34.16	0.606987	R
	Med		33.56	1.691873	R
	Low		31.97	1.272216	R
NSW2011	High	D	33.40	1.232234	R
	Med		34.08	0.595343	R
	Low		32.81	0.872296	R
Plasma		ND	0.00	0	NR

D = detected; ND = not detected; NR = nonreactive; R = reactive; S/CO = sample-to-cutoff ratio; SD = standard deviation.

TMA

The WNV TMA assay was used to test spiked samples.[14] This assay is a routinely used blood screening assay (Procleix WNV assay, Grifols, Emeryville, CA) that is commercially available in some countries. Samples spiked with infectious virus (n = 45) were tested at Hologic (San Diego, CA) as per the manufacturer's directions.

RESULTS

No neutralizing antibodies were detected in the FFP units used in this study (data not shown). All strains studied were detectable by RT-PCR (Table 2). All viral spiked samples were also reactive by WNV TMA (Table 2). The analyte S/CO from the TMA testing did not correlate with increased viral load. All control plasma samples were not detected by either method (Table 2).

DISCUSSION

Emerging infectious diseases pose an ongoing risk to transfusion safety. After the donor history questionnaire coupled with the exclusion of donors considered "higher

risk," the next tool in the blood safety arsenal is laboratory-based donation screening for infectious diseases. It is imperative that these assays have optimal sensitivity including the capability to detect emerging strains of their target agents. In this study we experimentally show that different strains of WNV are capable of being detected by the Procleix WNV assay. This assay has a high sensitivity, specificity, and reproducibility, with the 95% limit of detection for strains in Lineage 1 being 9.8 copies/mL on an automated system.[14] This assay has limited reactivity against other members of the JEV serogroup; however, some cross-reactivity with Usutu virus (also in the JEV complex) has been observed and the assay can detect one strain of $WNV_{KUN.}$[14,15] Here we extend this observation to four strains of WNV_{KUN}, including one that has been shown to have enhanced virulence and enhanced transmission by mosquitoes.[6,7] Given the viral titers used, this study may not capture differences in detection in the low concentration range. Nonetheless, this study has implications internationally where different strains of WNV may emerge.

New strains of an infectious disease may differ only in a single nucleotide, which, if in the area targeted by NAT, might reduce the sensitivity of the assay or, in the worst-case scenario, prevent amplification leading to a false-negative result, which may have disastrous consequences. This has been highlighted by the transfusion transmission of human immunodeficiency virus Type 1 (HIV-1) after HIV-1 NAT blood screening in Germany.[16] The detection failure of the NAT system in this case was proposed to be due to mismatches in the primer and probe binding regions, despite the test using primers that target a conserved region of the HIV-1 genome.[16] This limitation resulted in the recent recommendation that HIV-NAT assays be based on a "dual-target" design.[17] Like HIV, WNV exhibits genetic diversity,[3] and therefore constant due diligence is required by those involved in supplying commercial blood screening assays to ensure the maintenance of blood safety.

Another option for managing these emergent strains is pathogen inactivation, which involves the nonspecific targeting of nucleic acid, resulting in the prevention of pathogen replication. Different methods are available for manufactured plasma versus labile blood components; technologies designed for use with the latter utilize ultraviolet light with or without the addition of a photosensitizing agent and are effective against a range of arboviruses. Indeed, 3.5- to 4-log reduction in WNV infectivity was observed with the THERAFLEX UV-Platelets system (MacoPharma, Tourcoing, France),[18] 5.19 log was seen for the Mirasol system (TerumoBCT, Lakewood, CO, USA),[19] and more than 5.5 log for the INTERCEPT System for Platelets (Cerus Corporation BV, Amersfoort, the Netherlands).[20] These systems may have a place in maintaining a safe blood supply in the future.

With Australia considered endemic for WNV, it was essential that we understand the effectiveness of blood safety mitigation tools to be prepared in the advent that they may need to be implemented. Here we show that at least one commercially available blood screening method is able to detect strains of WNV currently circulating in Australia, including those with virulence factors similar to WNV_{NY99}, the strain that rapidly spread across North America resulting in considerable mortality and morbidity. Genetic variants of infectious diseases with changed virulence factors have the potential to emerge in many different regions. It is necessary therefore for the transfusion community to maintain a close watch for emergent agents that may escape detection through conventional blood screening methods.

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CONFLICT OF INTEREST

EO, JML, RC, and JAH are employed by the companies that developed (Hologic, Inc., San Diego, CA) and codeveloped (Grifols Diagnostic Solutions Inc., Emeryville, CA) the West Nile virus blood screening assay used in this study. The other authors have disclosed no conflicts of interest.

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