Journal of Medical Virology 80:557–563 (2008)

West Nile Virus and Blood Product Safety in Germany

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West Nile Virus (WNV) is a mosquito-transmitted flavivirus, widely distributed throughout Africa, Asia and the Middle East. WNV may cause epidemics of human meningoencephalitis. The unexpected emergence of WNV (New York, 1999) and its rapid spread throughout North America during the following years caused a number of blood transfusion- and organ transplant-associated transmissions of WNV. In order to estimate the potential WNV threat for Central Europe, we analyzed the anti-WNV prevalence and WNV-RNA incidence among 14,437 and 9,976 blood donors from Germany. There was a high rate of initially anti-WNV reactives (5.9%), but only a few cases (0.03%) were confirmed as anti-WNV positive by neutralization assay. No WNV-RNA positive blood donor was identified in this study. Whereas WNV-RNA was frequently detected in manufacturing plasma pools from the US, none was detected in pools of European or Asian origin. Virus inactivation steps integrated into the manufacturing process of plasma derivatives were shown to be sufficient to assure the WNV safety of plasma derivatives. A well-characterized WNV reference material was prepared, showing 340 WNV-RNA copies per infectious dose. ***J. Med. Virol. 80:557–563, 2008.***

KEY WORDS: West Nile Virus; WNV neutralization; anti-WNV IgG ELISAs; blood safety; plasma derivatives; virus inactivation

INTRODUCTION

West Nile Virus (WNV) was identified in 1937 in Uganda and is widely distributed throughout Africa, Asia, the Middle East and parts of Europe [Solomon et al., 2003]. This enveloped virus is classified under the virus family *Flaviviridae* in the genus *Flavivirus*, which includes more than 100 members that all are characterised by a complex replication cycle involving both insects and at least one additional animal species. This is why flaviviruses had been included in the previous taxonomic group of arboviruses (arthropod-borne viruses). The natural WNV replication cycle involves *culicine* mosquitoes and different bird species. Humans, horses and other mammalian species are so-called "dead-end" hosts characterised by WNV infections with potential clinical symptoms, but transient and low virus levels that are insufficient to establish a mosquito-mammalian WNV replication cycle. The vast majority of WNV infections in humans undergo an asymptomatic course. Approximately 20% of infected humans develop West Nile fever, a febrile illness of sudden onset, often associated with a long recovery period. Only a few cases (<0.2%) develop a neuroinvasive disease resulting in more serious symptoms, including meningitis or encephalitis, sometimes with fatal outcome [Petersen and Marfin, 2002]. The rate of serious outcome of WNV infection is much higher in immune-compromised patients, a status more frequently found in elderly persons or in recipients of blood transfusions.

After a flavivirus infection, the diagnostic differentiation of specific antibodies is complicated by a high rate of cross-reactivity between different members of the genus Flavivirus, for example, Dengue virus (DenV), tick-borne encephalitis virus (TBEV), and WNV [Allwinn et al., 2002; Koraka et al., 2002]. Cross-reactive antibodies are mainly directed against an envelope protein of flaviviruses, the E-protein.

Grant sponsor: German Ministry of Health (BMGS; Prevalence and incidence of West Nile Virus infections in Germany); Grant number: 115-1720-1/31.

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Accepted 12 November 2007
DOI 10.1002/jmv.21110
Published online in Wiley InterScience (www.interscience.wiley.com)

The unexpected emergence of WNV in New York in 1999 was associated with an encephalitis outbreak. The viral strain responsible for this epidemic was a lineage 1 virus already known from previous epidemics in other parts of the world, such as in Israel [Lanciotti et al., 1999]. In the following years, WNV spread across North America from East to West, causing the largest arbovirus epidemic in recorded history with more than 23,000 human infection cases until December 2006, including 893 deaths [Centers for Disease Control and Prevention, 2006]. During this WNV epidemic, new transmission routes, including breast-feeding [MMWR, 2002], organ transplantation [Iwamoto et al., 2003] and transfusion of blood components [Biggerstaff and Petersen, 2002; Hollinger and Kleinman, 2003], were recorded. To assure the safety of the US blood supply, the screening of all blood donations by nucleic acid amplification techniques (NATs) was recommended by the FDA beginning in July 2003. This measure resulted in the detection of WNV-RNA in more than 1,000 blood donations until the middle of 2005, which would otherwise have been used for transfusion of non-inactivated cellular blood components (red cells, platelets) or therapeutic plasma [Busch et al., 2005a; Stramer et al., 2005].

Despite some reports about sporadic WNV infections in humans and horses across Europe, for example, in France, Italy, and Romania [Zeller et al., 2004], no WNV epidemiology data were available for Germany. With the US epidemic still ongoing, the question arose as to whether a similar scenario could also affect Europe. The German Ministry of Health initiated an investigation into the prevalence and incidence of WNV infections in Germany. This publication relates to the assessment of WNV safety of blood and plasma products used in Germany.

MATERIALS AND METHODS

Human Serum and Plasma Samples

For the evaluation of WNV prevalence, 14,437 plasma or serum samples were collected during Summer 2004 from healthy blood donors from central Germany (Hesse) and 928 samples from Austrian blood donors (Carinthia and Vienna).

For the determination of WNV incidence, plasma samples from 9,978 healthy blood donors from central Germany (Hesse) were collected during the 2005 summer mosquito season, combined in pools of 8 using a Tecan robot, and tested for the presence of WNV-RNA.

Serum samples from German intravenous drug users (IVDUs) who tested positive for other blood-borne viruses (HIV and/or HCV) were collected during May 2002 and January 2004 at University Hospital Frankfurt/Main.

Anti-WNV IgG-positive plasma samples were obtained from confirmed clinical cases in the US. These plasma samples were obtained from BBI (West Bridgewater, MA) or kindly provided by H. Hofmann (Genzyme Virotech GmbH, Mainz, Germany).

One panel of WNV RNA-positive plasma samples from clinical cases in the US was provided by L. Tobler from the Blood Systems Research Institute, San Francisco.

Panels of anti-DenV and anti-TBEV positive specimens were designed with materials obtained from the respective clinical cases in Germany, which were kindly provided by Universität München, Abt. für Infektions und Tropenmedizin (T. Löscher), Universität Freiburg, Institut für Medizinische Mikrobiologie & Hygiene (D. Neumann-Haefelin), Universität Heidelberg, Hygiene Institut (P. Schnitzler) and Universität Frankfurt, Zentrum für Hygiene (R. Allwinn).

Aliquots of plasma pools collected for the fractionation of plasma derivatives were obtained from different plasma manufacturers. The respective plasma units had been collected in the US, in Europe or in Asia during the years 2004 and 2005.

ELISA Tests

The following ELISAs were performed strictly following the instructions given in the package insert: "Flavivirus IgG indirect ELISA" (PANBIO Brisbane, Australia), "Anti-West-Nile-Virus-ELISA IgG" (prototype version, Euroimmun, Lübeck, Germany), "West Nile Virus IgG DxSelect™" (Focus Diagnostics, Cypress, CA), and "Enzygnost anti-TBE Virus IgG and IgM" (DADE Behring, Marburg, Germany).

WNV NATs

Human plasma or serum and cell culture-derived samples were tested for WNV RNA using the "Procleix WNV assay" (Chiron Corporation, Blood Testing Division, Emeryville, CA). This test system is a qualitative nucleic acid amplification technique (NAT) based on TMA (transcription mediated amplification) technology. This NAT was performed after passing a training seminar organized by the manufacturer and strictly following the manufacturer's instructions.

WNV-RNA was quantified with the Artus Real Art™ WNV LC RT RCR Kit (QIAGEN, Hilden, Germany) using the QIAamp Viral RNA Mini Kit (QIAGEN) for viral RNA extraction. Quantification standards were provided with the test kit and run in parallel.

Viruses and Cells

Bovine viral diarrhea virus (BVDV), strain Osloss, was obtained from G. Pauli, Robert Koch Institut, Berlin, Germany, and was propagated and titered in MDBK cells (ATTC CCL-22). SFV was provided by J. Thiel (Institut für Virologie, Universität Giessen, Germany), and was propagated and titered in Vero cells (ATTC CCL-81). WNV (1999 New York isolate) was received from T.R. Kreil (Baxter, Vienna, Austria), and was propagated and titered in Vero cells in a biosafety level 3 laboratory.

Virus Titration

Virus infectivity was quantified by estimation of the tissue culture infectious dose ($TCID_{50}$) using standard cell culture conditions. Briefly, Vero and MDBK cells were grown to confluence in 96-well microtiter plates (MTPs). Threefold serial dilutions of the samples in

DMEM medium were prepared, and eight replicates per dilution were assayed by inoculation of a 50 µl sample per well. The cytopathic effects of WNV, Semliki Forest Virus (SFV), and BVDV were checked on days 3–6 post-infection. The $TCID_{50}$ was calculated according to the Maximum Likelihood statistical tool. If no virus was detected, the limit of detection was calculated according to the Poisson distribution.

WNV Neutralization Assay

For the WNV neutralization assay, 50 µl of a 1:3 serial dilution (1:10 to 1:270) of inactivated (56°C, 30 min) serum was mixed in eight replicates in MTP wells with 50 µl of 20 $TCID_{50}$ units of virus. After incubation for 60 min at 37°C, 50 µl of each well was transferred to another well containing 60% confluent (6,000–7,000) Vero cells. MTPs were incubated for 5–6 days at 37°C, and wells containing evidence of viral cytopathic activity were scored. The log ND_{50} was calculated according to the Maximum Likelihood function [Kundi, 1999]. For a high throughput version of the neutralization assay, only one serum dilution (1:30) was tested under the same conditions described above.

Virus Inactivation Studies

Pasteurization was performed at laboratory scale to assess this virus inactivation step. Stabilizer (1.1 g/ml saccharose, 0.3 g/ml glycine, 0.0162 g/ml $CaCl_2$ dihydrate) was added to 50 ml of a commercial 5% human normal immunoglobulin preparation. The solution was spiked with virus and heated to 60°C. Samples were taken after time intervals as indicated (0–10 hr) and immediately cooled. Cooled samples were subsequently titered. Before application on indicator cells, samples were diluted 1:100 in order to avoid cytotoxic and interfering effects of the test material. Absence of cytotoxic effects was verified by microscopic examination of control cells inoculated with non-spiked 1:100 diluted test material. Absence of interfering effects was verified by positive detection of virus from diluted (1:100) test material that had been spiked with a known amount of virus (100 $TCID_{50}$ per well).

RESULTS

Sensitivity and Specificity of Anti-WNV IgG ELISAs

To assess the relative diagnostic sensitivity and specificity of three anti-WNV IgG ELISAs, we used sera from well-characterized flavivirus-infected patients: 26 WNV-infected, 39 TBEV-infected, and 13 DenV-infected individuals.

Two assays (PANBIO, Focus) recognized all of the 26 anti-WNV-positive sera originating from U.S. patients. The third ELISA, the Euroimmun prototype ELISA version, missed three specimens, resulting in a relative sensitivity of 88% in this study (Table I).

The cross-reactivity rates with related flaviviruses were determined for the anti-WNV ELISAs using specimens from DenV or TBEV-infected patients. The highest cross-reactivity rate (lowest specificity) with these specimens was obtained for the PANBIO assay (92.3% and 79.5%, respectively) followed by the Focus ELISA (92.3% and 56.4%, respectively) and the Euroimmun prototype ELISA, which displayed cross-reactivity rates of 35.7% and 17.9%, respectively.

Based on this analysis of the relative sensitivity and specificity of the different WNV ELISA assays, the following test algorithm for determination of the WNV prevalence was chosen: after screening donor blood with the sensitive Focus ELISA test, a further analysis of all reactive specimens was performed with the more specific Euroimmun prototype WNV ELISA. Those samples that were reactive (positive or borderline) in the second anti-WNV ELISA were then tested in a WNV neutralization assay as a confirmatory test. Additionally, we characterised all samples reactive in the Focus WNV ELISA with an anti-TBEV IgG ELISA. However, this assay displayed 42% cross-reactivity when anti-WNV-positive specimens were tested.

WNV Prevalence

More than 14,000 healthy blood donors from Hesse/Germany were screened for anti-WNV specific IgG antibodies. 5.9% (852/14,437) of the tested donors were reactive in the Focus anti-WNV ELISA test. To estimate the potential impact of TBEV vaccination on the test results, more than 900 blood donors from Austria (where TBEV vaccination is a general public health measure) were also screened for anti-WNV. Seventy-two percent of these blood donors (669/928) were reactive in the anti-WNV ELISA.

All anti-WNV reactive samples from German blood donors were re-tested in the second anti-WNV ELISA and in the anti-TBEV IgG assay. Thirty-four percent of these samples were reactive in the second anti-WNV and 15% were non-reactive in the anti-TBEV ELISA, whereas 9.7% were reactive in the WNV-ELISA and non-reactive in the TBEV-ELISA.

TABLE I. Sensitivity and Cross-Reactivity of Different Anti-WNV IgG ELISAs (pos = Positive; React = Reactive)

		Anti-WNV IgG ELSA		
	Sera	PANBIO	Focus	Euroimmun
Sensitivity	Anti-WNV IgG positive	100% (26/26)	100% (26/26)	88% (23/26)
Cross-reactivity	Anti-dengue IgG positive	92.3% (12/13)	92.3% (12/13)	35.7% (5/14)
	Anti-TBE IgG positive	79.5% (31/39)	56.4% (22/39)	17.9% (7/39)

Two hundred two of the pre-selected anti-WNV suspicious blood donor samples were tested in the WNV neutralization assay. Most of these plasma samples (148/202; 73%) had no or very low WNV neutralization activity (log $ND_{50} < 1$), while 50 specimens (25%) neutralized WNV infection, with log ND_{50} titers between 1 and 2. Only four plasma samples from our blood donors exhibited relatively high neutralization titers (log $ND_{50} \geq 2$) equivalent to those titers observed with the anti-WNV IgG sera from US patients. To check whether any anti-WNV positive might have been missed by our test algorithm, 388 additional anti-WNV reactive specimens were tested in a high throughput neutralization assay. No further specimens with titers of log $ND_{50} > 2$ were identified by this approach.

WNV Incidence

The WNV incidence in a population of healthy German blood donors was investigated during the mosquito season of Summer 2005. In total, 9,976 blood donors were tested for the presence of detectable WNV-RNA using the Procleix WNV NAT assay in minipools of eight. All of the 1,247 minipools tested WNV-RNA negative, with the exception of one initially reactive test result for one minipool. This result was not confirmed on retesting and on testing of the individual plasmas. Furthermore, 198 of the anti-WNV reactive blood donor samples (see above) were tested as individual specimens, with negative test results. Plasma or serum samples collected from a population of German intravenous drug users (IVDU, n = 78), representing a population with increased risk for blood-borne pathogens also tested negative for WNV-RNA (Table II).

Plasma Derivatives

Plasma pools for manufacturing of plasma derivatives with the source plasma collected in the US, East Asia, or Europe were analyzed for the presence of WNV RNA using the Chiron TMA assay. All plasma pools from East Asia (n = 51) and Europe (n = 96) tested negative, while 32 out of 174 (18%) plasma pools from the US tested WNV RNA-positive in this qualitative WNV-NAT (Table II). These pools had been collected in different regions of the US during the years 2004 and 2005. Since the viral load in most pools was too low for accurate results in the quantitative WNV-NAT, viral load was analyzed after concentration of WNV particles by ultracentrifugation and subsequent extraction of the viral nucleic acids. Viral loads were calculated as ranging from 57 to 837 copies WNV-RNA/ml plasma, with 351 copies WNV-RNA/ml as an average value for the TMA-positive pools.

TABLE II. Detection of WNV RNA in Blood Specimens and Plasma Pools Using the Procleix WNV Assay (Chiron)

	Tested	WNV-RNA positive
Blood donors (pools of n = 8)	9,976	0
IVDUs	78	0
Anti-WNV reactive blood donors	198	0
Plasma pools (Europe)	96	0
Plasma pools (USA)	174	32
Plasma pools (East-Asia)	51	0

Virus inactivation steps are included in the manufacturing process of different plasma derivatives. For the production of human-derived medicinal products, a 10 hr heating step of a liquid product intermediate at 60°C (pasteurization) is often performed to inactivate a wide range of potential virus contaminants. The inactivation kinetics of WNV upon pasteurization of a sucrose-stabilized immunoglobulin preparation was compared with the inactivation kinetics of other commonly used model enveloped viruses. BVDV is a pestivirus that frequently serves as a model virus for hepatitis C virus and other members of the Flaviviridae. The inactivation kinetics of WNV were similar to the inactivation kinetics of BVDV. The inactivation kinetics (Fig. 1) confirm that WNV is effectively inactivated by this commonly used manufacturing step.

WNV Reference Preparation

We established and characterized a WNV reference preparation that may be useful for standardization and control of WNV-NATs and WNV-neutralization assays. Supernatant from WNV-infected Vero cells was harvested and characterized for its infectivity titer ($TCID_{50}$) and WNV-RNA content. WNV-RNA concentration was determined by replicate (24 per concentration) testing of limiting dilutions (factor of 2) using the qualitative WNV-NAT followed by calculation of the 95% cut-off concentration using Probit analysis. WNV-RNA concentration was also determined using the quantitative NAT test.

Both NAT approaches revealed a WNV-RNA concentration for the stock material of 6.5×10^9 or 8.1×10^9 copies/ml. Titration in Vero cells gave an infectivity titer of log 7.33 TCID/ml, correlating to approximately 340 copies WNV-RNA per infectious dose.

DISCUSSION

New emerging pathogens may be a threat to public health, not only because of their impact on the population, but also because of their potential to contaminate the blood or plasma supply and to be transmitted to recipients of blood products. Therefore, a research project was initiated by the German Ministry of Health after the huge WNV epidemic that followed the introduction of the virus to the New World in 1999. This research project was performed in cooperation among the Robert-Koch-Institut (RKI; Berlin), the Bernhard-Nocht-Institut (BNI; Hamburg), and the Paul-Ehrlich-Institut (PEI; Langen). In this study, we investigated whether WNV is or could become a threat to public health and the blood supply in Germany. Here, we focus on the prevalence and incidence of WNV among healthy blood donors and the potential for the transmission of the pathogen via plasma derivatives.

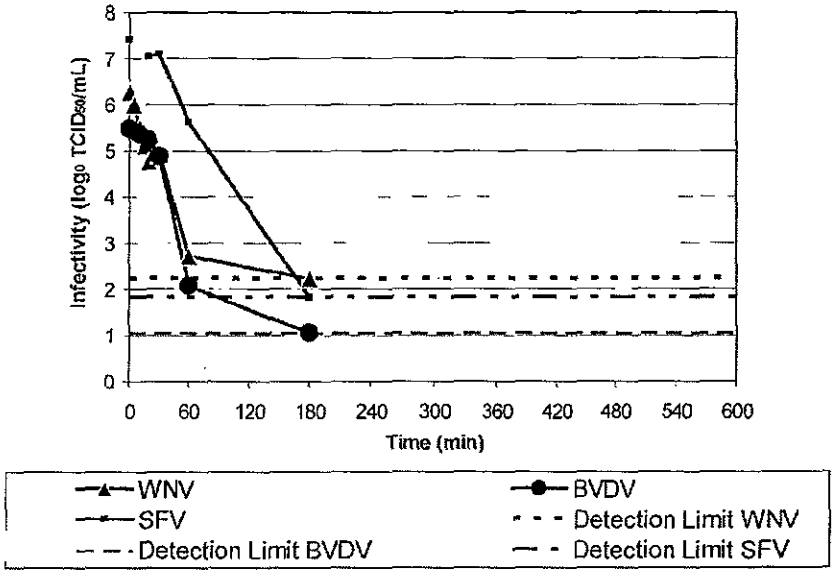

Fig. 1. Virus inactivation through heat inactivation (pasteurization). A sucrose-stabilized human immunoglobulin preparation (5%) was spiked with BVDV, SFV or WNV. After incubation at 60°C, samples were taken after time intervals as indicated (0–10 hr) and cooled immediately. Virus infectivity was quantified by calculation of the tissue culture infectious dose ($TCID_{50}$).

WNV Prevalence

First, we wanted to qualify the anti-WNV screening tests and define an appropriate test algorithm. We decided to use anti-WNV positive specimens from the US as decisive specimens for testing sensitivity for different reasons. First, the respective materials from clinically ill patients were easily available from the US. Second, WNV infections had been confirmed by the clinical course, the seasonal occurrence of the infection and the results of different diagnostic assays performed on individual follow-ups. Furthermore, TBEV, the human flavivirus most frequently found in Europe, is not yet present on the American continent. Therefore, cross-reacting TBEV antibodies should not be an issue for these samples.

The sensitivity was 100% for two anti-WNV assays and 88% for the third assay using such an anti-WNV positive serum panel. However, further panels composed of either anti-Dengue or anti-TBEV positive specimens showed high rates of cross-reactivity with the more sensitive anti-WNV assays and low cross-reactivity with the third assay. We decided to include an anti-TBEV assay as a further diagnostic tool because all of the anti-WNV positive specimens from the US that were missed by the third assay tested as anti-TBE negative, and only a few members of the entire US anti-WNV panel cross-reacted in the anti-TBEV assay. We aimed to preferentially choose anti-WNV reactive/anti-TBEV negative specimens for entering the "gold standard" WNV neutralization test. However, even with this standard, we experienced a high rate of cross-reactivity with anti-DenV-positive specimens. Fortunately, anti-TBEV-positive specimens, which are much more frequent in our region, showed cross-neutralization only at a low level. In conclusion, serological flavivirus diagnostics show high rates of cross-reactivity, and correct interpretation of test results requires extreme caution.

To determine the extent of past WNV infections, samples from more than 14,000 German blood donors were screened with the Focus anti-WNV IgG ELISA. Nearly 6% of the German blood donors were anti-WNV reactive in this assay. Many of the reactive test results were probably caused by cross-reactive antibodies originating from a related flavivirus infection or from vaccination. Though some parts of Southern Germany are TBE risk areas (as the clinical cases show), no reliable TBE incidence or prevalence data are available for Germany.

The TBE vaccination coverage of the Austrian population is in the range of 80%. Sera from Austrian blood donors had a similar reactivity rate in our anti-WNV screening assay. This illustrates the high rate of anti-TBEV cross-reactivity and confirms the similar results obtained in our test qualification study (see above).

Therefore, the anti-WNV reactive rate for blood donors in Hesse (5.9%) may primarily reflect the TBEV vaccination and/or infection level in donors from our region.

To narrow down the number of samples that had to be tested in a WNV neutralization assay, all blood donations that were reactive in the Focus ELISA were re-tested in the more specific prototype anti-WNV IgG ELISA test and in an anti-TBEV IgG assay.

Blood samples that were non-reactive in the anti-TBEV ELISA and reactive in the pre-market anti-WNV