

Photochemical inactivation of chikungunya virus in plasma and platelets using the Mirasol pathogen reduction technology system

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BACKGROUND: Chikungunya virus (CHIKV) is a reemerging mosquito-borne virus that has been responsible for a number of large-scale epidemics as well as imported cases covering a wide geographical range. As a blood-borne virus capable of mounting a high-titer viremia in infected humans, CHIKV was included on a list of risk agents for transfusion and organ transplant by the AABB Transfusion-Transmitted Diseases Committee. Therefore, we evaluated the ability of the Mirasol pathogen reduction technology (PRT) system (CaridianBCT Biotechnologies) to inactivate live virus in contaminated plasma and platelet (PLT) samples.

STUDY DESIGN AND METHODS: Plasma, PLTs, and phosphate-buffered saline controls were spiked with CHIKV and treated with riboflavin and varying doses of ultraviolet (UV) light using the Mirasol PRT system. Samples were tested before and after treatment for cytotoxicity, interference, and virus titer by titration and quantitative real-time reverse transcription-polymerase chain reaction.

RESULTS: A significant reduction in CHIKV titer of greater than 99% was recorded after treatment of plasma or PLTs with the Mirasol PRT system, and the titer reduction was directly proportional to the UV dose delivered to the samples. No cytotoxicity or interference was observed in any sample at any treatment dose.

CONCLUSION: These data indicate that the Mirasol PRT system efficiently inactivated live CHIKV in plasma and PLTs and could therefore potentially be used to prevent CHIKV transmission through the blood supply.

Many emerging infections are caused by blood-borne parasites, bacteria, or viruses and could therefore be transmitted through blood donation. The safety of the blood supply is currently reliant on specific antibody- and nucleic acid-based pathogen detection tests—with exclusion criteria for potentially infectious donors. However, specific tests are not currently available or approved to screen for all agents that have the potential for transfusion transmission and rapid screening and/or test deployment may not be possible in the early stages of an emerging epidemic of known or unknown etiology.^{1,2} Furthermore, the development and implementation of specific tests for many agents with sometimes sporadic or low-level transmission, or those circulating in resource-poor areas, such as chikungunya virus (CHIKV), has and will likely continue to be cost-prohibitive.³ Pathogen reduction technology (PRT) can circumvent these limitations through its potential applicability to a broad range of known or as yet unidentified threats.

ABBREVIATIONS: CHIKV = chikungunya virus; PAS = platelet additive solution; PRT = pathogen reduction technology; TCID₅₀ = tissue culture infectious dose 50%; UTMB = University of Texas Medical Branch.

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The Mirasol PRT system (CaridianBCT Biotechnologies, Lakewood, CO) uses ultraviolet (UV) light-activated riboflavin (vitamin B2) to alter functional groups of a pathogen's nucleic acids and prevent pathogen replication.⁴ This approach effectively inactivates multiple parasitic, protozoan, bacterial, and viral pathogens including *Leishmania donovani*, *Trypanosoma cruzi*, *Orientia tsutsugamushi*, *Babesia microti*, *Staphylococcus epidermidis*, *Escherichia coli*, human immunodeficiency virus (HIV), and West Nile virus in plasma and/or platelets (PLTs).^{2,4-7} Additionally, both in vitro and in vivo cell quality studies have demonstrated that Mirasol-treated PLTs retain acceptable performance after treatment.⁸⁻¹⁰

CHIKV is a single-stranded, enveloped RNA virus in the family *Togaviridae*. CHIKV is normally transmitted to humans via the bite of an infected *Aedes* species mosquito, but could be transmitted through blood transfusion. During a recent chikungunya outbreak, the risk of viremic blood donation was estimated to be as high as 1500 per 100,000 donations.¹¹ Since 2004, CHIKV has been responsible for large-scale epidemics in Africa, on several islands in the Indian Ocean, in India, and in other parts of Asia, along with a smaller outbreak in Italy. Imported cases have also been identified throughout Asia, Europe, and North America.¹² In the outbreak on the small island of La Réunion, roughly 40% of the population of 785,000 people were infected from 2005 to 2006.¹³ Of those that were infected, a mortality rate of 1 of 1000 was observed.¹⁴ The virus causes a high-titered viremia with fever and arthralgic disease. The peak viremia of CHIKV has been measured as high as 10^8 viral RNA copies/mL.¹³ Several risk factors for severe disease development, such as advanced age and cardiac disorders, are often present in recipients of blood transfusions.³ Its geographic expansion, among other factors, led to the inclusion of CHIKV in a list of agents that pose a safety risk to transfusion or transplant recipients by the AABB Transfusion-Transmitted Diseases Committee.¹ Petersen and colleagues³ concluded that even small chikungunya outbreaks will substantially impact transfusion medicine in the future. Therefore, we conducted this study to evaluate the efficacy of the Mirasol PRT system to reduce CHIKV titers in plasma and PLTs.

MATERIALS AND METHODS

PLTs, plasma, and media

Three different test conditions were studied using the Mirasol PRT system against CHIKV; they include 100% plasma; PLTs diluted in SSP+ (MacoPharma, Langen, Germany), an electrolyte solution containing phosphate, magnesium, and potassium buffer, which is used as an additive solution to replace plasma for PLT storage to a 35% plasma carryover; and phosphate-buffered saline (PBS) without calcium and magnesium (1× PBS, Cellgro, Manassas, VA). All plasma and PLT products were obtained from

an accredited blood banking facility. Plasma products were recovered plasma isolated from whole blood donations, shipped frozen, and stored at University of Texas Medical Branch (UTMB; Galveston, TX) until they were ready to be used. The PLT products were collected at Bonfils Blood Center (Denver, CO) using an apheresis blood collection device (Trima, CaridianBCT). The target collection was 3000×10^3 cells/ μ L in 105 mL of plasma. Postcollection products were immediately sent to CaridianBCT Biotechnologies for basic cell quality and cell count analysis, diluted in SSP+, and then shipped to UTMB overnight.

Virus

CHIKV strain LR2006 OPY1 was isolated from a febrile French patient returning from the island of La Réunion in the Indian Ocean.¹⁵ This isolate was obtained from the World Reference Center for Emerging Viruses and Arboviruses at UTMB. The strain was passed five times on Vero cell culture and once in suckling mice. Stock virus used in the Mirasol PRT treatments was produced after one additional passage in baby hamster kidney cells, and virus was stored at -80°C until needed. Titer of the stock virus was 7.7 log tissue culture infectious dose 50%/mL (TCID₅₀). Virus produced from a cDNA infectious clone of LR2006 OPY1 was utilized for the genomic equivalents studies.¹⁵

Treatment with Mirasol PRT

Study protocols were similar for the treatments of plasma products, PLTs stored in PLT additive solution (PAS), and PBS. All manipulations were performed aseptically and all treatments were performed in the standard 1-L Mirasol Illumination bag. Treatment volumes for units filled with plasma or PBS were 247 mL, whereas the PLTs stored in PAS had a treatment volume of 295 mL. The mean cell count for the PLT products treated in PAS was $998 \times 10^9 \pm 44 \times 10^9$ PLTs/L. Each unit contained 35 mL of 500 μ mol/L riboflavin and was inoculated with 4% to 5% (vol/vol) CHIKV stock or virus free media. Samples were taken immediately after inoculation to determine either the initial product viral load or product cytotoxicity. The illumination bags were weighed and the required product information was entered into the Mirasol Illuminator. Plasma and PLT units were dosed kinetically at 50, 100, and 150% of the set Mirasol PRT treatment dose for plasma and PLTs stored in PAS respectively, with the 100% energy dose being equivalent to the clinical dose. Plasma-based products treated with the Mirasol PRT system receive an energy dose of 6.24 J/mL, whereas PLT products treated in the presence of PAS receive a proportional dose based on the plasma percentage that was present. Treatment was stopped at each increment, and samples were removed for determination of viral load. The viral reduction in PBS was performed identically to the plasma

procedure except that the doses that were used were 10, 20, and 30% of the final Mirasol dose for plasma. Determination of final product cytotoxicity and interference was determined using virus-free media as an inoculum in either plasma or PLTs in PAS. Interference and cytotoxicity samples were collected before treatment and after treatment at 150% normal dose.

Virus titrations

Viral titers in plasma, PLTs, or media were quantified as TCID₅₀ endpoint titers (log TCID₅₀/mL) calculated by the method of Spearman-Kärber as described previously.¹⁶ Each sample was titrated on an individual 96-well flat-bottom tissue culture plate. The sample was pipetted into the eight wells in the first column and serially diluted in L-15 in a 10-fold series to the end of the plate at Row 12. Vero cells were seeded into all 96 wells on the plate at a density that produced a monolayer in each well after 7 days of incubation. The 96-well titration plates were incubated at 37°C. The plates were scored for cytopathic effect in each well and fixed using plate stain (0.1% naphthol blue black, 10% acetic acid, and 25% isopropanol in water).

Cytotoxicity

Cytotoxicity was performed by mock spiking units of PLTs stored in PAS or plasma with representative cell culture media and treating the product with the Mirasol PRT system. Both a pretreatment sample and a posttreatment sample (150% normal dose) were plated onto Vero cells and cultured for 7 days at 37°C. After 7 days samples were scored visually for the presence of cell cytotoxicity. Dilutions that were positive for cell death were considered to be cytotoxic and were excluded from all viral reduction calculations. This assay ensures that the cytopathic effect observed on plates containing virus is related to the actual presence of the virus and not from cell death induced by the test materials.

Interference

To determine if plasma or PLTs stored in PAS can interfere with the readout of the CHIKV assay, interference samples were collected from units that were mock spiked with virus-free cell culture medium and then treated with the Mirasol PRT system at 150% of the normal dose. Posttreatment samples were serially diluted and dosed with a concentration of CHIKV (1:5000 dilution from stock) designed to yield approximately 80% infection of tissue culture wells when inoculated into pure media. Four dilutions (0, 10⁻¹, 10⁻², and 10⁻³) of the plasma or PLTs stored in PAS posttreatment test samples were made with a total volume of 8 mL. Each posttreatment test dilution was inoculated with 30 µL of the 1:5000 virus dilution. Fifty-six replicates of each of the four

dilutions were plated on separate 96-well plates using 100 µL of sample per well. The interference control contained 8 mL of media and 30 µL of 1:5000 dilution virus using 56 replicates and 100 µL of sample per well. After 7 days of incubation at 37°C all plates were scored for cytopathic effect. The criteria used to determine if a given dilution was noninterfering was that it must have at least 50% of the number of wells infected compared to the interference control plate. Dilutions that have less than 50% of the wells infected compared to the interference control plate are considered positive for interference and that dilution was excluded from the TCID₅₀ calculation for CHIKV viral reduction samples. This assay ensures that the test material does not interfere with the virus-host cell interaction, thus potentially creating false negatives and causing a product to demonstrate more reduction than was actually achieved.

Real-time reverse transcription–polymerase chain reaction

Real-time reverse transcription–polymerase chain reaction (RT-PCR) was used to correlate virus infectious dose data obtained by titration to genome equivalents (geq) as described previously.¹⁷ RNA was extracted from 100 µL of serially diluted virus using a DNA-free total RNA isolation kit (Aurum total RNA 96 kit, Bio-Rad, Hercules, CA). Sixty microliters of isolated RNA were converted into cDNA using the Bio-Rad iScript cDNA synthesis kit. Five microliters of cDNA was used as the template for the real-time RT-PCR procedure and mixed with 12.5 µL of a proprietary blend of buffers, dNTPs, MgCl₂, and stabilizers (iQ SYBR Supermix, Bio-Rad) and 1.0 µL of 5 µmol/L forward and reverse primer. The primers used for the real-time RT-PCR assay, developed to detect a 151-bp fragment of CHIKV LR2006 OPY1, were as follows: forward, 5'-TCCTG ACCACCAACTCCTG, length 22 (location on Genomes 9405-9426); and reverse, 5'-ATACTTATACGGCTCGTTG-3', length 19 (location on Genomes 9538-9556).

Two dilution sets were analyzed (CHIK 1 and 2) using two replicates for each set. The dilutions for each set ranged from “neat” or undiluted virus to 10⁻¹⁰ dilution. These dilution sets were assayed by titration.

RESULTS

Inactivation of CHIKV in plasma

Three units of CHIKV-spiked plasma were tested separately with each unit exposed to UV light at three energy doses. Samples were removed for virus titration at the beginning of the experiment and after each UV exposure. The reduction of virus in plasma was found to be directly proportional to the amount of energy delivered. Samples exposed to riboflavin and 50, 100, and 150% of the Mirasol energy dose for plasma had reductions of 1.0, 2.1, and

TABLE 1. Reduction of CHIKV in plasma after Mirasol PRT treatment at three UV doses

Replicate	Dose (%)	Titer*	Log reduction
1	0 (initial)	6.4	
1	50	5.4	1.0
1	100	4.4	2.0
1	150	3.8	2.6
2	0 (initial)	6.0	
2	50	5.3	0.7
2	100	4.0	2.0
2	150	2.9	3.1
3	0 (initial)	6.5	
3	50	5.3	1.2
3	100	4.3	2.2
3	150	2.8	3.7
Mean	0 (initial)	6.3	
	50	5.3	1.0
	100	4.2	2.1
	150	3.1	3.2

* Titers are reported as log TCID₅₀/mL.

TABLE 2. Interference of CHIKV in plasma at 150% UV dose

Dilution	Infected replicates	Replicates tested	Interference
0	44	56	Negative
-1	43	56	Negative
-2	41	56	Negative
-3	37	56	Negative
Interference control	44	56	

3.2 log TCID₅₀/mL, respectively (Table 1). The cytotoxicity studies were all negative (data not shown). The interference study using and the posttreatment samples exposed to 150% of the standard Mirasol dose were also found to be negative for interference (Table 2).

Inactivation of CHIKV in PLTs

Three units of CHIKV-spiked PLTs stored in PAS were tested in a similar manner to the units tested in plasma. Each PLT sample was spiked with CHIKV and the samples were exposed to UV light at three energy doses. Samples were removed for virus titration at the beginning of the experiment and after each UV exposure, for a total of four samples for each of the three replicates. Before plating the samples were centrifuged to remove the PLTs. The mean reduction of virus in PLTs was found to also be directly proportional to the amount of energy delivered. Samples exposed to riboflavin and 50, 100, and 150% of the Mirasol energy dose for PLTs stored in PAS had mean reductions of 1.2, 2.2, and 3.5 log TCID₅₀/mL, respectively (Table 3). Cytotoxicity (data not shown) and interference (Table 4) were both determined to be negative.

Inactivation of CHIKV in cell culture media

CHIKV-spiked PBS was exposed to UV light and riboflavin in triplicate experiments at three kinetic energy doses. The

TABLE 3. Reduction of CHIKV in PLTs after Mirasol PRT treatment at three UV doses

Replicate	Dose (%)	Titer*	Log reduction
1	0 (initial)	5.5	
1	50	4.5	1.0
1	100	4.1	1.4
1	150	2.4	3.1
2	0 (initial)	5.6	
2	50	4.6	1.0
2	100	2.5	3.1
2	150	2.1	3.5
3	0 (initial)	6.4	
3	50	4.8	1.6
3	100	4.3	2.1
3	150	2.6	3.8
Mean	0 (initial)	5.8	
	50	4.6	1.2
	100	3.6	2.2
	150	2.4	3.5

* Titers are reported as log TCID₅₀/mL.

TABLE 4. Interference of CHIKV in PLTs at 150% UV dose

Dilution	Infected replicates	Replicates tested	Interference
0	26	56	Negative
-1	32	56	Negative
-2	28	56	Negative
-3	37	56	Negative
Interference control	50	56	

mean initial titer in these samples was 6.3 log TCID₅₀/mL. The mean reduction of virus in media after treatment with riboflavin and exposure to UV light ranged from 4.0 to 4.6 log TCID₅₀/mL or more, depending on the dose (Table 5).

Correlation of CHIKV infectious dose to geq

The viral infectious doses for the two independent replicates were determined to be 7.7 and 7.4 log TCID₅₀/mL, respectively. The mean copies/mL based on the two sets of samples used for the real-time RT-PCR assay ranged from 5.77×10^9 for the undiluted samples to 1.90×10^3 for the lowest detectable dilutions of 10^{-7} (Fig. 1). The ratio between the numbers of mean genome copies per mL for the undiluted virus (neat) through the 10^{-6} dilution compared to the mean infectious TCID₅₀ dose was calculated to be 200:1.

DISCUSSION

The current strategies employed by the blood bank community to prevent the transmission of blood-borne diseases rely on tests designed to screen the blood for the presence of pathogens, the presence of antibodies raised against those pathogens or on donor questionnaires that

aim to exclude donors that have a risk of being infected through risky behavior, past areas of residency, or travel. The above-stated methods have been highly effective at reducing the risk of many of the classic transfusion-transmitted diseases such as HIV, hepatitis C virus, and hepatitis B virus. However, these methods also have limitations in preventing transfusion-transmitted disease. The success of questionnaires depends on people understanding the questions they are being asked and honest answers. In the case of screening assays, for infection to be possible, certain pathogen concentration thresholds must be exceeded. Agents below this threshold concentration

may still initiate an infection during the “window period” if a unit tests negative and is allowed to be transfused. Another major weakness of screening assays is their ineffectiveness against emerging pathogens, since detection assays only work against the target agent for which they were designed. Once a new agent of concern has been identified, industry must determine if their investment costs for test development will be recovered through downstream revenues. If the emerging pathogen is endemic to financially poor countries or testing has not been mandated by the regulatory authorities, many companies may consider it too risky to undertake development of such assays. As seen in the La Réunion chikungunya outbreak, 40% of the population was infected over a 2-year period, demonstrating how rapidly some agents will spread through a sensitive population. The delays caused by the time needed to identify an agent and for blood banks to mandate the need for a potentially life-saving test leaves a hole where contaminated donations can enter the blood pool. There is little that can be done with current blood safety programs to prevent this from happening other than deferrals of all blood donations from areas experiencing outbreaks. If this area is large enough in scope this widespread deferral may cause strains to the blood supply.

Because the current testing strategy for screening blood relies on a reactionary principle to known pathogenic threats, current blood safety programs cannot prevent product contamination in the event of an unknown outbreak. Thus a new line of defense may be needed that can fill the void between organism identifica-

tion and test development. Such an approach could also provide protection from agents that are transient in nature and do not warrant the development of screening assays. This new proactive line of defense could be accomplished through the routine use of a PRT that has a demonstrated broad effectiveness against viruses, parasites, and bacteria. Several of these methods are currently in development or in clinical use in Europe.¹⁸⁻²¹ The technologies are based on the use of a photosensitizer, which can be activated by UV light in specific spectral regions and then carry out modifications to DNA and RNA that prevent their subsequent replication.²² These modifications can render agents that are present in these products as noninfectious.

One emerging agent making itself known in the transfusion community is CHIKV. The risk of transmission of CHIKV via blood transfusion has been

TABLE 5. Reduction of CHIKV in PBS after Mirasol PRT treatment at three UV doses

Replicate	Dose (%)	Titer*	Reduction
1	0 (initial)	6.5	
1	10	1.9	4.6
1	20		>5.0
1	30		>5.0
2	0 (initial)	6.3	
2	10		>4.8
2	20		>4.8
2	30		>4.8
3	0 (initial)	6.1	
3	10	2.1	4.0
3	20		>4.6
3	30		>4.6
Mean	0 (initial)	6.3	
	10	1.3	5.0
	20		>4.8
	30		>4.8

* Titers are reported as log TCID₅₀/mL.

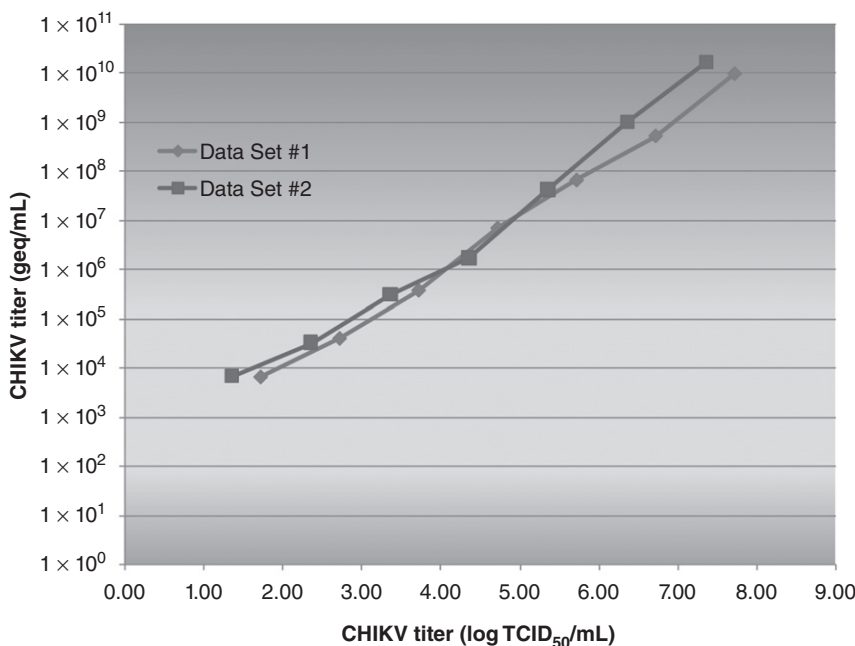


Fig. 1. Correlation of CHIKV geq/mL and TCID₅₀/mL in log space. Data are shown for two independent replicates.

increasing as this virus spreads to new geographical areas. The ongoing epidemic of CHIKV, which began in 2005 in the southern Indian Ocean, involved transmission by a new vector, *Aedes albopictus*.²³ This vector is common in Europe and in the United States, which has caused concern that this virus may establish in these areas. The 2005 epidemic involved more than 1.3 million people in India alone.²⁴ Additionally, a localized outbreak occurred in Ravenna, Italy, in July 2007 where a small transmission cycle was established. This was the first recorded local transmission of CHIKV in Europe.²⁵ Although the Italian outbreak was stopped, two cases of CHIKV infection were reported in nontravelers in Marseille, France, in September of 2010, implying a second incident of localized transmission in southern Europe.²⁶ In addition to these established transmission cycles, there have been reports of infected travelers returning to their respective countries with the virus, furthering concerns of possible extension of the range of this virus.

Evaluation of the amount of CHIKV in both plasma and PLTs stored in PAS after exposure to riboflavin and UV light using the Mirasol PRT system demonstrated the ability of this system to inactivate the virus sufficiently to significantly reduce viral titers in contaminated blood products. The reduction of CHIKV titers followed a roughly linear response based on the amount of energy delivered to the plasma or PLT units. Levels of infectious virus were reduced by more than 99% in both plasma and PLTs. Consistent with previous studies, no interference or toxicity was observed after treatment of blood component samples with riboflavin and UV light.

As part of this study, a quantitative real-time RT-PCR assay was developed and used to correlate virus infectious dose data obtained by titration with physical RNA copy numbers present. It was determined that the ratio for the mean genome copies/mL per TCID₅₀/mL is approximately 200:1. Another way to interpret these data is that CHIKV on average requires approximately 200 viral RNA copies to achieve one infectious dose in a cell model where the system is optimized to cause viral infection. When delivered to a human, the ratio may be even higher due to the probability of virus contacting a susceptible cell and subject having a functional immune system. When the above ratio is factored into the data obtained from studies to evaluate the effect of the Mirasol PRT treatment on CHIKV inactivation, this treatment may afford protection from CHIKV-contaminated blood with up to 4.5 log₁₀ geq/mL of virus present.

Even after factoring in that only 1 of 200 genomes are infectious, Mirasol PRT treatment still does not completely eliminate the potential risk that CHIKV may cause given that the literature suggests that during viremia 10^{5.5} PFUs/mL (equivalent to 10⁸ viral RNA copies/mL) can be found in the blood. Despite the presence of such high reported titers in blood of infected subjects, however, no

cases of CHIKV transmission via blood transfusions have been reported to date despite the lack of screening in areas where outbreaks have occurred or use of blood products that are not treated with a PRT method. One possibility to explain this apparent contradiction is that there is a relatively short asymptomatic period after infection when a blood unit may be drawn from an infected donor who is not screened out of the donation process during a routine pretransfusion health status check. Petersen and colleagues³ reported that most people become symptomatic within the first 36 hours after being bitten by an infected mosquito with persistence of the symptoms over a prolonged period of time after onset. If this is indeed the case, then use of a PRT method in these settings may only further reduce what is an already low probability event of disease transmission.

In summary, data from this study indicate that the Mirasol PRT system can effectively be used to inactivate CHIKV in contaminated plasma and PLTs. Combined with data previously obtained for other pathogens, this study reaffirms the utility of Mirasol PRT as a potential means for further protecting the blood supply from emerging infectious disease threats.

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CONFLICT OF INTEREST

DLV, KMH, RP, and SH report no conflict of interest. SDK and RPG are employed by CaridianBCT Biotechnologies (Lakewood, CO), the company that developed and sells the Mirasol pathogen reduction technology.

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