Inactivation of Ebola virus and Middle East respiratory syndrome coronavirus in platelet concentrates and plasma by ultraviolet C light and methylene blue plus visible light, respectively

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BACKGROUND: Ebola virus (EBOV) and Middle East respiratory syndrome coronavirus (MERS-CoV) have been identified as potential threats to blood safety. This study investigated the efficacy of the THERAFLEX UV-Platelets and THERAFLEX MB-Plasma pathogen inactivation systems to inactivate EBOV and MERS-CoV in platelet concentrates (PCs) and plasma, respectively.

STUDY DESIGN AND METHODS: PCs and plasma were spiked with high titers of cell culture–derived EBOV and MERS-CoV, treated with various light doses of ultraviolet C (UVC; THERAFLEX UV-Platelets) or methylene blue (MB) plus visible light (MB/light; THERAFLEX MB-Plasma), and assessed for residual viral infectivity.

RESULTS: UVC reduced EBOV (≥4.5 log) and MERS-CoV (≥3.7 log) infectivity in PCs to the limit of detection, and MB/light decreased EBOV (≥4.6 log) and MERS-CoV (≥3.3 log) titers in plasma to nondetectable levels.

CONCLUSIONS: Both THERAFLEX UV-Platelets (UVC) and THERAFLEX MB-Plasma (MB/light) effectively reduce EBOV and MERS-CoV infectivity in platelets and plasma, respectively.

Abbreviations: EBOV = Ebola virus; MB = methylene blue; MB/light = methylene blue in combination with visible light; MERS-CoV = Middle East respiratory syndrome coronavirus; PC(s) = platelet concentrate(s); PI = pathogen inactivation; RF = reduction factor; TCID₅₀ = 50% tissue culture infectious dose.

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has been transmitted through the transfusion of blood products. However, the fact that the mortality rate is high (>30%) and the transmission mechanisms are still not completely understood highlight the need for increased awareness of the potential threat to blood safety by MERS-CoV. MERS-CoV is an enveloped, positive-sense single-stranded RNA coronavirus. Interestingly, viral RNA was detected in the sera of asymptomatic patients during an outbreak of severe acute respiratory syndrome, which is caused by another coronavirus, severe acute respiratory syndrome-CoV. Consequently, there might be an asymptomatic viremic phase that could theoretically lead to transmission via blood transfusion. However, blood transfusions have not been implicated in the transmission of MERS-CoV to date.5

EBOV, a member of the Filoviridae family, is an enveloped pathogen containing negative-sense single-stranded RNA as its genetic material. EBOV disease is extremely fatal and is known to cause death rates of up to 90%. Recent outbreaks of EBOV disease in West Africa have devastated Guinea, Liberia, and Sierra Leone, killing more than 11,000 out of nearly 30,000 persons affected.6 EBOV is transmitted to humans from animals and spreads through human-to-human transmission, that is, via direct contact with infected blood and/or secretions.

Different pathogen inactivation (PI) techniques can effectively inactivate or incapacitate such viral agents, rendering them unable to replicate in blood components or products.7 THERAFLEX UV-Platelets (Macopharma), a novel ultraviolet C (UVC)-based PI system for platelet concentrates (PCs), is currently undergoing clinical efficacy and safety testing.8-10 In contrast to other PI technologies, it works without exogenously added photoactive substances. Shortwave UVC light (254 nm) interacts with nucleic acids directly, inducing the formation of cyclobutane pyrimidine and pyrimidine pyrimidone dimers that prevent the elongation of nucleic acid transcripts.11 The THERAFLEX MB-Plasma (Macopharma) is a photodynamic PI procedure for single units of fresh-frozen plasma. This system, based on the administration of methylene blue (MB) in combination with visible light (MB/light), was developed to increase the viral safety of plasma transfusions.12,13 More than 5 million units have been treated by this method and safely transfused to patients for more than 10 years.

Both systems have been shown to effectively inactivate a broad range of different DNA and RNA viruses, including arboviruses such as West Nile virus, Chikungunya virus, and Zika virus; recently, large outbreaks of the latter viruses have occurred outside of their previously known geographic ranges, seriously affecting thousands of people.9,13-18 This study aimed to investigate the efficacy of THERAFLEX UV-Platelets and THERAFLEX MB-Plasma to inactivate MERS-CoV and EBOV in PCs and plasma, respectively.

MATERIALS AND METHODS

Blood component preparation

All components were prepared from whole blood (500 mL) collected from screened, unpaid volunteers and stored in 70 mL of CPD according to German guidelines.19 Blood was separated into red blood cells (RBCs), plasma, and Buffy coat by standard blood banking procedures. Plasma units were leukoreduced by filtration (Plasmalex, Macopharma), pooled, split again, and stored at −20°C until further use. Plasma-reduced PCs were prepared from pools of five buffy coats as described by Eriksson and colleagues.20 The pools were mixed with 280 mL of PAS SSP+ (Macopharma), which is equivalent to PAS-E.21 The suspension was centrifuged for 7.5 minutes at 527 × g. PCs were isolated, leukoreduced with a high-efficiency filter system (AutoStop BC, Haemonetics), and stored under agitation at 22 ± 2°C. The platelet (PLT) concentration was approximately 10^9/mL, the plasma content approximately 35%, and the residual white blood cell (WBC) content was not more than 10^6 per unit. Air was routinely removed from all plasma units and PCs.

Antibody screening

The anti-MERS-CoV enzyme-linked immunosorbent assay (IgG; Euroimmun) was used to screen PCs and plasma units for the presence of antibodies against MERS-CoV. Only products tested for antibodies to MERS-CoV and confirmed to be negative were used in the inactivation experiments.

PI processes

UVC treatment (PCs)

PLTs were treated using THERAFLEX UV-Platelets, a PI system consisting of a UVC illumination device (MacoTronic, Macopharma) and a disposable set (REF XUV 4005 XI, Macopharma). Briefly, the PCs were irradiated with UVC light at a wavelength of 254 nm to a total dose of 0.2 J/cm², with vigorous agitation to ensure the uniform treatment of all compartments.9 The amount of UVC energy delivered was expressed in J/cm².

Visible light plus MB (plasma units)

Plasma units were MB/light-treated using THERAFLEX MB-Plasma (Macopharma), a PI system consisting of a THERAFLEX MB-Plasma kit and the MacoTronic B2 LED-based illumination device. Its closed-bag system contains a MB pill that delivers 85 μg of MB per unit of plasma or approximately 1 μmol/L per plasma unit. The standard treatment cycle includes initial filtration for leukoreduction (PlasmaFlex filter) followed by a second postillumination filtration step for the removal of MB and its photoproducts (BlueFlex filter).12,22 Plasma processing for PI efficacy testing deviated from the standard procedure.
in that the removal of MB and photoproducts (Blueflex filtration) was not performed so as to exclusively determine the virus inactivation effects of illumination. The amount of LED-based light energy delivered was expressed in J/cm². The total illumination dose was set at 120 J/cm², as is routinely recommended for this system.12

**Infectivity assays**

Endpoint titration assays for microtiter plates were employed to measure virus titers in eight serial 1-in-3 dilutions per parallel sample. Vero E6 cells were used as the indicator cells. Sample titration was performed at the initial dilution at which Vero cells exhibited no cytotoxicity. The plates were incubated at 37°C in a humid atmosphere with 5% CO₂. After 5 to 7 days of incubation, the cell layers were assessed for virus-induced changes in morphology (cytopathology). The 50% tissue culture infectious dose (TCID₅₀) was calculated according to the Spearman-Kärber method and was expressed as log TCID₅₀. The effectiveness of each individual process step for each virus was calculated as the log reduction factor (RF) using the formula RF = log A₀ − log Aᵣ, where R is the reduction factor, A₀ is the total virus load after spiking, and Aᵣ is the total virus load in the treated sample. The overall reduction factor was expressed as the sum of RFs for all steps. The limit of detection of the assay was defined as the lowest TCID₅₀ achievable at noncytotoxic sample concentrations.

**EBOV**

Vero E6 cells (ATCC CCL-22) together with the Zaire ebolavirus strain (Mayinga-76) were grown to approximately 80% confluence in Dulbecco’s modified Eagle’s medium (DMEM) with 2% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 mg/mL streptomycin, and 100 IU/mL penicillin. On Day 6, the viral supernatant was collected, centrifuged, aliquoted, and frozen at –80°C until further use (spiking experiments).

**MERS-CoV (HCoV-EMC, Ron A. Fouchier)**

Vero E6 cells (ATCC CCL-22) together with MERS-CoV (EMC/2012), obtained from Ron A. Fouchier, were grown to approximately 80% confluence in DMEM with 2% FBS, 2 mmol/L L-glutamine, 100 mg/mL streptomycin, and 100 IU/mL penicillin. On Days 3 to 5, the viral supernatant was collected, centrifuged, aliquoted, and frozen at –80°C until further use (spiking experiments).

**Spiking experiments**

For each virus, two bags of PCs (volume, 374 mL) and plasma (volume, 315 mL) were spiked with 10% viral supernatant and treated as described with UVC or MB/light, respectively. Each treatment was delivered incrementally up to the full light dose (0.2 J/cm² for UVC and 120 J/cm² for MB/light). At different process steps, samples were collected and virus titrations were performed. Reference samples were taken from each bag before treatment, stored at room temperature, and tested at the end of the experiments to account for any intrinsic virus inactivation by the blood product. Virus titers after spiking were below the expected dilution factor in six of the eight blood units used. In one PC and in one plasma unit used for EBOV inactivation and in all four blood units used for MERS-CoV inactivation, the virus titers before PI treatment were up to 1 log lower than expected from a 1-in-10 dilution. However, no additional reduction in virus titers was observed during the course of the experiment.

**RESULTS**

**UVC-based inactivation of EBOV and MERS-CoV in PCs**

The results of the infectivity assay demonstrated that UVC irradiation dose-dependently inactivated EBOV and MERS-CoV in plasma-reduced PCs (Table 1). At 0.15 J/cm² (75% of the full UVC dose), EBOV and MERS-CoV infectivity levels were below the detection limit, resulting in virus reduction factors of greater than 4.5 for EBOV and of greater than 3.7 for MERS-CoV.

**MB/light–based inactivation of EBOV and MERS-CoV in plasma**

MB/light effectively inactivated EBOV and MERS-CoV in all plasma units. As shown in Table 2, light doses as low as 30 J/cm², or 25% of the standard full light dose of 120 J/cm² for LED-based illumination, inactivated EBOV and MERS-CoV in plasma to levels below the detection limit. These results correspond to at least 4.6 and at least 3.3 log reductions of EBOV and MERS-CoV, respectively.

**DISCUSSION**

The THERAFLEX UV-Platelets and THERAFLEX MB-Plasma systems are designed to inactivate or remove pathogens in PLT and plasma products, as has been demonstrated in a wide range of pathogenic agents.9,12,17,18,23-25 The mechanism of THERAFLEX UV-Platelets is that short-wave UVC light penetrates blood fluids and cell membranes, causing direct covalent damage to the nucleic acids of pathogens and WBCs. This mechanism of action is broadly effective against extracellular and intracellular transfusion-transmitted DNA/RNA-containing pathogens, such as viruses, bacteria, and protozoa. THERAFLEX MB-Plasma combines MB—a phenothiazine compound that intercalates into viral nucleic acid—with visible light. The illumination of MB-treated plasma results in the generation of singlet oxygen, which leads to the destruction of viral nucleic acids and thus prevents viral replication.12 Because...
TABLE 1. Inactivation of EBOV and MERS-CoV in PCs by THERAFLEX UV-Platelets

| Light dose (cumulative) | EBOV |  |  | MERS-CoV |  |  |
|-------------------------|------|---|---|----------|---|---|
|                         | Bag 1 | Bag 2 | Bag 1 | Bag 2 | Bag 1 | Bag 2 |
| Virus stock             |      |      |      |       |      |      |
| 8.11 ± 0.16             | 7.99 ± 0.18 | 8.12 ± 0.20 | 8.06 ± 0.21 |
| Load                    |      |      |      |       |      |      |
| 6.84 ± 0.17             | 6.96 ± 0.17 | 6.43 ± 0.12 | 6.37 ± 0.16 |
| 0.05 J/cm²              | <2.37 | >4.5 | 2.43 ± 0.12 | 4.5 | <2.67 | ≥3.8 |
| 0.1 J/cm²               | <2.37 | ≥4.5 | 2.37 | ≥4.6 | <2.67 | ≥3.7 |
| 0.15 J/cm²              | <2.37 | ≥4.5 | 2.37 | ≥4.6 | <2.67 | ≥3.7 |
| 0.2 J/cm²               | <2.37 | ≥4.5 | 2.37 | ≥4.6 | <2.67 | ≥3.7 |
| Ref. sample             | 6.54 ± 0.16 | 0.3 | 6.96 ± 0.17 | 0.0 | 6.31 ± 0.17 | 0.1 |

Ref. sample = pretreatment reference sample.

TABLE 2. Inactivation of EBOV and MERS-CoV in plasma by THERAFLEX MB-Plasma

| Light dose (cumulative) | EBOV |  |  | MERS-CoV |  |  |
|-------------------------|------|---|---|----------|---|---|
|                         | Bag 1 | Bag 2 | Bag 1 | Bag 2 | Bag 1 | Bag 2 |
| Virus stock             |      |      |      |       |      |      |
| 8.83 ± 0.22             | 7.87 ± 0.21 | 7.86 ± 0.12 | 7.80 ± 0.16 |
| Load                    |      |      |      |       |      |      |
| 6.85 ± 0.16             | 6.99 ± 0.24 | 5.95 ± 0.12 | 6.13 ± 0.21 |
| 30 J/cm²                | <2.67 | >4.7 | 2.55 | 4.6 | 2.67 | ≥3.3 |
| 60 J/cm²                | <2.67 | >4.7 | 2.37 | ≥4.7 | 2.67 | ≥3.3 |
| 90 J/cm²                | <2.67 | >4.7 | 2.37 | ≥4.7 | 2.67 | ≥3.3 |
| 120 J/cm²               | <2.67 | >4.7 | 2.37 | ≥4.7 | 2.67 | ≥3.3 |
| Ref. sample             | 6.90 ± 0.12 | -0.1 | 7.11 ± 0.23 | -0.1 | 5.77 ± 0.18 | 0.2 |

Ref. sample = pretreatment reference sample.

MB only partially penetrates cell membranes, MB/light treatment is less effective against intracellular viruses. This is why two filters are integrated in the process chain. Although the second filter mainly removes MB and its photoactivated products after illumination, both filters have multiple layers of small-meshed membranes capable of removing residual WBCs, RBCs, and PLTs that may carry intracellular pathogens. It was recently shown that the two filters efficiently remove high levels (up to 5.9 log colony-forming units per milliliter) of viable bacteria and bacterial spores. It can be assumed that parasites of similar sizes can also be removed by the THERAFLEX MB-Plasma procedure. A previous investigation showed that the filtration step before the addition of MB and visible light illumination was critical to achieving the complete and reproducible elimination of Trypanosoma cruzi, the causative agent of Chagas disease.

It is estimated that there are more than 1400 known human pathogens, 13% of which are considered emerging or reemerging. The ability to provide proactive protection against emerging infectious agents complementary to the existing blood screening programs is a major additional benefit of PI systems for blood safety. Thus, it is imperative that manufacturers continuously challenge their PI systems with new infectious agents. To our knowledge, this is the first study investigating the efficacy of UVC and MB/light against MERS-CoV and EBOV or any other member of the Coronaviridae and Filoviridae families. Our results showed that these two PI systems reduced MERS-CoV and EBOV titers to below the limit of detection in the two blood units tested.

The maximum viral titer reduction detectable in a cell culture infectivity assay is based on the starting viral concentration of the blood product and the detection limit of the assay which, in turn, is determined by the susceptibility of the cell culture to plasma and PLT suspensions. As in a previous study, the observed reduction of EBOV titers and MERS-CoV titers in the present study was beyond the expected dilution factor in PCs and plasma. This phenomenon may occur due to the presence of non-specific immune mediators that neutralize viruses in plasma.

This study had a number of limitations. For example, the number of replicates was limited by safety constraints, as experiments with MERS-CoV and EBOV are subject to the number of replicates was limited by safety constraints, although this would have improved the detection limit and enabled more exact determination of log reduction factors. Although plasma
viral RNA loads in MERS-CoV patients may be up to 6 log genome copies/mL, and median viral RNA loads of up to 6.68 log genome copies/mL have been detected in the blood of Ebola patients.30,31 Virus titers in the plasma of asymptomatic infected or convalescent individuals recruited to donate blood may be significantly lower. As it is known for other infectious diseases, EBOV viral titers expressed in genome copies per milliliter do not necessarily reflect the infectivity titer. While viral titers measured by quantitative polymerase chain reaction are based on the detection of a small fragment of the viral genome, infectivity titers describe the number of intact, functional viral units required for replication and disease transmission. Although EBOV is highly infectious and low doses of less than 10 plaque-forming units of the virus are sufficient to cause disease, the ability of the UVC- and MB-light-based systems to reduce MERS-CoV and EBOV infectivity in blood products by several log steps may be sufficient to eliminate or significantly reduce the risk of transmission via the transfusion of PCs or plasma. In conclusion, this study further expands the list of pathogens that THERAFLEX UV-Platelets and THERAFLEX MB-Plasma can effectively inactivate in PCs and plasma, respectively.

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CONFLICT OF INTEREST

FT and SR are employees of Macopharma, the manufacturer and distributor of the THERAFLEX technologies for PI. UG, WH, THM, and AS received project grants from the “Forschungsgemeinschaft der DRK-Blutspendedienste e.V.” and from Macopharma for the development of the UVC-based PI technology for platelets. The other authors have disclosed no conflicts of interest.

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