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Amotosalen and ultraviolet A light efficiently inactivate MERS-coronavirus in human platelet concentrates

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SUMMARY

Objective: This study aimed to assess the efficacy of the INTERCEPT™ Blood System [amotosalen/ultraviolet A (UVA) light] to reduce the risk of Middle East respiratory syndrome-Coronavirus (MERS-CoV) transmission by human platelet concentrates.

Background: Since 2012, more than 2425 MERS-CoV human cases have been reported in 27 countries. The infection causes acute respiratory disease, which was responsible for 838 deaths in these countries, mainly in Saudi Arabia. Viral genomic RNA was detected in whole blood, serum and plasma of infected patients, raising concerns of the safety of blood supplies, especially in endemic areas.

Methods: Four apheresis platelet units in 100% plasma were inoculated with a clinical MERS-CoV isolate. Spiked units were then treated with amotosalen/UVA to inactivate MERS-CoV. Infectious and genomic viral titres were quantified by plaque assay and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). Inactivated samples were successively passaged thrice on Vero E6 cells to exclude the presence of residual replication-competent viral particles in inactivated platelets.

Results: Complete inactivation of MERS-CoV in spiked platelet units was achieved by treatment with Amotosalen/UVA light with a mean log reduction of $4{\cdot}48 \pm 0{\cdot}3$. Passaging of the inactivated samples in Vero E6 showed no viral replication even after nine days of incubation and three passages. Viral genomic RNA titration in inactivated samples showed titres comparable to those in pre-treatment samples.

Conclusion: Amotosalen and UVA light treatment of MERS-CoV-spiked platelet concentrates efficiently and completely inactivated MERS-CoV infectivity (>4 logs), suggesting that such treatment could minimise the risk of transfusion-related MERS-CoV transmission.

Key words: amotosalen, MERS-CoV, pathogen inactivation, platelets, UVA.

The treatment of acute bleeding due to trauma or surgery, or the prevention of bleeding in patients due to any indication, relies on the transfusion of blood components and has saved numerous lives. With these benefits comes the risk of pathogen transmission via the transfused blood components, making the supply of safe blood components a key mission of blood transfusion services. The introduction of screening tests for blood products strongly reduced the burden of known blood-borne pathogens such as hepatitis B and C viruses (HBV and HCV) and human immunodeficiency virus (HIV). However, cases of HBV and HIV transmission via blood from infected donors, which were not detected by serological screening or nucleic acid testing (NAT), have been described recently (Candotti *et al.*, 2018; Cappy *et al.*, 2019). In addition, other known or unknown pathogens pose a potential threat to blood safety, with regional differences. The number of different pathogen screening tests applied in blood banks is usually limited by economic factors and availability of reliable and affordable assays. Thus, an efficacious pathogen inactivation technology is a viable alternative to serological or NAT testing as the risk of transmission of a broad spectrum of pathogens can be reduced. It also reduces the need for implementing additional screening tests if proven efficient (Prowse, 2013).

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The Middle East respiratory syndrome-Coronavirus (MERS-CoV) has been described as an endemic zoonotic pathogen in Saudi Arabia and other countries of the Arabian Peninsula since 2012 (Zaki *et al.*, 2012). Dromedary camels are the most likely animal reservoir, and the virus can spread from animals and cause human infections (Azhar *et al.*, 2014). As of late May 2019, 2425 confirmed infections, including 838 deaths (~34·6% mortality rate), have been reported in 27 countries. The majority of confirmed cases (2037 total) were reported from Saudi Arabia, with a mortality rate of ~37·5% (WHO, 2019: https://www.who.int/emergencies/mers-cov/en/). Symptoms of MERS infections vary from asymptomatic to severe pneumonia (Zaki *et al.*, 2012; Mackay & Arden, 2015). Older age and comorbidities are the highest risk factors for the development of severe symptoms, such as extra-pulmonary manifestations and severe pneumonia, often leading to fatal outcomes (Almekhlafi *et al.*, 2016). Hospital and community outbreaks in Saudi Arabia and South Korea were reported as a result of nosocomial transmissions due to close contact with patients (Shehata *et al.*, 2016; Arabi *et al.*, 2017). Underreporting of asymptomatic cases is presumed, and thus, the real number of infected patients is likely to be much higher than the number of reported and confirmed cases (Lessler *et al.*, 2016), which together with mild cases may facilitate the spread of the virus and pose a possible risk for blood safety. Higher seropositivity in camel workers compared to the general population has been reported (Müller *et al.*, 2015; Alshukairi *et al.*, 2018). Therefore, asymptomatic donors may donate blood while they potentially carry the virus.

MERS-CoV genomic RNA was detected in respiratory, serum, plasma, urine and stool samples from infected patients (Memish *et al.*, 2014; Kim *et al.*, 2016; Min *et al.*, 2016). The detection of MERS-CoV viral RNA in plasma and serum was reported in around 30% of patients, and those cases were associated with more severe disease (Corman *et al.*, 2016; Kim *et al.*, 2016; Min *et al.*, 2016). CoVs are mainly transmitted via droplets, fomites, person-to-person contact and direct or indirect contact with zoonotic sources. Transfusion-transmitted MERS-CoV infections have not been reported so far (Stramer, 2014); however, the detection of viral RNA in the plasma and serum of acutely infected patients raises the possibility of such concern in endemic areas such as the Arabian Peninsula. Thus, use of pathogen inactivation technology may be crucial to prevent transfusion-transmitted MERS-CoV infections during an outbreak and could be implemented proactively in the prevision of potential outbreaks.

The INTERCEPT™ Blood System technology inactivates a broad spectrum of pathogens, including bacteria, viruses and protozoa, in platelet concentrates prepared for transfusion (Schlenke, 2014). Using amotosalen (a photoactive compound) and UVA light, pathogens' genomes are modified in a targeted and specific manner by cross-linking the genomic strands, preventing transcription and replication without affecting the platelet efficacy and patient safety as demonstrated in clinical evaluations (Cid *et al.*, 2012), national routine observations (Jutzi *et al.*, 2018) and by haemovigilance data from multiple countries (Benjamin *et al.*, 2017). As an additional effect, residual white blood cells of the donor are inactivated more efficiently than by gamma irradiation (Castro *et al.*, 2018), reducing the risk of immunological transfusion reactions and transfusion-associated graft-versus-host disease (TA-GvHD). The INTERCEPT Blood System is currently the only FDA-approved pathogen reduction system for platelets. Although we have recently shown that INTERCEPT treatment can efficiently inactivate MERS-CoV in human plasma (Hindawi *et al.*, 2018), there have been no data so far for the inactivation of MERS-CoV with INTERCEPT treatment in platelets. Here, we extend this work to evaluate inactivation of MERS-CoV in human apheresis platelet concentrates with INTERCEPT treatment.

MATERIALS AND METHODS

Cell line and MERS-CoV culture

Vero E6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) as previously described (Al-Amri *et al.*, 2017). The human MERS-CoV clinical patient isolate (MERS-CoV/Hu/Taif/SA/2015) described previously was used in all experiments in the Biosafety level 3 facility in the Special Infectious Agents Unit (SIAU), King Fahd Medical Research Center (KFMRC), King Abdulaziz University (KAU), Jeddah, Saudi Arabia.

Platelet preparation

Apheresis platelet units in 100% donor plasma (~380 mL, 4.6×10^{11} platelets per unit) were collected at King Abdulaziz University Hospital (KAUH), Transfusion Services, Jeddah, Kingdom of Saudi Arabia, from voluntary donors using a Trima™ apheresis collection unit (Terumo BCT, Japan). The platelets were stored at 20–22°C under continuous agitation. All platelet units were screened routinely for HCV-Ab/Ag, HBs-Ag, HBc-Ab, HIV (1/2)-Ab, HTLV (1/2)-Ab and syphilis, as well as for HCV, HBV and HIV by NAT. Donors were also confirmed to be negative for MERS-CoV-neutralising antibodies. After baseline sampling, the remaining volume was ~360 mL, and the remaining platelet count was $\sim 4.4 \times 10^{11}$.

MERS-CoV inactivation

Four units (A–D) of platelets were used in this study. Each platelet unit was inoculated with MERS-CoV stock in a 1:100 dilution. Platelet units were treated with the INTERCEPT Large Volume Processing Set and an INTERCEPT Illuminator (Cerus Corporation, U.S.A.). Residual amotosalen and free photoproducts were removed using the integrated compound adsorption device according to the manufacturer's instructions. The following samples were collected for testing: a positive control sample from the virus stock, a negative control sample from the platelets before inoculation with the virus, pre-treatment sample from the inoculated platelets and a sample from the treated platelets

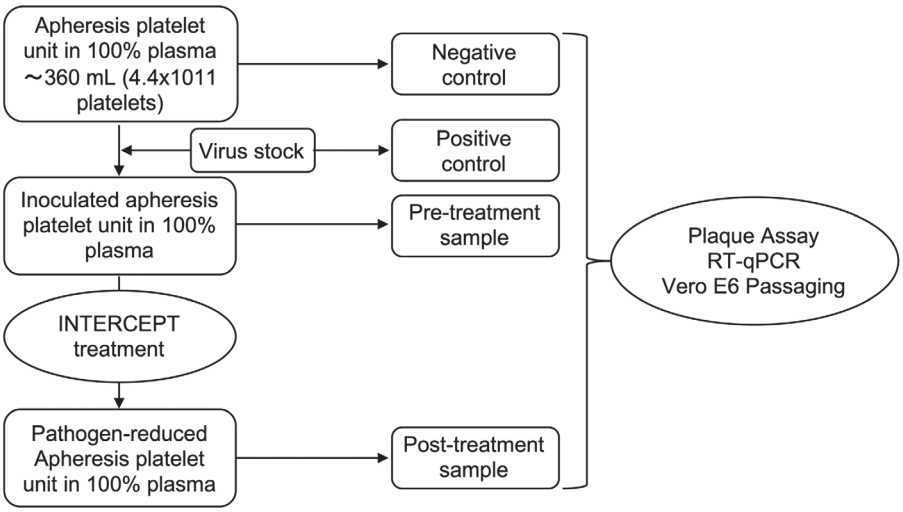

Fig. 1. Experimental design. Schematic view of the experimental design.

after INTERCEPT treatment (Fig. 1). All samples were stored at −80°C until testing.

Detection of replicative MERS-CoV

Detection of replicative MERS-CoV in INTERCEPT-treated platelets was performed as previously described with minor modifications (Pinna *et al.*, 2005; Hindawi *et al.*, 2018). Briefly, collected pre-treatment and inactivated samples were diluted at 1:10 dilution in DMEM with 10% FBS containing heparin sulphate (5 U/mL), inoculated on Vero E6 cells in 6-well plates in duplicates and incubated for 1 h at 37°C. Inoculum was then removed and replaced with 2 mL of DMEM with 10% FBS and incubated for 3 or 9 days at 37°C. On day three post-inoculation, supernatants were collected from plates, diluted at 1:10 in DMEM with 10% FBS and re-inoculated on Vero E6 cells for two more successive passages. Supernatants collected from each passage were also used for viral load quantification by quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). In some experiments, plates were maintained for up to nine days after inoculation and were observed for cytopathic effect (CPE) to ensure the absence of any residual infectious viruses.

Plaque assay

Plaque assay was performed as previously described (Hindawi *et al.*, 2018) with a minor modification. Briefly, samples were serially diluted in DMEM with 10% FBS containing heparin sulphate (5 U/mL) starting from 1:10, and 1 mL from each dilution was inoculated on confluent Vero E6 cell monolayers and incubated for 1 h at 37°C. Then, the inoculum was removed and replaced by overlay media (DMEM with 0·8% agarose) and incubated for 3 days at 37°C. After incubation, cells were stained with Neutral Red for 4 h at 37°C, and plaques were counted to calculate titre as plaque-forming unit (pfu)/mL.

RT-qPCR quantitation

Viral RNA was extracted from all samples collected directly from the platelet units (positive, negative, pre-treatment and post-treatment samples), as well as from the cell supernatants using the Qiagen Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Relative quantification of MERS-CoV viral load was performed as previously described (Hashem *et al.*, 2019) with some modifications. In brief, one-step real-time RT-qPCR was conducted using MERS-CoV-specific primers and probes targeting the upstream region of the E gene (upE) and QuantiTect Probe RT-PCR Kit (Qiagen, Germany) on an Applied Biosystems 7500 Fast Real-Time PCR System. Ct value for each replicate of each sample was analysed against Ct values of a standard curve of MERS-CoV with known mRNA copy number. The viral load was expressed as the equivalent of $\log_{10}$ equivalents per mL ($TCID_{50}$eq/mL) and compared to that of the pre-treatment sample. Each run included a positive viral template control and no-template negative control. Each sample was tested in duplicate, and the mean is reported as $TCID_{50}$eq/mL.

IRB approval

The study was approved by the biomedical ethics committee unit of the King Abdulaziz University Hospital (approval# 257-16).

RESULTS

Inactivation of MERS-CoV in human platelet concentrates

We spiked four units (A – D) of human platelets collected from healthy donors with MERS-CoV. Spiked units were then treated

Table 1. Reduction of infectious MERS-CoV titres in platelet concentrates after INTERCEPT treatment

	Viral infectivity titre, $\log_{10}$ PFU/mL				
Experiment	Positive control	Negative control	Pre-treatment sample	Inactivated sample	Log reduction
A	8·08	ND	4·30	ND	4·3
B	7·30	ND	4·18	ND	4·18
C	7·85	ND	4·85	ND	4·85
D	7·85	ND	4·60	ND	4·60
Mean ± SD	7·77 ± 0·33	ND	4·48 ± 0·3	ND	4·48 ± 0·3

ND, not detected.

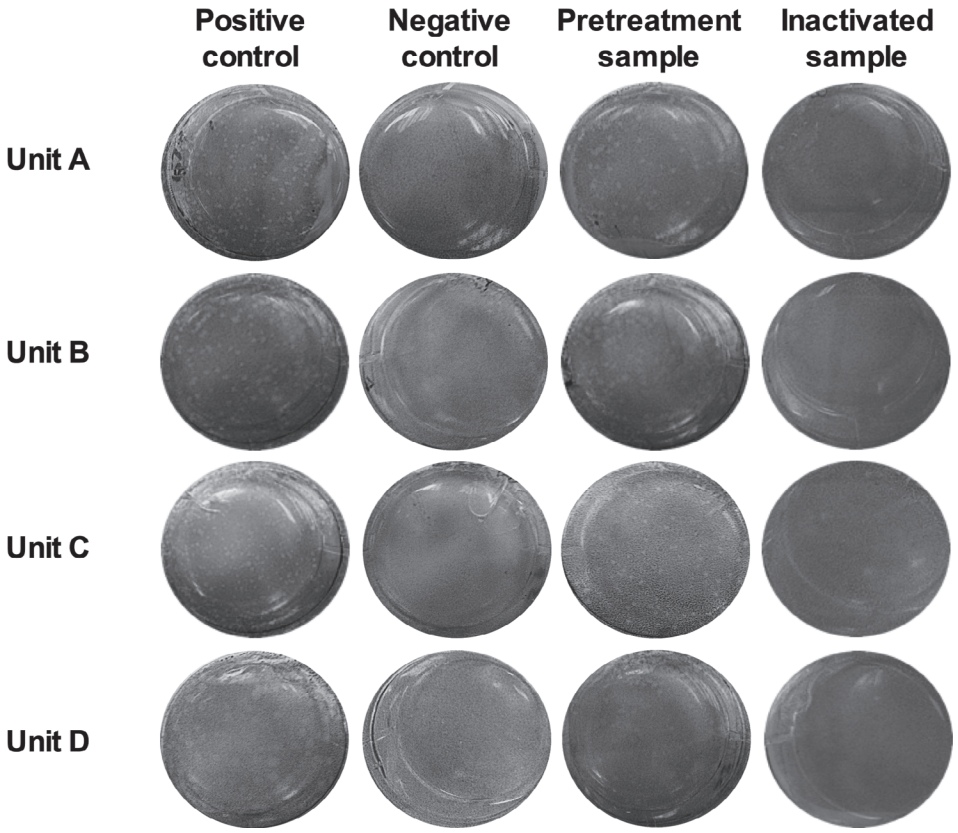

Fig. 2. Inhibition of MERS-CoV in platelets by amotosalen and UVA treatment. Representative wells from the plaque assay are shown for all tested platelets units.

with amotosalen and UVA light. Mean infectious viral titre in pre-treatment samples from the four units was 4·48 ± 0·3 $\log_{10}$ pfu/mL (range: 4·18 – 4·85 $\log_{10}$ pfu/mL) (Table 1). Treatment of spiked units resulted in a mean reduction of 4·48 ± 0·3 $\log_{10}$ pfu/mL as no infectious virus was detected by plaque assay (Table 1). Fig. 2 shows representative plaque assay results for all tested units. As expected, testing of negative control samples collected before spiking with MERS-CoV showed no replication-competent virus. Of note, the viral infectivity titre in the positive controls had a mean of 7·77 ± 0·33 $\log_{10}$ pfu/mL (range: 7·30 – 8·08 $\log_{10}$ pfu/mL), and it was reduced to 4·48 ± 0·3 $\log_{10}$ pfu/mL (range: 4·18 – 4·85 $\log_{10}$ pfu/mL) on spiking of platelets units, which is lower than the expected dilution of 1:100 by >1 log as we previously observed in plasma (Hindawi *et al.*, 2018), suggesting that platelets and/or plasma may have an impact on the MERS-CoV infectivity titre, most probably due to non-specific inhibition as the used samples were negative for MERS-CoV antibodies.

Impact of pathogen inactivation treatment on MERS-CoV genomic titre

To further confirm these results, the viral genomic titre was determined for all collected samples. As shown in Table 2, positive controls and pre-treatment samples from all units had a $\log_{10}$ $TCID_{50}$eq/mL equivalent to the infectious viral titre determined

by plaque assay as shown in Table 1. Importantly, post-treatment samples showed viral genomic titres that were only slightly reduced compared to titres in the pre-treatment samples. These results suggest that amotosalen and UVA light inactivated infectious viruses without any significant effect on the viral genomic titres as shown before for MERS-CoV, dengue virus and Zika virus (Musso *et al.*, 2014; Santa Maria *et al.*, 2017; Hindawi *et al.*, 2018).

Passaging of INTERCEPT-treated platelet concentrates to confirm complete inactivation

To exclude the possibility of any residual replicating MERS-CoV associated with the presence of viral genomic titres in the treated platelet units (Table 2), we inoculated the collected samples on Vero E6 cells and evaluated infectivity over three successive passages. Although culture of all pre-treatment samples showed complete CPE within 3 days post-inoculation, similar to that of the positive control, no CPE was observed in cells inoculated with inactivated samples similar to negative and

Table 2. MERS-CoV genomic titres in platelet concentrates before and after INTERCEPT treatment[1,2]

Experiment	Positive control	Negative control	Pre-treatment sample	Inactivated sample
A	7·60	ND	4·10	3·51
B	7·48	ND	4·03	3·57
C	7·58	ND	4·61	3·70
D	7·56	ND	4·44	3·98
Mean ± SD	7·56 ± 0·05	ND	4·29 ± 0·28	3·69 ± 0·21

ND, not detected.
[1]Data are shown as $\log_{10}$ $TCID_{50}$eq/mL.
[2]Titres were determined from the same samples used in Table 1.

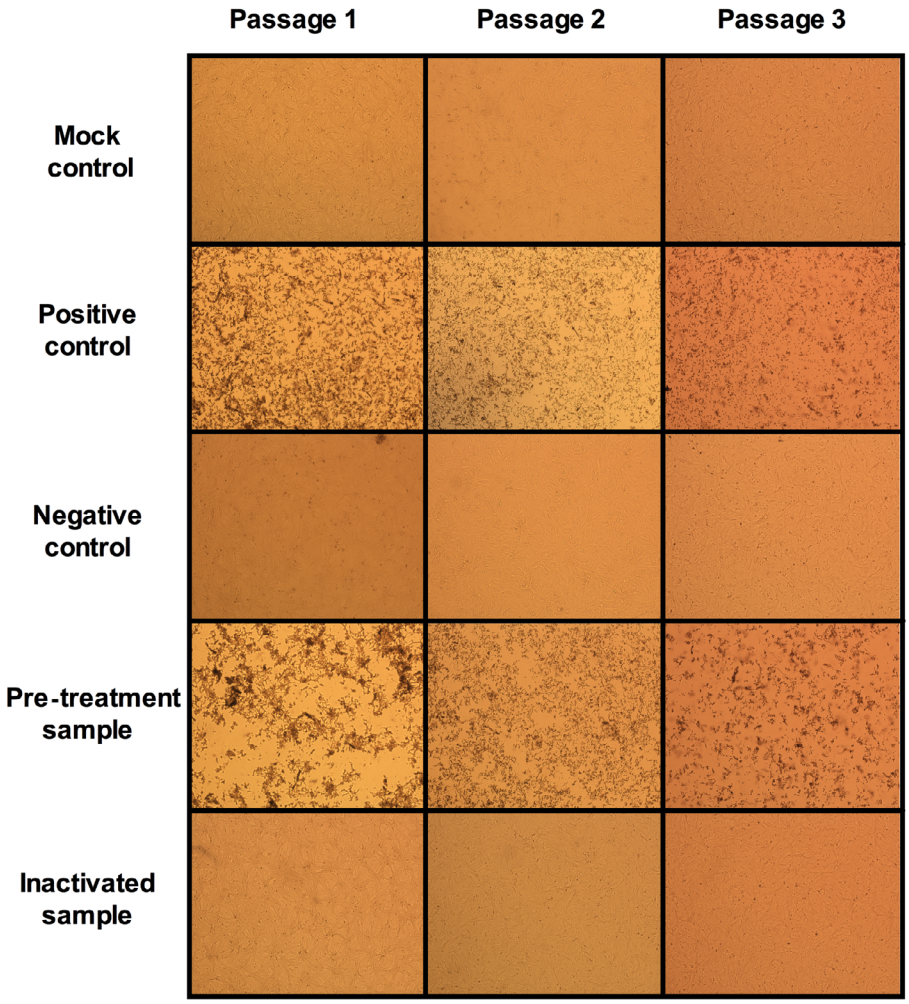

Fig. 3. Complete inactivation of replicative MERS-CoV by amotosalen and UVA treatment. Vero E6 cells were inoculated with DMEM only (Mock control), positive control, negative control, pre-treatment sample or inactivated sample and were passaged for three consecutive passages. Both the positive control and the pre-treatment sample caused extensive CPE by day 3 post-inoculation in all three passages. Mock control, negative control and inactivated sample did not show any CPE in Vero E6 cells. Photographs (4×) are shown from one representative platelet unit on day 3 post-inoculation in each passage.

Table 3. Results of passaging experiments of MERS-CoV in Vero E6 cells before and after inactivation of spiked platelets[1,2]

Experiment		Passage 1	Passage 2	Passage 3
A	Pre-treatment sample	7·00	7·04	9·28
	Inactivated sample	1·00	ND	ND
B	Pre-treatment sample	7·40	7·84	8·93
	Inactivated sample	1·75	0·39	ND
C	Pre-treatment sample	7·69	8·84	9·82
	Inactivated sample	1·26	ND	ND
D	Pre-treatment sample	8·37	8·45	9·84
	Inactivated sample	1·04	ND	ND

ND, not detected.

[1]Data are shown as $\log_{10}$ $TCID_{50}$eq/mL.

[2]Samples in Table 1 were used in this experiment. Samples were used at 1:10 dilution, and titre was determined on day 3 post-inoculation.

mock controls (Fig. 3) even after nine days of incubation in all three passages. For further confirmation, we determined the viral genomic titres from supernatants collected from all passages inoculated with either pre-treatment or post-treatment samples. As shown in Table 3, passaging of pre-treatment samples led to viral replication and an increase in viral titres upon passaging. On the other hand, viral infectivity titres in cells inoculated with inactivated samples decreased to lower or undetectable levels in culture supernatants. Together, these data confirm the complete inactivation of MERS-CoV in the tested platelets units and the absence of a replication-competent virus post-inactivation.

DISCUSSION

Outbreaks of emerging pathogens pose a high risk for blood safety as screening tests are often not available, and their occurrence, spread and impact are difficult to predict, making it hard to implement safety measures appropriately and in a timely manner. Recently, a chikungunya virus outbreak in Italy severely impacted blood availability in the Lazio region, causing an officially demanded halt in platelet supply. Only those centres in the region that had the INTERCEPT technology implemented were allowed to continue platelet product preparation and could thus prevent a complete stop in platelet supply by supporting the hospitals of the entire region (Vairo *et al.*, 2018). The Italian outbreak, the second after the Ravenna outbreak in 2008, was yet another example of recent emerging arbovirus outbreaks in the Mediterranean, likely enabled by the spread of vectors due to global warming or increased international travel and goods exchange. The 2015/2016 Zika virus outbreak in South and Central America is another example where the high number of asymptomatic carriers made it difficult to protect the blood supply (Lanteri *et al.*, 2016).

Although, to the best of our knowledge, transmission of MERS-CoV by blood transfusion has not yet been reported, the detection of MERS-CoV genomic RNA in the blood of infected patients raises concerns for blood safety (Stramer, 2014). Viral genomic RNA was detected in the plasma and serum of patients during MERS-CoV outbreaks in Saudi Arabia and South Korea, giving rise to concerns about potential transmission by blood transfusion. A viral load of 3·3 – 4·2 log genomic copies per mL in whole blood and 2·7 – 4·0 log genomic copies per mL in serum was reported in South Korea (Kim *et al.*, 2016). Viral loads up to 6 log genomic copies per mL, which correspond to ∼3 logs of infectious virus, in serum have also been reported during an outbreak in Saudi Arabia (Corman *et al.*, 2016). Although there are no reports on transfusion-related MERS-CoV transmission so far, viral RNA could be detected in patients' blood for almost two weeks post-diagnosis (Corman *et al.*, 2016), which may represent a possible route of transmission, especially that several studies have shown its replication in several blood cells (Chu *et al.*, 2014, 2016; Zhou *et al.*, 2014). Furthermore, the AABB lists MERS-CoV as a pathogen of concern for blood safety (Stramer, 2014). Therefore, it is important to investigate possible ways to reduce the risk of such transmission to avoid any effects on the blood supply, especially in endemic regions such as Saudi Arabia, which could lead to deferral of donations from confirmed and suspected cases as happened before during the SARS-CoV outbreak (WHO, 2019: https://www.who.int/csr/sars/guidelines/bloodsafety/en/).

We have previously described the inactivation of MERS-CoV in spiked human plasma samples (Hindawi *et al.*, 2018) with an average of 4·67 log reduction in infectious titre on amotosalen/UVA treatment. In this study, we report a mean of 4·48 log (4·18 – 4·85) reduction in the infectious titre of MERS-CoV in virus-spiked platelet samples, indicating that amotosalen/UVA treatment of MERS-CoV-spiked platelets can effectively inactivate the virus and potentially reduce the risk of MERS-CoV transmission through contaminated platelet units. This reduction is at least one log higher than the possible infectious titre in serum as reported previously (Corman *et al.*, 2016). Of note, the inactivation mechanism of the INTERCEPT treatment depends on cross-linking of nucleic acid strands, thereby preventing pathogen replication. Given the size of the amplicon, the detection of the viral genomic RNA by RT-qPCR was still possible after inactivation, and the genomic titre was only slightly affected by the pathogen inactivation treatment. This finding is not unusual as previously reported for other successfully inactivated viruses. Specifically, amotosalen/UVA light inactivation process results in cross-linking of the pathogen nucleic acid strands in a sequence-specific manner where an adduct is formed in the nucleic acids, thus inhibiting transcription and replication. However, this cross-linking is not necessarily at the site targeted by the RT-PCR. Thus, treatment is expected to affect viral replication, but the nucleic acid will remain detectable, which explains why the genomic titres are not reduced in a similar fashion as the infectivity reduction. Nonetheless, for additional safety, we assessed the presence of residual replication-competent particles. Inactivated platelet units were passaged three successive times for three days each, showing no CPE in the inactivated units. Viral genomes were

also not detected after nine days (three passages), showing the loss of replication-incompetent particles during passaging and confirming the total inactivation of all viral particles in the treated platelet units.

One major limitation of this study was the use of only four human platelets units, and subsequent studies should include larger number of samples. Although the addition of amotosalen alone without UVA light did not reduce viral titre in spiked samples (data not shown), the effect of the adsorption step alone without amotosalen and UVA light was not tested in this study and should be included in subsequent work to further determine the effect of all steps in the inactivation process. In addition, future studies should also investigate the possible effect of amotosalen and UVA light treatment on viral particles as inactivation may also be occurring via a path not directly associated with genomic alteration, such as covalent cross-linking of viral proteins or oxidative reactions induced by singlet oxygen generation from the process. Furthermore, possible virus attachment and adherence to platelets or even internalisation by platelets should be further tested. Nonetheless, our data show that the whole inactivation process is effective in reducing the risk of virus transmission via transfusion.

A recent study has reported a reduction of $\geq$3·7 log of MERS-CoV in spiked platelets prepared in 65% PAS and 35% plasma using UVC light (Eickmann *et al.*, 2018). In addition, a minimum reduction of $\geq$3·3 or $\geq$ 4·07 logs of MERS-CoV in plasma has been reported using methylene blue with visible light (Eickmann *et al.*, 2018) or riboflavin with UV light (Keil *et al.*, 2016), respectively. Although all these reports have shown marked reduction in infectious viral titres, which represent an acceptable safety margin according to the European Committee of Blood Transfusion, the presence of remaining or low levels of replication-competent virus after treatment should be investigated to confirm the complete inactivation of the virus. In addition, it would be interesting to observe the effect of these inactivation treatments on genomic viral load to correlate the results to the mean genomic titres of MERS-CoV in patients.

CONCLUSION

In this study, we showed efficient inactivation of MERS-CoV isolate spiked in human platelet concentrates in 100% plasma with amotosalen/UVA. Complete inactivation with a mean of 4·48 $\pm$ 0·3 log reduction of viral titre was confirmed by passaging experiments, excluding the remaining replication-competent particles. This technology represents an efficient method to reduce the possible risk associated with MERS-CoV, as well as other viruses, which may be transmitted via blood transfusion.

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A. M. Hash, A. M. Hass, A. M. T., M. A. A. and S. A. E. performed the research study. A. M. Hash, G. A. D., M. P. M., E. I. A. and S. I. H. designed the research study. Q. A. and S. I. H. contributed essential reagents and tools. A. M. Hash, A. M. Hass and M. P. M. analysed the data. A. M. Hash, M. P. M., E. I. A. and S. I. H. wrote the manuscript. All authors reviewed and approved the final draft.

CONFLICT OF INTEREST

QA and MPM are employees of the Cerus Corporation. The authors have no competing interests.

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