

ORIGINAL ARTICLE

Amotosalen photochemical inactivation of severe acute respiratory syndrome coronavirus in human platelet concentrates

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SUMMARY. A novel human coronavirus causing severe acute respiratory syndrome (SARS) emerged in epidemic form in early 2003 in China and spread worldwide in a few months. Every newly emerging human pathogen is of concern for the safety of the blood supply during and after an epidemic crisis. For this purpose, we have evaluated the inactivation of SARS-coronavirus (CoV) in platelet concentrates using an approved pathogen inactivation device, the INTERCEPT Blood System. Apheresis platelet concentrates (APCs) were inoculated with approximately 10^6 pfu mL⁻¹ of either Urbani or HSR1 isolates of SARS-CoV. The inoculated units were mixed with 150 µM amotosalen and illuminated with 3 J cm⁻² UV-A light. The viral titres were determined by plaque formation in Vero E6 cells. Mixing SARS-CoV

with APC in the absence of any treatment decreased viral infectivity by approximately 0.5–1 log₁₀. Following photochemical treatment, SARS-CoV was consistently inactivated to the limit of detection in seven independent APC units. No infectious virus was detected after treatment when up to one-third of the APC unit was assayed, demonstrating a mean log₁₀-reduction of >6.2. Potent inactivation of SARS-CoV therefore extends the capability of the INTERCEPT Blood System in inactivating a broad spectrum of human pathogens including recently emerging respiratory viruses.

Key words: amotosalen photochemical inactivation, platelet concentrates, SARS-coronavirus.

Platelet transfusions may result in the transmission of viral and bacterial diseases and cause adverse immune reactions. The advent of specific and sensitive tests for viral contamination has greatly reduced transfusion-associated transmission of selected viral diseases (Goodnough *et al.*, 1999). Nevertheless, multilayered testing has not eliminated all viral contamination because most tests are insensitive in detecting the presence of the pathogen during the window period before seroconversion of an infected donor (Dodd *et al.*, 2002). In this regard, collection of blood during this window period is likely to be the most important source of transfusion-associated human immunodeficiency virus (HIV) infections

(Busch *et al.*, 2000). Nucleic acid testing of mini-pools (MP-NAT) has reduced, although not eliminated, the window period for newly infected donors but may not detect those with a low HIV load during the acute phase (Phelps *et al.*, 2004). Some potential donors with silent chronic viral infections, such as hepatitis B virus (HBV), may have very low viral burdens without detectable antiviral antibodies, and thus remaining undetected by MP-NAT (Allain, 2004). With a combination of serological testing and MP-NAT in place, the residual risk of contamination for selected viruses has decreased to less than 1 : 2000 000 for HIV-1/2 and human T-cell leukaemia virus (HTLV-I/II), less than 1 : 1000 000 for hepatitis C virus and less than 1 : 200 000 for HBV (Goodnough *et al.*, 1999; Dodd *et al.*, 2002).

Although it has improved the safety of blood components, testing remains a reactive approach to blood safety. The contaminating organisms must be

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identified before sensitive tests can be developed. New pathogens, such as West Nile virus, continue to enter the donor population and may be transmitted before a sensitive test is in place (Harrington *et al.*, 2003). The recent emergence of new strains of pathogenic viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), demonstrates once again the susceptibility of the world's blood supply to previously unknown viruses that can spread globally in a short amount of time (Mahony *et al.*, 2004; Yu *et al.*, 2004).

The SARS has emerged in China and spread to several countries worldwide in a few months resulting in the infection of 8098 people and causing 744 deaths from November 1, 2002, to September 26, 2003 (World Health Organization, 2003). A novel human coronavirus (SARS-CoV) was consistently isolated from SARS patients in three different continents independently (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003; Vicenzi *et al.*, 2004). Human to human SARS-CoV transmission was contained in the same year (Lipsitch *et al.*, 2003); however, animal-human transmission was also documented implying that the virus could reemerge (Guan *et al.*, 2003). In fact, the disease was probably triggered by an animal virus that had acquired the ability to infect humans. In this case, it may be impossible to fully eradicate the virus, particularly if the natural host is a wild rather than a domesticated animal (Holmes, 2003). Human SARS-CoV transmission occurs by close contact with symptomatic infected individuals (Wu *et al.*, 2004). Thus far, there is no evidence that the virus is transmitted through the blood supply, although a viraemic phase has been described in the first week after the onset of illness (Grant *et al.*, 2003; Schmidt *et al.*, 2004). However, the possibility of introduction of the SARS-CoV into the blood supply should be prevented to avoid reemergence of this infectious agent by human to human route. In this regard, pathogen inactivation technologies offer a proactive approach and the potential to further improve blood safety. To reduce the risks associated with platelet transfusions, the INTERCEPT Blood System, a photochemical treatment (PCT) process utilizing a combination of amotosalen HCl and long-wavelength ultraviolet light has been developed and introduced into clinical practice in Europe (Wollowitz, 2001). *In vitro* studies and extensive clinical testing have demonstrated that PCT inactivates a broad spectrum of pathogens while preserving the haemostatic properties of platelets (van Rhenen *et al.*, 2003; McCullough *et al.*, 2004).

Our study demonstrates that the INTERCEPT Blood System inactivates high levels of SARS-CoV

inoculated into platelet concentrates in addition to the array of other human pathogens already proven to be killed by this system (Grass *et al.*, 1998; Lin *et al.*, 1998). Therefore, our observation extends the capability of the INTERCEPT Blood System in inactivating a broad spectrum of human pathogens to include recently emerging viral pathogens, such as SARS-CoV.

MATERIALS AND METHODS

Apheresis platelet concentrates

The experiments were conducted with apheresis platelet concentrate (APC) collected with the Amicus® Cell Separator (Baxter Healthcare, Deerfield, IL, USA). Each APC contained $3.0\text{--}6.0 \times 10^{11}$ platelets suspended in approximately 275–300 mL of 35% plasma and 65% InterSol® (also known as PAS III) in a PL 2410 plastic container (Baxter Healthcare).

PCT and UV-A illumination devices

PCT was performed using amotosalen HCl (150 µM) and UV-A light (320–400 nm). The processing device utilizes a closed system consisting of a series of plastic containers and an illumination source (Fig. 1). The experiments used a microprocessor-controlled Ultraviolet Illumination System capable of processing two platelet units at a time (Model R4R4007, Baxter Healthcare). The Ultraviolet Illumination System delivers a 3 J cm^{-2} UV-A treatment dose to each platelet unit in 3–6 min. The illumination device uses F15T12-BL fluorescent lamps mounted above and below the illumination tray and is air-cooled for temperature control. APCs were illuminated in the device while being agitated with reciprocal shaking.

Preparation of SARS-CoV isolates

The HSR1 viral isolate was obtained by inoculating VERO cells (ATCC, Rockville, MD, USA) with a sputum specimen from an Italian patient affected by a severe form of pneumonia of unknown aetiology with a history of travel from Vietnam to Italy in March 2003. By amplification of the second passage virus, the entire genome was sequenced and reported in GenBank under the accession number AY323977 (Vicenzi *et al.*, 2004). The viral stock was obtained in Vero E6 cells after two additional serial passages.

The Urbani strain of SARS-CoV (specimen number 809940, strain 200300592) was received from the

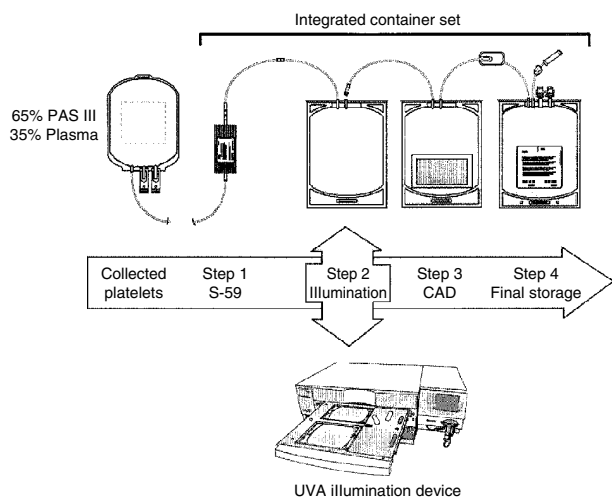


Fig. 1. Photochemical Treatment System – INTERCEPT Blood System for Platelets. The photochemical treatment system consists of an illumination device and a series of plastic containers. The disposable set provides a single-use, closed, integrated system for pathogen inactivation treatment of a platelet product (300 mL). The platelet product in a mixture of 35% plasma and 65% InterSol (also known as PAS III) is connected via a sterile connection device (SCD) and passed through a container of amotosalen HCl (also known as S-59) into an illumination container (Step 1). The integrated set is placed in the UV-A illumination device that provides a three J cm^{-2} dose of UV-A light to the mixture (Step 2). After illumination for 3–6 min, the platelets are transferred to a second container for treatment with a compound adsorption device (CAD) to lower the levels of residual amotosalen and free photoproducts (Step 3). After a minimum of 4 h of CAD treatment, the photochemical treatment platelets are transferred to a PL 2410 plastic container for storage up to 5 days (Step 4). The CAD step was not used in the viral inactivation experiments described in this report.

Centers for Disease Control (CDC), Atlanta, GA, USA. The viral stock was prepared from culture supernatant of infected Vero E6 cells (ATCC) after centrifugation to remove cell debris.

SARS-CoV inactivation experiments using the INTERCEPT blood system

Five replicate experiments were performed with SARS-CoV HSR1 strain in full-sized APC units. Each unit, in 275 mL of 35% plasma and 65% InterSol, was spiked with approximately 10^6 plaque-forming units per mL (pfu mL^{-1}) of SARS-CoV. An aliquot of this APC was tested after 5 min to determine the pretreatment viral infectivity titre. The spiked platelet mixture was then treated with

150 μM amotosalen and 3 J cm^{-2} UV-A light. Briefly, using a sterile connection device (SCD), the APC container spiked with SARS-CoV was sterile-connected in series with the amotosalen (S-59) container (Fig. 1, step 1). The APC in plasma/InterSol was passed through the amotosalen container into the illumination container. The air was expressed out of the illumination container, which contained the platelet concentrate, amotosalen and SARS-CoV and, subsequently, was separated from the amotosalen container by heat seal. In step 2, the amotosalen-platelet mixture was placed in the illumination device. The platelet mixture was exposed to a 3 J cm^{-2} treatment with UV-A with agitation ($60 \text{ cycles min}^{-1}$). After treatment, a sample was collected for measurement of the post-treatment viral infectivity titre. Each replicate experiment used an independent unit of APC. The infectious viral titre was measured using a standard plaque assay in Vero E6 cells. In the plaque assay, platelet samples were first diluted with tissue culture medium mixed with CaCl_2 at a final concentration of $50 \mu\text{M}$ and incubated at approximately 37°C for at least 1 h to allow time for clot formation, which was then removed by centrifugation. The clot-free supernatant was then used for the viral plaque assay. In two of the five replicates, up to 102 mL, or approximately one-third of the APC unit, were assayed for residual infectious virus. A larger volume was assayed to increase the dynamic range of the plaque assay.

The Urbani strain of SARS-CoV was evaluated for sensitivity to inactivation in APC in two independent experiments. The test unit contained 30 mL of APC suspended in 35% plasma and 65% InterSol instead of a full-sized (275 mL) APC unit. Each aliquot was spiked with approximately 10^6 pfu mL^{-1} of the virus and treated with $150 \mu\text{M}$ amotosalen and 3 J cm^{-2} UV-A light. After treatment, 3–13 mL of APCs were assayed for residual infectivity titres by plaque assay on Vero E6 cells. For these samples, heparin sulphate (5 U mL^{-1}) was added to the diluent in the plaque assay to prevent clotting.

SARS-CoV plaque assay

Samples of the test units containing the SARS-CoV were serially diluted in cell culture media (either Eagle's minimal essential medium with Earle's salts or Dulbecco's modified Eagle medium D-MEM, BioWhittaker, Verviers, Belgium) and supplemented with 10% fetal calf serum (HyClone or Perbio Science Erembodegem, Aalst, Belgium) and then inoculated onto Vero E6 cell monolayers for approximately 1 h at 37°C . The test samples were aspirated

and the adherent cell layers were overlaid with media containing either 0.75% agarose (Seaplaque, FMC Bioproducts, Rockland, ME, USA) or 1% carboxymethylcellulose (Sigma Chemical Corp., St. Louis, MS, USA). After 3–5 days of incubation at 37 °C, plaque numbers were scored visually after staining, if necessary, with neutral red (Sigma) or 1% crystal violet (Sigma) in 70% methanol.

Quantification of SARS-CoV genomes

Viral RNA, spiked into APC, was extracted using the Quiagen Viral RNA Mini Kit (Quiagen Inc., Santa Clarita, CA, USA) according to the manufacturer's instructions. The quantification of the viral RNA was performed using real-time polymerase chain reaction (PCR) in a TaqMan[®] assay after generation of cDNA (Vicenzi *et al.*, 2004). The following primer pair and probe: BNITMSARS1 TTATCACCCGCG AAGAAGCT, BNITMSARAS2 CTCTAGTTGCA TGACAGCCCTC and BNI-TMSARP 6-carboxyfluorescein-TGC GTG GAT TGG CTT TGA TGT-6-carboxy-tetramethylrhodamin were added to the universal PCR master mix (Applied Biosystems, Foster City, CA, USA) at 200 and 120 nM, respectively, in a final volume of 25 µL. The standard was obtained by cloning the 76-bp fragment into the pCR2.1 plasmid using the TA cloning kit (In Vitrogen Corp., San Diego, CA, USA). A linear distribution ($r = 0.99$) was obtained between 10^1 and 10^8 copies (data not shown).

Statistical analysis

The level of viral inactivation was calculated as \log_{10} -reduction using the formula: $\text{Log}_{10}\text{-reduction} = \text{Log}_{10}(\text{pretreatment viral titre}/\text{post-treatment viral titre})$.

After PCT, if no virus was detected, the viral titre was expressed as $<1 V^{-1}$ infectious units, where V is the total platelet volume assayed. Lack of virus detection indicated inactivation to below the limit of detection; thus, the \log_{10} reduction was expressed as greater than the input titre in the volume of platelet concentrate assayed.

RESULTS

Preliminary experiments were carried out in order to measure the SARS-CoV infectivity after mixing with APC in the absence of amotosalen and UV-A light treatments. For this purpose, SARS-CoV Urbani viral infectivity in culture medium was compared with viral infectivity after spiking into platelet concentrate and incubating in the platelet mixture for the duration of the experiment. The results showed a

mean decrease of viral infectivity titre of approximately $1 \log_{10}$ by mixing with platelets. Similarly, SARS-CoV HSR1 was inoculated into the platelet unit. In order not to exceed 1/10 of the total APC volume, 1 ml of viral stock was added to each 30 mL of platelet concentrate. After mixing the virus with the APC, aliquots were removed, and clot formation and retraction was carried out as described in *Materials and Methods*. The supernatant was then either tested in a plaque infectivity assay or subjected to RNA extraction for quantification of the viral genomes. Incubation of SARS-CoV HSR1 in control APC with no added amotosalen or UV-A light over the period of the experiment resulted in approximately 0.5 \log_{10} reduction in viral titre (from 5.38 ± 0.28 – $4.96 \pm 0.41 \log_{10}$ pfu mL⁻¹). The viral input (7.83 ± 0.41 copies/mL), as measured in real-time reverse transcription-polymerase chain reaction, was approximately 3 \log_{10} higher than the titre obtained from the plaque assay, confirming previous observations, which indicated that approximately 360 viral genomes were required to form a single plaque (Vicenzi *et al.*, 2004).

The INTERCEPT Blood System inactivated high titres of both the HSR1 and Urbani isolates of SARS-CoV (Table 1). In the five replicate experiments with the HSR1 strain of SARS-CoV, the pretreatment infectivity titre ranged from $10^{4.4}$ to $10^{5.4}$ pfu/mL in APC. After treatment, no viable virus was detected in test volumes of 3.5–102 mL, demonstrating a mean \log_{10} -reduction of $>6.1 \pm 0.9$ (range >4.9 to >7.4). Assaying 100–102 mL of the treated platelet sample increased the dynamic range of the plaque assay and demonstrated a \log_{10} -reduction of up to >7.4 . Similarly, in the two replicate experiments with the SARS-CoV Urbani strain, the pretreatment infectivity titre was $10^{5.4}$ and $10^{5.5}$ pfu mL⁻¹ in APC. After treatment, no viable virus was detected in test volumes of three and 13 mL, demonstrating inactivation of >5.9 and $>6.6 \log_{10}$ of viral infectivity, respectively. Taken together, in seven replicates, a mean \log_{10} -reduction of >6.2 was obtained. These results show that SARS-CoV is highly sensitive to PCT with amotosalen and UV-A.

DISCUSSION

In the present study, we have demonstrated that SARS-CoV was inactivated in platelet concentrates to below the limit of detection. A mean \log_{10} -reduction of $>6.2 \pm 0.2$ (range >4.9 to >7.4) was achieved by treatment with the INTERCEPT Blood System. The efficacy of pathogen inactivation of the INTERCEPT Blood System was demonstrated against two different SARS-CoV viral isolates. An

Table 1. Inactivation of severe acute respiratory syndrome-coronavirus (SARS-CoV) after treatment with 150 µM amotosalen and 3 J cm⁻² UV-A in apheresis platelet concentrate (APC)

SARS-CoV*	Pretreatment titre (pfu mL ⁻¹)	Volume (mL) assayed	Post-treatment titre†	Log ₁₀ -reduction‡ (pfu mL ⁻¹)
Replicate number 1 HSR1	10 ^{4.4}	3.5	<10 ^{-0.5}	>4.9
Replicate number 2 HSR1	10 ^{4.6}	17.5	<10 ^{-1.2}	>5.8
Replicate number 3 HSR1	10 ^{4.9}	17.5	<10 ^{-1.2}	>6.2
Replicate number 4 HSR1	10 ^{4.4}	100	<10 ^{-2.0}	>6.4
Replicate number 5 HSR1	10 ^{5.4}	102	<10 ^{-2.0}	>7.4
Replicate number 6 Urbani	10 ^{5.4}	3	<10 ^{-0.5}	>5.9
Replicate number 7 Urbani	10 ^{5.5}	13	<10 ^{-1.1}	>6.6
Mean ± SD	10 ^{4.9 ± 0.5}	36.6 ± 44.3	<10 ^{-1.2 ± 0.6}	>6.2 ± 0.7

*Inactivation experiments were performed in 30 mL aliquots of APC with the Urbani strain and in full-sized (275 mL) units of APC with the HSR1 strain.

†The '<' sign indicates that no infectious virus was detected in the volume assayed.

‡The '>' sign indicates inactivation to below the limit of detection.

effective pathogen inactivation system for blood components must have a broad-spectrum inactivation capability while maintaining the biological properties of the blood components. In the development of the INTERCEPT Blood System for platelets described in this report, studies were performed to demonstrate inactivation of bacteria, viruses, protozoa, and leucocytes in platelet concentrates (Lin *et al.*, 1998; VanVoorhis *et al.*, 2003). In this report, we demonstrate that amotosalen plus UV-A inactivates SARS-CoV, the novel human coronavirus that causes SARS. Effective inactivation was demonstrated against two independent viral isolates. The phylogenetic analysis showed that the two strains employed in this study have a common origin being more similar to those that originated in Hong Kong than those in North China and Taiwan (Vega *et al.*, 2004; Vicenzi *et al.*, 2004). By nucleotide sequence comparison, the Urbani isolate only differs by six nucleotide changes from the HSR1 isolate (Rota *et al.*, 2003; Ruan *et al.*, 2003; Vicenzi *et al.*, 2004). In this regard, the differences between the two isolates are consistent with the low mutation rate of SARS-CoV. SARS-CoV has been shown to have the lowest mutation frequency among RNA viruses (Yeh *et al.*, 2004).

The potential of SARS-CoV transmission through the blood supply is unknown, whereas close contact with symptomatic patients is the main route of transmission (Cheng *et al.*, 2004; Tong & Liang, 2004). SARS-CoV, however, has been detected in blood of infected individuals (Grant *et al.*, 2003; Yam *et al.*, 2003). The current NAT assays have, however, important limitations because they detect the SARS-CoV in only 75% of infected individuals during the

first week of symptoms (Yam *et al.*, 2003; Drosten *et al.*, 2004; Ng *et al.*, 2004). False-negative NAT results can occur due to PCR inhibitors or faulty RNA extraction. False-positive NAT results have also been reported with the consequence of triggering alarm bells worldwide (Yu, 2004). Real-time PCR assays have been modified in order to improve specificity and sensitivity (Lau *et al.*, 2003; Poon *et al.*, 2004). The window period for detection of SARS-CoV in blood remains undefined; in this regard, it has been postulated that the time course of SARS-CoV viremia may be relatively short, in that the viral load is too low to be detected 12–14 days after the onset of symptoms (Chen *et al.*, 2004; Ng *et al.*, 2004). In addition, the prevalence of individuals infected with the SARS-CoV in high-risk areas remains unknown. A recent report supports the existence of sub clinical or nonpneumonic SARS-CoV infections (Woo *et al.*, 2004). Nonpneumonic SARS-CoV infection was present in healthy blood donors during the SARS outbreak in Hong Kong (Woo *et al.*, 2004), although the levels of SARS-CoV viremia were not reported. Significant correspondence, however, was generated by respondents casting doubt on Woo's findings (Yip *et al.*, 2004; Theron, 2004; Young, 2004; Zhou, 2004). Nevertheless, pathogen inactivation is a proactive approach to blood safety for pre-emptively dealing with new emerging agents before they affect the safety of the blood supply without necessarily knowing the levels of infectious particles in the blood stream. The INTERCEPT Blood System utilizing amotosalen and UV-A light has been shown to inactivate bacteria, viruses, protozoa, and leucocytes (Lin *et al.*, 1997; VanVoorhis *et al.*, 2003; Lin *et al.*, 2004).

The INTERCEPT Blood System is a nucleic acid-targeted technology that does not require specific viral sequences. The aggregate data demonstrated that PCT has a broad-spectrum efficacy against a variety of enveloped and non-enveloped, RNA and DNA, known and emerging viruses in platelet concentrates, and therefore, offers the potential to prospectively prevent the majority of platelet transfusion-associated viral diseases.

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