Assessment of dengue virus inactivation in random donor platelets using amotosalen and ultraviolet A illumination

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Abstract:
OBJECTIVES: The study objective was evaluation of amotosalen and ultraviolet A (UVA) illumination-based inactivation of dengue virus (DENV) in blood platelets.

MATERIALS AND METHODS: Whole blood was collected from healthy donors and platelet concentrates were prepared at a tertiary care hospital in Gurugram, India. Platelet units collected from five blood group matched individuals were pooled and spiked with DENV. The spiked platelet units were subjected to amotosalen treatment followed by UVA illumination, to evaluate the efficiency of this method for viral inactivation. The treated platelet units were evaluated for the presence of infectious DENV. Amotosalen levels were quantified in the treated samples using high-performance liquid chromatography.

RESULTS: The presence of replicating DENV was not observed in spiked platelet units treated with amotosalen and UVA illumination, whereas untreated units contained actively replicating DENV. Amotosalen levels were found to be in the permissible range after photochemical inactivation.

CONCLUSIONS: Amotosalen/UVA pathogen inactivation treatment showed efficient inactivation of DENV in platelet components. Therefore, it seems to be a promising method for mitigating the risk of dengue transmission through transfusion of potentially contaminated platelet components in dengue-endemic countries such as India.

Keywords: Amotosalen, dengue virus inactivation, photoinactivation, random donor platelets, transfusion transmitted infection, transfusion

Introduction

Dengue virus (DENV) is a single-stranded positive sense RNA virus from the family Flaviviridae. It was first reported in 1789 in Philadelphia and since then, many outbreaks have occurred in different parts of the world. DENV is classified into four different serotypes based on their distinct antigenicity. While DENV is an arbovirus and primarily transmitted by Aedes aegypti mosquitoes, there have been reports of transfusion transmitted infections (TTIs).

In the last decade, numerous cases of asymptomatic dengue have been reported, which raised major concerns of transfusion transmitted dengue infection. For instance, in a study conducted in Pakistan, a total of 5230 individuals of age above 18 years (potential healthy blood donors) without any history of dengue fever (DF) were screened for the presence of anti-DENV IgG in blood serum. Overall, 1691 (32.3%) individuals were found to be cases of asymptomatic dengue transmission.
dengue infection.\(^1\) Also in various studies performed in different endemic areas, blood donors were screened for the presence of DENV RNA loads and were found to be positive for DENV RNA.\(^2\)–\(^4\) One study reported of a case in Puerto Rico where an individual developed dengue hemorrhagic fever after a blood transfusion, where the donor had no history of DF and not even reported any dengue-related symptoms after donating blood.\(^5\) All these studies emphasize the need to develop strategies to detect or inactivate pathogens in the blood prior to transfusion.

Primarily, three components of blood that have been reported to be a potential source of DENV during transfusions are fresh frozen plasma, platelets (random donor platelet [RDP]/single donor platelet), and red blood cells (RBCs). Among these components, platelets are highly active in metabolism and play an important role in maintaining hemostasis in circulation.\(^6\) Considering that platelet transfusion is one of the treatments for severe dengue cases as well as several other ailments, such mode of transmission is a major public health concern and hence it is essential to screen the collected blood components for pathogens. However, it is not possible to screen all the samples for all pathogens and it is crucial to devise efficient strategies for removal or inactivation of pathogens from the blood components before transfusions.

Several methods have been developed for inactivation of pathogens in blood components prior to transfusions.\(^6\) Depending on geographies, there are currently two commercially available pathogen inactivation systems, namely MIRASOL and INTERCEPT Blood System for platelets, and a third system, the THERAFLEX-UV platelet system is undergoing clinical trials.\(^7\)–\(^11\)

Photoinactivation in the presence of amotosalen using ultraviolet A (UVA) light illumination has been tested for several viruses such as Zika virus, chikungunya virus, and DENV (in plasma).\(^12\)–\(^15\) Amotosalen binds nonspecifically to the helical regions of DNA and RNA, and upon UVA light illumination undergoes a \((2 \pm 2)\) photoaddition reaction with pyrimidine bases, forming an interstrand crosslink.\(^8\)–\(^9\) These interactions restrict the replication of leukocytes and pathogens such as viruses, bacteria, and parasites. The present study was conducted to evaluate this photoinactivation method for DENV inactivation in whole blood-derived RDP pools.

### Materials and Methods

**Study design, donors, and preparation of platelet concentrates**

Fresh blood was taken for platelet collection and the platelets were processed immediately. Briefly, the platelets were harvested and subjected to DENV infection and UV treatment on the same day and further evaluated for infection for a period of 45 days. Figure 1 represents the schematic of workflow.
The initial procedure of blood collection and preparation of platelet concentrates was performed in a span of 3 months (April 2017–June 2017) at the department of Transfusion Medicine, Medanta, a tertiary care hospital in Gurugram, India. Healthy donors were informed and consented to the study. All donors were screened for DENV antigens using the rapid antigen test and only those who tested negative were included in the study.

Whole blood was collected from a total of 25 healthy blood donors, processed, and platelet concentrates were prepared by Single Buffy-Coat method in 100% plasma. Five RDP units of compatible blood group (ABO and Rh “D” matched) were prepared by pooling five platelet concentrates as a pooled platelet unit. The final units (PBC-01–05) were evaluated by swirling and 1 mL of platelet concentrate was taken from each unit to check for RBC, white blood cell (WBC), and platelet count, to meet INTERCEPT processing criteria, i.e., the processing volume of a platelet unit should be between 300 mL and 420 mL, with a platelet count of 2.5 × 10¹¹ to 7.0 × 10¹¹, while the RBC content should be <4 × 10¹¹. All the RDP units were stored at 22°C with constant agitation until further processing. The platelets were then transferred to the International Centre for Genetic Engineering and Biotechnology (ICGEB) for further processing. In addition, all the platelet units were screened for the presence of DENV RNA and active DENV, using reverse transcription-polymerase chain reaction and focus forming assay (FFA), respectively, at ICGEB. The study was approved by the Institutional Ethics Committee from ICGEB (ICGEB/IEC/2017/04, version 2) and Medanta (MICR-679/2016).

**Viruses propagation**

The DENV used in this experiment was a reference strain that originated from a clinical isolate during an outbreak in New Guinea in 1944, supplied from the American Type Culture Collection (ATCC) (ATCC VR-1584–DENV-2, Manassas, Virginia, USA). The virus was propagated in C6/36 cells (ATCC CRL-1660, Manassas, Virginia, USA) as per the instructions from the supplier guidelines and harvested after complete cytopathic effect was observed. Virus stock was concentrated using polyethylene glycol (P2139 Sigma-Aldrich St Louis, Missouri, USA) concentration method and stored at −80°C. DENV titer in the viral stocks was determined using FFA and was found to be 1.8 × 10⁸ focus-forming units (FFUs) per mL.

**Infection of platelets with dengue virus**

Three Hundred mL from each of the five RDP units was spiked with 10⁶ FFUs of DENV. And 20 mL of this mix was collected from each unit which served as a positive control (Pretreatment samples) and stored at −80°C, while the rest was used for further processing.

**Amotosalen and ultraviolet A treatment**

In this study, the INTERCEPT (large volume [LV]) (Cerus corporation, Concord, California, USA) set was used as previously described with a few modifications. The INTERCEPT LV set includes a 1 L PL 2410 illumination container, a 1 L PL 2410 container with a Compound Adsorption Device (CAD), an in-line filter, a small bag of 17.5 mL of 3 mM amotosalen solution, and a 1.3 L PL 2410 final storage container. As it was an evaluation study and processed RDP units were to be discarded, CAD and the final storage container were not used. The inactivation process was performed in two steps; addition of amotosalen, and UVA illumination. Each infected RDP unit was attached to the amotosalen bag using a sterile connecting device. The whole unit of infected platelet concentrates was passed through the amotosalen container. The entire contents passing through the amotosalen bag were collected in the illumination container. After collection, the illumination container was mixed gently and a sample was withdrawn to determine pre-UVA DENV titer (Control) and amotosalen concentration by high-performance liquid chromatography (HPLC). The entire illumination container was then illuminated with UVA light using the INT 100 Illuminator (INTERCEPT Blood System) (Cerus Corporation, Concord, California, USA). Post illumination, a 20 mL sample of platelet concentrate (test sample or posttreatment sample) was withdrawn from the illumination container and stored at −80°C. This sample was later used to determine post-UVA viral titer (Test) and post-UVA amotosalen concentration. The same process was followed for all the five infected RDP units.

**Detection of replicative dengue virus and viral quantification using focus-forming assay**

To evaluate DENV’s replication ability post-inactivation, all the five positive control (pretreatment) and five test (posttreatment) samples were used to inoculate C6/36 cells in 6-well plates, separately. These plates were incubated at 28°C with 5% CO₂ and 75% humidity for 7 days. After 7 days, the media from each well was collected and centrifuged for the removal of cell debris. These collected samples were used to inoculate another set of C6/36 and a similar process was repeated five times. After five rounds of propagation in C6/36 cells, all the samples collected were evaluated for DENV titer using standard FFA protocol. Vero cells (ATCC CCL-81, Manassas, Virginia, USA) were seeded in 96-well plates, followed by inoculation of the samples at different dilutions. Inoculated plates were incubated at 37°C for 2 h. Post incubation, the inoculums were aspirated and an overlay containing 5% fetal bovine serum (RM10681 Himedia, Mumbai, India) and 1% carboxymethyl cellulose (C4888 Sigma-Aldrich St Louis, Missouri, USA) in Dulbecco’s Modified Eagle...
Medium was added to the plates. These plates were incubated at 37°C for 72 h. After 72 h of infection, the overlay medium was removed from the wells, and cells were washed with phosphate-buffered solution (PBS), followed by fixation using acetone: methanol (1:1) for 30 min. After fixation and PBS washing, the cells were blocked with 5% skim milk (GRM1254 HIMEDIA, Mumbai, India) in PBS for 30 min. The infected cells were detected with a monoclonal anti-dengue antibody (SAB270223 Sigma-Aldrich St Louis, Missouri, USA). After washing with PBS, antibody-labeled cells were detected with Horseradish peroxidase conjugated secondary antibody (NB120-6808 Novus Biologicals, Littleton, Colorado, USA). The labeling was visualized with 3, 3', 5, 5'-tetramethylbenzidine (5510 Seracare, Milford, Massachusetts, USA). The FFUs were counted, and the viral titers were calculated.

Similarly, to validate complete inactivation of DENV by amotosalen and UVA treatment, posttreatment samples were also passaged in Vero cells for confirmation of results in a mammalian system. Vero cells inoculated with posttreatment samples were kept at 37°C with 5% CO₂ and 75% humidity for 7 days.

**High-performance liquid chromatography analysis for amotosalen quantification**

Photochemical inactivation was followed by HPLC-based quantification of residual amotosalen after illumination.[17] Pre-UVA and post-UVA amotosalen concentration for all the five DENV infected units was measured by HPLC.[16]

**Table 1: Mean of viral load quantified using focus-forming assay, during five passages in C6/36 and table Vero cells**

| Sample ID | Treatment | Passage 1 | Passage 2 | Passage 3 | Passage 4 | Passage 5 |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| PBC-1     | Pretreatment passaged in C6/36 cells | 7.78E+05 | 1.29E+06 | 2.10E+06 | 2.80E+06 | 3.03E+06 |
|           | Posttreatment passaged in C6/36 cells | -         | -         | -         | -         | -         |
|           | Posttreatment passaged in Vero cells | -         | -         | -         | -         | -         |
|           | Log inactivation | 5.89      | 6.11      | 6.32      | 6.44      | 6.48      |
| PBC-2     | Pretreatment passaged in C6/36 cells | 8.30E+05 | 1.29E+06 | 2.04E+06 | 3.13E+06 | 4.30E+06 |
|           | Posttreatment passaged in C6/36 cells | -         | -         | -         | -         | -         |
|           | Posttreatment passaged in Vero cells | -         | -         | -         | -         | -         |
|           | Log inactivation | 5.91      | 6.11      | 6.30      | 6.49      | 6.63      |
| PBC-3     | Pretreatment passaged in C6/36 cells | 6.80E+05 | 1.08E+06 | 1.92E+06 | 2.94E+06 | 3.40E+06 |
|           | Posttreatment passaged in C6/36 cells | -         | -         | -         | -         | -         |
|           | Posttreatment passaged in Vero cells | -         | -         | -         | -         | -         |
|           | Log inactivation | 5.83      | 6.03      | 6.28      | 6.46      | 6.53      |
| PBC-4     | Pretreatment passaged in C6/36 cells | 6.40E+05 | 9.60E+05 | 1.68E+06 | 2.49E+06 | 3.10E+06 |
|           | Posttreatment passaged in C6/36 cells | -         | -         | -         | -         | -         |
|           | Posttreatment passaged in Vero cells | -         | -         | -         | -         | -         |
|           | Log inactivation | 5.80      | 5.98      | 6.22      | 6.39      | 6.49      |
| PBC-5     | Pretreatment passaged in C6/36 cells | 7.20E+05 | 1.10E+06 | 1.79E+06 | 2.50E+06 | 3.20E+06 |
|           | Posttreatment passaged in C6/36 cells | -         | -         | -         | -         | -         |
|           | Posttreatment passaged in Vero cells | -         | -         | -         | -         | -         |
|           | Log inactivation | 5.85      | 6.04      | 6.25      | 6.30      | 6.50      |

PBC=Pooled buffy coat

**Results**

**Detection and quantification of active dengue virus**

Five pooled platelet units were prepared with the volume ranging from 308.73 mL to 372.13 mL (mean ±standard deviation [SD]) 336.88 ± 24.28205. RBC, WBC, and platelet counts in each unit were recorded (platelet concentration range [postsampling] [10^7/μL] 865–1084, mean ±SD] −1017.8 ± 112.7218; RBC concentration range postsampling [10^6/μL] 0.05–0.13, mean ±SD] −0.076 ± 0.051284; and WBC concentration range postsampling [10^5/μL] 0.1–0.5, mean ±SD] −0.3 ± 0.158114). DENV infectious titers in pretreatment samples ranged from 6.4 × 10^6 to 8.3 × 10^6 FFU/mL after first round of propagation in C6/36 cells and increased up to 3.03 × 10^9 to 4.3 × 10^9 FFU/mL after the fifth round of propagation in C6/36 cells. In contrast, no replicating infectious DENV was seen in posttreatment samples even after five rounds of propagation in C6/36 cells. Also, no replicating infectious DENV was detected in posttreatment samples in Vero cells. Hence, the presence of active DENV was assessed after each passage of 7 days, up to five passages in mosquito and mammalian cells, and no replicating DENV was detected in either system [Table 1].

**Amotosalen quantification using high-performance liquid chromatography analysis**

Amotosalen concentration was recorded to be ranging from 109.45 μM to 132.23 μM in the pre-UVA illumination
samples with an average of 122.8 ± 8.6 μM, which was reduced to an average of 69.5 ± 7.8 μM, ranging from 59.22 μM to 78.94 μM post UVA illumination. The average of the remaining amotosalen concentration, expressed as a percentage of the determined preillumination concentration, in the five platelet units was recorded to be 56.6% ± 4.6%.

The drop in the levels of amotosalen indicates appropriate photo-conversion of amotosalen to photoproducts.[8] The initial pre-UVA illumination and final post-UVA illumination concentrations of amotosalen are shown in Table 2.

### Discussion

Screening for pathogens in the blood components collected at the blood banks has become a necessity prior to any transfusions, as several studies have reported of TTIs around the globe.[218-20] Furthermore, for any pathogen detected in the blood components, the inactivation of those pathogens would be a boon for the patients in need of blood transfusions. For inactivation of DENV from blood components, amotosalen treatment followed by photo-inactivation using UVA illumination has been analyzed for inactivation of DENV from RBCs and blood plasma and has shown promising results with no active DENV being detected posttreatment.[14,21]

The present study further adds to the older reports that this method of pathogen inactivation efficiently inactivates DENV in platelets spiked with high titers of DENV and the virus is not able to replicate in either mosquito or mammalian cell systems. This study showed complete inactivation of DENV in platelets by amotosalen and UVA treatment, validated by culturing of treated samples in C6/36 and Vero cells five times. This negates the presence of active DENV in treated samples, as presence of even a single active DENV particle would have led to the replication of DENV, during five rounds of culture propagation in C6/36 or Vero cells. Also, results of amotosalen quantification suggest that amotosalen added to the platelets during the experiment is being reduced to the nontoxic levels.[8,22] In the present study, CAD unit was not used upon UVA and amotosalen treatment. Use of CAD further reduces the amount of amotosalen present in the treated platelets.[6]

With every such pathogen inactivation system arises the concern for biosafety in its clinical usage. In that regard, amotosalen has been tested for various toxic effects and end product accumulation in mammalian systems. It has been subjected to extensive toxicology studies involving genotoxicity, carcinogenicity, phototoxicity, and reproductive toxicity.[8,23] In this study, very high concentrations of amotosalen were used as compared to the actual clinical exposures.[23] Toxicological studies of this system has been evaluated by various international bodies such as Food and Drug Administration (North America), Paul Ehrlich Institute (Germany), Swiss Medics (Switzerland), and Afssaps (France).[8] This method of pathogen inactivation has also been tested for inactivation of several other pathogens such as chikungunya virus, Zika virus, cytomegalovirus, *Staphylococcus aureus*, *Escherichia coli* with considerable success.[12,13,15,16,24,25]

The present study is important, especially in a country setting such as India, for the following reasons: transfusion transmitted dengue infection is possible in only two conditions, one being receiving the blood from an asymptomatic carrier of DENV and other is receiving blood from an individual in whom the virus is in incubation phase prior to the onset of fever and other symptoms. During these two conditions, the asymptomatic donor is unaware of the presence of virus in his/her system and is a potential source of TTI, especially during outbreak conditions in endemic regions and a thorough prior analysis of the donor blood is important to prevent further spread of infections to transfusion patients.

This system of pathogen inactivation in blood components using amotosalen and UVA inactivation could be a masterstroke in preventing TTIs as it inactivates a broad spectrum of pathogens.

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**Conflicts of interest**

There are no conflicts of interest.

### References

1. Rafique I, Saqib MA, Munir MA, Qureshi H, Taseer IU, Iqbal R, et al. Asymptomatic dengue infection in adults of major cities of Pakistan. Asian Pac J Trop Med 2017;10:1002-6.

2. Chuang V, Wong TY, Leung YH, Ma E, Law YL, Tsang O, et al.  Asian Journal of Transfusion Science - Volume 16, Issue 1, January-June 2022

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### Table 2: Preultraviolet a and postultraviolet A amotosalen concentration

| Sample ID | Pre-UVA (µM) | Post-UVA (µM) | Percentage remaining |
|-----------|--------------|---------------|---------------------|
| PBC-01    | 132.23       | 68.26         | 51.6                |
| PBC-02    | 128.19       | 78.94         | 61.6                |
| PBC-03    | 121.70       | 65.86         | 54.1                |
| PBC-04    | 122.42       | 75.17         | 61.4                |
| PBC-05    | 109.45       | 59.22         | 54.1                |
| Average±SD| 122.8±8.6    | 69.5±7.8      | 56.6±4.6            |

SD=Standard deviation, UVA=Ultraviolet A, PBC=Pooled buffy coat
Review of dengue fever cases in Hong Kong during 1998 to 2005. Hong Kong Med J 2008;14:170-7.

3. Stramer SL, Linnen JM, Carrick JM, Foster GA, Krysztof DE, Zou S, et al. Dengue viremia in blood donors identified by RNA detection and detection of dengue transfusion transmission during the 2007 dengue outbreak in Puerto Rico. Transfusion 2012;52:1657-66.

4. Tambyah PA, Koay ES, Poon ML, Lin RV, Ong BK; Transfusion-Transmitted Dengue Infection Study Group. Dengue hemorrhagic fever transmitted by blood transfusion. N Engl J Med 2008;359:1526-7.

5. Ghoshal K, Bhattacharyya M. Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. ScientificWorldJournal. 2014;2014:781857.

6. Schubert P, Johnson L, Marks DC, Devine DV. Ultraviolet-based pathogen inactivation systems: Untangling the molecular targets activated in platelets. Front Med (Lausanne) 2018;5:129.

7. Goodrich RP, Doane S, Reddy HL. Design and development of a method for the reduction of infectious pathogen load and inactivation of white blood cells in whole blood products. Biologicals 2010;38:20-30.

8. Irsch J, Lin L. Pathogen inactivation of platelet and plasma blood components for transfusion using the INTERCEPT Blood SystemTM. Transfus Med Hemother 2011;38:19-31.

9. Irsch J, Seghatchian J. Update on pathogen inactivation treatment of plasma, with the INTERCEPT blood system: Current position on methodological, clinical and regulatory aspects. Transfus Apher Sci 2015;52:240-4.

10. Mohr H, Steil L, Gravemann U, Thiele T, Hammer E, Greinacher A, et al. A novel approach to pathogen reduction in platelet concentrates using short-wave ultraviolet light. Transfusion 2009;49:2612-24.

11. Seghatchian J, Tolksdorf F. Characteristics of the THERAFLEX UV-platelets pathogen inactivation system – An update. Transfus Apher Sci 2012;46:221-9.

12. Aubry M, Richard V, Green J, Broult J, Musso D. Inactivation of Zika virus in plasma with amotosalen and ultraviolet A illumination. Transfusion 2016;56:33-40.

13. Laughhunn A, Huang YS, Vanlandingham DL, Lanteri MC, Stassinopoulos A. Inactivation of chikungunya virus in blood components treated with amotosalen/ultraviolet A light or amustaline/glutathione. Transfusion 2018;58:748-57.

14. Musso D, Richard V, Broult J, Cao-Lormeau VM. Inactivation of dengue virus in plasma with amotosalen and ultraviolet A illumination. Transfusion 2014;54:2924-30.

15. Santa Maria F, Laughhunn A, Lanteri MC, Aubry M, Musso D, Stassinopoulos A. Inactivation of Zika virus in platelet components using amotosalen and ultraviolet A illumination. Transfusion 2017;57:2016-25.

16. Makroo RN, Sardana R, Mediratta L, Butta H, Thakur UK, Agrawal S, et al. Evaluation of bacterial inactivation in random donor platelets and single-donor apheresis platelets by the INTERCEPT blood system. Asian J Transfus Sci 2018;12:146-53.

17. Lin L, Conlan MG, Tessman J, Cimino G, Porter S. Amotosalen interactions with platelet and plasma components: Absence of neoantigen formation after photochemical treatment. Transfusion 2005:45:1610-20.

18. Dean CL, Wade J, Roback JD. Transfusion-transmitted infections: An update on product screening, diagnostic techniques, and the path ahead. J Clin Microbiol 2018;56:e00352-18.

19. Peterman T, Lui KJ, Lawrence DN, Allen JR. Estimating the risks of transfusion-associated acquired immune deficiency syndrome and human immunodeficiency virus infection. Transfusion 1987;27:371-4.

20. Walsh JH, Purcell RH, Morrow AG, Chanock RM, Schmidt PJ. Posttransfusion hepatitis after open-heart operations. Incidence after the administration of blood from commercial and volunteer donor populations. JAMA 1970;211:261-5.

21. Aubry M, Laughhunn A, Santa Maria F, Lanteri MC, Stassinopoulos A, Musso D. Pathogen inactivation of Dengue virus in red blood cells using amustaline and glutathione. Transfusion 2017;57:2888-96.

22. Tice RR, Gatehouse D, Kirkland D, Speit G. The pathogen reduction treatment of platelets with S-59 HCl (Amotosalen) plus ultraviolet A light: Genotoxicity profile and hazard assessment. Mutat Res 2007;630:50-68.

23. Ciavarino V. Preclinical safety of a nucleic acid-targeted helinx TM compound: A clinical perspective. Semin Hematol 2001;38:12-9.

24. Roback JD, Conlan M, Drew WL, Ljungman P, Nichols WG, Preiksaitis JK. The role of photochemical treatment with amotosalen and UV-A light in the prevention of transfusion-transmitted cytomegalovirus infections. Transfus Med Rev 2006;20:45-56.

25. Tsatsarkin KA, Sampson-Johnasses A, Sawyer L, Kinsey J, Higgs S, Vanlandingham DL. Photochemical inactivation of chikungunya virus in human apheresis platelet components by amotosalen and UVA light. Am J Trop Med Hyg 2013;88:1163-9.