

Pathogen-reduction methods: advantages and limits

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Pathogen-reduction (inactivation) provides a proactive approach to reducing transfusion-transmitted infection. Pathogen-reduction technologies have been successfully implemented by plasma fractionators resulting in no transmission of human immunodeficiency, hepatitis C, or hepatitis B viruses by US-licensed plasma derivatives since 1987. Fractionation technologies cannot be used to treat cellular blood components. Although blood donor screening, deferral and disease testing have drastically reduced the incidence of transfusion-transmitted diseases, the threat of new or re-emerging pathogens remains. Of particular concern is the silent emergence of a new agent with a prolonged latent period in which asymptomatic infected carriers would donate and spread infection. The ultimate goal of pathogen-inactivation is to reduce transmission of potential pathogens without significantly compromising the therapeutic efficacy of the cellular and protein constituents of blood. The acceptable technology must not introduce toxicities into the blood supply nor result in neoantigen formation and subsequent antibody production. Several promising pathogen-inactivation technologies are being developed and tested, and others are currently in use, but all of them have limits. Pathogen-reduction promises an additional 'layer of protection' from infectious agents and has the potential to impact the safety of blood transfusions worldwide.

Key words: Blood safety, pathogen inactivation, pathogen reduction, transfusion, transfusion safety.

The first decade of the 21st century remains an age of emerging and re-emerging pathogens that threaten the blood supply. Blood collectors appreciate the dramatic reduction in risk of transfusion-transmissible infections, even as they are sobered by the failures of the 20th century safeguards to prevent widespread transmission of human immunodeficiency virus (HIV) and hepatitis viruses [1]. The specter of a new, as yet undiscovered agent with an extended latent phase raises the concern that the current system of overlapping safeguards that protects patients from infectious blood components is still disturbingly vulnerable.

Until recently, the approach to blood safety depended upon a combination of donor education, screening, testing for selected agents and discarding components in inventory if

donor exposure or illness was reported post-donation. This strategy, while effective, is reactive. Pathogen reduction of blood components represents a proactive approach to blood safety [2]. Inactivation technologies promise an additional layer of protection both from infectious agents that are known as well as from those not yet recognized as threats to the blood supply (Table 1). A method with broad antimicrobial activity could eliminate emerging agents before they become recognized as transfusion-transmitted pathogens. However, because blood contains numerous labile proteins and fragile cells, and because there is a wide array of potentially infectious agents, no single method of pathogen-inactivation will likely preserve all blood components, yet effectively remove all viruses, bacteria, spores, protozoa and prions. Furthermore, any chemical or physical process applied to blood must be 'safe' or at least less toxic to recipients than the infectious risk of blood.

Many pathogens that have the potential to invade the blood supply are not yet screened by testing because of low prevalence in the general population, unknown transmission rate of infection through transfusion or the lack of a readily

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Table 1 Human blood-borne viral infectious agents

Viral pathogens	Family	Genome	Enveloped	Size (nm)	Transmitted by	
					Cellular products	Plasma products
Human immunodeficiency 1/2	Retro	ss-RNA	+	80–100	Yes	Yes
Human T cell leukaemia I/II	Retro	ss-RNA	+	80–100	Yes	No
Hepatitis B	Hepadna	ds-DNA	+	40–48	Yes	Yes
Hepatitis C	Toga	ss-RNA	+	40–50	Yes	Yes
West Nile	Flavi	ss-RNA	+	40–60	Yes	Yes
Hepatitis A	Picornia	ss-RNA	–	27–32	Yes	Yes
Human erythro B19	Parvo	ss-DNA	–	18–26	Yes	Yes
Hepatitis G	Flavi	ss-RNA	+	40–60	Yes	Yes
Hepatitis E	Calci	ss-RNA	–	35–39	?	?
Hepatitis δ	Delta	ss-RNA	+	36	Yes	Yes
TT	Circo	ss-DNA	+	30–50	Yes	Yes
Epstein Barr	Herpes	ds-DNA	+	120–220	Yes	Yes
Cytomegalovirus	Herpes	ds-DNA	+	180–200	Yes	No
Human herpes 8	Herpes	ds-DNA	+	120–200	Yes	No

ss, single stranded; ds, double stranded; +, presence of an envelope; –, no envelope; ?, uncertain.

available test for the agent (Table 1). Agents with the potential to infect the blood supply are numerous and include the known viral pathogens – more than 35 arboviruses such as the Flaviviruses Den-1 through Den-4 and St Louis encephalitis virus; the Togaviruses western and eastern equine encephalitis and Chikungunya; the Coronavirus severe acute respiratory virus; the Circovirus TT and its variant SEN; and the Deltavirus hepatitis D. Other blood-borne viruses include the herpes viruses such as the Epstein–Barr virus and human herpes viruses-6, -7 and -8, as well as the human Parvovirus B19 virus. Numerous animal blood-borne agents (zoonotics) have been able to cause infection across species barriers; most do not cause disease, but have the potential to do so. Of particular concern are the simian viruses such as the foamy viruses and SV 40 that have been reported in animal handlers and in some vaccine recipients [3]. Protozoa that threaten the blood supply include the four malarial, *Babesia microti*, found primarily in the northeastern USA, *Toxoplasma gondii*, the causative agent of toxoplasmosis, *Leishmania donovani*, and numerous other subspecies that result in a high disease burden in the developing world [4]. *Borrelia burgdorferi*, the cause of tick-borne Lyme disease, has the potential for blood transmission and another tick-borne illness, human granulocytic ehrlichiosis has been reported from transfusion-transmission of the bacterial pathogen *Anaplasma phagocytophilum* [5]. Four instances of transfusion transmission of the infectious protein or prion Pr^{Sc} have been reported in the UK, at least three of which almost certainly caused the human equivalent of ‘mad cow disease’, variant Creutzfeldt–Jakob disease [6]. There is no blood screening test for the prion diseases.

Most of the world does not have access to safe blood [7]. Most developing countries do not screen donor units for all

viral markers, because the technology is sophisticated, the cost prohibitive and/or the incidence of infected individuals so high that little blood would be available if all marker-positive donors were excluded. Approximately 50% of blood donations in developing nations are from family members or paid donors [8]. Each year, unsafe blood transfusions in third world countries result in an estimated 8–16 millions hepatitis B virus (HBV) infections, 2.3–4.7 million hepatitis C virus (HCV) infections and 80 000 to 160 000 HIV infections. Pathogen-reduced blood could have a dramatic impact on blood safety in these circumstances.

Pathogen inactivation of plasma and plasma derivatives – early successes

In 1946, Edwin Cohn introduced what has become the most widely used commercial fractionation method for plasma proteins involving multiple steps of precipitation and physical separation by centrifugation or filtration of the precipitant and effluent using changes in pH, temperature ionic strength and ethanol concentration gradients. As pathogen-reduction occurs after different steps of precipitation and filtration in the fractionation process, proteins isolated later in the schematic were generally safer from infectious agents [9]. The albumin and globulin fractions proved extremely safe, particularly regarding transmission of hepatitis viruses and HIV. However, the process was not infallible and deviations from proper processing have resulted in disease transmission. Some of the most important plasma protein derivatives such as factors VIII and IX are separated early in the fractionation process and do not reap the benefits of added layers of fractionation and processing.

Pasteurization has been used effectively to inactivate viruses in albumin fractions stabilized with small chain fatty acids from as early as 1948 [10]. Other proteins, especially clotting factors, denature during the pasteurization process unless additional stabilizers are added. Terminal heat inactivation of lyophilized clotting factors has brought varied results depending upon temperature and processing time. Dry heat treatment of lyophilized clotting factors at 60–68°C does not prevent transmission of HBV, HCV and HIV. Increasing the dry heat temperature to 80°C for 72 h destroys HIV, HBV and HCV, although the non-enveloped viruses, especially HAV and human Parvo B19, may not be completely inactivated. Adding humidity (vapor heating) improves viral kill. Heat inactivation at 100°C for as little as 1 h has resulted in viral inactivation of both lipid enveloped and non-enveloped viruses. When this process is applied to intravenous immunoglobulin concentrates, little protein is lost. Exposure to low pH inactivates many enveloped viruses, however among commercial proteins, only the immunoglobulins are stable under the necessary acid conditions (pH 4.0).

Use of organic solvents and detergents in the processing of coagulation factors inactivates the lipid-enveloped viruses, but not the non-lipid enveloped viruses [11]. The solvent and detergent combination disrupts the lipid envelope and prevents the virus from binding to cells and replicating. Incorporation of a virucidal detergent and solvent (solvent 1% tri-n-butyl phosphate and the detergent 1% Triton-X-100 for 4 h at 30°C) into the processing of plasma from pools of approximately 12 000 donors produces a product known as solvent-detergent plasma [12]. The tri-n-butyl phosphate is removed by oil extraction, and Triton-X-100 is removed by chromatographic adsorption. Plasma protein concentrates have been made from solvent-detergent-treated plasma, and fresh-frozen plasma (FFP) equivalent has been licensed in the USA and Europe. As with the heat-inactivated coagulation factors, solvent-detergent technology does not inactivate the non-enveloped viruses. Solvent-detergent treatment also results in some loss of integral plasma proteins such as α -2 antiplasmin and protein S [12,13]. Alpha-2 antiplasmin is crucial in maintaining haemostasis especially in patients with liver dysfunction. Decreased levels of the natural anticoagulant protein S can lead to a hypercoagulable state especially when massive solvent-detergent plasma transfusions are used as in massive traumas or therapeutic plasma exchanges. These concerns, along with economic factors, resulted in the removal of solvent-detergent plasma from the US market. Solvent-detergent plasma is still used widely in Europe. Recently, two new solvent-detergent treatment procedures have been developed for single unit or mini-pools of 10–12 units of plasma that yield > 90% mean recovery of coagulation factors, anticoagulants (including Protein S), protease inhibitors (including α -2 antiplasmin), total protein, albumin

and immunoglobulins. Single unit and mini-pool solvent-detergent treatment technologies show promise and have the potential to overcome some of the drawbacks of the original industrial solvent-detergent treatment processes [14].

Nanofiltration has proven effective in removing a wide range of viruses including the non-enveloped viruses, and may even remove viruses smaller than the filter pore size [15]. Many currently licensed plasma derived coagulation factors and immunoglobulins that are subjected to heat, pasteurization and/or solvent-detergent treatment are also nanofiltered. All classes of plasma protein fractions such as antithrombin, C-1 inhibitor, protein C, fibrinogen and ceruloplasmin have been nanofiltered without apparent change in the protein characteristics.

Methylene blue (MB) is a photoactive phenothiazine dye that has been used in Europe for more than 15 years for the pathogen-inactivation of single units of plasma. MB has an affinity for nucleic acids and the surfaces of viruses [16]. When MB-treated plasma is exposed to ultraviolet light, most enveloped viruses are easily inactivated; however non-enveloped viruses are more resistant. Intracellular viruses are not inactivated by MB/ultraviolet light, but freezing and thawing plasma often disrupts the cell membranes of leucocytes, thus liberating the viral particles and leaving them susceptible to MB pathogen-inactivation. Residual intact white blood cells containing viruses are removed by a micropore filter. Neither protozoa nor bacteria are inactivated by MB treatment. Plasma proteins are moderately affected; fibrinogen and FVIII activity is reduced by up to 30% [17]. MB treatment can be used for pathogen-inactivation of single units of plasma, thus eliminating the risk of large plasma pools currently used to manufacture solvent-detergent plasma. MacoPharma uses an in-line system consisting of a membrane filter, MB dye, illumination bag, elimination filter and storage bag. The 0.65 μ M membrane filter removes platelets, leucocytes and debris. The plasma then passes through tubing containing an MB pill that dissolves as the plasma flows through the tubing into the illumination bag. The MB-containing plasma is subjected to double-sided illumination by sodium high-intensity low-pressure lamps emitting yellow light at a wavelength of 590 nm for 15–20 min. Plasma is then passed through an MB elimination filter that removes greater than 95% of the residual dye and photoderivative by-products [18]. Millions of MB single unit of plasma have been transfused in Europe without unexpected adverse outcomes.

Ongoing development of pathogen inactivation – plasma components and platelets

Until recently, attempts at pathogen-reduction for cellular blood components have achieved little success. Leucoreduction

of blood has reportedly decreased the risks of transfusion transmitted cell-associated viruses, such as cytomegalovirus, human T-lymphotropic virus I/II and probably Epstein-Barr virus and Human Herpesvirus-8 (HHV-8), as leucocytes are the principal reservoir for these infectious agents.

Psoralens

Psoralens are small, planar molecules that cross cell membranes and viral capsids and intercalate between the bases of the nucleic acids. Upon illumination with ultraviolet A (320–400 nm), the psoralens react with the DNA or RNA pyrimidine bases to form covalently bonded intranucleic and internucleic acid cross-links. This cross-linking prevents replication and transcription of the RNA or DNA [19]. Psoralen treatment with ultraviolet A light results in the reduction of a broad array of viruses, bacteria and protozoa to a level unlikely to transmit infection. Aminomethyl-trimethyl psoralen, a three-ringed synthetic psoralen known as amotosalen hydrochloride or S-59, has been extensively tested in the pathogen-reduction of platelets and plasma. Amotosalen and photochemical treatment have demonstrated an acceptable safety profile through extensive toxicological studies for acute toxicity, repeat dose toxicity, reproductive toxicity, phototoxicity, and mutagenic and carcinogenic potential. In order to pathogen-inactivate with psoralens, the platelet concentrate must be volume reduced and re-suspended in 30–45% plasma and 70–55% platelet additive solution. The amotosalen is added to the platelet and incubated for 3–5 min [20]. The product is then exposed to ultraviolet A light after which approximately 80% of the psoralen have been photodegraded to by-products. The remaining psoralen and by-products are removed by a 'compound absorption disc'.

INTERCEPT, an S-59 amotosalen system, has been evaluated in three clinical trials in Europe (euroSPRITE) involving 166 thrombocytopenic patients. Two of these trials evaluated whole blood-derived buffy-coat platelets, and one assessed single donor apheresis platelets. These studies demonstrated that when equal platelet doses were transfused, the INTERCEPT and conventional platelet transfusions resulted in comparable post-transfusion platelet count increments without significant differences in adverse reactions.

In the USA, the SPRINT trial evaluated the haemostatic efficacy and safety in 645 thrombocytopenic oncology patients receiving INTERCEPT single donor apheresis platelets collected on the Amicus separator [20]. A total of 4719 platelet transfusions were given; 2678 INTERCEPT platelets and 2041 conventional platelets. The incidence of World Health Organization grade 2 bleeding between the groups was comparable, and the incidence between the groups of World Health Organization grade 3 or 4 bleeding was equivalent. Patients receiving the INTERCEPT platelets had lower post-transfusion platelet count increments, required more platelet transfusions

and had a shorter interval between transfusions. Explanations for the differences in post-transfusion platelet count increments in the photochemical-treated (PCT) platelets were partly explained by the lower mean platelet dose and disproportionate number of transfusions containing platelet doses less than 3.0×10^{11} cells. Transfusion reactions were fewer with PCT platelets most likely attributed to the reduced volume of plasma in the PCT units as well as increased leucocyte inactivation resulting in less cytokine production during storage [21]. However a trend towards a higher frequency of respiratory complications has been noted and is of great concern to the US Food and Drug Administration, but apparently less so for other national regulatory bodies.

Amotosalen and ultraviolet A light has been used to pathogen-inactivate plasma in a system much like that used for platelets [22]. Post-thaw coagulation studies of the PCT plasma demonstrate that most coagulation factors are well-preserved in the range of 73–98% of control thawed plasma. Factor VIII levels, while decreased to 73%, are still sufficient for therapeutic use. There were no significant differences in the quantity and activity of the von Willebrand factor, the pattern and distribution of the von Willebrand multimers, or the activity of the ADAMTS-13. Fibrinogen maintained functional activity of approximately 87%. Protein C and S as well as antithrombin were maintained at > 95% pre-treatment activity levels. There was no evidence of coagulation factor activation as a result of treatment. The factor VII kinetics of post-transfusion PCT plasma was compared to standard FFP in a crossover study [23]. In a study of 34 patients with congenital coagulation factor deficiencies, coagulation factor kinetics and therapeutic efficacy of FFP treated with amotosalen and ultraviolet A light has been shown to be consistent with that of conventional FFP [24]. Randomized controlled trials of PCT-FFP supported haemostasis for the treatment of acquired coagulopathy of liver disease and liver transplantation have revealed outcomes similar to that of conventional FFP [25]. Additional studies utilizing PCT-FFP for plasma exchanges in thrombotic thrombocytopenia purpura demonstrated similar results as those with conventional plasma. Cryoprecipitate can also be produced from amotosalen and ultraviolet A-treated plasma. Preliminary studies indicate that PCT cryoprecipitate coagulation factor levels are acceptable.

Riboflavin/ultraviolet light

Riboflavin, vitamin B2 a naturally occurring essential nutrient, has been used as a pathogen-inactivating agent for platelets and plasma. Riboflavin is a 3-ringed planar structure that binds to nucleic acids and intercalated between DNA and RNA bases. Upon activation of cross-linked riboflavin with ultraviolet or visible light, guanosine bases are oxidized resulting in single strand breaks in the nucleic acids. The damaged and disrupted nucleic acids are incapable of repair

and replication. Toxicities of riboflavin and its photoderivative by-products do not appear to cause concern, because riboflavin and its breakdown products are present in many food and natural products. Removal of the spent riboflavin and products post-illumination may not be necessary in a pathogen-inactivation system using riboflavin. The US Food and Drug Administration has classified riboflavin as a 'generally regarded as safe' compound.

The Mirasol PRT System contains 30 ml of riboflavin (500 µM) in a light protective pouch and a pathogen-reduction illumination/storage bag. Platelets or plasma are sterilely connected to the system, and 250 ml of plasma or platelet product is transferred to the bag containing the riboflavin diluting it to a final concentration of 50 µM. The riboflavin-treated product is subjected to double-sided ultraviolet illumination [27]. Riboflavin/ultraviolet light treatment has been evaluated in preclinical studies and found to result in reduction of infectivity by many pathogens including west Nile virus, intracellular HIV, bacteria and protozoa. The Mirasol system demonstrated successful pathogen reduction of selected pathogen-spiked platelet units after treatment and storage for 5 days. The viral reduction of platelets was sufficient to close the window period of transmission of HIV and chronic phase of Parvo B19, eliminate the viraemic period of west Nile virus, prevent infection due to *Staphylococcus epidermidis* and *Escherichia coli*, and result in a 5–6 log reduction of *Leishmania donovani infantum* [27]. Additionally, *Leishmania*-spiked plasma units treated with riboflavin/ultraviolet light demonstrated a 5–7 log reduction in parasites. Studies have demonstrated significant differences in control and treated platelets after 5 days of storage in regards to accelerated changes in platelet morphology, increased platelet activation and induced partial platelet aggregation. Riboflavin/ultraviolet light-treated plasma has been shown to retain acceptable levels of clotting factors without evidence of increased complement activation.

Ongoing development of pathogen-inactivation – whole blood and red blood cells

Pathogen-inactivation of components containing red blood cells presents a particularly challenging dilemma. Methods utilizing photoactivation must do so in the red wavelength region of the light spectrum above that of haemoglobin in order to avoid absorption or scattering of the light by the red blood cell. Many potential methods of pathogen-inactivation easily alter or disrupt the red blood cell membrane resulting in decreased red cell survival, haemolysis or immunogenicity.

S303

S303 (Helinx), a small molecule designed for pathogen-inactivation treatment of red blood cells, is an alkylating

agent derived from a quinacrine mustard that belongs to a class of 'frangible anchor linker effectors' (FRALE) compounds. FRALE compounds contain an intercalator group that inserts into the helical region of DNA and RNA, an effector group that permits covalent attachment of nucleic acids and a central frangible bond that orchestrates the degradation of the compound [28]. S-303 is a positively charged planar structure that easily intercalates into the helical regions of the negatively charged nucleic acids. The process does not depend on light for activation. FRALE compounds are activated by a shift from lower pH storage environment to the higher neutral pH of red blood cells causing hydrolysis and generating S-300 the primary degradation product; cross-linking of the DNA and RNA ensues. S-300 is rapidly metabolized and excreted leaving no detectable parent compound. The remaining free degradation products are absorbed and removed by a compound removal step. S-303 binds to other proteins and cell membranes as well as nucleic acids, and up to 20% can potentially remain bound to the surface or contained within the red blood cells.

S-303 has demonstrated pathogen-inactivation of a wide range of viruses, bacteria and protozoa. No unexpected toxicities have been described. Assays for red blood cells storage lesions (extracellular potassium leakage, plasma-free haemoglobin, adenosine diphosphate, 2,3-diphosphoglycerate, glucose and lactate) are comparable to control red blood cells. The red blood cell function appears to be normal, and *in vivo* ⁵¹Cr-labelled survival studies exceed the standard of 75% at 24 h.

Two randomized, controlled trials involving patients either undergoing first-time cardiovascular surgery or with haemoglobinopathies were in progress when antibodies to residual red blood cell bound S-303 were discovered in two subjects [29]. The trials were suspended as a consequence of these findings. Additional studies have revealed that 1% of patients and healthy donors that had never been exposed to S-303 had naturally occurring antibodies that reacted with S-303 treated red blood cells. Modifications have been made to the S-303 treatment process to reduce the amount of red blood cell bound S-303 in attempts to eliminate immunoreactivity and immunogenicity. Preliminary finding indicates that red blood cells from the modified S-303 treatment process were cross-match-compatible with the anti-S-303 antibodies formed after exposure to the original S-303 formulation as well as the anti-S-303 antibodies found in the patients and donors never exposed to S-303. The antibodies do not appear to impair transfusion or to pose any clinical problem. New clinical trials have been initiated.

Riboflavin-based pathogen-inactivation systems for red blood cells are currently under development. If found to be a successful means of pathogen-inactivation of red blood cells, riboflavin may serve as the one material to inactivate pathogens in three blood components (red cells, platelets and plasma).

New prototype leucoreduction filter

Current leucoreduction filters are effective in removing cell-associated viruses, but remove only 42% of total prion infectivity in endogenously infected blood. A leucoreduction filter under development removes prions in exogenously and endogenously infected blood more effectively [30]. Exogenous infectivity studies were conducted using 270 ml of red blood cells and 30 ml of 10% (wt/vol) with high titre brain homogenates from hamsters infected with scrapie for a final 1% homogenate concentration. Endogenous infectivity studies utilized red blood cells processed from 500 ml of whole blood collected from 120 scrapie-infected hamsters. The new prototype leucoreduction, prion reduction filter was effective in removing 3.7 logs of scrapie infectivity from exogenously infected red blood cells and all of the detectable PrP^{Sc} from the endogenously infected hamsters. In the endogenous infectivity study, the pre-filtered-infected red blood cells transmitted disease to six of 43 animals, and the post-filtration red blood cells did not transmit disease to any of 35 animals.

Summary

Allogeneic blood is a critically important therapeutic, but also an inherently risky biologic source material. Among the dangers is transmission of a wide range of pathogens. Donor selection and blood testing have reduced this risk dramatically and will remain the cornerstone of blood safety programmes. Nevertheless, infectious units still elude screening and testing; testing errors and product release errors are probably impossible to eliminate. The largest current infectious risks involve pathogens for which we do not test, for which we have no test, or for which demographic screening and testing are inadequate. The greatest fear concerns the emergence of a 'new' transmissible agent, particularly one that has not previously been associated with human disease, has a long silent period, can infect others by secondary spread and is highly lethal – as was the case with HIV. Ideally, pathogen-inactivation techniques would provide an additional safeguard. That has been the experience in the plasma fractionation industry.

No single pathogen-reduction method will likely be effective for every class of agent and for every blood component. Some combination of techniques that remove and inactivate infectious agents will probably be needed. These technologies are generally expensive and have the potential to profoundly escalate the cost of blood, but this cost may be partially offset by the elimination of some testing markers. However, if potent pathogen-inactivation techniques that preserve blood function and do not evidence some new toxic risk can be created, the developed world, which embraces the myth of zero-risk transfusion, will likely adopt them almost regardless

of cost. For the developing world, in which low-risk blood donors are at a premium and elegant testing methods often not feasible, a good but not perfect pathogen reduction method, especially if relatively inexpensive and easy to implement, could save millions of lives.

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