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New strategies for the control of infectious and parasitic diseases in blood donors: the impact of pathogen inactivation methods

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Abstract

Around 70 infectious agents are possible threats for blood safety.

The risk for blood recipients is increasing because of new emergent agents like West Nile, Zika and Chikungunya viruses, or parasites such as *Plasmodium* and *Trypanosoma cruzi* in non-endemic regions, for instance.

Screening programmes of the donors are more and more implemented in several Countries, but these cannot prevent completely infections, especially when they are caused by new agents.

Pathogen inactivation (PI) methods might overcome the limits of the screening and different technologies have been set up in the last years.

This review aims to describe the most widely used methods focusing on their efficacy as well as on the preservation integrity of blood components.

Keywords: blood donors, viruses, bacteria, protozoa, Pathogen inactivation methods, plasma, platelets, red cells

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Introduction

Blood transfusion (BT) is a life-saving procedure which is essential especially in management of critical patients.

Safe and efficacious blood and blood components for transfusion or manufacturing use need several processes, which include: i) the selection of blood donors; ii) the collection, processing and testing of blood donations, iii) the testing of patient samples; iv) the identification of compatible blood and v) its administration to the patient (1).

Actually, BT is a medical procedure which is crucial in many clinical situations. However it can be also associated to risks and to certain complications, which could have important effects on public health and confidence in blood safety.

Complications commonly associated with transfusion include immune-mediated (haemolytic and febrile non haemolytic reactions, transfusion-related acute lung injury, allergic reactions, post transfusion purpura and transfusion-secondary graft versus host disease) and non-immune transfusion reactions (transfusion related circulatory overload and, particularly, transfusion transmitted infections, TTIs) (2).

Generally, the risk of TTIs per unit trasfused has been greatly reduced nowadays but risks still vary substantially even between high-income and low-income countries as well as between low-endemic and high-endemic areas around the world (3).

In high-income countries, the infectious risks are usually low and non infectious pathologies have become the most frequent complications observed in clinical practice, after a transfusion (4, 5).

Indeed, transfusion-trasmitted (TT) viral pathogens have been dramatically reduced over the last 30 years and the risk for HBV, HCV and HIV to recipient per unit transfused

is now less than 1 in 1 million. On the contrary, sepsis due to bacterial contamination of platelets may occur more frequently (6, 7).

For example, in Australia no HIV or HCV transfusion-trasmission has been identified since the nucleic acid testing (NAT) was introduced in 2000 and only 3 cases (probable) of HBV have been observed in the 2005-2016 period (8).

Since 2010 up to 2019, in USA, the National Healthcare Safety Network documented 54 TTIs: the most frequently reported pathogens were bacteria (primilarly *Staphylococcus aureus*) present in platelets and *Babesia* spp. in red blood cells (RBCs) (9).

Since 1996, in UK 80 confirmed cases of TTIs have been documented, involving 91 recipients. Bacteria were the primary cause of TTI (49% of all cases) and HBV was the most commonly documented viral infection, partly because of longer window period than for HCV and HIV, despite blood donations were screened with NAT. Only 3 TTI were recorded as probable or confirmed (HEV, HBV and *Staphylococcus epidermidis*) in 2018 (10). Moreover, after the bacterial screening for platelet products was introduced in the UK in 2011, there has been only one case of proven bacterial TTI (11).

Unlike this situation, either not screened for all the major TTIs or not screened within a reliable quality system (3, 12), in low-income countries the risk of TTIs is still considerable and donated blood remains unsafe in a significant proportion. This is a great problem in areas where a high percentage of population could carry an infectious agent. For instance, in many sub-Saharian regions, where more than an half of the population is parasitised by *Plasmodium* spp., whose rate of transmission is around 14-20% (13).

Data on blood safety indicators provided in 2007 by ministries of health to the WHO Global Database on Blood Safety (GDBS) indicate that, out of the 155 countries reporting to perform 100% screening for HIV, in only 45% of them the screen is carried out in a quality-assured manner (12).

A substantial number of countries still require concerted efforts to achieve 100% TTI screening of donated blood within quality systems (3).

Finally, the lack of post-transfusional surveillance makes it difficult to recognize a TTI as such and these infections are frequently attributed to a non-transfusion acquisition (14).

Several strategies are implemented in order to reduce the risk of TTIs.

Pre-donation health screening with increasingly donor eligibility criteria, diversion of the first mL of blood collected, screening of donated blood for major transfusion-trasmitted pathogens with more sensitive detection methods, process control, pre-release contamination screening have attenuated the risk of TTIs significantly but a residual risk remain (15, 16, 17). In fact, very-low pathogen concentration in the peripheral blood and escaped mutants can determinate false-negative test results. Moreover, not targeted pathogens are not detected and new agents responsible for emerging infectious disease (EID) are potential threats to blood safety.

Blood safety is today ensured by a reactive principle: introduction of a new safety system after the identification of an evident threat. Recent outbreaks and documented TTIs caused by EID agents highlight that current approach may not be appropriate in this scenario (18).

The developement and the approval of pathogens inactivation technologies for cellular bood products, in accordance with precautionary principle, could be considered a key mesure to change strategy towards a proactive preventive approach to blood safety (19).

If applied to all separate blood components or whole blood, these technologies, may let a restructure of current screening tests with a possible reduction of redundant investigations (for example HBV core antibodies where this test is carried out), modified questionary and/or deferral and simplified handling of postdonation information while preserving the safety of the blood supply (20).

In this context EIDs, in the last decades, have had a relevant effect on perceived, and real, blood safety (21, 22).

EIDs can be defined as "those whose incidence in humans is increased within the past twenty years or threatens to increase in the near future" (23). Many of such infections are possibly transmitted by blood transfusion.

In 2009, AABB's Transfusion Trasmitted Disease Commitee identified around 70 infectious agents identified as possible threat for blood safety. The list included viruses which cause Dengue (DENV), Chikungunya (CHIKV), protozoa like *Trypanosoma cruzi* and other pathogens that have proven to be a serious problem for blood collector and national blood organizations (24).

As years go by new agents add to this list (Zika virus, Middle East Respiratory Syndrome Coronavirus and others).

Several zoonotic and re-emerging pathognes have caught extensive attention for outbreaks in the last decade (25, 26, 27).

In 2005, the distribution of CHIKV spread throughout the Indian Ocean islands, some areas of Southern Asia and India (28). The major outbreak affected Reunion Island, France, where between 2005 and 2007 approximately 30-40% inhabitants were infected (29). During the peak of the outbreak, blood collections were stopped but this strategy was not applied for labile blood components such as platelets. Platelet products were then photochemically inactivated with the aim to decrease the risk of CHIKV transmission while maintaining blood availability in cases of the need of platelet transfusion (30). CHIKV outbreaks were also reported in 2006 in India, in 2007 in Italy, and in Thailand in 2009 (31). In later times, a great outbreak of CHIKV involved Caribbean (32). In Europe, more limited outbreaks interested Italy (Lazio and Calabria) (33, 34) and France (Var department) in 2017 (35).

ZIKV is known since 1947 but only infrequent sporadic cases have been reported in tropical areas until 2007. In 2007, an outbreak was reported in Micronesia with about 75% of the population of Yap Island resulting infected (36) and an outbreak of a syndrome of ZIKV fever has been reported in French Polynesia.

The ZIKV epidemic in 2015 has marked the beginning of an international public health emergency when the virus reached the American continent (33 countries reporting autochthonous transmission of ZIKV infection) and Europe, with imported cases.

Puerto Rico has been heavily affected in 2015-2017. With the aim to reduce the risk for TT ZIKV infection, blood collection ceased and blood safety interventions in Puerto Rico consisted only in importating blood units from US areas where the virus was absent and treatment of plasma and apheresis platelets with pathogen-reduction technology until FDA in April 2016, authorized the use of NAT test carried out on individual donation (37).

In period 2015-2017, between 400,000 and 1.5 million cases of ZIKV infection has been reported. Since 2017, the number of cases declined, although the virus remains circulating in many countries (38).

West Nile virus (WNV) is widely distributed throughout Africa, the Middle East, southern Europe, western Russia, south western Asia, and Australia, the reason is due to its ability to infect numerous mosquito as well as bird species. Until the early 1990s, outbreaks involving humans were observed infrequently from Middle Est and Africa. In America, WNV spread from its discovery in 1999 (39). A total of more than 15000 patients with WNV neuroinvasive and 1,500 fatalities have been recorded in the United States. In consideration of the widespread outbreaks occurring in the last twenty years, WNV should be considered global threat, not only for animals, but also for humans, at global level (40). As regards blood safety, the preventive measures which generally consist in a 28-day deferral and the use of WNV-NAT, can control effectively the risk of WNV. Nucleic acid amplification testing is used, in the United States, and Canada, for blood donor screening. In Europe WNV-NAT is implemented where viral circulation is proven by animal and vector WNV surveillance. However, recently, a possible case of TTI is reported from an apheresis platelet that resulted negative by NAT. Authors noted that implementatoin of NAT cannot eliminate the risk of TT WNV infection, which could best be controlled by PI (41).

In endemic areas, parasites are a current and real threat to blood safety. This threat interests also non-endemic countries.

TT malaria (TTM) is an accidental *Plasmodium* spp. infection of the recipent, caused by the transfusion of whole blood or a blood component from a malaria infected donor.

The risk of TTM differs considerably between countries with low-endemicity, where the infection is "imported" from outside (e.g. travel to or immigration of individuals from highly endemic regions) and countries with high prevalence in the general population of infection.

The reliability of TTI is due to the ability of *Plasmodium* spp. to persist in blood donor for many months before being cleared and to the capacity to survive several days in conditions of storage. Moreover experimental evidence indicates that as few as 10 infected RBCs can transmit the infection (42). These amounts are too low to be detected by direct methods.

A typical non-endemic country such as the USA trusts in a pre-donation questionnaire for the screening of potentially infected donors. Other countries, including Italy, France, and Australia, use serologic testing on those donors considered at risk. So, appropriate diagnostic tools are necessary in order to improve the blood safety. However, different serological kits showed highly variable results and different responses are possible, depending on the laboratory which performs the test (43). In Sub-Saharian countries where malaria is endemic microscopy is the most frequent diagnostic tool used but it has a low sensitivity and it often detect only parasitemia of >600 parasites/μL (44).

TTM is may cause morbidity and mortality particularly in non-endemic areas where individuals are not semiimune to malaria.

A recent review reported around 100 cases described in the Literature, since the first documented case in 1911, of TTM in non-endemic areas for malaria, mainly caused, as expected, by whole blood and/or RBCs transfusion, but also although rarely, by platelets as well as by plasma (45).

Finally, american trypanosomiasis (Chagas disease, CD) is a potentially life-theatening infection which is caused by protozoan *Trypanosoma cruzi*. WHO estimates that around 8 million people are infected at global level, mostly in South America where CD is endemic and transmitted by triatomine bugs (46). Several studies estimated number of people affected by CD in non-endemic countries: 33,000-330,000 in USA, 12,000-250,000 in Spain (47), 6,000-12,000 in Italy (48).

TT Chagas disease has been reported from endemic countries in Latin America (49) and transfusion is an important modality of transmission in non-endemic regions (50). Platelets are the most frequently reported blood components responsible for TT Chagas disease (51).

After the parasite enters in the organism, the acute phase, characterized by a high-level parasitemia, occurs being, in most cases, without symptoms. During the chronic phase which developes after 4-8 weeks, parasitaemia decreases and it usually results not detectable and inconstant. Thus, serology is preferred to direct detection methods (52).

For patients, at least two serological tests based on different principles must be carried out to search anti-*T. cruzi* IgG antibodies. However in case of blood screening in blood banks, it is sufficient to decide on blood exclusion, on the basis of a single test (53).

Different strategies have been adopted to reduce the risk of TT American trypanosomyasis. Currently, in endemic areas all donations should be screened for specific antibodies for the parasite, whereas in countries with no endemicity interventions are different: exclusion of high risk donors (Sweden), *T. cruzi* serology screening of at-risk blood donors (France, Italy and Portugal) or one-time testing of all donors (USA).

To face these new or known threats, different strategies have used in different country in order to mitigate risks of this blood-borne infections: nationwide screening of donors for

new agents in addition to classic TTIs, testing on donors that visited endemic regions, deferral.

The report on blood safety and availability of the WHO described different screening strategies applied in different countries and different sanitary contexts, the reader is referred to the report (3).

Several outstanding reviews are present in literature and describe the present situation of emerging infectious diseases as well as their impact on "Blood system". A detailed description of epidemiology, pathogenesis and clinical characteristics of EIDs is out of the scopes of this review.

This review will focus on new PI technologies and their efficacy and safety profile.

PI technologies for plasma

Plasma, for the first time treated with solvent detergent (S/D) was used in Europe in 1992 and only six years later, in North America (54).

S/D plasma is a pathogen-inactivated blood plasma developed aiming at reducing the risks due to the use of untreated fresh frozen plasma (FFP). In comparison to FFP, S/D plasma, in fact, brings to a lower incidence of allergic reactions, transfusion-related acute lung injury (TRALI) and viral transmission (55).

The S/D-plasma manufacturing process involves pooling hundreds or thousands of single donor plasma derived by apheresis or whole blood to which an organic solvent (such as 1% tri-η-butyl phosphate) and a non-ionic detergent (generally 1% Triton X-100) are added to inactivate lipid-enveloped viruses. After 24 hours the organic solvent and the non-ionic detergent are removed *via* oil extraction and/or chromatography (56, 57).

Dilution and the presence of neutralising antibodies in the pool contribute to reduce the virus load but also the titer of possible alloantibodies directed against blood components (58).

Manufacturing process allows a more standardised content of certain plasma proteins than FFP. However, a reduction of fibrinogen, factor V, factor VIII, factor XI, ADAMTS13, protein S, antiplasmin and antitrypsin is also documented (59).

S/D-plasma may be preferable to standard plasma in specific clinical situations in which it is important reduce the amount of plasma infused (for example orthotopic liver transplantation) (60).

In the United States, in patients with liver disease and related coagulopathies, an association between the use of S/D plasma and thrombosis with pulmonary embolism was reported. In Europe no thrombotic complications in similar patient groups was documented. These different results explains the different approach to this product (61).

Treatment has an high inactivation rate for enveloped viruses (HIV, HBV, HCV, WNV, CHIKV, etc..) but it is not active on non-enveloped viruses (like for instance, HAV and parvovirus B19) (62). Neutralizing antibodies in plasma pools may provide additional safety against non-enveloped pathogens but it is not clear whether the antibody content can prevent transmission of the infection. Thus, cases of clinically apparent parvovirus B19 infection have been described after using S/D plasma containing high amounts of parvovirus B19 DNA (63).

PI technologies for cellular products

The use of PI for cellular products has several advantages, in fact they can inactivate most clincally relevant pathogens and, for this reason, decrease the residual risk of infection in the window period (when screening tests could provide false-negative results) and reduce the risk against unknown pathogens, EID and recognizable pathogens which still cannot be prevented completely (bacteria). Two other possible advantages of PI are the elongation of shelf life up to one week, allowing for better management of the inventory (64) and the inactivation of residual lymphocytes in the product. In particular, γ irradiation with 25 Gy is currently used as the standardised treatment of blood products to prevent T-cell proliferation and graft versus host disease (GVHD). Several studies documented that PI treatments have the same efficacy as that of γ irradiation in stopping T-cell proliferation (65). This finding suggests, theoretically, that γ irradiation could be replaced with PI treatment to prevent transfusion-associated GVHD.

At present, all avaible treatments illuminate cellular products with UV light with or without photoactive chemicals.

The following technologies for cellular products are suitable or have been object of phase III trials.

Intercept Blood System

The INTERCEPT Blood System (Cerus Corporation, Concord CA, USA) exploits the properties of the psoralen compound amotosalen HCl (S-59), a photoactive compound activated by low-energy UVA light (320-400 nm).

Amotosalen penetrates through the membranes (cellular and nuclear) forming a non-covalent bond between pyrimidic residues in DNA and RNA chains. Illumination with UVA rays (320–400 nm) leads to a photochemical reaction which converts the non-covalent links into irreversible covalent bonds. Covalent bonds prevent DNA replication and RNA transcription (see **Fig. 1**).

After illumination, residual amotosalen and its breakdown products must be removed. Therefore, this procedure includes a recapture step during wich the excess amotosalen is removed by a compound adsorption device containing a resine chelates. The adsorption step lasts up to 16 hours (66).

Operationally, the procedure includes a first step (via one sterile connection) through the bag containing amotosalen. Inside the illuminator an UVA around 3 J/cm^2 is applied for around 5 minutes. After illumination, a novel step into the container with the compound adsorbtion device for a variable period (10–20 min for plasma or 4–16 h for platelets). Finally, the product is transferred into the storage bag.

The amotosalen/UVA procedure is not suitable for RBCs because of UVA light absorption by hemoglobin. In fact, the peak of absorption by hemoglobin is greatest in the UV

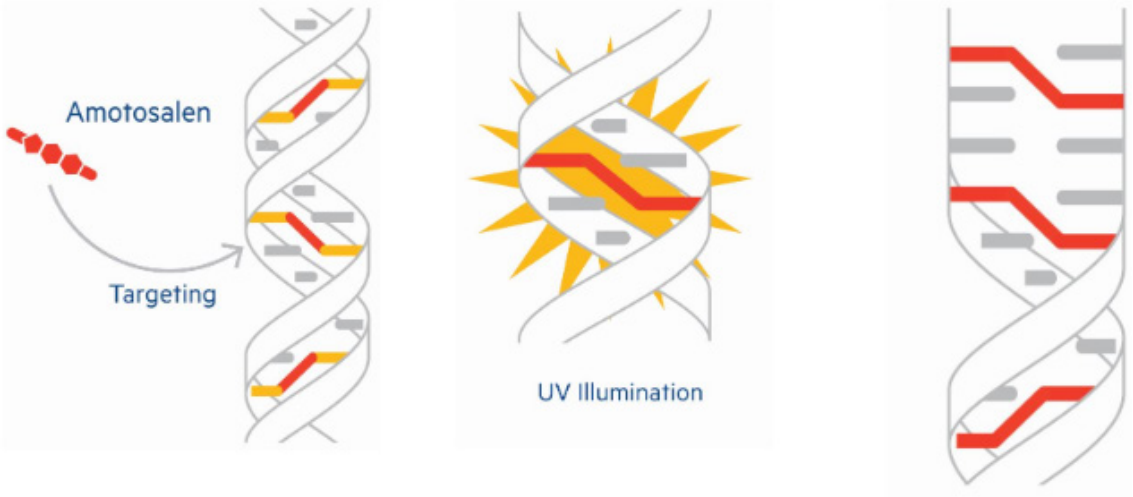

Figure 1. Amotosalen/UVA technology mechanism of action. Photoactive compound targets nucleic acids; UVA illumination activates photoactive compuond causing permanent cross-links between the double-stranded chains and blocks the replication of DNA and RNA. From: http://www.intercept-usa.com.

region, particurlarly around 400 nm, therefore amotosalen activation is inefficient in presence of hemoglobin. For this reason INTERCEPT Blood System is suitable only for plasma and platelets. Amotosalen/UVA method can inactivate several viruses (either enveloped or non enveloped), bacteria and parasites (67, 68, 69, 70). However, HEV and parvovirus B19 trasmission by treated platelet units are described (71, 72).

Amotosalen is also active against residual T-cells, therefore it can reduce risk of transfusion-associated GVHD and infection with intracellular pathogens such as citomegalovirus (73).

Amotosalen/UVA is adopted in more than 40 countries worldwide, in America, Europe and Asia (74).

Mirasol PRT System

MIRASOL PRT System (Terumo BCT, Lakewood, CO, USA) uses a photosensitizing agent (riboflavin, vitamin B2) and broad-spectrum UV light (UVA and UVB, 280–400 nm; for a nominal dose around 6.2 J/mL of product).

Riboflavin associates with nucleic acids, after exposition to UV light, mediates oxygen-independent electron transfer, causing irreversible damage to guanidic bases in nucleic acids chains (75).

Riboflavin and its photodegradation products are not toxic or mutagenic, for this reason they are not removed at the end of the procedure (76) (see **Fig. 2**).

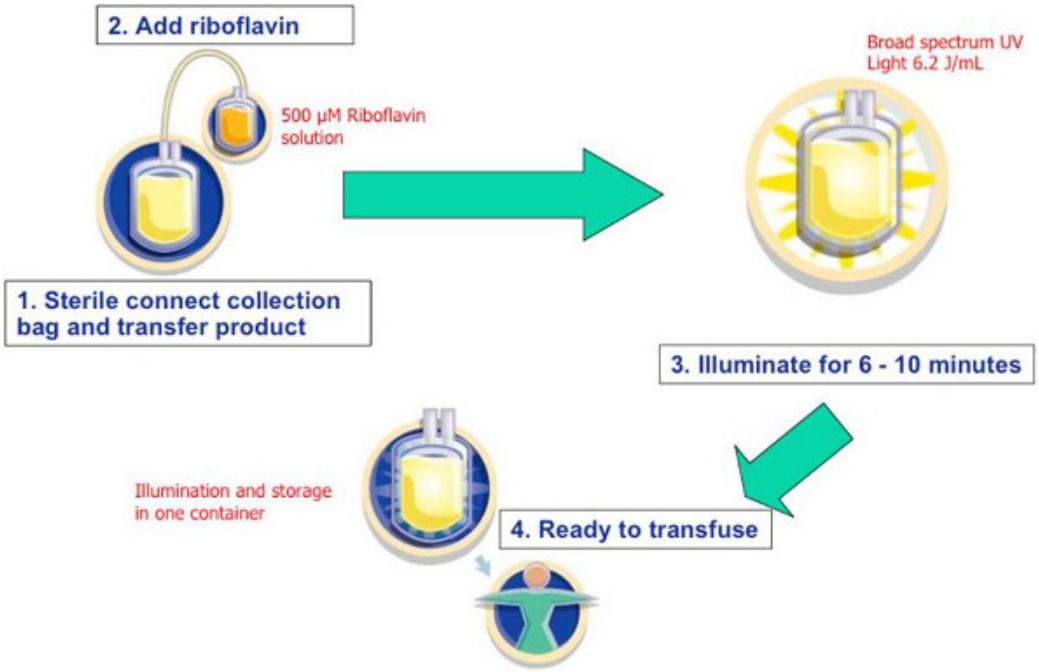

Figure 2. Mirasol mechanism of action. From Ref. (77), with permission.

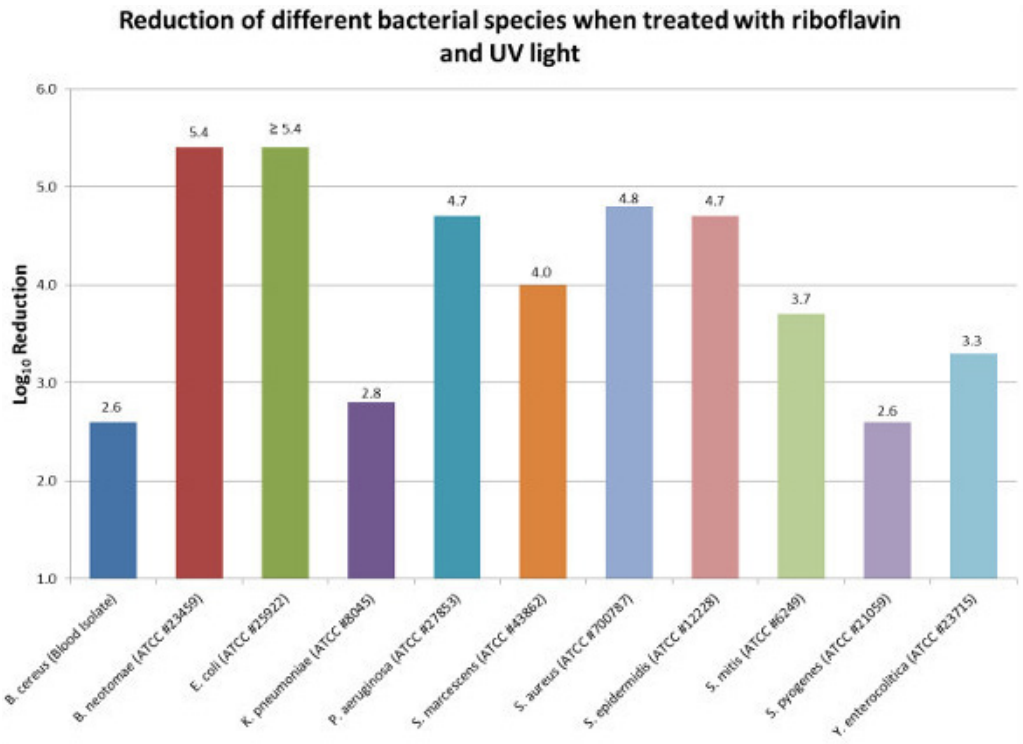

Figure 3. Reduction of various bacterial species when treated with Mirasol PRT System. From Ref (77), with permission.

This system is suitable for plasma and platelets but studies for extension of the MIRASOL system to whole blood are under way. In AISM study the estimated incidence of transfusion-trasmitted malaria by whole blood units in non-affected patients is substantially reduced (from 16% to 4%) (78).

This method is effective against pathogens such as bacteria, *Plasmodium* spp, *Leishmania*, Ebola virus (4.5 log reduction), MERS-CoV (around 3.5 log reduction) and Chikungunya virus (78, 79, 80, 81, 82) but treatment still does not eliminate the potential risk of some viruses which may remain viable (83). In **Figure 3** the effectiveness on bacteria are shown.

Similarly to amotosalen/UVA technology, riboflavin/UV method is effective in inactivation of residual lymphocytes in the blood product (65).

Riboflavin/UV is adopted in around 20 countires worldwide (74).

Theraflex-UV

THERAFLEX-UV (Macopharma, Tourcoing, France) is a method that does not employ any photoactive subastance but it is based on UVC irradiation.

Shortwave UVC light (254 nm) affects directly nucleic acids forming intra- and inter-strand cyclobutane pyrimidine and pyrimidine pyrimidone dimers that block the transcription and replication of nucleic acids. Wavelenght of 254 nm is next to the peak of absorption of nucleic acids (260 nm). Plasma protein absorption is very low for this wavelinght. The nominal UVC dose applied to the product is around 0.2-0.3 J/cm^2 (84).

As no photoactive compounds are involved, there is no product to remove and the treatment is simple and fast. Operationally the whole procedure takes less than ten minutes, requiring a sterile connection and two pack transfers (85). The transfer in a provided bag is necessary to allow a considerable UVC penetration. Then, the treated product is transferred into the storage bag, and it does not require further processing before being released.

UVC method is effective in inactivation of lymphocytes, enveloped virus, protozoa and bacteria (84, 86, 87, 88, 89) . However, its efficacy against HIV is limited (90).

THERAFLEX-UV is in development stage. The system was originally developed for platelets but it is also suitable for plasma and RBC units.

Intercept RBC System

The INTERCEPT RBC system (Cerus Corporation, Concord, CA, USA) has been specifically developed for RBC units.

It employs amustaline (S-303) which is a small modular compound composed by an acridine anchor (that targets nucleic acids non-covalently), an effector (a bis-alkylator group that reacts with nucleophiles), and a linker (a carbon chain containing a labile ester bond that hydrolyzes to release non-reactive products) (91).

After S-303 addition to RBC unit, it forms covalent bonds and adducts with nucleic acids whithin 2-4 hours of treatment. The compound decomposes by hydrolysis to a non-reactive product (S-300) that has a short half-life (around 25 minutes). Finally, incubation and centrifugation permit to remove non-reactive breakdown products. This final step takes up to 20 hours.

The S-303 system does not require UV light. It requires the presence of glutathione (GSH) in order to prevent non-specific reactions between amustaline and other nucleophiles (such as proteins and phosphates) present in the RBC unit.

The INTERCEPT RBC system showed effectiveness against enveloped virus, bacteria and protozoa (92, 93, 94). It can inactivate also residual lymphocytes (95). At the moment, this technology as well as THERAFLEX system is still in development.

Efficacy

Efficacy of the various inactivation technologies, described above, has been evaluated against several pathogens. Inactivation effectiveness on a specific pathogen is variable, thus results obtained against a specific pathogen are different using different methods and performances cannot automatically be transposed from one to another (96, 97).

In general, the suitable methods showed good performances against bacteria, enveloped viruses and parasites, but results are often insufficient against spores, nonenveloped viruses and prions .

The efficacy of different technologies against a group of selected pathogens is summarized in **Table 1**.

In order to assess a PI technology's effectiveness in reducing the risk of TTI, it is important to understand pathogen dynamics during infection, to evaluate the load of infectious

Table 1. Reduction of infectious disease agent logs induced by different PI technologies*

Pathogen		Amotosalen/UVA	Riboflavin/UV	UVC	Amustaline-GSH
HBV	Enveloped virus	>5.5 (66)	2.5 (76)	na**	>5.1 (105)
HCV	Enveloped virus	>4.5 (66)	3.2 (76)	>4.9 (103)	na**
HIV (cell free) latent	Enveloped virus	>6.2 (66)	>4.5 (76)	1.4 (90)	>6.5 (92)
HIV (cell-associated) active	Enveloped virus	>6.1 (66)	>5.9 (76)	na**	>5.9 (91)
CMV	Enveloped virus	>5.9 (66)	2.1 (76)	na**	>6 (105)
WNV	Enveloped virus	>6.0 (66)	>5.1 (76)	3.4 - 4 (85)	> 6.0 (93)
CHIKV	Enveloped virus	>6.9 (31)	2.1 - 4 (83)	6.34 (86)	> 6.78 (31)
Influenza A virus	Enveloped virus	>5.9 (66)	>5 (76)	na**	na**
HAV	Nonenveloped virus	0.6 (98)	1.8 (76)	4.2 (87)	na**
Parvovirus B19	Nonenveloped virus	3.5-5.0 (99)	>5 (76)	5.4 (31)	na**
Zika Virus	Enveloped virus	> 6 (68)	na**	5.7 (104)	5.99 (94)
Dengue Virus	Enveloped virus	> 4.3 (100)	1.8 (101)	>4.6 (86)	6.61 (106)
Staphylococcus aureus	Bacteria (Gram positive)	>6.6 (67)	4.8 (6)	>4.4 (88)	5.1 (91)
Staphylococus epidermidis	Bacteria (Gram positive)	>6.6 (67)	4.2 (102)	4.6 (88)	> 6.9 (92)
Pseudomonas aeruginosa	Bacteria (Gram negative)	4.5 (67)	4.5 (6)	4.9 (84)	4.5 (92)
Escherichia coli	Bacteria (Gram negative)	>6.4 (67)	4.4 (102)	7.3 (88)	>6.7 (91)
Serratia marcescens	Bacteria (Gram negative)	> 6.7 (67)	4 (6)	> 5 (88)	5.1 (91)
Yersinia enterocolitica	Bacteria (Gram negative)	> 5.9 (66)	3.3 (6)	na**	>6.8 (93)
Trypanosoma cruzi	Protozoa	>5.3 (66)	>5 (76)	na**	> 5.4 (93)
Plasmodium spp	Protozoa	>6 (66)	>3.2 (76)	na**	> 6.8 (93)
Babesia spp	Protozoa	> 5.3 (66)	>4 (76)	> 5 (89)	>5.5 (93)

* Results are expressed as > number indicate that the pathogen concentrations was reduced to the limit of detection of the assay; ** not available.

agents in blood products and, preliminarily, to determine the transfusion-transmission relevance of a given pathogen.

Several consideratios suggest that evidences derived from laboratory studies have to be confirmed and evaluated in the light of the epidemiological and clinical data.

Some considerations should be made:

i) the estimate of "log titer reduction" is commonly used to determine effectiveness of a method and to compare different technology. However, to obtain high rates of agent titer reduction could often be unnecessary and in some cases "log reduction" may not be a good parameter to estimate the blood safety (107). In fact, it is important to consider that bacterial concentration is not very high shortly after collection and production (generally < 100 CFU/product), so a high log reduction capacity is not necessary (108, 79). Instead, possible toxins produced during proliferation or lysis of pathogens are not inactivated by any avaible technology. Then, in this regard the timing of treatment (earliest opportunity after production) is more important than log titer reduction obtained in laboratoty studies. However, a partial inactivation may allow the growth of microbes at life-threatening concentrations during storage period (6);

ii) the minimum infective dose of pathogen particles and genome copies varies from one pathogens to another and from one isolate/strain to another of the same agent. Furthermore, numbers of genome copies in peripheral blood varies between different period of infection (109, 110, 51). Consequently, it is important to consider that results obtained with a method are based on a specific laboratory strain and comparison of technologies based on this parameter should be carefully evaluated;

iii) several criteria exist to determine whether a pathogen could be a real threat to blood safety: 1) a long asymptomatic phase of infection in donor (therefore unidentifiable during the selection process); 2) ability to infect humans; 4) transfusion trasmissibility; 5) abilty to survive during blood components processing and storage conditions. These requested characteristics could explain why several potential pathogens are rarely implicated in TTIs or can be implicated occasionally even when the endemicity is high. For instance, the risk of transmission of *Treponema pallidum* is very low as the spirochaetae have an intermittent bacteriemia during the infection, they cannot survive more than 72 hours at storage temperature and a good screening test is avaible and generally mandatory (1, 111).

Furthermore, despite large documented outbreaks of Zika virus or Chikungunya virus infection, reported cases of transfusion transmission of the former virus have been scarce (27), and none for CHIKV have ever been documented (83). However, it has to be considered that it may also be difficult to identify and prove transfusion-associated transmission in areas affected by large-scale community outbreaks (29). Thus, TT cases could be understimated.

So, it seems reasonable to consider that efficacy inactivation is more important for non screened pathogens, for bacteria (which most frequently contaminate platelets) and viruses that represent a known high transfusion risk (as, for example, HEV) than pathogens with low probability of trasmission. This is especially true in low-income countries where high prevalence "classic" pathogens (malaria, HBV and others) are a frequent complication of blood transfusion.

Safety and Quality

The document entitled "Guide to the preparation, use and quality assurance of blood components" by European Committee on Blood Transfusion contains specific indications regarding quality parameters for production and use of different blood components (64).

Platelet products

In order to assure quality of platelets, standards are indicated for volume, platelet content, leukocytes and red blood cells content and pH level until last day of storage (64).

Several reports hightlight that all PI systems increase the platelet storage lesions, platelet metabolism and impair the platelet function *in vitro* (99, 112, 113, 114). The mechanisms leading to this impaired function differ for different systems and they are still unclear. However, despite these alterations, all pathogen reduction technologies meet good quality criteria with platelet quality parameters mantained throughout platelet storage.

The signs of platelet activation include increased metabolism, increased expression of phosphatidylserine (PHOS) in the outer layer of membrane and changes in the profile of platelet surface receptors (CD62P, P-selectine, expression) (115).

Particularly, an increased glycolitic flux, associated with increased glucose and bicarbonate consumption, lactate accumulation and consequently a progressive reduction of pH, is described after all pathogen reduction treatments. PH level after treatment meets the Council of Europe and FDA reccomandations of ph>6.4 and 6.2 respectively (2, 64, 116, 117, 118).

The expression on platelet membrane of CD62P and that of PHOS in the outer layer of platelet membrane are other changes associated to platelet activation. Increased externalization of PHOS expression is also associated with formation of microparticles and a reduced membrane responsiveness to osmotic stress (HSR) that is associated with post-transfusion efficacy. After amotosalen/UVA (119), riboflavin/UV (120) and UVC (121) treatments, CD62P expression increase over time of storage. HSR is immediatly modified after UVC irradiation, but it is not reduced after amotosalen/UVA treatment and effects of riboflavin/UV treatment are variable (122).

PI treatment seems to induce platelet apoptosis by activation of pro-apoptotic (Bcl-xl, Bak, cleavage of caspase-3 (123, 124) and inibition of anti-apoptotic pathways (125). However, many of these features are prominent only after 5 or 7 days of storage and may only need to be significant in case

of prolonged platelet storage (114).

Mitochondria in platelets have an important role in metabolism but play also a well known role in apoptosis and in preservation of platelet function during storage (126). Amotosalen/UVA treatment modifies expression of platelet mitochondrial DNA (127) and riboflavin/UV treatment causes discharge of free mitochondria, mainly at the last days of storage. However, UVC treatment does not increase mitochondria membrane polarization during storage (117, 122), as a matter of facts, it does not negatively influence oxidative metabolism despite enhanced glycolisis.

Interesting, some evidences indicate that release of mitochondrial DNA during storage correlate with adverse events after transfusion, particularly respiratory distress post-transfusion (128).

Finally, PI treatment is associated with reduced post-transfusional survival of platelets and shedding and/or desyalilation of adhesion receptors such as glycoprotein Ibα (GPIbα) and glycoprotein V (GPV) with important effects on platelet adhesion to subendothelial matrix and activated endothelial cells, interaction with coagulation factors and thrombin-dependent platelet activation (129, 130, 131, 132). Therefore, these consequences of treatment could explain the impaired adhesion of treated platelets in *under flow* conditions and the accelerated platelet clearance (133).

By virtue of the different development phases of these technologies, most data are available for amotosalen/UVA treatment whereas data about UVC technology are more limited. However, the consequences on the quality of platelet concentrates after UVC treatment seem to be less important than those of amotosalen/UVA and riboflavin/UV and markers of platelet metabolism are only moderately influenced by UVC irradiation (90, 115).

Several studies have evaluated the consequences of PI on platelet quality on *in vitro* parameters, but few studies have investigared the impact *in vivo* of these treatments.

Generally, clinical studies document that platelets retain their hemostatic efficacy after treatment.

In fact, no differences in incidence of severe bleeding have been observed for treated, compared to conventional platelets (134).

A recent meta-analysis by Estcourt et al (135) have investigated the effectiveness and safety profile of platelets treated with PI (PRP) compared with conventional platelets in the prevention of bleeding. The analysis has confirmed that no differences subsist between PRP and standard platelets in the incidence of clinically significant bleeding complications or life-threatening bleeding. Moreover, no differences have been observed in the incidence of serious adverse events, acute transfusion reactions, or minor adverse events. However, patients who have received PRP transfusions have showed a lower 24-hour corrected count increment (CCI) and have required more platelet transfusions. These patients had a greater risk of developing platelet refractoriness and an increased risk of alloimmunisation.

Red Blood Cells

Amustaline/GSH system, which is in clinical development, is the only PI method available specifically for RBCs. Riboflavin/UV system is under study for an extension of use to whole blood.

First generation of amustaline system did not affect significantly RBC quality and functions but reports showed an alloimmunization against treated RBCs, with a low-titer of antibodies which did not cause clinical hemolysis but resulted reactive versus the acridine moiety of amustaline (136). For this reason, the quencher concentration of GSH was increased from 2 to 20 mmol/l in order to decrease the aspecific activity of S-303 on the surface molecules of RBCs to lower the potential for immune response, resulting in promising results.

After Amustalin/GSH treatment, ATP containted in RBCs appears adequate but very low levels of 2,3 DPG are reported, likely consequence of room temperature storage rather than the treatment process itself (137). Moreover a decreased deformability of treated-RBCs compared to untreated cells is documented (138). The clinicial significance of these findings is unkown.

Aydinok et al. (139) showed that amustaline/GSH treatment of RBCs did not significantly increase RBC consumption in transfusion-dependent thalassemia (TDT) patients, appearing tolerated by patients and logistically manageable for chronic transfusion therapy. No differences were documented in safety profile between treated and untreated RBCs and no immune responses specific against treated units or alloantibodies were reported. Moreover, a phase III study investigated efficacy and safety profile of S-303 treated RBCs during and after a cardiac surgery. It demonstrated that treated units contained a normal Hb concentration, compared to conventional RBCs, and met EDQM criteria guidelines for RBCs quality parameters.

Clinically, no significant differences in effectiveness and safety profile was observed between treated units and conventional products (140).

In a phase II study, RBCs from WB treated with amustaline/GSH and transfused after 35 days of storage met the FDA guidance criteria for post-transfusion recovery and no significantly differences between treated RBCs and untreated RBCs were detected (141).

Cancelas et al. reported an acceptable RBCs quality of RBCs derivered by WB treated with riboflavin/UV irradiation. There was a decreased *in vivo* viability of RBCs treated and a reduced 24-hours recovery (around 10%) compared to untreated RBCs. However RBC units derived by treated WB met FDA criteria for 24-hour recovery after 21-day storage (142).

However, some studies show that immunization against amustaline-coated RBCs still occurs after modification of the system and antibodies against treated cells were detected in healthy blood donors who were never been transfused with pathogen-reduced RBCs (143).

These data seems to show that the use of chemical agents for PI of cellular products may increase the risk of immune responses against blood components in transfusion recipients.

Conclusions

The difficult road towards the target of a pathogen-free blood has made great progress in the last two decades.

Great differences persist in different countries and emergent pathogens threaten the obtained results. Thus, especially in low-income Countries, the goal of provison of safe blood is still hard to reach.

The developement and the implementation of PI technologies for blood components, may enable to have an additional tool to gain the aim. National and regional experience give back encouraging results.

In 2009, Belgium nation parliament has been the first to approve a bill that mandates nationwide PI for all platelet units distributed (74). After two years, the Swiss Agency for Therapeutic Products (Swissmedic) decided the universal diffusion of PI in Swizerland for platelet products and analysis of hemovigilance data revealed that without PI a fatal case of TTI might occur in Swizerland every two years (19). Regional Blood Transfusion Center in Warsaw (Poland) implemented Riboflavin/UV technology in order to increase safety of platelet concentrates produced by pooling of buffy coats (2009) and apheresis (2010). In 2015, an hemovigilance survey concluded that treatment of these blood components was safe and effective, and not associated with increased incidence of adverse events (144).

In an international survey conducted by Reesnik et al. (145), no TTIs were identified after approximately 200,000 transfused PRP products and no increased adverse events were documented compared to conventional products. However a complete evaluation of efficacy of these technologies is difficult in a context where the risk of TTIs is already low.

We tried to illustrate pros (inactivation of most clinically relevant pathogens and reduction of the residual risk of infection during the window period, when screening tests could provide false-negative results; reduction of the risk against unknown pathogens; elongation of shelf life from 5 to 7 days for platelet concentrates; inactivation of residual lymphocytes in the product) and cons (additional costs of implementing new technologies; impairment of platelet metabolism and of the platelet function *in vitro*, worsening of the platelet storage lesions; increased risks of alloimmunization against treated RBCs, decreased *in vivo* viability of RBCs, reduction of shelf life for treated RBCs) of PI treatment. In this regard, only long-time follow up studies, still absent, will guarantee the complete safety of these PI procedures.

It could be envisaged the implementation of a registry for recipients of blood components treated with PI, with the aim to monitor the occurrence of possible side effects, even in late periods.

Further studies should be conducted in order to finally document clinical efficacy and safety profile of treated products.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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