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**REVISED RECOMMENDATIONS FOR REDUCING THE RISK OF HUMAN
IMMUNODEFICIENCY VIRUS TRANSMISSION BY BLOOD AND BLOOD
PRODUCTS; DRAFT GUIDANCE FOR INDUSTRY**

REFERENCE 16

Pathogen inactivation and removal methods for plasma-derived clotting factor concentrates

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Pathogen safety is crucial for plasma-derived clotting factor concentrates used in the treatment of bleeding disorders. Plasma, the starting material for these products, is collected by plasmapheresis (source plasma) or derived from whole blood donations (recovered plasma). The primary measures regarding pathogen safety are selection of healthy donors donating in centers with appropriate epidemiologic data for the main blood-transmissible viruses, screening donations for the absence of relevant infectious blood-borne viruses, and release of plasma pools for further processing only if they are nonreactive for serologic markers and nucleic acids for these viruses. Despite this testing, pathogen inactivation and/or removal during the manufacturing process of plasma-derived clotting factor concentrates is required to ensure prevention of transmission of infectious agents. Historically, hepatitis viruses and human immunodeficiency virus have posed the greatest threat to patients receiving plasma-derived therapy for treatment of hemophilia or von Willebrand disease. Over the past 30 years, dedicated virus inactivation and removal steps have been integrated into factor concentrate production processes, essentially eliminating transmission of these viruses. Manufacturing steps used in the purification of factor concentrates have also proved to be successful in reducing potential prion infectivity. In this review, current techniques for inactivation and removal of pathogens from factor concentrates are discussed. Ideally, production processes should involve a combination of complementary steps for pathogen inactivation and/or removal to ensure product safety. Finally, potential batch-to-batch contamination is avoided by stringent cleaning and sanitization methods as part of the manufacturing process.

Human plasma is a source of clotting factor concentrates used for treatment of bleeding disorders caused by coagulation factor deficiencies. Such disorders include hemophilia A (Factor [F]VIII), hemophilia B (F IX), and von Willebrand disease (von Willebrand factor [VWF]); rarer disorders such as fibrinogen, FXIII, and FX deficiencies; and a number of acquired bleeding disorders. For von Willebrand disease, some rare bleeding disorders, and acquired bleeding, plasma-derived clotting factor concentrates, but not recombinant concentrates, are available. A four-factor prothrombin complex concentrate (Kcentra [Beriplex P/N in Europe], CSL Behring, Marburg, Germany) has been recently approved in the United States for reversal of warfarin.¹

Efforts to prevent transmission of infectious agents were accelerated after the recognition of human immunodeficiency virus (HIV) transmission to hemophilia patients in 1983 and the identification of hepatitis C virus (HCV) thereafter. By the 1990s, there was substantial clinical evidence of a lack of transmission of lipid

ABBREVIATIONS: B19V = parvovirus B19; BVDV = bovine viral diarrhea virus; EMA = European Medicines Agency; HAV = hepatitis A virus; PARV4 = human parvovirus 4; PPV = porcine parvovirus; PRV = pseudorabies virus; QSEAL = Quality Standards of Excellence, Assurance, and Leadership; SINV = Sindbis virus; vCJD = variant Creutzfeldt-Jakob disease; WNV = West Nile virus.

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membrane-enveloped viruses (hepatitis B [HBV], HCV, and HIV) using solvent/detergent (S/D) treatment.² Nonenveloped viruses, including hepatitis A virus (HAV) and parvovirus B19 (B19V), presented additional challenges, as they are not inactivated by this technique.³⁻⁵ In Germany, pasteurization (heating at 60°C for 10 hours in a stabilized aqueous solution) was developed in the late 1970s for a clotting factor concentrate as a dedicated virus reduction method; this FVIII and VWF concentrate was licensed in 1981 and no proven cases of virus transmissions were reported.⁶

More recently, prion transmission has become a concern.⁷⁻⁹ Manufacturing processes have evolved to include multiple steps to prevent transmission of both known and emerging blood-borne pathogens.¹⁰ The objective of this review is to examine current methods to prevent pathogen transmission via plasma-derived clotting factor concentrates by state-of-the-art selection of plasma, and effective pathogen inactivation and removal by the manufacturing process.

PATHOGEN REDUCTION REGULATIONS FOR PLASMA-DERIVED PRODUCTS

Clotting factor concentrates are derived from pools consisting of large numbers of donations.² The fractionation pool size varies depending on the size of the vessel used to pool the plasma. A batch of final product can include several fractionation pools. Donor screening and testing of donations and fractionation pools are the first steps in product safety, followed by pathogen inactivation and/or removal (i.e., reduction) during manufacturing. Source plasma from apheresis commonly comes from compensated donors, while recovered plasma is from whole blood donors not compensated in cash.^{11,12} The manufacturing process of clotting factor concentrates from both starting materials and their safety records are virtually identical.^{11,12}

The European Medicines Agency (EMA) published a guideline focusing on the safety of plasma-derived products;¹⁰ the fourth version details measures necessary to prevent transmission of viruses to recipients of plasma-derived products. These measures include selection of donors, screening of donations and plasma pools for markers of infection with known viruses, and a manufacturing process with a high capacity to inactivate and/or remove viruses by selected steps validated for their virus reduction capacity. The guidelines state that “effective steps for the inactivation/removal of a wide range of viruses of diverse physicochemical characteristics” are required during manufacture. Both the EMA and the World Health Organization (WHO) recommend that two distinct steps with complementary modes of action be used,^{10,13} helping to ensure that any virus surviving Step 1 is inactivated and/or removed in Step 2. At least one step

should inactivate or remove nonenveloped viruses. The EMA guideline further states that if a manufacturing process contains a step that reliably inactivates and/or removes a wide range of enveloped and nonenveloped viruses and additional manufacturing steps reliably contribute to inactivation and/or removal of viruses, the second step might not be required.¹⁰ In the United States, the manufacture of plasma-derived products must include at least one effective step for removal and/or inactivation of viruses.¹⁴ Further, careful validation is required for each inactivation or removal step. Various methods of virus inactivation and removal used in concentrates available in many countries are presented in Table 1.¹⁵⁻³²

To estimate the potential of specific steps for reducing prion infectivity, the EMA recommends evaluation studies for prions.^{7,9} Established processes for the production of clotting factor concentrates have been shown to reduce prion infectivity.^{7,33,34}

Biologic products, including recombinant clotting factor products, are required to undergo complementary pathogen reduction steps analogous to those of plasma-derived clotting factor concentrates.^{35,36} Validation requirements are also similar for recombinant proteins.^{35,36} The safety focus in plasma-derived products has received greater attention because of the history of virus transmissions before 1990. However, the potential for virus contamination is an issue for all biologic products.

PLASMA SCREENING

Adherence to strict donor selection criteria and screening of donations effectively reduces the virus load to be inactivated and/or removed from the plasma pool for fractionation. Currently, no screening tests are able to detect prion infectivity in donors,³⁷⁻³⁹ although an experimental blood test for prion infection was recently developed.⁴⁰ As a precaution, the EMA, the US Food and Drug Administration (FDA), and WHO recommend deferral of donors at risk of developing Creutzfeldt-Jakob disease (CJD) and its variant (vCJD) using a series of questions to exclude donations at risk from pooling and further processing.⁷⁻⁹

Although screening for viral markers by serology and virus nucleic acid by nucleic acid testing (NAT) ensures that nearly all plasma units entering production are free of HBV, HCV, and HIV, inactivation and removal steps are necessary to reduce any viruses that may enter the plasma pool during a “window period” before markers can be detected.⁶ In 1999, it was estimated that this window for serologic testing could result in the identification of up to 1 in 10 plasma pools containing an HIV-infected unit and up to one in five containing an HBV- or HCV-infected unit.² For a particular NAT assay and pool configuration, NAT has been shown to decrease the diagnostic window from 22 to 11 days for HIV-1, 59 to 34 days for HBV, and 82 to 23 days for HCV, thereby decreasing the amount of virus

TABLE 1. Virus inactivation and/or removal methods for selected plasma-derived clotting factor products^{1,15-32}

| Brand name (manufacturer) | Concentrated coagulation factor(s) | Virus inactivation and removal methods | | | | | | | Virus filtration |
|--------------------------------------|------------------------------------|----------------------------------------|----------------|----------------|-----|----------|----------------|------------|------------------|
| | | Precipitation | Chromatography | Pasteurization | S/D | Dry heat | Lyophilization | Vapor heat | |
| Alphanate SD (Grifols) | FVIII, VWF | x | x* | | x | x | x | | |
| Biostate (CSL Ltd.) | FVIII, VWF | x | x† | | x | x | x | | |
| Humate-P/Haemate-P (CSL Behring) | FVIII, VWF | x | | x | | | x | | |
| Koate DVI (Talecris Biotherapeutics) | FVIII, VWF | | | | x | x | x | | |
| Wilate (Octapharma) | FVIII, VWF | | x† | | x | | x | | |
| Hemofil M (Baxter) | FVIII | | x† | | x | | x | | |
| Monoclote-P (CSL Behring) | FVIII | | x* | x | | | x | | x |
| Alphanine SD (Grifols) | F IX | | x† | | x | | x | | x |
| Bebulin VH (Baxter) | F IX | | x† | | | | x | x | x |
| Monoline (CSL Behring) | F IX | | x* | | | | x | | x |
| Profilnine SD (Grifols) | F IX | | x† | | | | x | | |
| Fibrogammin P/Corfact (CSL Behring) | FXIII | | x† | | x | | x | | x |
| Beriplex P/N/Kcentra (CSL Behring) | FII, FVII, F IX, FX | x | x† | x | | | x | | x |
| Octaplex (Octapharma) | FII, FVII, F IX, FX | x | x† | x | | | x | | x |
| Haemocomplettan P (CSL Behring) | Fibrinogen | x | x† | x | x | | x | | x |

* Immunoaffinity chromatography.

† Size-exclusion chromatography.

‡ Ion-exchange chromatography.

entering the plasma pool for fractionation.⁶ This reduces the residual risk by decreasing both the number of potentially infected units in the pool and the concentration of virus. However, screening does not sufficiently ensure the safety of products, as tests have limits of detection and are target specific.¹³ In addition, the size of the minipool for NAT impacts its effectiveness.

INDUSTRY STANDARDS

The plasma industry developed two sets of standards through the Plasma Protein Therapeutics Association.⁴¹ The International Quality Plasma Program focuses on donor and center management. The second standard, known as Quality Standards of Excellence, Assurance, and Leadership (QSEAL), addresses manufacturing.⁴¹ Part of QSEAL is the inventory hold standard, which was introduced to address the “window period” described previously; this standard allows removal of previously donated units from donors who subsequently test positive for a virus (i.e., removal of window donations). This has been highly effective in reducing the risk of donations from infected donors who are nonreactive in serology (and NAT) at the time of donation from entering a manufacturing pool.⁴² The inventory hold is not a quarantine, as it would not interdict an infectious unit in the NAT window period if the donor did not return. The effectiveness of the hold is related to the large number of donors who donate frequently. All source plasma donations used for manufacturing are equivalent to donations for transfusion in terms of pathogen safety. In addition to manufacturing standards, QSEAL includes qualified donor and virus marker standards identical to those in International Quality Plasma Program.⁴¹

VALIDATION STUDIES

Validation of production steps is necessary to estimate pathogen inactivation and/or removal capacity and provides confidence in the safety of a plasma-derived clotting factor product.^{6,43} The virus reduction capacity of a manufacturing process is tested in a dedicated laboratory using a validated scaled-down version of the manufacturing process by employing process intermediates spiked with high titers of an appropriate virus and then determining the virus reduction factor achieved.⁶ Viruses selected for validation should closely resemble those that pose the greatest threat of contamination, such as hepatitis viruses and HIV. For some viruses, no practical cell culture system is available; thus, specific model viruses are used. The selected viruses should include different genome, size, and envelope characteristics to represent the wide range of the physicochemical properties of viruses. This allows manufacturers to fully test the virus reduction capacity of the manufacturing process and to provide indirect

TABLE 2. Virus reduction factors (log) for relevant viruses reported for individual process steps*

| Process step evaluated | Virus reduction factors (log) | | | | | | | | | |
|---------------------------------------------------------------------------------------|-------------------------------|-----------------|----------------|-------|--------------------|-----------------------|----------------------|------|------|-------|
| | Enveloped viruses | | | | | Non-enveloped viruses | | | | |
| | HIV, HIV-1 | HCV model, BVDV | HBV model, BHV | PRV | HAV and model, HAV | POL | B19V and model, B19V | PPV | CPV | MMV |
| Lyophilization ^{17,45} | 1.2 | 1.7 | 1.3 | — | 2.1 | 3.4 | — | — | <1.0 | — |
| Precipitation | | | | | | | | | | |
| Cryoprecipitation ³² | — | — | — | 1.6 | 1.5 | — | — | — | 1.5 | — |
| 3.5% PEG precipitation ¹⁷ | <1.0 | <1.0 | <1.0 | — | — | 3.3 | — | — | 1.2 | — |
| Al(OH) ₃ adsorption/glycine precipitation/NaCl precipitation ³² | 3.6 | 2.4 | — | 3.7 | 2.4 | — | — | — | 3.4 | — |
| S/D ^{17,46} | ≥11.1 | ≥4.5 | ≥8.0 | ≥8.5 | NA | NA | — | NA | NA | NA |
| Dry heat ^{17,45,46} | 5.2† | ≥4.9‡ | 2.1‡ | 4.9§ | ≥5.8‡ | ≥2.5‡ | — | 4.1§ | 4.1‡ | — |
| Pasteurization ³² | ≥6.4 | ≥8.9 | — | 4.6 | 4.2 | — | ≥3.9 | — | 1.1 | — |
| Chromatography ¹⁷ | ≥2.0 | <1.0 | 7.6 | — | — | <1.0 | — | — | <1.0 | — |
| Virus filtration ^{19,47} | >6.4¶ | 6.6** | — | >6.0¶ | 6.8** | — | — | — | — | ≤1.0¶ |
| Vapor heat ^{19,48} | >6.8 | >7.1 | — | >7.4 | >4.5 | — | ≥3.5†† | — | — | ≤1.0 |

* Dashes indicate that no data are available.

† 100°C for 30 min.

‡ 80°C for 72 hr.

§ 100°C for 120 min.

|| In aqueous solution at 60°C for 10 hr.

¶ 35-nm filtration.

** 35- to 15-nm sequential filtration.

†† 60°C for 500 min, followed by 80°C for 60 min.

BHV = bovine herpes virus (a nonspecific model virus for HBV); CPV = canine parvovirus (a model virus for B19V); MMV = mice minute virus (a model virus for B19V); NA = not applicable; PEG = polyethylene glycol; POL = poliovirus Sabin type 2.

evidence that it reduces viruses in general.^{13,14,43} Reduction factors greater than 4 log are generally considered effective.^{43,44} Some virus reduction factors achieved by individual inactivation and/or removal steps are presented in Table 2.^{17,19,32,45-48}

Removal of prions from plasma-derived products is evaluated similarly to virus validation studies, based on a valid scaled-down version of the manufacturing process.^{33,49,50} For prion detection, the quantitative animal bioassay is considered most appropriate, although the in vitro methods of Western blot assay, enzyme-linked immunosorbent assay, and conformation-dependent immunoassay are also used for the detection of misfolded prion protein.

PATHOGEN INACTIVATION METHODS

Pasteurization

Pasteurization by heating the protein in aqueous stabilized solution at 60°C for 10 to 11 hours inactivates both lipid membrane-enveloped and a range of nonenveloped viruses (Table 3).¹³ Coagulation factors are heat sensitive; therefore, stabilizers (usually sugars, amino acids, or acetate) are added to preserve protein integrity and are removed after pathogen inactivation. Homogeneity of temperature throughout pasteurization must be validated by temperature mapping techniques. Studies on inactivation of HIV, HAV, and B19V by pasteurization in a FVIII/VWF concentrate have demonstrated virus reduction

factors of at least 6.4, 4.2, and at least 3.9 log, respectively.^{6,32} Table 4 is an example of virus reduction factors observed in an actual product. Additional studies have demonstrated that pasteurization inactivates a wide range of enveloped and nonenveloped viruses, including bovine viral diarrhea virus (BVDV; a specific model virus for HCV), pseudorabies virus (PRV; a nonspecific model virus for HBV), herpes simplex virus-1, West Nile virus (WNV), and poliovirus.⁶

S/D treatment

Lipid membranes of enveloped viruses are disrupted by S/D mixtures, thereby preventing binding to and infection of cells by these viruses.⁵¹ Organic solvents (e.g., tri(*n*-butyl)phosphate), combined with a nonionic detergent (polyethylene glycol octylphenyl ether [Triton X-100] or polysorbate 80), are typically used for treatment; S/D compounds are removed by chromatography or oil extraction.⁵¹ Product solutions are filtered to remove viruses entrapped in particles and thus protected from exposure to S/D.¹³ Homogeneous mixing and maintenance of a consistent temperature throughout incubation must be validated. Plasma Protein Therapeutics Association member companies have compiled data on effective virus inactivation by S/D treatment for FVIII, FIX, intravenous immunoglobulin, and intramuscular immunoglobulin.⁵² Virus inactivation was evaluated in 308 studies reflecting production conditions, as well as variables significantly beyond process specification; the results demonstrated

TABLE 3. Common methods for virus inactivation*

| Treatment | Treatment conditions | Advantages | Limitations | Relevant properties to be recorded |
|-------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Pasteurization | <ul style="list-style-type: none"> Heat treatment in aqueous solution at 60°C for 10 hr Stabilizers (sugars, amino acids, or acetate) added | <ul style="list-style-type: none"> Inactivates enveloped and some nonenveloped viruses, including HAV and B19V Relatively simple equipment | <ul style="list-style-type: none"> Protein stabilizers may also stabilize viruses Stabilizers usually need to be removed after process is completed HBV is relatively heat stable Process validation required | <ul style="list-style-type: none"> Temperature Temperature homogeneity Duration Stabilizer concentration |
| Terminal dry heat | <ul style="list-style-type: none"> Heat treatment of lyophilized product at 80°C for 72 hr or Heat treatment of lyophilized product at 100°C for 30-120 min | <ul style="list-style-type: none"> Inactivates enveloped and some nonenveloped viruses, including HAV Treatment applied on final container | <ul style="list-style-type: none"> At least 80°C usually required for reduction of hepatitis viruses Requires strict control of moisture content Freezing and lyophilization and dry heat treatment conditions require extensive validation | <ul style="list-style-type: none"> Temperature Temperature homogeneity Duration Moisture content |
| Vapor heat | <ul style="list-style-type: none"> Heat treatment of lyophilized product at 60°C for 10 hr, with water content adjusted to 7%-8% (wt/wt) Additional heating at 80°C for 1 hr for some products | <ul style="list-style-type: none"> Inactivates enveloped and some nonenveloped viruses, including HAV | <ul style="list-style-type: none"> Freezing and lyophilization and heating conditions require extensive validation | <ul style="list-style-type: none"> Temperature Temperature homogeneity Duration Moisture during heating |
| S/D | <ul style="list-style-type: none"> Typical conditions: 0.3% TNBP and 1% nonionic detergent, either Tween 80 (≥6 hr) or Triton X-100 (≥4 hr) at 24°C | <ul style="list-style-type: none"> Very efficient against enveloped viruses Does not denature proteins High process recovery Relatively simple equipment Not generally affected by buffers | <ul style="list-style-type: none"> Nonenveloped viruses unaffected S/D reagents must be removed | <ul style="list-style-type: none"> Temperature Duration Reagent concentration Lipid levels must be controlled In-process solution free from gross aggregates potentially protecting viruses |

* Adapted from the World Health Organization.¹³
TNBP = tri(*n*-butyl)phosphate.

TABLE 4. Pathogen reduction factors for a pasteurized FVIII and VWF concentrate^{6,32}

| | Virus reduction factors (log) | | | | | | | Prion reduction factors (log) | |
|---------------------------------------------|-------------------------------|-------|------|------|-----|------|-----|-------------------------------|-------------------|
| | HIV | BVDV | WNV | PRV | HAV | B19V | CPV | Microsomes | PrP ^{Sc} |
| Cryoprecipitation (Stage 1) | | | | 1.6 | | | 1.9 | } | 3.9 |
| Al(OH) ₃ /glycine/NaCl (Stage 2) | 3.8 | 2.8 | | 3.9 | 2.3 | | 3.0 | | |
| Pasteurization (Stage 3) | ≥6.4 | ≥8.9 | ≥7.8 | 4.7 | 4.2 | ≥3.9 | 1.1 | } | 4.0* |
| Lyophilization (Stage 6) | | | | | 1.3 | | | | |
| Overall reduction factor | ≥10.2 | ≥11.7 | ≥7.8 | 10.2 | 7.8 | ≥3.9 | 6.0 | 6.4 | 7.9 |

* Reduction factor for the combined steps of pasteurization, second NaCl precipitation, dialysis, ultracentrifugation, and sterile filtration.
CPV = canine parvovirus (a model virus for B19V); PrP^{Sc} = disease-associated prion protein.

that product class, process temperature, protein concentration, and pH were not substantially involved in virus inactivation. In contrast, the concentration of S/D was critical when it was significantly below that specified for the process.⁵² For a FVIII and VWF concentrate, S/D treatment studies demonstrated reduction factors of more than 7.5 log for HIV, Sindbis virus (SINV), and PRV;⁴⁶ for a

F IX concentrate, reduction factors were greater than 4.5 log for HIV, SINV, vesicular stomatitis virus, BVDV, and PRV.⁴⁷

An important limitation of S/D treatment is specificity for lipid-enveloped viruses;^{4,53} there have been reports of patients with hemophilia with HAV or B19V infections after receiving S/D-treated FVIII concentrates.^{3,5,54-56}

TABLE 5. Selected common methods for virus removal*

| Treatment | Treatment conditions | Advantages | Limitations | Relevant properties |
|------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Chromatography | <ul style="list-style-type: none"> • Ion exchange • Size exclusion • Antibody-mediated affinity chromatography | <ul style="list-style-type: none"> • Purifies protein • Can remove both enveloped and nonenveloped viruses, including HAV and B19V | <ul style="list-style-type: none"> • Virus removal highly dependent on choice of resin, protein solution, and buffers • May be highly variable from one virus to another • Degree of virus removal may change as resin ages • Resin must be sanitized between lots | <ul style="list-style-type: none"> • Resin packing • Protein elution profile • Flow rate, contact time, and buffer volumes • Composition of buffer • Number of cycles of resin use |
| Virus filtration | <ul style="list-style-type: none"> • Filters with pores of approximately 15, 20, 35, and 50 nm | <ul style="list-style-type: none"> • Does not denature or activate proteins when validated production conditions are used⁶⁷ • High recovery of "smaller" proteins such as coagulation F IX | <ul style="list-style-type: none"> • Degree of virus removal depends on the pore size of the filter • Removal of small viruses may be incomplete | <ul style="list-style-type: none"> • Pressure • Flow rate • Filter integrity • Protein concentration • Ratio of product volume to filter surface area • Buffer composition |

* Adapted from the World Health Organization.¹³

Therefore, a second virus reduction step is required in the manufacture of such plasma-derived products to close this gap in virus safety.

Dry heat treatment of lyophilized products

Most plasma-derived concentrates are lyophilized and, especially those treated with S/D, subsequently treated with dry heat to inactivate nonenveloped viruses that resist S/D treatment. Lyophilization confers a certain degree of virus inactivation;^{2,57-59} the moisture content of lyophilized products undergoing dry heat treatment should be kept low (typically <2%), as residual moisture may affect product stability,⁴⁵ although higher levels may enhance inactivation of some viruses.⁶⁰

Dry heat treatment of lyophilized products has demonstrated favorable results for inactivation of relevant or model viruses of HAV, HBV, HCV, and HIV.^{45,46,61,62} Dry heating of a lyophilized FVIII product at 80°C for 72 hours inactivated a wide range of viruses,⁶⁰ and dry heating of FVIII or F IX concentrates reduced the risk of HCV transmission.⁶¹ An additional study demonstrated that terminal dry heating of a lyophilized FVIII concentrate at 100°C for 30 minutes inactivated HAV and HIV to below detectable levels within 10 minutes, while retaining approximately 95% of FVIII activity,⁴⁵ whereas heating a lyophilized FVIII and VWF concentrate at 100°C for 120 minutes reduced a wide array of viruses (HIV, SINV, PRV, reovirus type-3, HAV, and B19V) by more than 4 log.⁴⁶ Although B19V was reduced by dry heat in validation studies, the reduction factor may not be sufficient for complete inactivation of the virus load in the final product; asymptomatic B19V infection was detected in a patient who received FVIII concentrate treated at 80°C for 72 hours.⁶³

Vapor heat

One drawback to lyophilization is that in addition to stabilizing coagulation factors, the removal of water can also stabilize potential viruses in the product.⁶² By adding water vapor to lyophilized products before heating, higher levels of virus inactivation can be achieved at equivalent temperatures.¹³ Vapor heating of lyophilized products targets enveloped and nonenveloped viruses and has demonstrated inactivation of hepatitis viruses and HIV.¹⁹ Lyophilization and vapor heat at 60°C inactivated approximately 6 log of HAV in spiked FVIII concentrates within 8 to 10 hours and similarly reduced HAV titers in F IX concentrates within 3 hours.⁶⁴ Among 20 patients with hemophilia who received vapor-heated F IX infusions, none developed markers for infection with HCV or HIV during 6 to 15 months of follow-up, suggesting that vapor heat-treated concentrates may be associated with a low risk of viral infection.⁶⁵ However, vapor heat at 60°C may be insufficient to inactivate HBV completely, depending on the virus load in the starting material (batch related) and the amount of product given to patients not vaccinated against HBV; there are reports of four hemophilia patients not vaccinated against HBV who became infected with HBV after receiving vapor-heated FVIII concentrate.⁶⁶

METHODS OF PATHOGEN REMOVAL

Partition processes: fractionation and chromatography

In addition to virus inactivation, viruses can also be physically removed from plasma-derived products by fractionation (precipitation or chromatography) and filtration (Table 5). Before the 1980s, plasma fractionation was mainly considered a step in protein purification; no dedicated virus reduction step (except for pasteurization of

TABLE 6. Virus reduction factors for a pasteurized FXIII concentrate utilizing ion-exchange chromatography and virus filtration⁷²

| Stages | Virus reduction factor (log) | | | | | | |
|--------------------------------------------------------------|------------------------------|-------|-------|-------|----------------------|------|------|
| | Enveloped viruses | | | | Nonenveloped viruses | | |
| | HIV | BVDV | WNV | PRV | HAV | CPV | B19V |
| Adsorption to Al(OH) ₃ /Vitacel and defibrination | | | | 6.9 | | | |
| Ion-exchange chromatography | 5.0 | 3.3 | | ≥8.0 | 3.4 | 3.7 | |
| Pasteurization (heat treatment at 60°C for 10 hr) | ≥7.7 | ≥8.1 | ≥7.4 | | 4.3 | 1.0 | ≥4.0 |
| 20N/20N virus filtration | ≥6.1 | ≥5.0 | ≥7.4 | ≥6.4 | ≥5.6 | 6.1 | |
| Overall virus reduction factor | ≥18.8 | ≥16.4 | ≥14.8 | ≥21.3 | ≥13.3 | 10.8 | NA |

CPV = canine parvovirus (a model virus for B19V); NA = not applicable; PRV = pseudorabies virus (a nonspecific model virus for HBV).

human albumin) was implemented during the manufacturing process. Later virus validation studies showed that fractionation reliably contributes to the overall virus reduction capacity for plasma-derived proteins.^{47,68,69} Although ethanol precipitation is the most widely used plasma fractionation method worldwide,¹³ it is not used for the production of coagulation factor concentrates, as this treatment denatures the desired proteins.

After initial cryoprecipitation, many clotting factor concentrates are purified chromatographically. In a study by Roberts and colleagues,⁶⁹ purification of F IX concentrate using metal chelate affinity chromatography reduced poliovirus and SINV by reduction factors of 4.0 and 6.5 log, respectively. Some products are further purified using affinity chromatography mediated by an antibody against the protein of interest.⁴⁴ Affinity chromatography has been used to purify FVIII concentrates, resulting in reduction factors of 8.3 log for encephalomyocarditis virus,⁶⁸ 4.3 log for SINV,⁶⁸ 4.2 log for porcine parvovirus (PPV; a model virus for B19V),²¹ and 5.3 log for HAV.²¹ Purification of a FVIII concentrate using monoclonal antibody (MoAb) affinity chromatography contributed to overall virus reduction capabilities of 5 to 6 log for HIV, SINV, vesicular stomatitis virus, and PRV before pasteurization.²³ For a F IX concentrate, MoAb affinity chromatography alone resulted in reduction factors of at least 5.8 log for enveloped viruses HIV, BVDV, PRV, and WNV and reduction factors of 3.3 and 6.6 log for nonenveloped viruses HAV and parvovirus, respectively.²⁴ An important consideration exists in the reuse of chromatography columns;¹³ viruses adhere to resins and cannot be completely washed out. Therefore, equipment and material must be sanitized with validated chemical and/or physical treatments to inactivate and remove viruses to ensure no cross-contamination of subsequent batches of product.¹³

Virus filtration

Viruses may be removed from clotting factor concentrates via filtration employing retentive filters with pores smaller than the virus diameter (virus filtration, also called

nanofiltration).⁶⁷ Filtration may not be feasible for products containing proteins of a comparable or larger size to the viruses or pore size of the filter; thus, filtration is mainly limited to smaller-molecular-weight coagulation factors (e.g., F IX or FVIII). However, a VWF concentrate filtered through 35-nm pore membranes resulted in reduction factors of at least 5.0 log of enveloped viruses HIV, BVDV, and PRV, although filtration removed large VWF multimers as well.⁷⁰ Filtration of a FVIII concentrate using 35- and 15-nm sequential filtration resulted in removal of enveloped (HIV, BVDV, and PRV) and nonenveloped (HAV and PPV) viruses.⁷¹ Virus filtration alone resulted in virus reduction factors of at least 3.6 log, while combination of S/D, chromatography, and virus filtration yielded reduction factors of at least 5.1 log for all tested viruses. Virus filtration of a FXIII concentrate using a 20-nm filter resulted in a reduction of enveloped (HIV, BVDV, PRV) and nonenveloped (HAV) viruses by at least 5.5 log and a reduction of a nonenveloped parvovirus by 3.4 log.⁷² Table 6 is an example of virus reduction achieved through the addition of a filtration step. In a safety study on F IX concentrate, a final virus filtration step was added following fractionation, chromatography, and S/D treatment.⁴⁷ The 35- to 15-nm sequential filtration step resulted in a reduction factor of at least 6.8 log for HAV and at least 6.6 log for BVDV.⁴⁷ Filtration of another F IX concentrate using two filters (20-nm mean pore size) in series very effectively removed enveloped and nonenveloped viruses.²⁴

RISK ASSESSMENT REGARDING VIRUS TRANSMISSION

An appropriate margin of safety with respect to blood-borne viruses is achieved when the overall virus reduction factor clearly exceeds the amount of virus potentially entering the manufacturing pool. In Europe, quantitative risk assessments are required.¹⁰ An estimate of virus particle content of the finished product is determined based on the worst case scenario of virus concentration in the starting material and the amount of plasma required to produce one vial, divided by the virus reduction factor

demonstrated in validation studies. For this calculation, the following variables must be considered:¹⁰

- Virus load in the plasma volume needed for production of one vial of product
 - Volume of manufacturing pool
 - Potential virus load in a donation from an infected donor remaining below the limit of detection of a NAT assay
 - Volume of donation
 - Number of viremic donations entering a manufacturing pool taking into account
 - Epidemiology in donor population
 - Inventory hold and look-back procedure
 - Virus doubling time, if more than one donation from an infected donor could enter the manufacturing pool
 - Product yield
- Virus reduction factors demonstrated in validation studies

A high margin of safety is documented when fewer than one virus particle per million doses is expected; this is based on the sterility assurance level with respect to bacteria, molds, and yeasts by pharmacopoeias worldwide. As viruses cannot replicate in a cell-free medium such as the dissolved final product, application of the sterility assurance level for viruses is conservative.

PRION RISK REDUCTION

Transmissible spongiform encephalopathies are a group of prion-associated, fatal neurodegenerative diseases. Three possible cases of prion transmission resulting in vCJD and one probable case of asymptomatic infection have been reported in patients in the United Kingdom who received non-leukoreduced red blood cells from asymptomatic infected individuals.⁷³ To date, no recipients of pooled plasma-derived products have developed vCJD;³⁹ however, there is one report of asymptomatic prion detection at autopsy in a hemophilia patient,⁷⁴ probably due to prion-contaminated FVIII concentrate.⁷⁴ As prion screening tests are currently unavailable, donor selection and manufacturing processes for prion removal are important.³⁹ As requested by the WHO, EMA, and FDA, restrictions on plasma donation have been imposed in many countries.^{6-9,33} Plasma-derived proteins are leukoreduced by plasma preparation, decreasing the risk for prion transmission. Additionally, manufacturing steps for plasma fractionation and virus removal, including precipitation, chromatography, and filtration (Table 4), have also proven efficacy in evaluation studies.^{6,34,75} Inactivation of prions in plasma-derived clotting factor concentrates is not possible, as the needed techniques denature coagulation factors.³³ Appropriate cleaning and sanitization methods for material and equipment used in the pro-

duction of plasma-derived products inactivate and remove prion proteins; thus, batch-to-batch segregation can be achieved and batch-to-batch contamination excluded.⁷⁶⁻⁷⁹

EMERGING THREATS

Emerging microbial pathogens or parasites are less of a threat because of sterile filtration, which, in combination with other steps before preparation of the finished product, removes these pathogens. However, “new emerging viruses” must be assessed very carefully with regard to potential impact on the safety of the plasma pool for fractionation and the final products. New emerging viruses may be novel, zoonotic viruses that are encountered as humans enter new geographic areas with previously undiscovered animal viruses that cross the species barrier to enter the human disease chain (e.g., HIV, yellow fever virus, severe acute respiratory syndrome coronavirus, Menangle virus, Hendra virus, Nipah virus, hantaviruses, monkeypox virus). Hepatitis E virus has been detected in different regions of the world and is considered a zoonosis; in particular, Genotype 3, mainly detected in domestic and wild pigs, has been found in humans in developed countries.^{80,81} Human parvovirus 4 (PARV4) and related parvoviruses have been identified in cows, pigs, primates, and humans.⁸²⁻⁸⁶ Furthermore, a “new” virus can emerge and/or reemerge in new geographic regions; for example, WNV was known in Africa and the Middle East but emerged in North America in 1999.⁸⁷ Improved diagnostic methods have resulted in detection of previously unknown viruses (which should not be considered as emerging), such as B19V in 1975,⁸⁸ HCV in 1989,⁸⁹ human herpesvirus Type 8 in 1996,⁹⁰ and TT virus in 1997.⁹¹ To meet this challenge, the epidemiology of emerging pathogens in the donor population and the potential infectious virus load in a donation during asymptomatic incubation must be addressed. Diligent surveillance of available information may result in a (temporal) deferral of donors based on geographic risk.

PARV4 can reach a high load in an infected person,⁹² but the prevalence of the virus is low in many regions.^{93,94} Contamination of plasma pools for fractionation with PARV4 has been reported,⁹⁵ resulting in final products with PARV4 DNA. However, PARV4 DNA in current products is undetectable⁹⁶ or lower than in products manufactured between 1970 and 1980,^{97,98} possibly because of thorough screening of donors and testing of donations for pathogens that may be associated with PARV4. Some manufacturing processes for coagulation factor concentrates remove and/or inactivate low levels of PARV4.⁹⁸ Dedicated virus reduction steps in the manufacture of coagulation factor concentrates also reduced arthropod-transmitted viruses such as WNV,⁹⁹ other flaviviruses (dengue virus),¹⁰⁰ and togaviruses (Chikungunya virus);¹⁰¹

reduction of these viruses during manufacture of clotting factor concentrates was also demonstrated using model viruses for validation. These data demonstrate that the measures currently taken are also relevant for emerging viruses.

DISCUSSION AND CONCLUSIONS

The combination of inactivation and removal methods, in addition to donation screening, has essentially eliminated transmission of viruses of principal concern, namely HAV, HBV, HCV, HIV, and B19V. As shown in validation studies, a strategy involving different steps for inactivation and removal is optimal for targeting viruses with different physicochemical properties. For instance, although S/D is highly effective against enveloped viruses, it is ineffective against nonenveloped viruses (e.g., HAV and B19V); thus, a second step, often dry heating of the lyophilized product, is implemented. Procedures for purification and concentration of the desired protein are usually less effective in virus removal than dedicated reduction steps, such as inactivation by heat and S/D treatment or removal by virus filtration. As diagnostic techniques advance, emerging pathogens may be identified,⁹² and their genomes may be detected in the final product (e.g., PARV4), depending on the manufacturing process.^{96,98} The methods employed make it extremely likely that any new pathogen would be inactivated or removed to a high degree, but diligence must be maintained.

Immune globulin and other plasma products, such as α_1 -proteinase inhibitor and C1 esterase inhibitor, are subject to similar pathogen reduction measures. In addition, low pH can be utilized for immune globulin products to inactivate certain viruses. Thus, the same high safety record has been observed with these products in recent years. Albumin has always been pasteurized to prevent pathogen transmission.

A product similar to fresh-frozen plasma (FFP), but pooled and treated with S/D, has been available in Europe and was recently licensed in the United States (Octaplas).¹⁰² Enveloped viruses are effectively inactivated by S/D treatment, and chromatographic methods reduce prion transmission potential. This product relies on testing of the donations for protection against nonenveloped virus, specifically HAV, B19V, and more recently, hepatitis E virus. Improved virus safety compared with FFP is based solely on the reductions observed with S/D methods for blood products.¹⁰³

Over the past 20 years, fears of potential infection and the introduction of recombinant factor products have fueled a shift toward recombinant products as the first choice for treatment of patients with hemophilia. However, opinions on the role of recombinant and plasma-derived concentrates for treatment vary. The United Kingdom Hemophilia Center Directors Organiza-

tion recommends recombinant products for all patients with hemophilia, whereas other European countries recommend them as the first choice for children only. In contrast, in Germany and Austria, there are no clear recommendations on use of recombinant factor concentrates in patients with hemophilia. Furthermore, patients with other factor deficiencies for which no recombinant product is currently available (e.g., von Willebrand disease) must use plasma-derived concentrates.

Physicians currently have fewer safety concerns using plasma-derived products than in the past; in fact, 45% of FVIII consumption and approximately 65% of F IX consumption in Germany are plasma-derived. For patients with hemophilia A and inhibitors against FVIII, plasma-derived FVIII products containing VWF are widely used as second-line treatment for immune tolerance induction. Due to the improved and comprehensive pathogen reduction steps, physicians and patients should have confidence in the safety of plasma-derived products for the treatment of bleeding disorders.

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CONFLICT OF INTEREST

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