Estimating the pathogen safety of manufactured human plasma products: application to fibrin sealants and to thrombin

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BACKGROUND: Plasma fractionators have implemented many improvements over the past decade directed toward reducing the likelihood of pathogen transmission by purified blood products, yet little has been published attempting to assess the overall impact of these improvements on the probability of safety of the final product.

STUDY DESIGN AND METHODS: Safety margins for human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), hepatitis A virus (HAV), parvovirus B19, and variant form of Creutzfeldt-Jakob disease (vCJD) were calculated for the two fibrin sealants licensed in the United States and for thrombin. These products were selected because their use in a clinical setting is, in most cases, optional, and both were relatively recently approved for marketing by the US Food and Drug Administration (FDA). Moreover, thrombin and fibrinogen both undergo two dedicated virus inactivation steps and/or removal steps in accord with the recommendations of regulatory agencies worldwide. Safety margins were determined by comparing the potential maximum viral loads in contaminated units to viral clearance factors, ultimately leading to the calculation of the residual risk per vial.

RESULTS: The residual risk of pathogen transmission per vial was calculated to be less than 1 in 10^{-15} for HIV, HCV, HBV, and HAV for both fibrinogen and thrombin. Owing to the greater quantities that can be present and its greater thermal stability, the calculated risk for parvovirus transmission was 1 in 500,000 vials for fibrinogen and less than 1 in 10^7 per vial for thrombin. Assuming that vCJD is found to be present in plasma donations, its risk of transmission by these purified and processed plasma derivatives would appear to be very low.

CONCLUSIONS: The pathogen safety initiatives implemented by plasma fractionators over the past 10 to 20 years have resulted in products with excellent pathogen safety profiles. Of the agents examined, parvovirus continues to have the lowest calculated margin of safety. Despite this, parvovirus transmissions should be rare. Manufacturers are encouraged to continue exploring processes to further enlarge parvovirus safety margins and to continue exploring ways of eliminating prions.

Products derived from human plasma have important therapeutic uses, including substitution therapy for hemophilia and primary immune deficiency disorders, plasma expanders after trauma and surgery, and as hemostatic agents.[1-3] Plasma proteins and their functions are so diverse that new applications for currently licensed plasma protein products continue to be investigated[4] and novel plasma protein products continue to be developed.[5-7] Consequently, there has been an increase in the quantity of plasma processed worldwide, and significant improvements have been made in manufacturing procedures and in plant design and operation. Many of these improvements were implemented with the goal of assuring safety of plasma derivatives from transfusion-transmissible pathogens. These include 1) improved selection of donors, 2) use of plasma only from "qualified" donors who repeatedly pass viral screening procedures, 3) use of nucleic acid amplification testing (NAT) methods to detect and eliminate virus before the pooling of donor units, 4) inventory hold policies that allow interdiction of "window-phase"

ABBREVIATIONS: BVDV = bovine viral diarrhea virus; HAV = hepatitis A virus; ID(s) = infectious dose(s); PRV = pseudorabies virus; SARS = severe acute respiratory syndrome; S/D = solvent/detergent (method of virus inactivation); TNBP = tri-(*n*-butyl)phosphate (the solvent in S/D treatment); vCJD = variant form of Creutzfeldt-Jakob disease that infects man and presumptively has arisen from the epidemic of bovine spongiform encephalopathy in cattle; WNV = West Nile virus.

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units before pooling, 5) employment of purification procedures shown to remove virus or prions should they be present, 6) the use of two complementary or "orthogonal" methods of virus inactivation, and 7) the engineering and design of facilities so as to prevent contamination of downstream process streams with upstream fractions.

Products that promote hemostasis and tissue sealing following trauma and surgery are among the more recently licensed human plasma products in the United States. Two fibrin sealants, one from Omrix (New York, NY) and one from Baxter (Deerfield, IL), are licensed in the United States by the Food and Drug Administration (FDA), and Omrix also recently received approval of a topically applied thrombin. While they cannot be used in all surgical settings, such as to control high-pressure (arterial) bleeds, these products have been shown to improve surgical outcomes, reduce the time to hemostasis, reduce blood loss, and reduce surgical complications.[8] Substitutes for these human plasma-derived hemostatic agents have also been developed, including bovine thrombin and recombinant-derived human thrombin. Bovine thrombin is antigenically distinct from human thrombin and has been shown to elicit antibodies when used in man.[9] These antibodies, as well as antibodies elicited to bovine impurities in the product, especially antibodies to coagulation factor (F)V, have resulted in severe bleeding complications due to cross-reaction with their human counterparts.[10-13] Higher purification has reduced this complication, although a recent report[14] indicates that antibody formation still occurs. Products made by recombinant technology have their own, somewhat unique, issues. Depending on the gene construct used and the cell line chosen, the amino acid sequence may differ from that which occurs naturally, and differences in posttranscriptional processing often result in altered patterns of glycosylation or other molecular changes.[15-19] Consequently, immunogenicity is a potential problem that needs to be continually assessed. Also, depending on specific production details, manufacturing procedures must employ steps designed to inactivate and/or remove viral contaminants (and other potential pathogens) known to be present in the cell line and/or in the culture medium employed.[20] Additionally, in many circumstances, the higher cost associated with recombinant proteins limits their use.

In the past decade, many estimates of the viral safety of transfused whole blood and its components (i.e., red blood cells, platelet concentrates, and fresh-frozen plasma) have been published, with each passing year showing improved viral and bacterial safety.[21,22] In the same time frame, aside from monitoring clinical outcomes and despite the aforementioned improvements, little has been published to assess the parallel increase in safety of manufactured plasma products. A recent publication by Janssen and colleagues[23] used a probabilistic, Monte Carlo model to estimate the risk of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) in a hypothetical plasma derivative subjected to what appears to be a single method of viral inactivation. Based on their assumptions, they calculated that the risk per vial approximated 1 in 1 million. Given these improvements, the recent licensure of human plasma-derived topical thrombin, and the frequent surgical use of fibrin sealants, consisting of fibrinogen in addition to thrombin, it is timely to estimate their pathogen safety. These estimates are especially useful since the fibrinogen component of fibrin sealants is among the least processed blood derivatives, while the manufacturing procedures for thrombin, whether part of a fibrin sealant kit or used by itself, are typical of those employed with most newer plasma derivatives. Thrombin and fibrinogen both undergo two dedicated virus inactivation steps and/or removal steps. Fibrinogen and thrombin from each company are each treated by solvent/detergent (S/D). Additionally, Omrix pasteurizes its fibrinogen and nanofilters its thrombin, while Baxter vapor heats both components following lyophilization. The fibrinogen preparation cannot be nanofiltered without suffering large losses in fibrinogen and fibronectin due to their large size. The presence of fibronectin may be important since it contributes to cell adhesion.[24] For HIV, hepatitis C virus (HCV), and HBV, this report updates estimates made by one of us (BH) in 1990[25] using better information on viral loads than was available then and enlarges the pathogen list to include hepatitis A virus (HAV) and parvovirus, both of which are nonenveloped viruses, and the prion that causes variant form of Creutzfeldt-Jakob disease (vCJD). It is anticipated that the method of approach reported here can be applied to other existing or experimental blood protein products.

MATERIALS AND METHODS

Two distinct methods can be used to calculate pathogen safety. The first is to calculate safety margins by comparing the number of infectious units or doses of pathogen in the starting material to the clearance capacity of the manufacturing process. The second is to measure clinical outcomes, comparing the incidence of transmission to the quantity of product infused. Transmissions in a clinical setting should be considered the gold standard since they involve actually measuring what we want to know, and with this information, one can back-calculate clearance capacities of processes for known pathogen burdens. On the other hand, clinical studies of the type required can be extremely lengthy and expensive and the results possibly misleading. The former method has the advantage that estimates of safety can be made in advance of clinical testing. Moreover, the safety margins calculated for a wide range of viruses likely will also be applicable to unstudied and newly emerging viruses.

Product safety margins can be calculated by comparing potential viral loads with the viral clearance capacity by the formulas

$$VL = N \times C,$$

where VL is viral load, N is the number of units in a plasma pool containing infectious virus, and C is the concentration of virus in those units, and

$$\text{Safety Margin} = CC/VL,$$

where CC is the clearance capacity or the ability of the process to remove or inactivate the infectious agent being studied.

Viral load

The pathogens of interest for manufactured plasma products are largely viruses that are present in blood predominantly as cell-free virions (e.g., HBV, HCV, HIV, HAV, and parvovirus B19). Other examples include West Nile virus (WNV) and dengue viruses. The newly described vCJD agent, presumably a prion, is also a potential concern despite the absence of evidence that it is transmitted by purified plasma protein products.[26,27] Cell-associated viruses like cytomegalovirus, Epstein-Barr virus, and human herpes virus 8 are not a concern since infected cells are removed by the apheresis and filtration procedures in common use. Bacteria and fungi are also effectively removed by the terminal sterile filtrations applied to all biologic products, including plasma products, recombinant products, monoclonal antibodies, and so forth, and therefore will not be addressed here.

For the major transfusion-transmitted viral pathogens, the viral loads are typically measured as genome-equivalents (geq) per mL of plasma based on results of quantitative NAT. These loads vary dramatically during the progressive stages of infection with the highest viral loads seen transiently during the acute preseroconversion (i.e., so-called window period) stage of infection; moreover, infectiousness is also highest during this same period.[28-30] Subsequent to antibody seroconversion (and coincident with innate and adaptive cellular immune responses to infection), the agents are 1) eliminated from the body (e.g., eradication of infection, as occurs with WNV, HAV, and dengue); 2) cleared from plasma but with persistence of cell-associated virus in tissues (e.g., latent infections such as herpes viruses, parvovirus B19, and "occult" HBV infections); or 3) persistent at reduced concentrations in plasma (i.e., so-called set-point viremia after establishment of chronic HBV, HCV, and HIV infections). In addition to variations in viral load measured by NAT, as infections evolve the infectivity of viruses change profoundly.[31-39] For HIV (and its model agent simian immunodeficiency virus), HBV, HCV, and WNV, it is now well established that during the acute preseroconversion phase of infection (pre–ramp-up and ramp-up stages), virion particles in plasma are highly infectious, with 10 or fewer geq in the entire volume of plasma sufficient to transmit infection following parenteral injection. In contrast, viral particles present in plasma from the same infected individuals have significantly (10- to 1000-fold) reduced infectious potential weeks to years after seroconversion.[29-35,40-43] The reduced infectivity of plasma virus from postseroconversion phases of chronic infection is attributable to a combination of factors, including presence of endogenous neutralizing antibodies, generation of defective virions (i.e., lacking full genomes or other required infectivity factors), and immune selection of virions with reduced fitness. Hence, viral load distributions observed during acute versus chronic stages of infection need to be adjusted by a factor to account for the relative infectivity of virion particles to derive estimates for the functional viral load during each stage.

All donated blood in the United States, whether for the preparation of components or for use in manufactured plasma products, is screened by serologic assays for HIV-1 and -2, HBV, HCV, and human lymphotropic virus-1 and -2 and by NAT for HIV and HCV. Donors are also excluded if they have certain risk factors that make their exposure to viruses or prions more likely. Additionally, plasma manufacturers screen donated plasma in a minipool format for HBV, HAV, and parvovirus by NAT. The use of NAT greatly reduces viral loads since positive units missed by serologic screening procedures typically have the highest concentrations of virus, which is also highly infectious. Consequently, with very rare exceptions of concordant testing errors in serology and NAT screening, only units that test both serologically negative (i.e., window-phase units) and that have relatively low titers of infectious virus (<500-5000 infectious doses [IDs]/mL) are pooled. Furthermore, manufacturing pools are retested by NAT before fractionation to assure that high-titer viremic units were not missed as a result of erroneous testing. As a result, the probability that a fractionation pool contains a significant level of virus is extraordinarily remote.

Pathogen infectious load estimates are given in Table 1. To estimate the number of positive units missed by the screening procedures employed, currently observed NAT yields, expressed as number of positive samples per million donations, were adjusted to account for the amount the window period is believed to be closed through the use of NAT. From this, we conclude that few plasma pools will contain HIV, HCV, or HAV while contamination by HBV and parvovirus B19 will be considerably more frequent. Based on the analytical sensitivity of the NAT assays, the dilution factors during assay, and the volume of an individual donor unit, we calculated the

TABLE 1. Viral load estimates

Virus	NAT yield (number/million donations)* (A)	Percent NAT closes window period† (B)	Number of positive units missed by NAT/million donations (C) = (A/B – A)	NAT analytic sensitivity‡ (geq/ml)	NAT operational sensitivity‡ (geq/mL in the donor unit)	Maximum genomic load (log geq/ fractionation pool)§	Ratio of IDs to geq¶	Maximum viral load (log ID/pool)
HIV	0.58	48% (11/23)	0.63	1.40	717	5.7	1:1	5.7
HCV	4.08	88% (50/57)	0.56	3.10	1,587	6.0	1:1	6.0
HBV	13	23% (10/43)	44	0.66	338	5.4	1:10	4.4
HAV	0.30	71% (5/7)	0.12	2.0	1,024	5.9	1:1,000	2.9
Parvovirus B19	50.5	71% (5/7)	20.6	22.6	5,120,000	9.6	1:1,000	6.6
vCJD						4.3	1:1	4.3

* For HIV, HCV, and HBV, a NAT yield unit is defined as an antibody- or hepatitis B surface antigen (HBsAg)-negative donation detected by RNA and/or DNA screening using pooled NAT systems. Rates presented are published rates from United States and European whole-blood donor screening programs.[76-78] Although NAT yield rates for these viruses among source plasma donors are higher, this is offset by source plasma policies that stipulate that only plasma from "qualified donors" be released for fractionation and that frozen units be held in inventory enabling interdiction of quarantined potential window-phase units when donors later test reactive for infectious disease markers or are deferred for other reasons.[79] For HAV and parvovirus B19, we use the rate of detection of high titer viremic donations by low sensitivity NAT screening of whole-blood and plasma donors, irrespective of serostatus of viremic units.[80,81]

† The percentage is determined by dividing the number of days NAT detects positive samples by the number of days from when a donor becomes infectious until there is sufficient antibody to be detected serologically (HIV, HCV, HBV) or there is sufficient antibody to render the donation noninfectious (HAV, parvovirus; see Busch et al.[82] for conceptual basis for this approach and Kleinman and Busch[83] for application of this approach to HBV). The residual infectious window periods are defined as the number of days from viremia reaching the minimal infectious threshold (set as 1 copy per 20 mL of plasma; Busch et al.[84]) to the level of viremia detected by pooled-sample NAT, using the viral doubling-times during the acute ramp-up phases established for each agent (20.5 hr for HIV, 10.8 hr for HCV, 2.6 days for HBV, and approx. 1 day for HAV and parvovirus B19). This yielded pre-NAT infectious window periods of 12 days for HIV, 7 days for HCV, 33 days for HBV, and 2 days for HAV and parvovirus B19. The NAT detection windows are based on time from reaching the 50 percent sensitivity of the NAT screening assays to the point of seroconversion for HIV (11 days), HCV (50 days), and HBV (10 days) or the duration of the estimated NAT yield window period for HAV (5 days) and parvovirus B19 (5 days).

‡ We assumed that the 50 percent sensitivity levels for assays used by source plasma donors are in the same range as those reported by the National Genetics Institute (NGI). NGI NAT assays are used by approximately 60 percent of the source plasma sector for all five viruses, as well as by the American Red Cross for HAV and parvovirus B19. For HIV-1, HBV, and HCV, the analytic sensitivity quoted is that of the assay itself without taking sample dilutions or pooling into account (Schreiber et al.[79]). Operational sensitivity takes these dilutions into account and refers to the maximum quantity that could be present in the contaminated donor unit. For parvovirus, Omrix's acceptance requirement for a pool of 512 units is less than 10,000 geq per mL, and thus for operational sensitivity we used $10{,}000 \times 512$ (the number of units in the minipool).

§ NAT operational sensitivity was multiplied by 700, the assumed volume of the donation. Based on the number of units missed by NAT per million donations and a pool size of 6000 L, we assume that only one positive unit will enter a fractionation pool. For vCJD, we assumed 30 ID per mL in a contaminated unit.

¶ For HIV and HCV, the infectious load is considered to be equivalent to the viral load expressed in geq or copies, given that we are restricting consideration to the acute preseroconversion viremic phase, which is known to be highly infectious (see text), and that seropositive units from other donors, which might contain neutralizing antibodies, have been detected by serologic screening and excluded from the manufacturing pools. For HBV, we similarly assume high-level infectivity of window-phase donations[35,85] but reduce this to a ratio of 1 in 10, in part, because of the likely presence of anti-HBsAg in the plasma pool. We used a ratio of 1:1000 for HAV and parvovirus. We believe this to be justified since the neutralization capacity of anti-HAV is well established. While the ratio of infectious units to geq for parvovirus is unknown, results from tissue culture infectivity studies indicate that the ratio is 1:5000 for genotype 1 and 1:260,000 for genotype 2[86] for products devoid of parvovirus antibody, the lowest ID that has been reported on infusion into a seronegative recipient is 2×10^4 geq,[70] and the infectivity of products containing parvovirus antibody has been shown to be reduced considerably.[83,87,88]

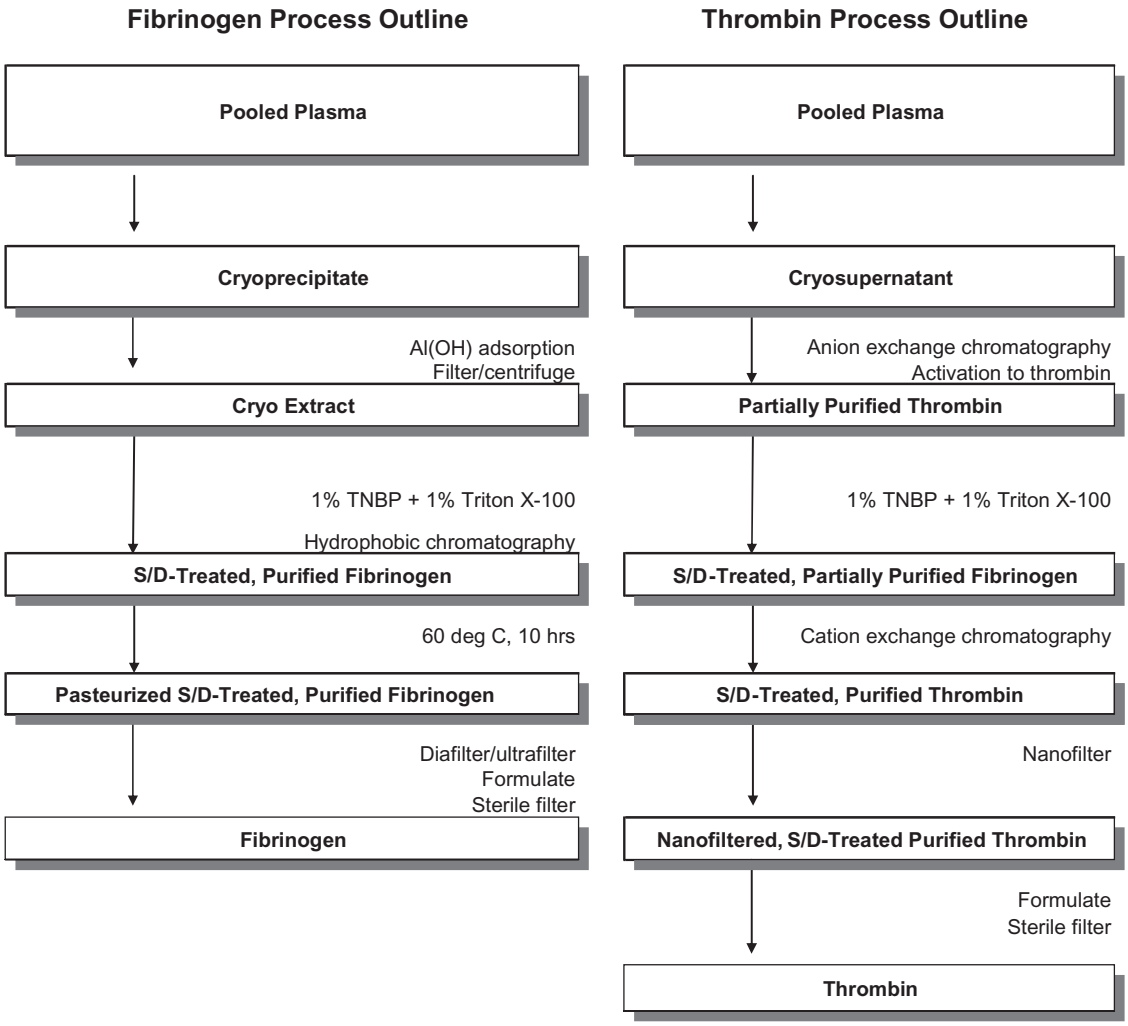

Fig. 1. Process outlines for fibrinogen and thrombin.

maximum genomic load likely to be present in a fractionation pool (Table 1, Column 7). This was adjusted downward for HBV, HAV, and parvovirus B19 to account for the reduced infectivity of virus that occurs as a result of neutralizing antibodies derived from other donors in the pool, since antibody screening is not performed for these prevalent agents. No adjustment was applied to HIV or HCV since later-stage infections with potential neutralizing antibodies are interdicted by the currently deployed serologic tests. Although attempts to transmit vCJD by human plasma have failed,[44] epidemiologic evidence supports its transmission by whole blood and blood components.[45-47] Based on animal models, its concentration is likely to be quite low, estimated at 20 to 30 IDs per mL.[48] With the use of this estimate, the maximum concentration in the plasma pool approximates 0.003 IDs per mL (3 IDs/L), and the total maximum load in a 6000-L plasma pool will approximate $10^{4.3}$ IDs (Table 1).

Clearance capacity

The clearance capacity for pathogens is a function of the extent to which pathogen is removed during steps designed to purify the protein of interest, the inclusion within the manufacturing process of dedicated viral inactivation and removal steps, the presence of neutralizing antibody in final product, and serendipitous inactivation that occurs. The process steps for fibrinogen and thrombin used by Omrix are outlined in Fig. 1. As is typical of modern plasma protein products, each process includes two dedicated, viral elimination steps: fibrinogen is treated with S/D and is pasteurized, and thrombin is treated with S/D and is passed through a purposefully designed, virus removal filter (so-called nanofiltration). Additionally, each chromatographic step and filtration in the presence of filter aid can contribute to pathogen removal. Baxter's Tisseel is processed similarly except, when first introduced in 1998, it utilized vapor heating as

TABLE 2. Validated viral elimination when processing fibrinogen: Omrix process

		Log kill or removal				
	Virus:	HIV-1	BVDV	PRV	HAV	CPV
	Model for:	HIV	HCV	HBV	HAV	Parvovirus B19
Step	Enveloped virus?:	Yes	Yes	Yes	No	No
Cryoprecipitation + Al(OH) treatment		ND	ND	ND	1.5	1.5
S/D treatment		>4.4*	>4.4†	>4.4†	ND	0
Pasteurization		>4.4‡	>5.5‡	ND	>5.8‡	1.3
	Sum:	>8.8	>9.9	>4.0	>7.3	2.8

* No infectivity after 5 minutes. Treatment is for 4 hours.
† No infectivity after 10 minutes, the first time point taken.
‡ 9 to 10 hours were required to achieve reported kills. Treatment is for 10 hours.
Al(OH) = aluminum hydroxide; CPV = canine parvovirus; ND = not done.

TABLE 3. Validated viral elimination when processing thrombin: Omrix process

		Log kill or removal						
	Virus:	HIV-1	BVDV	Sindbis	PRV	EMCV	CPV	MMV
	Model for:	HIV	HCV	HCV	HBV	HAV	Parvovirus B19	Parvovirus B19
Step	Enveloped virus?:	Yes	Yes	Yes	Yes	No	No	No
Cryo removal		ND	ND	ND	ND	ND	ND	ND
Anion-exchange chromatography		ND	ND	ND	ND	ND	ND	ND
S/D treatment		>5.8*	>4.7†	>5.3‡	>4.3†	ND	0	ND
Cation-exchange chromatography		ND	ND	ND	ND	ND	ND	ND
Nanofiltration		>4.4	ND	>5.3	>5.5	7.0	5.9	5.8
	Sum:	>10.2	>4.7	>10.6	>9.8	7.0	5.9	5.8

* No infectivity after 5 minutes. Treatment is for 6 hours for thrombin.
† No infectivity after 10 minutes, the first time point taken.
‡ No infectivity after 15 minutes, the first time point taken.
EMCV = encephalomyocarditis virus; MMV = mouse minute virus; ND = not done.

its sole, dedicated virus inactivation step; S/D treatment has been added recently. Another difference is that its thrombin component is isolated starting with Baxter's activated prothrombin factor complex.

The FDA and other applicable regulatory authorities demand that formal viral inactivation and/or removal studies be performed and that these adhere to international standards as they relate to the selection of viruses to be used, the conduct of these studies under Good Laboratory Practice guidelines and the calculations provided. We need not reiterate those guidelines here, except to say that the model viruses selected were chosen to represent multiple viral types and, in particular, the viruses of concern for products derived from human blood. Thus, viral elimination studies typically use HIV, bovine viral diarrhea virus (BVDV; model for HCV), pseudorabies virus (PRV; model for HBV), HAV or another picornovirus such as encephalomyocarditis virus, and canine parvovirus (or another model for human parvovirus B19).

The results from these formal studies for the fibrinogen and thrombin components of Omrix's and Baxter's fibrin sealant products are given in Tables 2 and 3 and Table 4, respectively (see product package inserts, with updates from manufacturers; see Acknowledgments). The clearance factors for enveloped viruses and the models for HIV, HCV, and HBV exceed the challenge dose for each of the dedicated viral elimination steps (i.e., S/D, pasteurization, nanofiltration, and vapor heating). Consequently, when the same virus has been studied in each of the two dedicated steps, the validated clearance factors exceed 9 log, and where higher doses of virus have been used or more steps validated, clearance factors as large as 18 log have been reported. The validated clearance of nonenveloped viruses is significantly less than for enveloped viruses since only one of the two dedicated viral elimination methods is effective against these viruses. Parvoviruses are a special case since they are especially heat-stable, and only 1 to 2 log of animal parvoviruses are inactivated by either pasteurization or vapor heating. It should be noted, however, that human parvovirus B19 may be more heat-sensitive than the models used here.[49] Nanofiltration is significantly more effective, and Omrix has shown for its thrombin preparation that nanofiltration removes approximately 6 log of parvoviruses.

A more complete estimate of safety margin needs to take into account the contribution of the other steps in the process that contribute to safety despite not being formally validated. It is commonly accepted that immune neutralization contributes to HAV and parvovirus B19 safety and that the neutralization capacity of antibodies to

TABLE 4. Validated viral elimination when processing fibrinogen and thrombin: Baxter process

		Log kill or removal				
	Virus:	HIV-1	BVDV or TBEV	PRV	HAV or ERV	MMV
	Model for:	HIV	HCV	HBV	HAV	Parvovirus B19
Process and step	Enveloped virus?:	Yes	Yes	Yes	No	No
Fibrinogen						
Cryoprecipitation + wash		2.6	1.3	1.5	1.8	ND
Lyophilization + vapor heating		>6.2	>6.8	>7.1	>6.5	1.5
S/D treatment		>6.6	>6.5	>6.7	NA	NA
	Sum:	>15.4	>14.6	>15.3	>8.3	>1.5
Thrombin						
Cryoprecipitation + wash		1.4	ND	1.1	ND	ND
Anion-exchange chromatography		2	ND	3.1	ND	ND
Lyophilization + vapor heating		>5.3	>5.9	>7.0	>4.7	1.0
S/D treatment		>5.2	>6.0	>6.9	NA	NA
	Sum:	>13.9	>11.9	>18.1	>4.7	1.0

MMV = mouse minute virus; NA = not applicable; ND = not done.

TABLE 5. Assignment of additional virucidal activity based on reserve capacity

Time required for complete kill (% of total)	Estimated minimal additional cidal power (log)
>100	0
76-100	1
51-75	2
26-50	3
≤25	4

these viruses is at least 3 to 4 log.[50] Since fibrinogen is purified by simple precipitations, it, like intermediate-purity FVIII preparations, likely benefits from the copresence of antibody in the final preparation.[51] Ion-exchange chromatography typically removes 2 to 3 log of virus.[52-55] Finally, some contribution to the calculation of safety margins should be ascribed to the "reserve capacity" of the viral inactivation method(s) employed, defined as the ability to achieve complete virus kill in a fraction of the treatment time allotted. While numerous publications make clear that linear extrapolation of virus inactivation curves overstates inactivation potential,[56,57] assigning no benefit to reserve capacity when calculating safety margins clearly underestimates inactivation capacity. Unless data indicate otherwise, we propose adopting the scheme described in Table 5. Although seemingly arbitrary, this scheme has the value of simplicity. Its use is supported by the dozens if not hundreds of times results with S/D and heat treatment methods have been reproduced, thereby increasing the quantity of virus subjected to challenge. Also, for S/D treatment methods, viral kill has been shown to be complete even when using tri-(*n*-butyl)phosphate (TNBP) together with sodium cholate, a combination that provides far slower kill kinetics than the more frequently employed TNBP-Tween 80 or TNBP-Triton X-100 combinations (Fig. 2), and the fact that reagent concentration can be halved without affecting viral kill (data not shown). We have not made reserve capacity estimates for vapor heating since much of the loss in viral infectivity occurs before initiation of the heat cycle (Fig. 3).

Taking these factors into account, along with published information on the inactivation of HIV, HCV, and HBV,[58] for fibrinogen, we estimate that 15 to 17 log of enveloped viruses, 10 to 11.5 log of HAV or most other nonenveloped viruses, and 7 log of parvovirus are eliminated. For thrombin, we estimate that 17 to 22 log of enveloped viruses, 11 to 13 log of HAV, and 7 to 10 log of parvoviruses are eliminated (Table 6).

Calculation of safety margin

The calculated margins of safety are given in Table 7. For enveloped viruses, safety margins are exceedingly large, estimated at about 100 billion-fold for fibrinogen and 1 trillion-fold for thrombin. Although lower, the safety margins for HAV for both fibrinogen and thrombin exceed 1 million-fold. Owing to the potentially significantly higher content of parvovirus and its greater resistance to inactivation, fibrinogen enjoys only a small safety margin while that for thrombin is approximately 2000-fold. Even still, when expressed as risk of transmission per vial, a calculation typically required by regulatory authorities, the risk with fibrinogen is calculated at 1 in 500,000 vials and that with thrombin is approximately 1 in 100 million vials or less. Thus, parvovirus transmission should still be an infrequent event.

Clinical experience

Tisseel has been marketed in the United States since 1998, and in Europe it was introduced clinically more than a decade earlier. Evicel and, except for a formulation change, its identical predecessor product Crosseal have been marketed in the United States since 2003 and were available in Europe several years earlier. Throughout this use, there have been no known cases of hepatitis or HIV

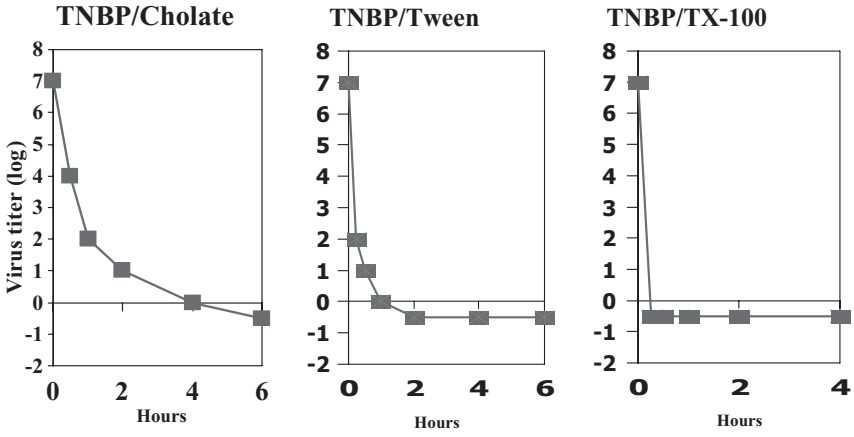

Fig. 2. S/D inactivation of vesicular stomatitis virus added to an antihemophilic factor concentrate.

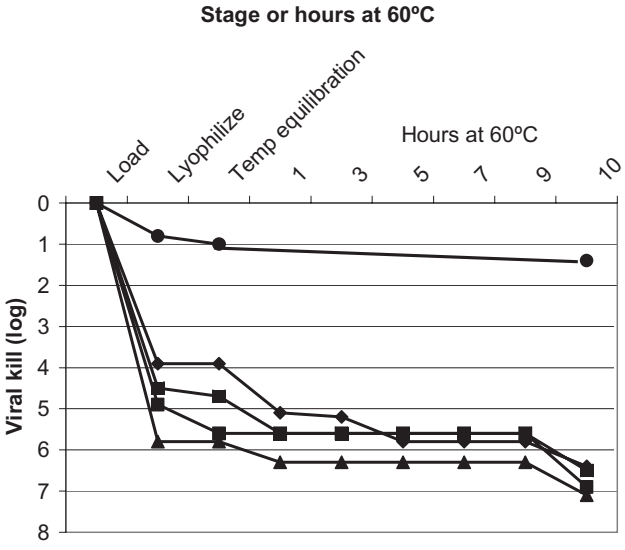

Fig. 3. Validated viral kill on vapor heating of fibrinogen (currently used Baxter process). BVDV (■) and PRV (▲) were undetectable upon reaching 60°C, and HIV (◆) and HAV (■) were undetectable after 5 and 1 hour, respectively, at 60°C. (●) Mouse minute virus.

transmission associated with commercial fibrin sealants.[59] This is notable since, for much of this time, the manufacturing process for Tisseel utilized only one dedicated viral elimination step while modern processes utilize two. On the other hand, epidemiologic evidence suggests that among patients who do not have parvovirus antibody at the time of fibrin sealant application, approximately one-fifth have reduced reticulocyte counts 12 to 20 days after surgery and develop parvovirus antibodies 12 to 48 weeks after surgery.[60] This finding is consistent with the calculations presented above since the study was performed using fibrin sealant prepared from plasma pools that were not screened by NAT for parvovirus, thereby potentially starting with 10,000-fold higher parvovirus loads. Experience with S/D-plasma indicates that reducing the quantity of parvovirus DNA to no more than 10^4 geq per mL (10^6 geq/patient exposure) eliminates parvovirus transmission as measured by DNA replication or seroconversion when the patient also receives product containing parvovirus antibody.[61] Additional clinical studies are needed to show whether the fibrin sealants manufactured today can still transmit parvovirus B19 or one of the newly described, human blood-borne parvoviruses.[62]

New viral threats

In the past 5 to 8 years, three new pathogens, WNV, Chikungunya virus, and vCJD, have emerged as potential threats to the blood supply. Other infectious agents, like H5N1 influenza virus, the strain of corona virus that causes severe acute respiratory syndrome (SARS); dengue virus; and vaccinia virus are potentially transmissible by blood and blood products. With the exception of vCJD, all are enveloped viruses and would be expected to be completely cleared by the processes now in place for manufactured blood products like fibrin sealant or thrombin. As shown in Table 8, WNV, H5N1 influenza virus, SARS-associated corona virus, and Chikungunya virus were all inactivated completely to the extent of challenge by the methods of viral inactivation discussed above.

The infectious agent of vCJD is believed to be a protein that resists most methods of inactivation including all that are applied to manufactured plasma products. Although there is evidence that it can be found in blood (see above), despite years of surveillance there have been no reported transmissions by manufactured plasma products. Model studies indicate that significant quantities would be removed by the purification processes now in use, including cryoprecipitation, depth filtration with filter aids, nanofiltration, and ion-exchange or affinity chromatography.[25,63-69] Based mostly on published findings, compared with a total maximum load of about 4 log of vCJD per plasma pool, the fibrinogen and thrombin processes should remove greater than 7.6 and greater than 13 log, respectively (Table 9), providing large safety margins.

DISCUSSION

The safety of modern plasma-derived products with respect to HBV, HCV, and HIV has been proven clinically over the past decade or more, mostly using manufacturing procedures employing only one dedicated method of

TABLE 6. Estimated viral elimination when processing fibrinogen and thrombin: based on both Omrix's and Baxter's processes

Step		Log reduction HIV	HCV	HBV	HAV	Parvovirus B19
Fibrinogen						
Cryoprecipitation + Al(OH) or wash		1.5	1.5	1.5	1.5	1.5
Immune neutralization					3*	3*
S/D treatment		>6	>6	>6	0	0
Heat treatment		>6	>6	>6	>5.8 to >6.5†	1.3
Greater heat sensitivity of B19 than CPV						1
Reserve capacity of virucidal methods‡		4	4	2	0	0
	Sum:	17.5	17.5	15.5	10.3-11.5	6.8
Thrombin						
Cryo removal		1	1	1	1	1
Immune neutralization		0	0	0	0	0
Initial fractionation (Baxter)§		2	2	2	2	2
Chromatographic purification		3	3	3	3	3
S/D treatment		>6	>6	>6	0	0
S/D reserve capacity		4	4	2	NA	NA
Heat treatment (Baxter)		>6	>6	>6	6.9	1.3
Nanofiltration (Omrix)		>4.4	>5.3	>5.5	7	5.9
	Sum:	18-22	19-22	17.5-20	11-13	7.3-9.9¶

* Virus neutralization is predicated on the fibrinogen containing antibody.
† The lower number applies to Omrix's fibrinogen and the higher number applies to Baxter's fibrin.
‡ The reserve capacity of vapor treating is estimated at zero since most of the reported viral kill takes place prior to initiating the heat cycle. The estimate of HBV reserve capacity with S/D treatment comes from studies with duck HBV added to whole plasma.
§ A mean of 2 log removal during cold alcohol fractionation is assumed.
¶ The lower number applies to Baxter's thrombin and the higher number applies to Omrix's thrombin.
NA = not applicable.

TABLE 7. Calculation of viral safety margins

	HIV	HCV	HBV	HAV	Parvovirus
Viral load (log; from Table 1)	5.7	6.0	4.4	2.9	6.6
Fibrinogen					
Viral clearance capacity (log)	17.5	17.5	15.5	10.3	6.8
Safety margin (fold)	6.3×10^{11}	3.2×10^{11}	1.3×10^{11}	2.5×10^{7}	1.6
Risk/vial (with virus at maximum load)*	3×10^{-16}	5×10^{-16}	1×10^{-15}	7×10^{-12}	1×10^{-4}
Adjusted risk/vial (all lots)†	1×10^{-19}	3×10^{-19}	5×10^{-17}	2×10^{-16}	2×10^{-6}
Thrombin					
Viral clearance capacity (log)	18	19	17	11	7.3-9.9
Safety margin (fold)	2×10^{12}	1×10^{13}	4×10^{12}	1×10^{8}	2×10^{3}
Risk/vial (with virus at maximum load)‡	8×10^{-18}	2×10^{-18}	4×10^{-18}	1×10^{-13}	3×10^{-6}-8×10^{-9}§
Adjusted risk/vial (all lots)†	4×10^{-21}	8×10^{-22}	2×10^{-19}	3×10^{-18}	7×10^{-8}-2×10^{-10}§

* Assumes 1 vial per L of plasma.
† The risk was adjusted to include lots without virus (calculated from Table 1, Column 4) and further assumes that the average viral load of contaminated lots is 1 log lower than the maximum load.
‡ Assumes 10 vials per L of plasma.
§ The larger number applies to Baxter's thrombin and the smaller number applies to Omrix's thrombin.

virus inactivation.[70] The safety record of fibrin sealant products, composed of both fibrinogen and thrombin, matches the safety record of other manufactured plasma products.[57] This suggests that for these and other enveloped viruses, the safety margins of fibrin sealant or stand-alone thrombin should be much higher than required since both employ an additional, dedicated method of viral elimination and, indeed, our calculated safety margins for enveloped viruses are extremely high. As a consequence, when new threats from enveloped viruses (e.g., WNV, pandemic influenza, and dengue viruses) are identified, the procedures in place for manufactured blood products are sufficient to ensure safety. This contrasts with the record of so-called labile blood components that have been shown to transmit, for example, WNV, at least until new screening tests are developed and deployed. From a patient safety perspective, it is also important to note that the safety margins for fibrin sealant and thrombin exceed those for labile blood components by many orders of magnitude. This is a direct consequence of the multiple improvements adopted by manufacturers of purified blood products over the past 20 years including deploying robust methods of virus inactivation.

TABLE 8. Inactivation of new viral threats

Virus	Preparation	Treatment	Log kill	First time point where infectious virus was not detected	Reference
WNV	α1-proteinase inhibitor	Pasteurization at 60°C for 10 hr	≥6.5	5 hr	Remington et al.[89]
	Antihemophilic factor concentrate	S/D (0.3% TNBP/1.0% Tween 80) at 28°C for 6 hr	≥5.9	1 hr	
WNV	FEIBA	Vapor heating (60°C for 10 hr and 80°C for 1 hr)	>7.6	6 hr	Kreil et al.[90]
	Antihemophilic factor concentrate	S/D (0.3% TNBP and 1% Triton X-100) at 20°C for 60 min	>6.0	<1 min	
H5N1 influenza virus	FEIBA	Vapor heating (60°C for 10 hr and 80°C for 1 hr)	>5.3	10 hr	Kreil et al.[91]
	IVIG	S/D (0.3% TNBP, 1% Triton X-100 and 0.3% Tween 80) at 18°C for 60 min	>4.7	<2 min	
SARS-associated corona virus	Haptoglobin, AT III, or IVIG	Pasteurization at 60°C for 10 hr	>3.3 to >6.5	1 hr	Yunoki et al.[92]
Chikungunya virus	IVIG	Pasteurization at 60°C for 10 hr	>5.2	1 hr	Uemura et al.[93]

AT III = antithrombin III; FEIBA = factor VIII bypassing activity produced by CSL Behring; H5N1 = the strain of influenza virus that causes SARS; IVIG = intravenous immune globulin.

Calculated safety margins for nonenveloped viruses are smaller since the manufacturing procedures for many plasma proteins, including both fibrinogen and thrombin, typically employ only one dedicated viral inactivation and/or removal method effective against these viruses, and parvoviruses are especially stable to thermal inactivation. There are no reports of HAV transmission by fibrin sealants even before adoption of NAT screening procedures. This is in accord with the finding that coagulation FVIII preparations did not transmit HAV provided they were either heat-treated or affinity-purified.[71] Additionally, given the modest processing fibrinogen undergoes and the known presence of immunoglobulin G in cryoprecipitate, it is reasonable to assume that fibrinogen is further protected by anti-HAV, present as a "contaminant."

Numerous reports describe the transmission of parvovirus B19 by coagulation factor concentrates[72] and its transmission by fibrin sealant has also been reported.[58] Frequent transmission results from the high concentration of virus that can be present in plasma pools containing units from donors with acute-phase viremia[73] and because parvovirus is not inactivated by S/D and is relatively stable to heat treatment methods. Beginning around 2001, commercial manufacturers of plasma products began employing NAT to screen incoming plasma units in a minipool format to limit viral loads. Originally, testing was performed to ensure that titers did not exceed 10^5 geq per mL; for Omrix's and Baxter's fibrin sealant products, a standard of not more than 10^4 geq per mL has been adopted for the minipool being tested. A recent article by Geng et al.[51] confirms the benefits of screening incoming plasma for parvovirus B19 by NAT. Despite this improvement, the maximum load of infectious virus that might be present remains considerable, and it would appear that the fibrinogen component might still transmit parvovirus B19, albeit at very low frequency. Clinical studies will be required to confirm this since the antibody content of fibrinogen might provide adequate protection, or parvovirus B19 might be more sensitive to heat treatment than the animal parvovirus models used, as has been suggested.[74] The greater safety margin calculated for the thrombin component arises from the use of more vigorous purification procedures and the overall effectiveness of nanofilters in removing parvovirus. Because of its size and shape, fibrinogen cannot be nanofiltered successfully.

All evidence to date indicates that vCJD is not transmitted by manufactured plasma products. Safety may result from the geographic restrictions that have been instituted to eliminate individuals who are at high risk of exposure, the very low levels in blood, and its removal by steps in common use including precipitations, filtrations, and column chromatography. If a vCJD contaminated unit was included in the plasma pool, the calculated safety margin for fibrinogen and thrombin is very high. Unlike viruses used in spiking studies, however, the structure of

TABLE 9. Clearance of prions: Omrix process

	Prion load (log; from Table 1): 4.3 Log removal	Reference*
Fibrinogen		
Cryoprecipitation	1.6 mean (0.6-2.6)	Foster, 2000[66] and 2006[69]
Al(OH) extraction and filtration	>2	Omrix validated study
Oil extraction of S/D reagents	2	Omrix preliminary study
Hydrophobic chromatography	2	Foster, 1999[63]
Clearance capacity (log)	>7.6	
Safety margin (fold)	1995	
Adjusted risk/vial†	7×10^{-10}	
Thrombin		
Cryo removal	1	Foster, 2000[66] and 2006[69]
Filtrations	2	Foster, 2000[66] and 2006[69]
Anion-exchange chromatography	3	Foster, 2000[66] and 2006[69]
Cation-exchange chromatography	3	Foster, 2000[66] and 2006[69]
Nanofiltration	4.4 mean (1.6 to >5.9)	Foster, 2000[66] and 2006[69]
Clearance capacity (log 10)	13.4	
Safety margin (fold)	1.3×10^{9}	
Adjusted risk/vial†	1×10^{-16}	

* See also additional Foster references.[61,64,65,67] It should be noted that the form that the infectious vCJD agent takes in plasma is unknown and that, should it be present in plasma pools, its behavior may differ from the materials used.

† We employed the same assumptions as used in Table 7 plus assumed risk of vCJD presence was the same as for CJD, i.e., 1 per 1 million donations, and mean load was same as maximum load.

the causative agent of vCJD is unknown and may differ significantly from the models in use. Because of this uncertainty and the devastating nature of the disease, the authorities in the United Kingdom have concluded that recipients of plasma-derived FVIII, F IX, and antithrombin prepared using donations from individuals who subsequently developed vCJD should be told that they may be at increased risk for developing the disease.[75] This emphasizes the importance of donor exclusion criteria implemented for all products licensed in the United States or Europe. With these exclusions taken into account, the risk for vialed product should remain well less than 1 in 1 million for fibrinogen and less than 1 in 1 trillion for thrombin.

Finally, we should comment about the methods employed in making these calculations. In addition to the validated studies presented to the FDA and other regulatory agencies, we examined other steps in the manufacturing process that were likely to contribute to safety together with published information from other related processes. We also have taken into account the benefit of using viral inactivation methods that have a large reserve capacity. Our intent in employing this approach was not to replace the stricter approach taken by regulatory authorities, but simply to more completely assess safety. Nonetheless, we acknowledge that our calculations are estimates and actual findings may differ. Acknowledging these shortcomings but based on our calculations, we encourage manufacturers and other interested parties to continue seeking ways to enlarge the safety margin, especially for nonenveloped viruses and to address the theoretical risk posed by vCJD

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