Hepatitis E virus and the safety of plasma products: investigations into the reduction capacity of manufacturing processes

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BACKGROUND: Hepatitis E virus (HEV) has been transmitted by transfusion of labile blood products and the occasional detection of HEV RNA in plasma pools indicates that HEV viremic donations might enter the manufacturing process of plasma products. To verify the safety margins of plasma products with respect to HEV, virus reduction steps commonly used in their manufacturing processes were investigated for their effectiveness to reduce HEV.

STUDY DESIGN AND METHODS: Detection methods for HEV removal (by reverse transcription quantitative polymerase chain reaction) and inactivation (using an infectivity assay) were established. Immunoaffinity chromatography and 20-nm virus filtration for Factor (F)VIII, cold ethanol fractionation, and low-pH treatment for immunoglobulin, heat treatment for human albumin, and 35-nm nanofiltration for FVIII inhibitor-bypassing activity (FEIBA) were investigated for their capacity to reduce HEV or the physicochemically similar viruses feline calicivirus (FCV) and hepatitis A virus (HAV).

RESULTS: For FVIII, HEV reduction of 3.9 and more than 3.9 log was demonstrated for immunoaffinity chromatography and 20-nm nanofiltration, respectively, and the cold ethanol fractionation for immunoglobulin removed more than 3.5 log of HEV, to below the limit of detection (LOD). Heat treatment of human albumin inactivated more than 3.1 log of HEV to below the LOD and 35-nm nanofiltration removed 4.0 log of HEV from the FEIBA intermediate. The results indicated HAV rather than FCV as the more relevant model virus for HEV.

CONCLUSION: Substantial HEV reduction during processes commonly used in the manufacturing of plasma products was demonstrated, similar to that previously demonstrated for HAV.

Hepatitis E virus (HEV) is a small (27-34 nm) nonenveloped, single-stranded RNA virus (*Hepevirus*, *Hepeviridae*).[1,2] Genotypes 1 and 2 cause large epidemics of acute viral hepatitis in many countries in tropical and subtropical regions, where HEV is transmitted via the fecal–oral route.[2] More recently, Genotypes 3 and 4 have been recognized as zoonotic, primarily food-borne pathogens in industrialized countries and transmissions have occurred mainly after consumption of raw or undercooked pork products.[2-4] HEV seroprevalence rates between 10 and 53% have been described in blood donors in several European countries and in the general population in the United States,[3,5-7] providing evidence that a significant proportion of the population in industrialized countries has experienced—typically asymptomatic—HEV infections.[2,5] HEV has been transmitted by blood transfusion[8-11] and although a screening of coagulation factor concentrates did not find HEV RNA in these plasma-derived products,[12] low

ABBREVIATIONS: FCV = feline calicivirus; FEIBA = Factor VIII inhibitor-bypassing activity; GE = genome equivalents; IFA = immunofluorescence assay; LOD = limit of detection; RF(s) = reduction factor(s); rHEV = recombinant hepatitis E virus; RT qPCR = reverse transcription quantitative polymerase chain reaction; $TCID_{50}$ = 50% tissue culture infectious dose; wtHEV = wild-type hepatitis E virus.

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concentrations of HEV RNA were detected in up to 10% of plasma pools for fractionation.[4,13,14] This apparent disparity between plasma pool and final product testing is, however, consistent with higher test sensitivity at plasma pool level, that is, before any virus reduction has taken place in the manufacturing process.

With the emergence of any new virus of concern, verification studies with that particular agent are conducted as soon as possible, to provide for adequate reassurance to plasma product users and regulators that such a virus is indeed reduced during the manufacturing processes, as done in the recent past for West Nile virus,[15] Chikungunya virus.[16] and the H5N1 influenza virus, for example.[17] Ensuring high margins of safety with respect to transmission of blood-borne viruses is a priority for products made from human plasma. Careful donor selection and plasma testing do substantially reduce the associated risks, but cannot totally ensure absence of viruses. Therefore, effective and robust virus removal and inactivation steps are incorporated into the manufacturing processes of plasma-derived products, which generically reduce any potentially present viral load. The efficacy of these virus reduction steps has been shown in virus clearance studies using relevant viruses such as, for example, hepatitis A virus (HAV) and human immunodeficiency virus or physicochemically similar model viruses, when the target viruses were not available for study.

HEV has been difficult to grow in cultured cells, and while some success has been reported[18-20] the infectivity protocols proved difficult to establish at other laboratories (own observation).[21] With the availability of a recombinant expression system for cell culture–adapted HEV[22,23] the production of higher-titer virus stocks has become possible, but the experimental complexity continues to be a limiting factor for virus clearance studies. Members of the *Picornaviridae* (e.g., HAV) and *Caliciviridae* (e.g., feline calicivirus [FCV]) are taxonomically closely related as they share sequence conservation of capsid gene and nonstructural gene sequences[24] and are otherwise similar to HEV in physicochemical properties; that is, they are small, icosahedral ssRNA viruses with similar virion buoyant densities and are non–lipid enveloped, although more recently a lipid-enveloped form has been reported for HEV and HAV.[25,26] HAV and FCV were therefore included in the virus clearance studies and evaluated as potential model viruses for HEV.

MATERIALS AND METHODS

Cells

HepG2/C3A (CRL10741), CRFK (CCL-94), and FRhK-4 (CRL-1688) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and BSC-1 cells (85011422) from the European Collection of Cell Cultures (Wiltshire, UK). Cells were maintained in Eagle's MEM (HepG2/C3A, CRFK) and D-MEM (BSC-1, FRhK-4) supplemented with fetal calf serum (FCS, SAFC, Irvine, UK), L-glutamine, gentamycin, NEAS, and sodium-pyruvate (Life Technologies, Carlsbad, CA). For maintenance and titration of HepG2/C3A cells ultralow IgG FCS (Life Technologies) was used.

Viruses

Recombinant and wild-type HEV

For the expression of recombinant HEV (rHEV), plasmid p6 was obtained from the National Institutes of Health (NIH, Bethesda, MD) and RNA transcripts generated and transfected into HepG2/C3A cells as described.[23] HEV-producing cells were cloned by limiting dilution and rHEV stocks produced from cell culture supernatants by ultracentrifugation (200,000 × *g*, 73 min, 4°C). To remove a potentially present lipid envelope, rHEV stock was solvent/detergent (S/D) treated by addition of Triton X-100:tri-*n*-butyl-phosphate:polysorbate 80 to 1, 0.3, and 0.3%; vigorous mixing; and incubation at room temperature for 60 minutes. The S/D chemicals were subsequently removed by solid-phase extraction (approx. 6 mL of virus stock per Waters Sep-Pack Long C18 cartridge containing 820 mg of sorbent in 1.6 mL, WAT023635, Waters Corp., Milford, MA).

Liver samples from Austrian rearing pigs that had tested positive for HEV RNA by reverse transcription quantitative polymerase chain reaction (RT qPCR)[27] were obtained from the Austrian Agency for Health and Food Safety (Department of Molecular Biology, Austria) and wild-type HEV (wtHEV) stocks prepared using a method previously described.[20] These wtHEV stocks were used in all experiments with the exception of Factor (F)VIII nanofiltration, where a wtHEV stock originating from Japanese swine feces (isolate swJB-M5),[28] obtained from the Hirakata Laboratory, BENESIS Corp. (Osaka, Japan) was used.

FCV and HAV

FCV strain F-9 (VR-782, obtained from the ATCC) was propagated and titrated on CRFK cells. The origin, propagation, and titration of HAV strains HM175/18f and HM175/24a has previously been described.[29]

Virus detection methods

HEV RT qPCR

Quantitative HEV nucleic acid detection was done on a sequence detection system (ABI Prism 7900 HT, Applied Biosystems, Carlsbad, CA) using an optimized HEV RT qPCR assay.[30] RNA spiked with an internal positive control was extracted with a viral RNA mini kit (QIAamp, Qiagen) and amplifications were performed in duplicates in 50-μL reaction volumes containing 20 μL of template. The HEV RT qPCR assay was calibrated against the World Health Organization standard for HEV RNA nucleic acid

amplification techniques, Paul Ehrlich Institute Code 6329/10, and 1 IU corresponded to 1.44 genome equivalents (GE) of the in-house RNA standard. The assay had a limit of quantification of 2.8 log GE/mL and a limit of detection (95%; LOD_{95}) of 2.1 log GE/mL.

HEV immunofluorescence assay

For the heat treatment of HEV, infectious virus titers were determined by immunofluorescence assay (IFA) and quantified by calculation of the 50% tissue culture infectious dose ($TCID_{50}$). The HEV stock used for all experiments had a titer of 4.5 log $TCID_{50}$/mL. HepG2/C3A cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well the day before infection with log or half-log sample dilutions in quadruplicates. After infection for 4 to 5 hours the cells were washed and further incubated for 6 to 7 days (36°C) before being fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% goat serum. Viral proteins were stained with a mouse monoclonal antibody specific for HEV capsid protein (LS-C67674, LifeSpan BioSciences, Seattle, WA) for 1 hour, followed by washing and incubation with Alexa Fluor 488 goat anti-mouse antibody (Molecular Probes A11029, Life Technologies) for 1 hour. Cells were visualized under a fluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY) and wells were scored as positive or negative for HEV infection. HEV infectious titers were calculated as described below.

HAV RT qPCR

Quantitative HAV nucleic acid detection was performed using a validated HAV RT qPCR assay, as described earlier.[31]

FCV and HAV infectivity

FCV and HAV infectious titers were determined by $TCID_{50}$ assays, using serial half-log sample dilutions that were titrated in eightfold replicates on CRFK (FCV) or FRhK-4 (HAV) cells in 96-well plates. After incubation for 7 (FCV, 36°C) or 21 days (HAV, 33°C) wells were scored for virus infection by evidence of a cytopathic effect and infectious virus titers (expressed as log $TCID_{50}$/mL) calculated according to the Poisson distribution and the maximum likelihood method.[32]

Downscaled manufacturing processes for plasma derivatives

All virus reduction studies were designed to support the manufacturing processes for Baxalta (Bannockburn, IL) products. Downscaled models were established with the target of identity to the manufacturing processes and the equivalence of the laboratory models to the respective large-scale processes was demonstrated by comparing critical process and selected biochemical variables. Virus reduction factors (RFs; reported in log values) were calculated in accordance with regulatory guidelines[33] and with unrounded values, only the final RF was rounded. For nanofiltration experiments the virus stocks were passed through 0.1-μm filters immediately before spiking the process intermediate with virus, to ensure absence of virus aggregates. Results from virus removal studies followed by RT qPCR are presented as log GE, whereas virus inactivation determined by $TCID_{50}$ assay is given as log $TCID_{50}$.

Production process of FVIII

HEMOFIL-M is Baxalta's purified FVIII preparation isolated from pooled human plasma by immunoaffinity chromatography. For the downscale of the immunoaffinity chromatography step, a validated liquid chromatography system (ÄKTA Explorer 10 S, GE Healthcare, Buckinghamshire, UK) was used and each chromatography run consisted of the following steps: column preparation, equilibration, 1:100 virus spike of approximately 160 mL of HEMOFIL-M intermediate, pre-filtration, loading, washing, elution, and storage.

During the manufacturing of HEMOFIL-M, the affinity chromatography eluate is passed through 20-nm virus filters (nanofiltration). The validated downscaled model of the nanofiltration step used the fully automated filtration equipment recently reported.[34] Approximately 30 or 60 mL of 1:11 (for rHEV) or 1:60 (for wtHEV from swine feces) spiked process intermediate, respectively, was applied to 0.1-μm prefilters (Durapore PVDF, Merck Millipore Corp., Darmstadt, Germany) followed by 0.001-m^2 20-nm virus filters (Planova, Asahi Kasei Corp., Tokyo, Japan), similar to that described.[35]

Production process of immunoglobulin

Gammagard liquid (in the United States) and KIOVIG (in Europe) are Baxalta's 10% liquid human immune globulin infusions (in the following referred to as GG/KIOVIG), manufactured from pooled human plasma by the same process.[36,37] The virus removal capacity of the extraction of fraction II+III paste[38] was evaluated as previously described.[37] Virus removal was investigated at the different pH extremes that are defined for manufacturing (pH 6.6 and 6.8). Fraction II+III was precipitated from approximately 180-mL volumes of fraction I supernatant that had been spiked 1:21 with virus stock. The precipitate was resuspended in acidic phosphate/acetate buffer and filtered through a depth filter in the presence of filter aid; the filter cake was resuspended in phosphate-buffered saline. Samples were removed at appropriate steps to investigate virus removal (Table 3) and results were compared to already existing removal data for HAV.[37]

The virus inactivation capacity of the low-pH treatment, which is done at 31 ± 1°C for 21 to 22 days in the manufacturing process,[37] was investigated for FCV as previously described,[16] at the upper pH range (pH 4.9), using a target temperature of 29 ± 1°C in 50-mL volumes of process intermediate spiked 1:12.5 with virus stock. Virus

reduction was investigated at periodical intervals until Day 20 (Fig. 1), where samples were diluted immediately with cold cell culture medium to prevent further virus inactivation before titration and results were compared to already existing HAV inactivation data.[37]

Production process of human serum albumin

Human serum albumin (HSA) is manufactured from human plasma by the modified Cohn-Oncley cold ethanol fractionation followed by pasteurization of the final product at 60 ± 1°C for 10 to 11 hours. The capacity to inactivate FCV during this heat treatment was investigated in 1:20 spiked 50-mL volumes of process intermediate, as previously described.[15-17] HEV was heat treated in volumes of 0.5 mL using a validated heating block with an additional temperature sensor in a control vial, as 1:2 spike into 25% HSA (i.e., in 12.5% HSA) or as virus stock with or without prior S/D treatment. Results were compared to already existing HAV inactivation data,[29] which was selected and reanalyzed as mean of experiments performed in 5 and 25% HSA, to allow direct comparison to the HEV experimental set-up.

Production process of FVIII inhibitor-bypassing activity

FVIII inhibitor-bypassing activity (FEIBA) NF (anti-inhibitor coagulant complex) is Baxalta's human plasma fraction with FEIBA, for which the nanofiltration was investigated. The validated downscaled model of the nanofiltration step used 0.1-μm and 75-nm prefilters (Ultipore N66, Pall, Port Washington, NY; and Asahi Planova, respectively) followed by virus filtration with 0.001-m^2 35-nm virus filter units (Asahi Planova) and the fully automated filtration equipment recently reported.[34] Approximately 300 mL of FEIBA intermediate was spiked at a ratio of 1:300 with wtHEV, rHEV, rHEV that had been treated with S/D, or with HAV, and virus reduction was investigated in single runs for each of the HEV preparations and duplicate runs for HAV.

RESULTS

For each of the downscaled virus removal and inactivation steps equivalence with the respective manufacturing-scale process could be confirmed through the analysis of selected variables, thereby confirming validity of the different scale processes. Generally, conditions least favorable for virus reduction were used, such as shorter incubation times or treatment at slightly lower temperatures than those specified for the manufacturing process.

Production process of FVIII

The virus removal capacity of the immunoaffinity chromatography step during the manufacturing of HEMOFIL-M was investigated for HEV, FCV, and HAV. HEV was effectively removed from the HEMOFIL-M intermediate, with RFs of 4.1 or 3.6 log GE obtained for rHEV or wtHEV, respectively (Table 1). Similarly, effective reduction of FCV infectivity was seen (mean RF of 3.9 log $TCID_{50}$, n = 2)

TABLE 1. Removal of FCV, HAV, rHEV, and wtHEV by affinity chromatography during the manufacturing of FVIII (HEMOFIL-M)

	Log $TCID_{50}$ (log GE)*		Log GE	
Sampling stage	FCV load	HAV load	rHEV load	wtHEV† load
Virus-spiked material‡	8.8/8.3	6.1/7.9 (10.5/10.8)	8.9	7.9
Flow through	8.8/8.5	<4.8/<5.3 (10.7/10.8)	8.8	7.7
Wash	5.9/5.8	<3.7/<3.7 (8.9/9.2)	7.4	6.3
FVIII elution‡	4.7/4.6	<1.6/<1.6 (7.6/7.5)	4.8	4.3
Virus RF	4.2/3.7	>4.5/>6.3 (3.0/3.3)	4.1	3.6
Mean RF	3.9	>5.4 (3.1)	3.9	

* Values in brackets are log GE results from RT qPCR testing.
† wtHEV stock from porcine liver was used.
‡ Fractions used for the calculation of the virus RF.

TABLE 2. Removal of FCV, HAV, rHEV, and wtHEV by 20-nm nanofiltration during the manufacturing of FVIII (HEMOFIL-M)

	Log $TCID_{50}$		Log GE	
Sampling stage	FCV load	HAV load	rHEV load	wtHEV* load
Virus-spiked material	8.2/8.3	8.1/8.2	7.7/7.9	7.4
20-nm pooled nanofiltrate	<2.7/<2.7	<2.8/<2.8	<3.6/<3.6	<3.8
Virus RF	>5.5/>5.5	>5.3/>5.4	>4.1/>4.3	>3.6
Mean RF	>5.5	>5.4	>3.9	

* wtHEV stock from swine feces was used.

TABLE 3. Removal of FCV, HAV, rHEV, and wtHEV by extraction of fraction II+III paste during the manufacturing of immunoglobulin (Gammagard liquid/KIOVIG)

	Log $TCID_{50}$		Log GE	
Sampling stage	FCV load	HAV load	rHEV load	wtHEV* load
Virus-spiked material†	8.9/8.8‡	7.7	9.3/9.2‡	7.1/6.9‡
Supernatant after EtOH addition, incubation, and centrifugation	6.3/7.4	6.0	8.6/9.3	<6.1/<5.8
Resuspended precipitate	8.2/8.2	7.0	8.8/9.0	6.5/6.4
Resuspended filter cake	7.7/7.5	7.0	8.6/8.5	5.5/5.2
Filtrate†	5.5/4.5	<3.7	<5.3/<4.9	<4.2/<4.2
Virus RF	3.4/4.4	3.9§	>4.0/>4.3	>2.9/>2.7
Mean RF	3.9	3.9§	>3.5	

* wtHEV stock from porcine liver was used.
† Fractions used for the calculation of the virus RF.
‡ Results for Run 1/Run 2 at different extremes of pH that are defined for this step (pH 6.6 or 6.8).
§ Data from Poelsler et al.[37]

and virus recovery data showed that the mechanism of action was virus removal, rather than inactivation (Table 1). A combination of virus inactivation and removal was seen for HAV, as contact with the product matrix resulted in loss of HAV infectivity to below the LOD with a mean RF of more than 5.4 log $TCID_{50}$, whereas RT qPCR testing showed HAV removal of 3.1 log GE.

During the manufacturing of HEMOFIL-M, affinity chromatography is followed by a 20-nm nanofiltration step, which was investigated for HEV, FCV, and HAV. As expected, effective removal to beyond the LOD was shown for all viruses tested, with RFs of more than 4.2 log GE (mean of n = 2) and more than 3.6 log GE (n = 1) for rHEV and wtHEV, respectively (Table 2). For FCV a mean RF of more than 5.5 log $TCID_{50}$ (n = 2) and for HAV a mean RF of more than 5.4 log $TCID_{50}$ (n = 2) were determined and virus recovery data showed that the mechanism of action was by virus removal, as there was

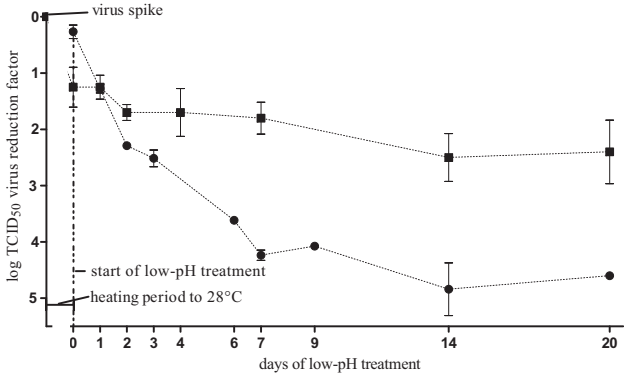

Fig. 1. Low-pH treatment of Gammagard liquid/KIOVIG. Virus inactivation kinetics for incubation at pH 4.9 and 29 ± 1°C (FCV) or pH 4.65 buffer and 30 ± 1°C (HAV) for up to 20 days. Mean log RFs and SD for two runs are shown. (●) FCV; (■) HAV.

no indication for loss of infectivity during this process (data not shown).

Production process of immunoglobulin

Extraction of fraction II+III paste was investigated for HEV and FCV and the results compared to already existing data for HAV.[37] HEV was removed from the intermediate to below the LOD_{95} irrespective of the pH (Table 3), with mean RFs of more than 4.2 and more than 2.8 log GE for rHEV or wtHEV, respectively (n = 2). FCV infectivity was reduced by 3.4 or 4.4 log $TCID_{50}$ at the different pH extremes and virus recovery data suggested that the reduction was mainly due to virus removal, as most of the infectious virus was detected in the precipitate (Table 3). In comparison, the process step resulted in effective reduction of 3.9 log $TCID_{50}$ HAV infectivity.[37]

Low-pH treatment of GG/KIOVIG was investigated for FCV and the results compared to already existing data for HAV.[37] FCV inactivation was effective but not complete (Fig. 1), with a mean RF of 4.6 log $TCID_{50}$ (n = 2) at the end of the 20-day incubation period. Inactivation of HAV in low-pH buffer progressed slowly over the 20-day incubation period (Fig. 1) and resulted in 2.4 log $TCID_{50}$ (n = 2) inactivation.[37]

Production process of human serum albumin

Heat treatment in 12.5% HSA at 58 ± 1°C inactivated HEV to below the LOD within 180 minutes, with a mean RF of more than 3.1 log $TCID_{50}$ (n = 2; Fig. 2). Inactivation was more rapid when HEV was heat treated as virus stock or as S/D-treated virus stock, when the LOD was reached at 60 minutes and RFs of more than 3.7 log $TCID_{50}$ (n = 2) and more than 3.5 log $TCID_{50}$ (n = 1) obtained (Fig. 2A). There was no difference in heat sensitivity between S/D-treated and untreated HEV stock (Fig. 2A). FCV was inactivated more rapidly, with no infectious virus detected after 120 minutes of incubation and a mean RF of more than

7.0 log $TCID_{50}$ (n = 8; Fig. 2B). In comparison, inactivation of HAV by heat treatment was incomplete, with mean RFs of 2.4 log $TCID_{50}$ (n = 6) and 4.4 log $TCID_{50}$ (n = 6) determined in the same experimental downscale for heat-resistant and heat-sensitive HAV variants, respectively (Fig. 2B).[29]

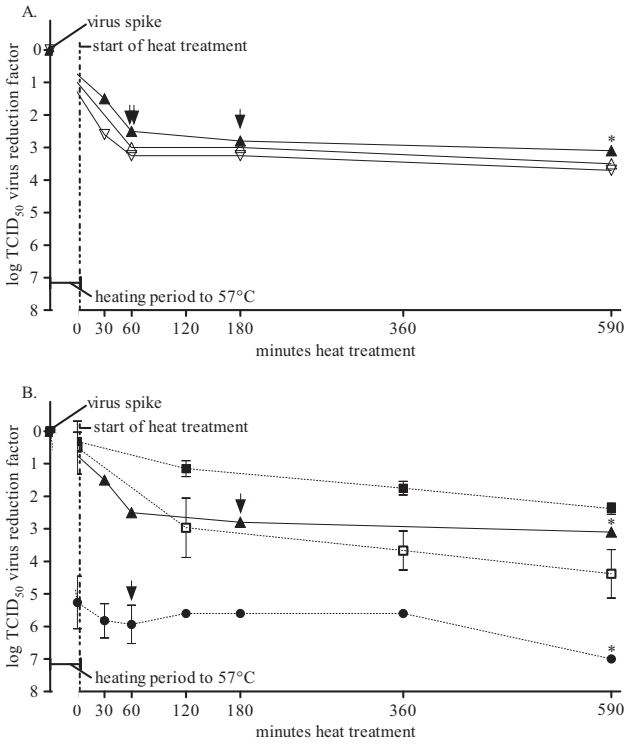

Fig. 2. Heat treatment of human serum albumin. Virus inactivation kinetics for incubation at 58 ± 1°C for 590 ± 10 minutes. Mean log RFs and (where applicable) SD are shown, the number of replicates (n) is indicated; arrows (↓) indicate time point when the LOD was reached; (*) lower detection limit toward the end of treatment due to cumulative negative samples. (A) Heat treatment of HEV (▲) in HSA, n = 2; (▽) as virus stock (without HSA), n = 2; (△) as S/D-treated virus stock (without HSA), n = 1. (B) Comparison of heat inactivation in HSA of (▲) HEV, n = 2 versus the HEV-model viruses (●) FCV, n = 8; (■) HAV strain HM175/18f (heat resistant), n = 6; and (□) HAV strain HM175/24a (heat sensitive), n = 6.

Production process of FEIBA

The nanofiltration step in the manufacture of FEIBA NF was investigated for its HEV and HAV removal capacity. rHEV was removed from the process intermediate with a mean RF of 4.0 log GE (n = 2) and S/D treatment of the HEV stock had no effect on virus removal (Table 4); wtHEV was removed by more than 2.2 log GE (n = 1), that is, to below the LOD_{95}. HAV infectivity was reduced by a mean of 2.6 log $TCID_{50}$ (n = 2). The validated downscale of the 35-nm nanofiltration step included 0.1-μm and 75-nm prefilters, where no significant virus removal was seen at the 0.1-μm filtration for HEV or HAV. The 75-nm prefilter removed 1.6 (mean of n = 2) and more than 1.7 (n = 1) log GE of rHEV and wtHEV, respectively, whereas no significant removal of HAV infectivity (mean RF of 0.5 log $TCID_{50}$, n = 2) was seen at this stage (Table 4).

DISCUSSION

To investigate the safety of plasma products with respect to HEV, virus reduction steps that are commonly used in the manufacturing of plasma products were evaluated for their potential to remove or inactivate HEV and/or the possible HEV model viruses FCV and HAV. For the detection of infectious HEV after 1 week of incubation on HepG2/C3A cells the IFA rather than RT qPCR was used, as preliminary experiments had shown that RT qPCR detected an increase in HEV RNA also in cells that were nonpermissive for HEV infection (e.g., Chinese hamster ovary cells), whereas such false-positive results were not seen with the IFA (data not shown). HEV infectivity results that are based purely on RT qPCR should therefore be evaluated carefully and are not reported in this work.

For the manufacturing process of HEMOFIL-M, effective HEV reduction was demonstrated for the two dedicated virus removal steps investigated, that is, 3.9 log GE

TABLE 4. Removal of HAV, rHEV, and wtHEV by 35-nm nanofiltration during the manufacturing of FEIBA NF

		log GE		
Sampling stage	log $TCID_{50}$, HAV load	rHEV load	rHEV (S/D)* load	wtHEV† load
Virus-spiked material‡	6.7/6.4	9.4	9.3	7.2
0.1-μm filtrate	6.0/5.7	9.4	9.2	6.9
75-nm filtrate	5.7/5.0	7.7	7.7	5.2
35-nm pooled nanofiltrate‡	4.0/<4.0	<5.5	5.5	<5.0
Virus RF	2.7/>2.4	>4.0	3.9	>2.2
Mean RF	2.6		4.0	

* rHEV stock that had been treated with S/D.
† wtHEV stock from porcine liver was used.
‡ Fractions used for the calculation of the virus RF.

for immunoaffinity chromatography and more than 3.9 log GE for 20-nm nanofiltration. The extraction of fraction II+III paste substantially removed HEV to below the LOD_{95} and heat treatment for final product HSA inactivated HEV to below the LOD. Finally, the 35-nm nanofiltration during the manufacturing of FEIBA NF substantially removed HEV with a mean RF of 4.0 log GE for rHEV or below the LOD_{95} for wtHEV.

Reduction of HEV during the different manufacturing steps was similar to that obtained for the two potential HEV model viruses FCV and HAV, with the exceptions of the heat treatment of HSA and 35-nm nanofiltration of FEIBA intermediate. Similar to a previous study,[39] HEV was more sensitive to heat inactivation than HAV and the slower inactivation of HEV in HSA rather than as virus stock (Fig. 2A) was in agreement with previous results.[28] FCV was not a suitable HEV model for the pasteurization of HSA, as comparably more rapid inactivation to below the LOD was seen for this virus. There was a difference in HEV removal by 35-nm nanofiltration of FEIBA intermediate when compared to HAV (Table 4), as the 75-nm prefilter contributed substantially to the overall HEV reduction, whereas this effect was not seen for HAV infectivity. Within the limitations of the experimental set-up, treatment of the HEV virus stock with S/D to remove a potentially present lipid envelope did not alter the effective removal of HEV (Table 4), indicating either that rHEV does not possess a lipid envelope, treatment with S/D alone might not be able to fully convert this atypical lipid-enveloped virus into a nonenveloped phenotype, as previously reported,[26] or that the low level of HEV antibodies present in the FEIBA intermediate (data not shown) did not suffice to enhance the nanofiltration process. The observed rHEV removal of more than 2 log GE by the 35-nm nanofilter corresponds with previously reported results of 1.1 to at least 3.6 log reduction of a 75-nm prefiltered HEV stock in buffer.[28] Some differences were seen between HAV and FCV, for example, a FVIII matrix effect on HAV inactivation during immunoaffinity chromatography and a greater resistance of HAV to low-pH treatment during the manufacture of GG/KIOVIG.

The presence of antibodies in the process intermediate has an effect on virus reduction, where HAV antibodies contributed, for example, to virus removal during 35-nm nanofiltration[31,37] and resulted in immediate virus neutralization during low-pH treatment of GG/KIOVIG.[37] As HEV-specific antibodies have been reported in plasma pools,[4,13] a contribution to virus removal and inactivation similar to that seen for HAV seems feasible, but has yet to be confirmed.

In conclusion, substantial HEV reduction during processes that are commonly used in the manufacturing of Baxalta plasma products was demonstrated. Partial utility of FCV and HAV as model viruses for HEV was shown by equivalent virus removal during nanofiltration, immunoaffinity chromatography, and a part of the cold ethanol fractionation process, whereas HAV rather than FCV inactivation during heat treatment in HSA was similar to the inactivation achieved for HEV (Fig. 2B).[28] Considering that HEV and HAV are human pathogens, substantial HEV antibody content of plasma pools has been shown[4,13] and antibody-mediated effects on HAV removal and inactivation have been reported,[31,37] HAV appears to be the physiologically more relevant HEV model virus. For FCV, these factors may be less applicable. The similarity between HEV and HAV is further supported by reports of lipid-enveloped forms for both of these non–lipid-enveloped viruses,[25,26] the effect and relevance of which, however, remains to be elucidated in further studies.

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CONFLICT OF INTEREST

All authors are employees of Baxalta. PR, AF, JM and TRK have stock interest.

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