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BRIEF REPORT

TRANSFUSION

Antibody-enhanced hepatitis E virus nanofiltration during the manufacture of human immunoglobulin

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Abstract

Background: Circulation of hepatitis E virus (HEV) in areas where plasma is sourced for the manufacture of plasma-derived medicinal products (PDMPs) has prompted verification of HEV clearance. HEV exists as quasi lipid-enveloped (LE) and non–lipid-enveloped (NLE) forms, which might be of relevance for HEV clearance from manufacturing processes of antibody-containing PDMPs with solvent/detergent (S/D) treatment upstream of further clearance steps.

Study Design and Methods: Presence of different HEV particles in stocks used in clearance studies was investigated, with nanofilters graded around the assumed HEV particle sizes and by gradient centrifugation. HEV removal by 35-nm nanofiltration was investigated in the presence or absence of HEV antibodies, in buffer as well as in immunoglobulin (IG) manufacturing process intermediates.

Results: HEV particles consistent with LE, NLE, and an "intermediate" (IM) phenotype, obtained after S/D treatment, were seen in different HEV stocks. In the absence of HEV antibodies, log reduction factors (LRFs) of 4.0 and 2.5 were obtained by 35-nm nanofiltration of LE and IM HEV, consistent with the larger and smaller sizes of these phenotypes. Addition of HEV antibodies enhanced IM HEV removal around 1000-fold (LRF, 5.6). Effective (LRF, >4.8 and >4.0) HEV removal was obtained for the nanofiltration processing step for IG intermediates with varying HEV antibody content.

Conclusion: HEV spikes used in clearance studies should be carefully selected, as differences in physicochemical properties might affect HEV clearance. Antibody-mediated enhancement of HEV nanofiltration was

Abbreviations: ccHEV, cell culture–adapted hepatitis E virus isolate; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; HEV, hepatitis E virus; GG LQ, Gammagard Liquid; hsHEV, human stool derived hepatitis E virus; IG, immunoglobulin; IM, intermediate; LE, lipid-enveloped; LOD, limit of detection; LRFs, log reduction factors; NLE, non–lipid-enveloped; PBS, phosphate-buffered saline; PDMPs, plasma-derived medicinal products; rHEV, recombinant hepatitis E virus; RT qPCR, reverse transcription quantitative polymerase chain reaction; S/D, solvent/detergent; WHO, World Health Organization.

demonstrated in IG process intermediates even at low HEV antibody concentration, illustrating the robustness of this manufacturing step.

1 | INTRODUCTION

Hepatitis E virus (HEV) is one of the leading causes of acute viral hepatitis worldwide. While transmitted via the fecal-oral route in developing countries, HEV has been recognized as a zoonosis in industrialized countries, where it is primarily transmitted through consumption of raw or undercooked pork products. The virus has been transmitted by transfusion of blood components (plasma, erythrocytes, thrombocytes).[1,2] Although low HEV RNA concentrations in plasma pools for fractionation have been detected,[3,4] no transmission of HEV through plasma-derived medicinal products (PDMPs) has been reported to date. In contrast to blood components, substantial virus clearance is achieved by dedicated viral reduction steps in the PDMP manufacturing processes. With the emergence of a new virus or scientific evidence that alters previously accepted concepts, studies are required to verify safety margins.

HEV is a small (27-34 nm) positive-sense, single-stranded RNA virus,[5] taxonomically classified as non–lipid enveloped (NLE). However, the virus also exists as 40- to 50-nm quasi lipid-enveloped (LE) particles.[6–9] An intermediate (IM) phenotype, obtained following treatment of the virus with a lipid solvent, has a different buoyant density than either the LE or NLE forms,[7] but with a virion diameter similar to NLE particles (approx. 30 nm).[6,7] LE HEV particles are not recognized by antibodies, however, removal of the LE allows virions to be bound and neutralized by monoclonal antibodies and immune sera.[6,7,9]

The existence of different forms of HEV particles may impact virus clearance. Previous studies confirmed the HEV clearance capacity by virus reduction steps commonly implemented during the manufacture of PDMPs. However, few studies considered the effect that different physicochemical properties of HEV particles might have on virus clearance.[10–12] Particularly, where a manufacturing process includes treatment with solvent/detergent (S/D) upstream of further virus reduction steps, the type of the HEV particle (ie, the LE or NLE form) together with the presence of HEV-specific antibodies may affect virus clearance as a result of antibody binding to NLE particles. This is of relevance for antibody-containing plasma fractions, for example, immunoglobulin (IG) products, which are fractionated from human plasma containing antibodies to a variety of pathogens and for which the manufacturing pathway commonly includes an S/D treatment.[13]

Here, we aimed to characterize the different phenotypes in HEV stock preparations used for virus clearance studies, firstly by size, using a series of nanofilters with pore sizes graded around the assumed sizes of the different HEV particles and by density in isopycnic gradient centrifugation. HEV removal by nanofiltration using 35 nm filters was then investigated in presence or absence of HEV-specific antibodies, that is, situations of relevance in the manufacture of antibody-containing plasma products prior to or following S/D treatment.

2 | MATERIALS AND METHODS

2.1 | Hepatitis E virus preparations

HEV-positive plasma was obtained from Haema AG (Leipzig, Germany), virus particles were concentrated by ultracentrifugation at 4°C and 100 000*g* for 75 minutes and resuspended in phosphate-buffered saline (PBS). Stocks of recombinant HEV (rHEV) were produced with use of HepG2/C3A cells (CRL10741, American Type Culture Collection, Rockville, MD) and plasmid "p6" that had been obtained from the National Institute of Health (NIH, Bethesda, Maryland), as previously described.[10] HEV stock originating from Japanese swine feces (isolate swJB-M5)[11] was obtained from the Hirakata Laboratory, Japan Blood Products Organization (Osaka, Japan). A sample of HEV-containing human stool was kindly provided by H. Dalton,[14] and stocks were prepared by suspension in Dulbecco's Modified Eagle Medium (DMEM), vortexing with glass beads, low-speed centrifugation, and passage of the supernatant through 0.45-μm and 0.2-μm filters (hsHEV). A cell culture–adapted HEV isolate was obtained from a human stool-derived preparation after serial passage on HepG2/C3A cells (ccHEV).

To remove any virus-associated LE, HEV stocks obtained from cell culture (rHEV, ccHEV) and from HEV-positive plasma were S/D treated. The virus stocks were mixed with the S/D reagents (Triton X-100: Tri-n-Butyl-Phosphate: Polysorbate 80) to give final concentrations of 1, 0.3 and 0.3% (v/v), respectively. The mixtures were vortexed and incubated at room temperature or 30°C (for serial nanofiltration) for 60 minutes. Alternatively, to remove LE and proteins associated with the HEV capsid surface, S/D reagent and 0.1% pronase E (Sigma-Aldrich, St. Louis, Missouri) were added to rHEV and

samples incubated at 37°C for 2 hours. The S/D reagents were subsequently removed by solid phase extraction (Waters Sep-Pack Long C18 Cartridge, WAT023635, Waters Corp, Milford, Massachusetts), as previously described[2] or by castor-oil extraction (1 hour at room temperature) and adsorption to C18 resin (WAT020595) in suspension for 1 hour at room temperature (for serial nanofiltration). C18 resin beads were removed by low-speed centrifugation and filtration through 0.45 μm cellulose acetate filters (Sartorious Minisart, Sartorius AG, Göttingen, Germany).

2.2 | HEV characterization by serial nanofiltration

Filtration of hsHEV and ccHEV stock through a series of nanofilters with defined pore sizes was performed as previously described.[15] Briefly, DMEM was spiked 1:10 with HEV and processed in dead-end mode through 0.1-μm polyethersulfone filters (Sartorius Minisart). The filtrate was processed in dead-end mode through a series of Planova filters with different average pore diameters: 75 nm (Planova 75N, Asahi Kasei Medical, Tokyo, Japan), 40 nm (Asahi, not commercially available), 35 nm (Planova 35 N), and 30 nm (Asahi, not commercially available). After each filtration, a sample was withdrawn for analysis. Asahi filters were 0.001 m^2. Filter loads were equivalent to 100 L/m^2 or 200 L/m^2 with constant pressure between 0.9 and 1.0 bar.

2.3 | Isopycnic gradient centrifugation

HEV stocks were loaded onto the surface of a 15% to 50% iodixanol (Optiprep, Sigma Aldrich) step gradient and centrifuged at 141 000*g* in an SW32 Beckman Coulter rotor for 48 hours at 4°C. Fractions of 1 mL were collected, and buoyant density was determined with a refractometer (Digital refractometer RX-5000, Atago). For analysis of HEV RNA content, two sequential fractions were pooled before RNA extraction.

2.4 | Detection of HEV RNA

HEV RNA was extracted from volumes of 120 μL with a viral RNA isolation kit (QIAamp; Qiagen, Hilden, Germany). Samples were eluted in 80 μL of buffer provided with the kit and reverse transcription quantitative polymerase chain reaction (RT qPCR) was performed on a RT qPCR system (ABI 7900HT Real-Time PCR; Applied Biosystems, Life Technologies, Carlsbad, California) with an optimized HEV RT qPCR assay, as previously reported.[10] Results were expressed as genome equivalents per milliliter and the lack of matrix interference on the detection of HEV RNA in IG intermediate was verified in control studies. For the serial filtration experiment, HEV RNA was extracted from 200-μL samples with a purification kit (QIAamp MinElute Kit, Qiagen), eluted in 70 μL buffer, of which 10 μL were used for the RT qPCR. Amplification and detection were done using an HEV RT-PCR kit (RealStar 1.0; Altona Diagnostics GmbH, Hamburg, Germany) and an RT PCR system (LightCycler 480, Roche Deutschland Holding GmbH, Germany).

Both HEV RT qPCR assays were calibrated against the World Health Organization (WHO) International Standard for HEV RNA nucleic acid amplification techniques (Paul-Ehrlich-Institut code 6329/10).[16]

2.5 | Detection of HEV IgG antibodies

HEV IgG antibodies were detected with an HEV-IgG enzyme-linked immunosorbent assay (ELISA) kit (Wantai, Beijing, China) following the manufacturer's instructions and an ELx808 Ultra Microplate Reader (Bio-Tek Instruments Inc., Vermont) at 450 nm. The kit is suitable for detection of HEV antibodies in human serum and plasma samples and was qualified for detection of HEV antibodies in IG intermediate and IG product. Using the WHO HEV antibody standard reference reagent 95/584 (NIBSC, Hertfordshire, UK) assay linearity ranged between 0.39and 1.56 WHO units/mL (U/mL). One EU plasma-derived KIOVIG lot (protein content, 100 mg/mL) as well as six EU and eight US plasma-derived 10% IG intermediates (protein content, approx. 10 mg/mL) were diluted in human serum negative for HEV antibodies and analyzed in duplicate.

2.6 | Investigation of nanofiltration enhancement

Gammagard Liquid (name: GG LQ in the US and KIOVIG in Europe), represents Takeda's 10% liquid human IG infusions, which are fractionated by the same process that includes three dedicated virus reduction steps, ie, an S/D treatment followed by 35 nm nanofiltration, and finally by low pH incubation at elevated temperature.[17] For initial investigation of HEV reduction by nanofiltration, rHEV, or S/D-treated rHEV (without pronase) was spiked at a ratio of 1:10 into approximately 20 mL of PBS. A 10% IG lot for which the HEV antibody content had been determined by ELISA

was added at a final concentration of 1%, which corresponded to 0.76 U/mL. The nanofiltration step used 0.001 m^2 35-nm virus filter units (Planova) in dead-end mode and fully automated filtration equipment as previously described.[18] The process conditions were: matrix pH, 7.2 to 7.4; mean differential pressure, 0.8 bar; product intermediate feed load, 20 L/m^2; and room temperature. Virus reduction was investigated in duplicate runs, and results were calculated as log factors of viral RNA loads before and after filtration.

For investigation of HEV reduction during nanofiltration in the manufacturing of GG LQ/KIOVIG, a validated scaled-down model was established, and comparison of critical process and selected biochemical variables confirmed that the model reflected the manufacturing process. Planova 0.001 m^2 35 nm filters were used in combination with a VR06 depth prefilter (Cuno VR06, nominal retention rating of 0.2 μm; Bangalore, Karnataka, India), in a cross-flow mode at constant transmembrane pressure using fully automated filtration equipment.[18] Virus stocks were passed through 0.2-μm filters immediately before process intermediate spiking, to ensure the absence of virus aggregates. Approximately 1200 mL of GG LQ intermediate and 800 mL of KIOVIG intermediate, respectively, were spiked at a ratio of 1:300 with S/D-treated (without pronase) rHEV and incubated at room temperature for 1 hour before starting the nanofiltration process. The process conditions were: matrix pH, 6.2 to 6.4; matrix conductivity, 1.9 to 2.3 mS/cm; mean differential pressure, 1.0 bar; mean flow rate, 53 to 57 L/m^2 and hour; product intermediate feed load, 800 to 1200 L/m^2 and 8.1 to 8.7 kg protein/m^2; buffer chase, 50 to 60 L/m^2; temperature, 19 to 21°C. Virus reduction was investigated in single runs and virus reduction factors (LRFs; reported in log values) were calculated in accordance with regulatory guidelines[19] and with unrounded values (only the final LRF was rounded).

3 | RESULTS

3.1 | HEV characterization by serial nanofiltration

Initial characterization of crude HEV preparations for particle size through use of a cascade of nanofilters with defined pore size confirmed ccHEV without S/D treatment, ie, LE HEV, to be somewhat larger, with the majority of virus being removed by filters with pore sizes in the range of 40 to 75 nm. S/D treatment of HEV increased penetration though 75-nm filters, and similar removal was obtained for S/D-treated ccHEV (IM) and NLE hsHEV particles, ie, at 35-40 nm (Table 1), which confirmed the smaller size of these particle phenotypes compared to LE HEV. As expected, S/D treatment of NLE particles from stool did not further enhance passage through the filters (data not shown) and mean reduction values were calculated from runs with both, non–S/D-treated and S/D-treated stool samples, respectively (Table 1).

3.2 | Phenotype of HEV preparations

Only LE particles were detected in HEV-positive human plasma and rHEV stocks, in which viral particles banded at a density of 1.06 to 1.10 g/cm^{-3} (fractions 9-12) (Figure 1A,B). S/D-treated human plasma and rHEV stock did not contain NLE particles, but particles of IM density of 1.15 to 1.17 g/cm^{-3} (fractions 16-18) (Figure 1A,B), still below a buoyant density of 1.19 to 1.22 g/cm^{-3}, which would be typical for NLE particles (Figure 1C). When HEV preparations were treated with S/D reagent and protease, particles of 1.19 to 1.22 g/cm^{-3} buoyant density were obtained (fractions 20-22) (Figure 1A,B), similar to HEV particles derived from a porcine fecal extract, which was used as a NLE control (Figure 1C).

TABLE 1 Characterization of HEV particle size by serial nanofiltration (mean logarithmic virus reduction factors [LRF] ± SD; n = number of experiments)

	Cell culture HEV		Stool HEV
Pore size (nm)	LE[a]	IM[b]	NLE[c]
100	0.0 ± 0.1; n = 2	0.3; n = 1	0.1 ± 0.5; n = 5
75	2.8 ± 0.4; n = 3	0.5 ± 0.0; n = 2	0.5 ± 0.1; n = 5
40	>1.6 ± 0.6; n = 3	2.5 ± 0.7; n = 2	3.0 ± 1.2; n = 5
35	1.0; n = 1	1.5 ± 0.7; n = 2	1.3 ± 1.1; n = 5
30	>0.4; n = 1	>0.4 ± 0.4; n = 2	>0.1 ± 0.0; n = 4

Note: HEV detection was done by reverse transcription quantitative polymerase chain reaction; boxes indicate main virus removal.

[a]LE: lipid-enveloped particles from cell culture supernatant (ccHEV).

[b]IM: "intermediate particles" from S/D-treated cell culture supernatant (ccHEV).

[c]NLE: non–lipid enveloped particles from human stool (hsHEV).

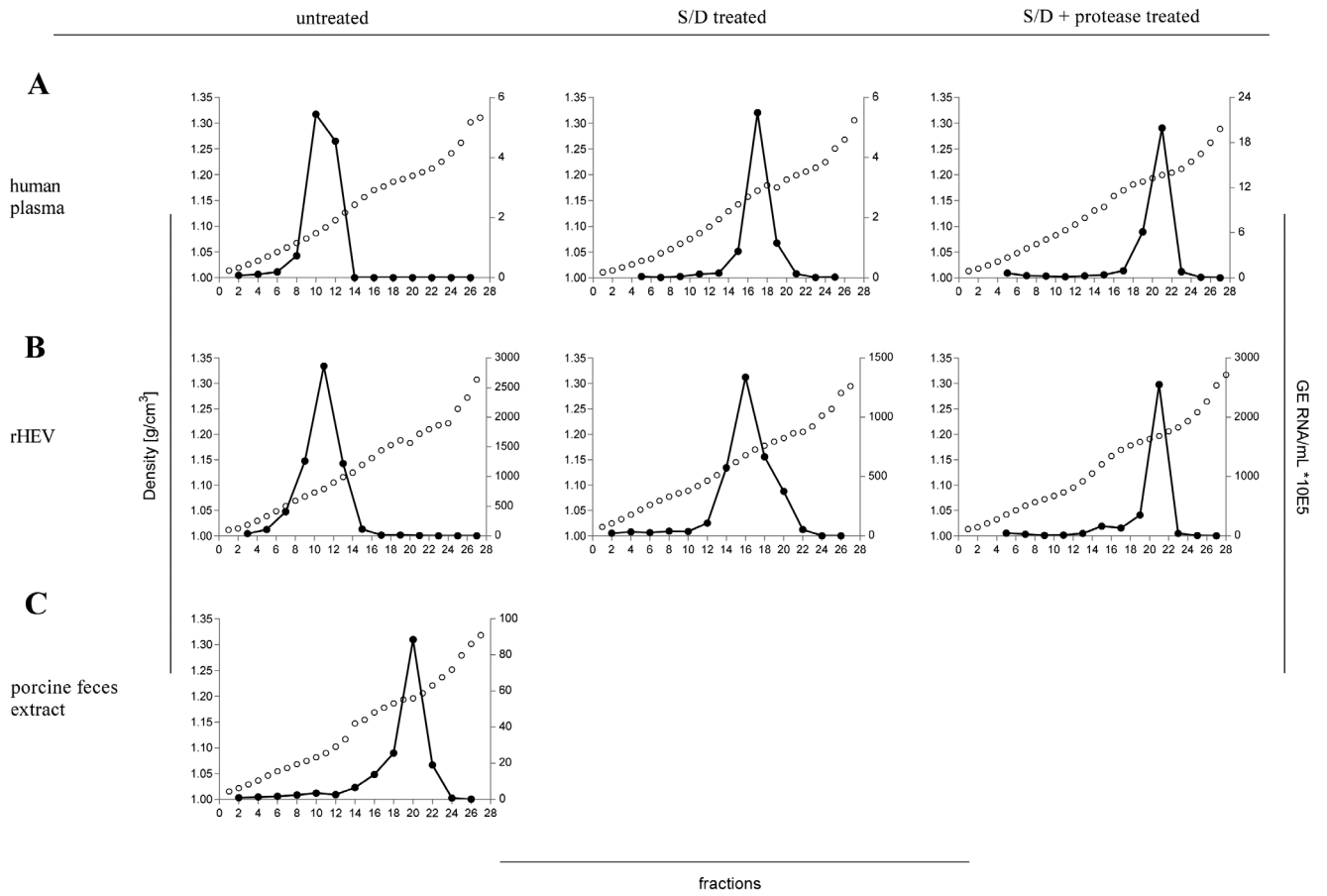

FIGURE 1 Characterization of untreated, S/D-treated and S/D and protease–treated HEV. Buoyant densities of, A, wild-type HEV from human plasma, B, rHEV, and C, wild-type HEV from porcine feces without any further treatment (untreated), following S/D treatment (S/D treated), or after S/D and protease (pronase E) treatment. Virus was resolved in isopycnic gradients and RNA was detected by RT qPCR. LE particles have a density of 1.06 to 1.10 g/cm^3, virions of IM band at 1.15 to 1.17 g/cm^3, whereas NLE virions have a density of 1.19 to 1.22 g/cm^3

TABLE 2 Removal of LE rHEV and IM rHEV by 35 nm nanofiltration (log GE)

Matrix	PBS								IG intermediate	
Antibody	–		+[a]		–		+[a]		European Union[b]	United States[b]
HEV phenotype	LE		LE		IM		IM		IM	IM
spiked material	10.1	9.7	9.9	10.0	9.8	10.1	9.8	10.0	9.9	9.8
35-nm filtrate	6.1	5.8	6.4	5.2	7.8	7.2	4.6	4.1	<5.1	<5.8
LRF	4.0	3.9	3.5	4.8	2.0	2.9	5.2	5.9	>4.8	>4.0
mean LRF	4.0 ± 0.1		4.1 ± 0.9		2.5 ± 0.6		5.6 ± 0.5		>4.4 ± 0.4	

Abbreviations: GE, genome equivalent; HEV, hepatitis E virus; IG, immunoglobulin; LRF, log reduction factor; PBS, phosphate-buffered saline.
[a]IG was added to a final concentration of 1%, resulting in an HEV antibody concentration of 0.76 U/mL.
[b]IG intermediate fractionated from EU (HEV antibody 1.11 U/mL) or US (HEV antibody <0.39 U/mL)-derived plasma was used.

3.3 | Detection of HEV IgG antibodies

Six EU and eight US plasma-derived 10% IG intermediates at the nanofiltration manufacturing stage as well as one final product KIOVIG lot fractionated exclusively from EU plasma were tested for HEV-binding antibody by the Wantai ELISA. All of the eight US plasma-derived and one of the EU plasma-derived intermediates tested

below the limit of detection (LOD; ie, <0.39 U/mL) for HEV antibodies, whereas five of six EU plasma-derived intermediates contained HEV antibodies in concentrations that were quantifiable, with titers ranging between 1.02 and 1.32 U/mL. For the final product KIOVIG lot, a titer of 7.6 U/mL HEV antibodies was determined, which was subsequently used for antibody spiking in the 35-nm nanofiltration experiments in PBS as the matrix.

3.4 | 35-nm nanofiltration of HEV

When 35-nm nanofiltration was performed in the PBS matrix and in absence of HEV antibodies, mean LRFs of 4.0 and 2.5 were obtained for rHEV (LE) and for S/D treated rHEV (IM), respectively (Table 2), consistent with the larger and smaller particle sizes of the different phenotypes. Addition of HEV antibodies at a concentration of 0.76 U/mL did not affect the removal of LE rHEV (mean LRF, 4.1), whereas enhanced removal was seen for S/D-treated IM rHEV in the buffer substrate, for which a mean LRF of 5.6 was obtained (Table 2).

When the HEV removal capacity of the IG manufacturing process step nanofiltration was investigated, S/D treated rHEV removal was effective, with LRFs of >4.8 and >4.0 for rHEV (IM) spiked in EU plasma and US plasma intermediate, respectively (Table 2). Nanofiltration was done in the presence of 1.11 U/mL HEV antibodies in EU plasma-derived intermediate, whereas the HEV antibody level in US plasma-derived nanofiltrate was below the LOD, that is, less than 0.39 U/mL. However, the obtained LRF for rHEV indicated that even levels of antibodies below the LOD as still present in the matrix were sufficient to effectively remove HEV.

4 | DISCUSSION

HEV from human plasma was confirmed to occur as LE virus particles (Figure 1A),[6,7] and this phenotype was also obtained in cell culture (Figure 1B).[6–9] HEV stocks prepared from cell culture, rather than from fecal samples that contain the NLE phenotype (Figure 1C),[6,7] are therefore the appropriate HEV spike for clearance studies of PDMPs upstream of any S/D treatment in the manufacturing process. S/D treatment resulted in HEV particles of IM rather than NLE phenotype (Figure 1A, B), which differ in buoyant densities (Figure 1A,B) and the presence of the open reading frame 3 encoded protein on the IM but not on the NLE particle surface.[7] The HEV phenotypes differ in particle size, where LE HEV size was estimated at 52 ± 15 nm and following detergent treatment smaller particles of 28 ± 3 nm, identical in size to those found in stool, were reported.[6,8,9,20] The relevance of this size difference was confirmed by nanofiltration, where LE HEV particles were effectively removed already by 75-nm and 40-nm filters during serial nanofiltration (Table 1) and by 35-nm filtration (Table 2), whereas S/D-treated particles were removed mainly by the 40-nm and 35-nm filters during serial nanofiltration (Table 1) and removal by the single 35-nm filter was somewhat less efficient (Table 2).

There were differences in the recognition of the various HEV particles by antibodies. The presence of an LE prevented antibodies binding to HEV, while both the IM and NLE HEV phenotypes were bound and neutralized.[6,7,9] This difference was of relevance during 35-nm filtration in PBS, where addition of polyclonal antibodies to a concentration of 0.76 U/mL did not alter clearance of LE HEV, which is not bound by antibodies (Table 2), but significantly enhanced removal of the IM HEV phenotype approximately 1000-fold, with an LRF of 5.6 for IM HEV in the presence of HEV antibodies, compared to an LRV of 2.5 for IM HEV in the absence of HEV antibodies (Table 2). These experiments have certain limitations, as spike or unspecific IgG effects were not evaluated.

Antibody-mediated enhancement of nanofiltration, previously reported for hepatitis A virus and human parvovirus B19,[21] was also seen for 35-nm nanofiltration in the GG LQ and KIOVIG intermediates. Of note, HEV removal to below the LOD was obtained in both runs (Table 2), even in the presence of undetectable HEV antibody titers, that is, below the LOD of the ELISA (<0.39 U/mL) in the US plasma-derived GG LQ intermediate. Matrix interference was noted for the ELISA in IG intermediates and final product, which resulted in a narrow working range (ie, 0.39-1.56 U/mL), in contrast to the 0.25-5 U/mL range reported for the detection of HEV antibodies in rhesus macaque serum that used the same ELISA in a similar setup.[22] The narrow range of reliable HEV antibody detection in the IG matrices warrants future evaluation of low HEV antibody titers in IG final product.

The difference in HEV antibody titers in EU plasma- and US plasma-derived intermediates is reflective of HEV prevalence in the respective regions. Greater HEV seroprevalence, frequency of HEV blood transfusion transmission, and detection of HEV RNA in pools for fractionation was reported for Europe than for the United States.[1–4,23] The lower HEV prevalence in the United States was reflected in HEV antibody titers below the LOD in the eight US plasma-derived intermediates tested, whereas only one in six EU plasma-derived intermediates was below the LOD and HEV titers of 1.02 and 1.32 U/mL

were determined for five EU plasma-derived intermediates. A recent study determined HEV antibody titers in IG preparations and found all products reactive for HEV IgG.[24] This included a lot of KIOVIG, for which a titer of approximately 2.8 U/mL was determined, however, without information on geographic origin of the plasma used in manufacture,[24] whereas the titer of the KIOVIG lot fractionated exclusively from EU plasma that was used in the PBS experiment reported here was 7.6 U/mL.

The current work demonstrated that the virus spike used in HEV clearance studies needs to be chosen carefully, as differences in HEV particle size and accessibility by HEV-specific antibodies might affect the reduction obtained in HEV clearance studies. In the current example, following an upstream S/D treatment in the manufacture of IG, the appropriate spike of IM HEV at the nanofiltration step resulted in substantial HEV removal during 35-nm nanofiltration, even when HEV antibody levels were below the LOD (Table 2). Effective HEV clearance was seen at the antibody concentrations present in EU plasma and also US plasma-derived IG intermediates (Table 2). Effective HEV removal during nanofiltration suggests that the substantially higher HEV antibody titers in final-product IG additionally contribute to HEV clearance through neutralization. A recent study reported the presence of HEV antibodies in all IG products investigated and suggested passive seroprotection from persistent HEV infection in immune-deficient patients as associated with the presence of HEV antibodies in these IG products.[24] The HEV neutralization potency of IG lots manufactured from plasma collected in the European Union or the United States would still benefit from further studies. Although limited to antibody containing PDMPs, the results reported here contribute further evidence that the virus clearance capacity of PDMP manufacturing pathways are adequate to maintain safety margins for HEV.

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CONFLICT OF INTEREST

A.M.K., M.R.F., A.W. and T.R.K. were employees of Baxter AG, Vienna, Austria, now part of the Takeda group of companies. M.R.F. and T.R.K. have Takeda stock interest. T.M. is employed by Asahi Kasei. M.Q.A., S.A.B., and J.B. declare no conflict of interest.

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