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BLOOD COMPONENTS

TRANSFUSION

# Hepatitis E virus: Efficacy of pasteurization of plasma-derived VWF/FVIII concentrate determined by pig bioassay

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## Abstract

**Background:** Hepatitis E virus (HEV) is the leading cause of acute hepatitis throughout the world. Increasing blood component transfusion-associated HEV infections highlight the need for reliable virus inactivation procedures for plasma derivatives from pooled plasma donations.

**Study Design and Methods:** An animal infection study was conducted to evaluate the efficiency of HEV inactivation by pasteurization during the manufacturing process of the von Willebrand Factor/Factor VIII (VWF/FVIII) concentrate Haemate P/Humate-P (CSL Behring, Marburg, Germany). For this purpose, groups of pigs were inoculated with stabilized VWF/FVIII intermediate spiked with HEV-positive liver homogenate and exposed to increasing incubation times of 0, 3, 6, and 10 h at 60°C. Animals were evaluated for virus replication over 27 days and in a subsequent trial over 92 days.

**Results:** Virus replication was detected in animals up to the 6-h pasteurization group. In contrast, pasteurization for 10 h did not reveal virus detection when the observation period was 27 days. In an additional experiment using the 10-h pasteurized material, two individuals started virus excretion and seroconverted when the observation period was extended to 92 days. Based on the total infection rate (2 of 12) of the animals inoculated with the sample pasteurized for 10 h, a virus reduction factor of at least 4.7 $\log_{10}$ is calculated.

**Conclusion:** This study demonstrates that pasteurization at 60°C for 10 h of an HEV-positive plasma derivative leads to the effective reduction of infectivity, resulting in a VWF/FVIII product with an appropriate margin of safety for HEV.

KEYWORDS

hepatitis E virus, inactivation, infectivity, pasteurization, plasma product

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## 1 | INTRODUCTION

So far, four genotypes of the Hepatitis E virus (HEV), assigned to the genus *Orthohepevirus*, species *Orthohepevirus A*,[1] have been found to be human pathogenic. The most prevalent HEV genotype in developed countries is HEV genotype 3 (HEV-3), which is zoonotic and is responsible for sporadic HEV cases in humans. The infection mainly occurs through food-borne transmission routes. The main reservoirs are pigs and wild boar. Numerous studies revealed the ubiquitous presence of HEV-3 in these animals.[2–4] Human infections with HEV can range from subclinical mild to more severe clinical courses with jaundice and fulminant liver failure.[5] Extrahepatic manifestations and chronic courses in immunocompromised patients have been reported.[6–9] A possible source of transmission in these cases was contaminated blood products that came from viremic blood donors.[10] The risk of infection should not be neglected as HEV-positive blood donations (based on serology or nucleic acid amplification techniques) were detectable with up to 0.08%[11] to 0.12%[12] in Germany, 0.035% in the United Kingdom,[13] 0.04% in Denmark,[14] 0.045% in France,[15] 0.14% in The Netherlands,[16] and 0.002% in the United States.[17] HEV transmission through various blood components like fresh frozen plasma (FFP; including amatosalen-treated FFP), red blood cell concentrates, and platelet concentrates, as well as solvent/detergent (SD) plasma, has been reported.[18,19] Therefore, the European Pharmacopoeia requires screening of pooled SD plasma for a minimal amount of HEV RNA ($\leq$ 2.5 log10 IU/ml).[18]

This underlines the need for reliable virus inactivation/removal procedures to ensure the safety of plasma-derived products as the starting material of these products are pools of plasma with up to several thousand donations. The incidence rate in the donor population (HEV RNA reactive donations) differs between geographic regions; the rate varies between approximately 1 in 700, 1 in 4500, 1 in 7000, and 1 in 8000 for some European countries and 1 in 43 000 donations for the United States.[17,20–22]

Up to now, there has been only little information about the impact of chemical and/or physical treatments on the stability of HEV due to limitations of cell culture models. Only specific clones from cell lines and only specific HEV isolates—typically with additional insertions—can be used as the starting material for cell culture-derived HEV replication.[23–25] Depending on the virus strain used, approximately 27 000 and 1000 HEV RNA copies, respectively, are required for one cell culture infectious dose, demonstrating the low sensitivity of a cell culture for HEV infection.[23,25] Thermal stability of HEV has been analyzed in a few in vitro studies primarily covering food contaminated with HEV; the inactivation capacity varies depending on genotypes, used temperature, and duration of heat exposure,[26–32] but the parameters are not related to the pasteurization of plasma-derived products. This highlights the need for alternative and sustainable models to monitor HEV infectivity and to validate inactivation methods for these medicinal products.

A plasma-derived product (von Willebrand Factor/Factor VIII [VWF/FVIII] concentrate [Haemate P/Humate-P; CSL Behring, Marburg, Germany]), purified, stabilized by sucrose/glycine to prevent protein denaturation during pasteurization (heat treatment in stabilized aqueous solution at 60°C for 10 h), and finally lyophilized, was employed in this study. The stabilized VWF/FVIII intermediate was mixed with liver homogenate from an HEV-infected pig and pasteurized for 3, 6, and 10 h at 60°C and subsequently inoculated to pigs according to an established pig infection model.[33] Pigs were analyzed for fecal virus shedding, viremia, antibody production, and virus distribution in tissues to determine the potential reduction of infectivity by pasteurization.

As HEV is a quasienveloped virus, the relevance of the HEV spiking material derived from pig liver was compared to HEV from human plasma and pig feces. This membrane-associated quasi-enveloped virus is released from cells into the bloodstream via the host cell ESCRT mechanism and is mediated by HEV open reading frame 3 (ORF3) protein, which binds to proteins of the endosomal sorting complex required for transport (ESCRT) pathway. The virus capsids are covered by lipid membranes that resemble the lipid membranes of exosomes, which is removed after entering the bile and is subsequently shed in the feces as nonenveloped virion.[34,35]

## 2 | MATERIAL AND METHODS

### 2.1 | Preparation of inoculum

The inoculum used consists of a sucrose-/glycine-stabilized VWF/FVIII (Haemate P/Humate-P) intermediate spiked with the liver homogenate of an experimentally HEV-infected wild boar from a previous study.[33] The liver material was minced, ground, and dissolved in phosphate-buffered saline (PBS) to obtain a 20% liver homogenate, which was subsequently sterile filtered using a 0.22-µm MILLEX-GP Syringe Filter Unit (Millipore, Ireland). An aliquot was set aside to function as native control and stored at −80°C. The remaining homogenate was thoroughly mixed at a ratio of 1:11, with the VWF/FVIII intermediate stabilized to 110% stabilizer concentration to achieve a stabilizer concentration equivalent to production conditions (100% stabilizer concentration). The mixture

**TABLE 1** Experimental setup

| Inoculum | Pasteurization time [hours] | Hepatitis E Virus RNA concentration in inocula [copies/ml] | Number of animals | | Gender | Age at inoculation [weeks] | Observation period | Animal identification | Group of animals |
|---|---|---|---|---|---|---|---|---|---|
| | | | Inoculated | Horizontal controls [c] | | | | | |
| Native liver homogenate | n.a. | 3.90E+06 | 3 | 1 | f/m | 8 | 28dpi | P2-97, P2-98, P2-99, P2-100c | A |
| Haemate P spiked with liver homogenate 11:1 | 0 | 2.12E+06 | 3 | 1 | f/m | 8 | 28dpi | P1-01, P1-02, P1-03, P1-04c | B |
| | 0 | 3.59E+05 | 6 | 0 | f/m | 10 | 28dpi | P3-15, P3-16, P3-17, P3-18, P3-19, P3-20 | |
| | 3 | 3.85E+05 | 6 | 2 | f/m | 8 | 27dpi | P2-09, P2-10, P2-11, P2-12, P2-13, P2-14, P2-15c, P2 -16c | C |
| | 6 | 3.04E+05 | 6 | 2 | f/m | 8 | 28dpi | P2-01, P2-02, P2-03, P2-04, P2-05c, P2-06, P2-07, P2-08c | D |
| | 10 | 2.96E+05 | 6 | 2 | f/m | 8 | 28dpi | P1-05, P1-06, P1-07, P1-08, P1-09, P1-10, P1-11c, P1-12c | E |
| | 10 | 2.90E+05 | 3 | 1 | f | 11 | 91dpi | P4-01, P4-02, P4-03, P4-04c | F |
| | 10 | 7.13E+05 | 3 | 1 | m | 11 | 91dpi | P4-05, P4-06, P4-07c, P4-08 | |

was divided into four aliquots. Besides one untreated control (0 h control), stored at 80°C after preparation, the additional aliquots were pasteurized at 60.0°C ± 0.5°C in a calibrated water bath for 3, 6, and 10 h. After reaching 60 ± 0.5°C (warm-up period of 25 min), the timer was started. The temperatures of the water bath and of a mock spiked sample in this water bath were monitored with a calibrated thermometer during the pasteurization experiment. Aliquots were taken at 3, 6, and 10 h after heat treatment; were cooled on ice; and were stored at −80°C after generation; all pasteurized samples, as well as the untreated control (0 h control), were stored at −80°C until inoculation in pigs was performed (Table 1).

## 2.2 | Equilibrium centrifugation in a sucrose density gradient

HEV particles from different sources were tested for the presence of a quasienvelope. HEV-3-positive serum and feces samples, as well as liver homogenate, were derived from individual pigs, originating from existing animal experiments (LALLF M-V/TSD/7221.3-2.1-017/13). Human samples were derived from a plasma donation of a donor infected with HEV (CSL Behring). An equilibrium centrifugation of plasma, liver, and feces material by sucrose gradient was carried out according to a previously published study.[36] Samples were left native or incubated with 2% NP-40 at 37°C for 1 h prior to ultracentrifugation. NP-40 (Sigma-Aldrich) is a nonionic detergent that solubilizes plasma membrane. In short, 13.2-ml Ultra-Clear tubes (Beckman&Coulter, Krefeld, Germany) were filled with a sucrose density gradient with increasing concentrations of sucrose (60%, 40%, 30%, and 20% sucrose; 10% sucrose in Tris-NaCl-EDTA (TNE) buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid) followed by 3 ml TNE buffer). Centrifugation was performed in an SW-41TI rotor (Beckman&Coulter) in an L7-55 ultracentrifuge (Beckman&Coulter) at 21,0000 g at 4°C for 4 h. Overall, 18 fractions were recovered, starting from the top with seven 1-ml fractions followed by eleven 500-µl fractions, and analyzed via HEV-specific quantitative real time reverse transcription polymerase chain reaction (qRT-PCR). Sucrose concentration in fractions was monitored using a Refractometer (Brix scale).

## 2.3 | Experimental design of the animal study

The competent authority of the Federal State of Mecklenburg Western-Pomerania approved all described animal experiments based on national and European laws (approval number of the local authorities: LALLF M-V/TSD/7221.3-2.1-017/13). For this experiment, 46 large white pigs from a commercial breeder were used. The animals were maintained under containment conditions in a Biosafety Level 3 facility during the study. Prior to acquisition, all animals were tested for the absence of signs of HEV by RT-qPCR and ELISA in blood and feces and were retested prior to inoculation. Furthermore, all animals were tested free of porcine reproductive and respiratory syndrome virus and porcine circovirus 2, as well as for African swine fever virus, foot-and-mouth-disease virus, and suid herpesvirus 1, employing a quantitative multiplex RT-PCR[37] The investigation was performed twice at the stable of origin, as well as at the facilities of Friedrich-Loeffler-Institut (FLI). The animals were randomly divided into groups (Table 1). Untreated horizontal controls were housed with the inoculated animals except in group r0 h (second set of experiment). Sentinel (control) animals were used to demonstrate the productive HEV infectivity of the inoculated animals through fecal–oral transmission[38,39] After stabling, the animals were divided into the corresponding groups and held in quarantine for 2 weeks in separate stable units. Complete change of protective clothes, as well as wearing of filtering face piece 3 (FFP3) masks, was mandatory before entry into each of the rooms. Daily animal manipulation always began in the 10-h pasteurization group, followed by the 6-, 3-, and 0-h pasteurization groups.

The inoculation was performed with a 2-ml dose of inoculum intravenously into the *vena cava cranialis*. Animal behavior and rectal body temperature were checked daily. As described in previous studies, body temperatures over 40°C for at least two consecutive days were considered a febrile response.[33,40] Depression, diarrhea, vomitus, icterus, ascites, and neurological symptoms were considered signs of acute hepatitis and would have led to immediate removal and euthanasia of the animal.

During the experiments, blood and fecal samples were taken regularly every 2–3 days. The corresponding serum samples were stored at −20°C. From the fecal samples, a 10% fecal suspension was made using 0.89% NaCl solution. After vortexing and centrifugation (4400 g, 4°C, 20 min), the supernatant was sterile filtered using a 0.22-µm MILLEX-GP Syringe Filter Unit (Millipore, Ireland) and stored at −20°C. To further evaluate the distribution of HEV in the inoculated animals, at the end of each an experimental setup, the animals were euthanized (electro stunning followed by exsanguination). Necropsies were performed, and samples from blood, feces, bile, and different tissues were aliquoted for RNA extraction and stored at −20°C. Tissue samples were treated with 4% neutral buffered formalin for further histological and immunohistochemical studies (see Supplement).

## 2.4 | RNA and antibody detection

RNA extraction of serum, fecal filtrate, and bile was performed manually using the QIAmp Viral Mini Kit (Qiagen) following manufacturer's instruction. Manual RNA extraction from tissue samples was performed with the RNEasy Mini Kits (QIAGEN GmbH, Hilden Germany). A heterologous internal control was added to each extraction sample. Obtained RNA was stored at −80°C until further use. To monitor the course of the infection and to quantify viral shedding and viremia, quantitative real-time RT-PCR (qRT-PCR) with standard curves was performed as described previously.[41] The immunological response was monitored using the Priocheck HEV Ab porcine enzyme-linked immunoassay (ELISA) (Mikrogen GmbH, Neuried, Germany), a porcine-specific anti-HEV IgG ELISA; the cut-off was calculated as mean $OD_{450}$ of the cut-off control (component of the test kit) multiplied by 1.2 according to manufacturer's instructions.

## 2.5 | Statistical evaluation of the correlation of pasteurization time and time to detection of HEV infectivity

To assess a correlation between the pasteurization time (in hours) and the length of time (in days) of the first detection of HEV replication within the pigs of one group, Pearson's correlation was applied (calculated using SYSTAT); no linear relationship of the two continuous variables exists at Pearson's correlation coefficient of near 0, and a strong linear relationship exists at ±0.8 or higher.

## 2.6 | Calculation of virus titers and virus reduction factor

As published previously in an accompanying study, the liver tissue used for spiking had an infectivity titer of 5.8 $\log_{10}ID_{50}$/ml calculated according to Spearman–Kärber as outlined in additional file 3 of a previous publication.[44] As 20% liver homogenate was used for spiking, a 1:5 dilution and a further 1:11 dilution by spiking with VWF/FVIII intermediate has to be considered for the titer of the inoculum. The logarithmic titer of the spiked untreated sample (0 h, Group B) was, thus, calculated as follows:

$$5.8\log_{10}\mathrm{ID}_{50}+\log_{10}(1:5)+\log_{10}(1:11)=5.8\log_{10}-0.7\log_{10}-1.0\log_{10}=4.1\log_{10}\mathrm{ID}_{50}/\mathrm{ml}$$

The virus reduction factor is calculated according to the relevant European guideline, Appendix II,[42] taking into consideration the virus load in the starting material divided by the virus load in the final sample of manufacturing step. As the volume of the product intermediate does not change during pasteurization, for the calculation of the virus reduction factor, the virus load is equivalent to the virus titer. Thus, the virus reduction factor (inactivation factor) is calculated as the difference of the logarithmic factor of the spiked starting material and of the final sample (10 h pasteurization time).

The infectious titer of the 10-h pasteurized HEV-spiked product intermediate was calculated according to Spearman–Kärber based on the reactivity rate of animals positive for HEV RNA in feces during the first 27 days after inoculation. Rules for titer calculation were the same as described in the study for the determination of the liver tissue.[43] The virus reduction factor (VRF) by pasteurization was calculated based on logarithmic titers of the 0-h and the 10-h samples: $\log_{10}$ titer (0 h) - $\log_{10}$ titer (10 h) = VRF [$\log_{10}$].

# 3 | RESULTS

## 3.1 | Density and composition of HEV virions from human plasma, porcine liver, and feces

To analyze HEV virions derived from human plasma and porcine liver, corresponding samples were applied to equilibrium centrifugation in a sucrose gradient (10%-60% w/v) and subsequently tested for HEV-derived RNA via PCR (Figure 1). In human plasma, most of HEV-derived RNA was found in an interval of sucrose concentrations of 15%–40%, with the highest concentrations between 22% and 32% sucrose (Figure 1(A)). The bulk of HEV in liver material was found in the range of 21%–43% sucrose, with the highest HEV RNA concentrations between 27% and 43% sucrose (Figure 1(B)). In contrast, HEV-derived RNA from feces peaked at 37%–47% sucrose, indicating a different density of feces-derived virions (Figure 1(C)). Application of detergent NP-40 led to a change in density regarding plasma and liver-derived particles to 44%–47% sucrose. However, particles from feces only slightly changed density, indicating the lack of a quasienvelope in feces material (Figure 1(A)–(C)).

## 3.2 | HEV RNA concentration in inocula

Inocula were prepared according to the Material and Methods section and have undergone subsequent heat inactivation treatment for increasing pasteurization times at 60°C. The HEV copy numbers in the liver homogenate had an HEV load of $3.9*10^3$ RNA copies/µl, and the

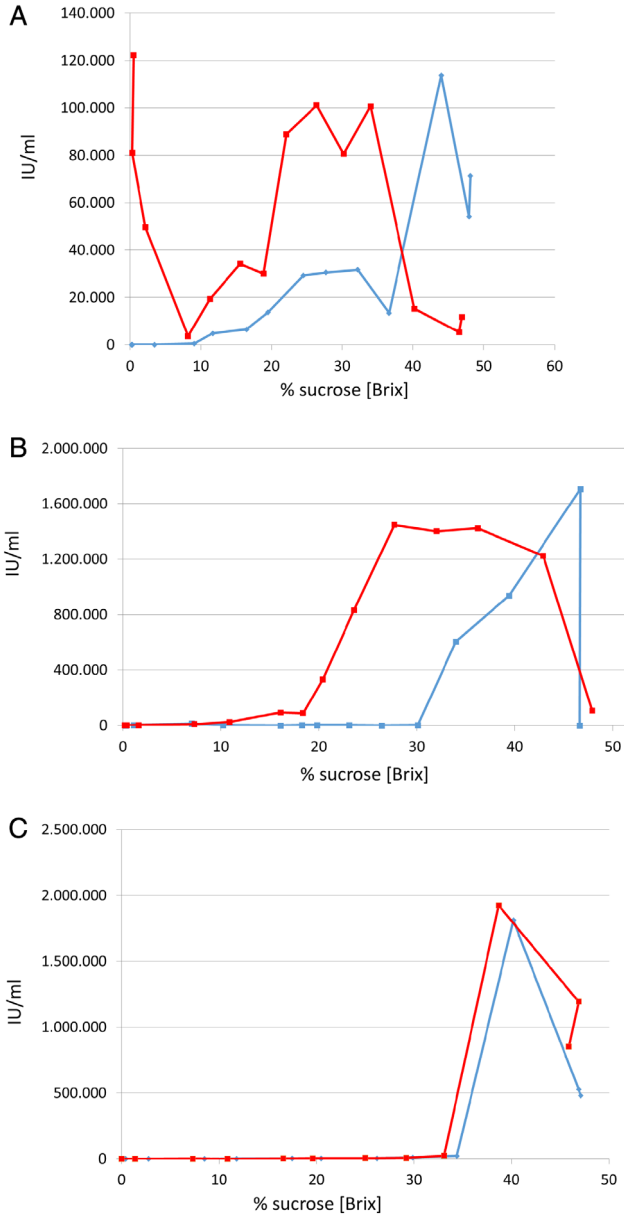


**FIGURE 1** Sucrose density gradient fractionation of Hepatitis E virus genotype 3-positive human plasma (A), pig liver homogenate (B), and pig feces (C) with (blue line) or without (red line) prior treatment with NP-40 [Color figure can be viewed at wileyonlinelibrary.com]

intermediate spike of 1:11 had an approximately 10-fold lower HEV RNA concentration; the HEV RNA concentration did not decrease considerably by pasteurization. A general summary including individual pig designation and the observation/incubation period is shown in Table 1.

## 3.3 | Clinical parameters and pathology

None of the animals showed a febrile response or any clinical signs consistent with hepatitis throughout the whole experiment. Animal P3-20 showed signs of regurgitation resulting in poor growth but could otherwise be considered clinically inconspicuous. No signs of viral hepatitis could be detected on gross examination at necropsy (data not shown).

## 3.4 | Virus detection in feces and serum

Feces and serum of inoculated pigs were tested regularly via HEV-specific qRT-PCRs to investigate productive virus replication. Starting from the control group (native liver homogenate) up to the 6-h pasteurization group, all inoculated animals displayed virus shedding within the observation period albeit at different time points. The Pearson correlation showed a high association between the parameter pasteurization time (0, 3, 6, and 10 h) and time

to detection of HEV replication in the inoculated pigs demonstrated by HEV RNA positivity in feces and by seroconversion. HEV antibodies in the serum of inoculated pigs are detected, on average, 7 days (range 0–17 days) after HEV shedding is demonstrated in feces. Delayed virus detectability related to increased pasteurization times implies an inactivation of HEV (Table 2).

In groups of pigs where HEV shedding in feces was observed, the sentinel (contact) animals also started shedding HEV (except for the sentinel animal in the "0h pasteurization time" group) but with a delayed onset of at least 10 days (Figure 2), indicating a fecal–oral transmission.

In the control group (diluted liver homogenate, untreated, Figure 2(A)), all inoculated animals started shedding viral RNA in their feces within 3–6 days post infection (dpi). A fecal–oral transmission occurred as HEV RNA was observed at 13 dpi in the feces of the contact animals. Animals inoculated with spiked product intermediate not pasteurized (0 h) and pasteurized for 3, 6, and 10 h (Figure 2(B)–(F))) started shedding viral RNA in their feces within 6–15 dpi, 9–13 dpi, 13–15 dpi, and 27–30 dpi, respectively. In the 10-h pasteurization groups, six and three animals did not show any signs of HEV infection (incubation period up to 27 days and 92 days, respectively), and in the third group of three animals, two showed HEV replication due to inoculation of the spiked treated material. As the infection of the contact animal was observed by HEV shedding in the feces starting on day 45, the third inoculated animal with virus shedding from day 55 dpi onward is considered to have been secondarily infected.

Details on HEV detection in tissues and body fluids (bile, liver, gall bladder, lymph nodes, spleen, and pancreas) for each animal in the study after necropsy are compiled in Supplement (Table S1).

### 3.5 | Infectivity titers of pasteurized samples and HEV reduction factor for the pasteurization of VWF/FVIII

Test results of animals for HEV RNA in feces during the incubation time of 27 days are documented in Figure 2. As for the spiked samples after 0, 3, and 6 h of heat treatment only, 2-ml aliquots of the undiluted samples were tested in groups with nine, six, and six inoculated animals, and as all animals of the respective groups became reactive for HEV RNA in feces within the 27-day observation period, no infectivity titer could be calculated based on Spearman–Kärber. Nevertheless, for the 0-h sample, a theoretical titer of 4.1 $\log_{10}$ $ID_{50}$/ml could be calculated using the infectivity titer of the liver homogenate used for spiking, known from a published accompanying study (see Material and Methods). On the other hand, in groups E and F inoculated with 2-ml aliquots of the sample after 10-h pasteurization, zero and 2 of six animals became reactive for HEV RNA within the 27-day observation period. Thus, a Spearman–Kärber titer could be calculated based on the additional conservative assumption that a 10-fold concentrated sample would have led to a reactivity rate of 1. Consequently, the titer of the 10-h sample was calculated to be $\leq -0.6$ $\log_{10}ID_{50}$/ml. According to the European guideline [CPMP/BWP/268/95],[42] the titer of the final sample ($\leq -0.6$ $\log_{10}$, 10-h sample) has to be subtracted from the titer of the starting material (4.1 $\log_{10}$, 0 h sample), resulting in a VRF of $\geq 4.7$ $\log_{10}$ for the pasteurization of VWF/FVIII.

## 4 | DISCUSSION

In the last few years, transfusion-associated HEV infections from HEV-positive blood donations raise questions about plasma product safety against HEV as HEV can be detected in plasma donations, as well as in plasma pools for fractionation.[22,44–47] The virus load in plasma pools for fractionation is low, never exceeding 1000 copies/ml.[47] Heat treatment has only a minor impact on the titer of virus nucleic acids[48]—this fact is also observed in our current study. Therefore, the absence of detectable HEV RNA in the final preparations of plasma-derived coagulation factors[49] may be attributed mainly to the capacity of protein purification steps and virus filtration steps in some manufacturing processes[50] to reduce the HEV particle load. Consequently, the destruction of HEV infectivity by heat treatment adds a safety margin on top of a nondetectable HEV RNA load.

**TABLE 2** Correlation of pasteurization time and first detection of hepatitis E virus (HEV) replication in pigs

| First time of HEV detected in an animal of each category [dpi] | | | | | |
|---|---|---|---|---|---|
| Sample studied | Pasteurization time | | | | Pearson's correlation coefficient |
| | 0 h (group B) | 3 h (group C) | 6 h (group D) | 10 h[a] (group E-F) | |
| Feces (HEV PCR) | 6 | 9 | 13 | 27 | 0.959 |
| ELISA (HEV antibodies) | 17 | 21 | 27 | 27 | 0.932 |

[a]Prolonged observation period (92 days).

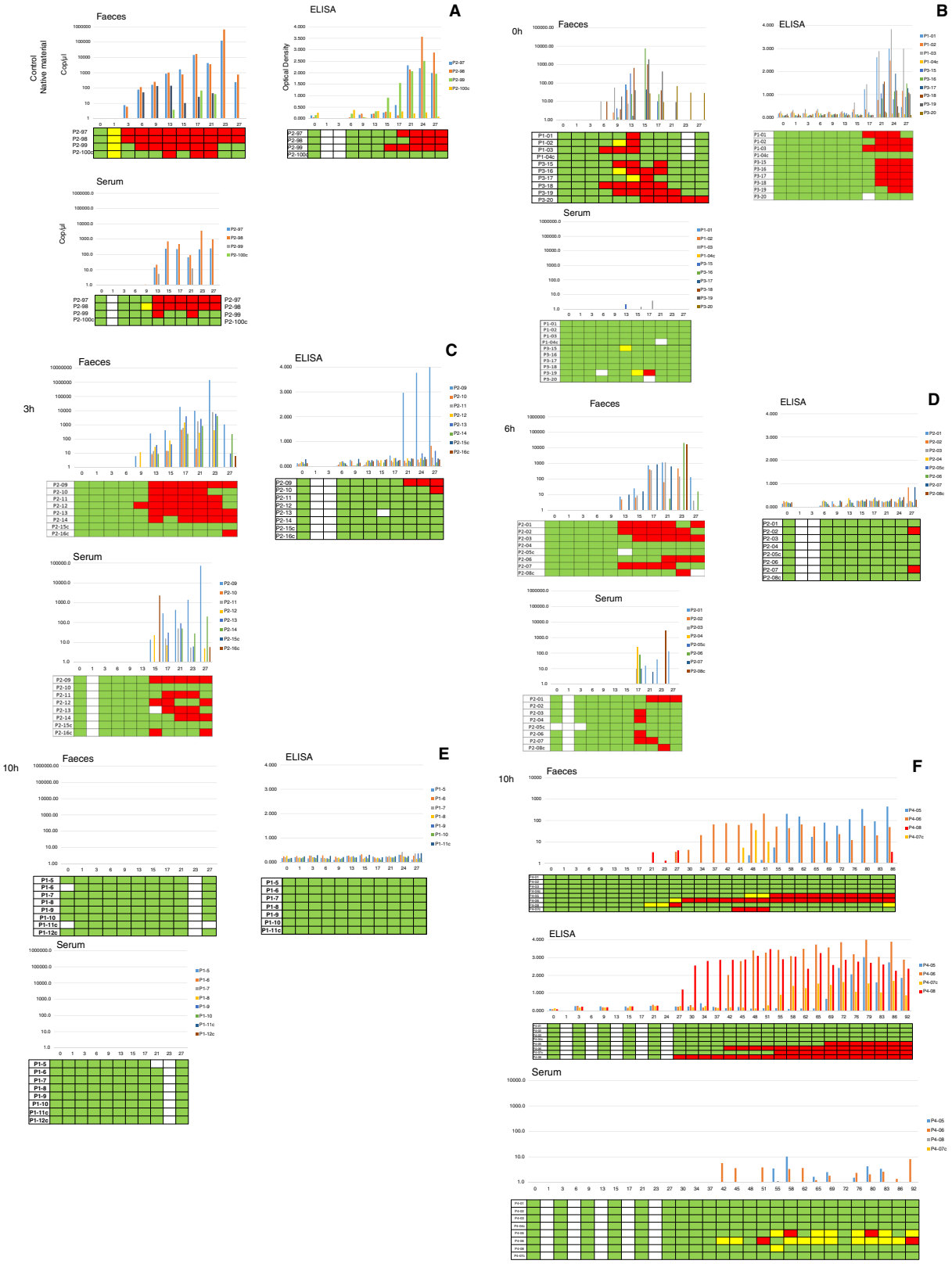


**FIGURE 2** Results of the RT-qPCR from feces and serum and the results of Priocheck ELISA from serum of all inoculated animals from the control (A) and the 0-h (B), 3-h (C), 6-h (D), 10-h (E), and the elongated 10-h (F) groups. The figures display the time course (days after inoculation) of all individuals, indicating copy number and corresponding optical density. The color code green represents not detected, yellow for borderline, and red for detection [Color figure can be viewed at wileyonlinelibrary.com]

The considerable heat resistance of HEV is known from preparing food: incubation of liver material at 56° for 1 h induced an HEV infection in four of five animals,[26] and heating at 62°C for 120 min resulted in the infection of three of four pigs, whereas exposure to 71°C for 20 min completely inactivated HEV.[27] Using a cell culture infectivity study, cell culture supernatant treated at 60°C caused complete inactivation of the virus after 10 min.[32]

A widely used method for virus inactivation in plasma-derived medicinal products is pasteurization at 60°C for 10 h; CSL Behring's pasteurization step is performed in intermediates stabilized commonly by high sucrose concentration, together with glycine or potassium acetate, to protect the plasma proteins from heat denaturation.[51] The efficacy for this thermal treatment has been demonstrated previously for a large variety of viruses, including human immunodeficiency virus, bovine viral diarrhea virus as a model virus for hepatitis C virus and West Nile virus, herpesviruses, Hepatitis A virus, parvovirus B19, and others.[51] Such pasteurization studies were previously not performed for HEV except for human serum albumin (HSA), which is not stabilized by sucrose and glycine or potassium acetate[50,52] but only by caprylate and N-acetyltryptophan, and these components do not affect the inactivation kinetics of a range of viruses. HEV inactivation data in HSA are not consistent. In 12.5% HSA, HEV was inactivated at 58 ± 1°C below the limit of detection of a cell culture infectivity assay within 180 min, resulting in a virus reduction factor of more than 3.1 $\log_{10}$.[50] In a different study in 25% HSA, different strains of HEV genotypes 3 and 4 showed considerable heat resistance with inactivation factors between 1.0 and ≥2.2 $\log_{10}$ within 5 h at 60°C[52]; in contrast to these data in HSA, the authors demonstrated a rapid HEV inactivation in PBS, resulting in reduction factors of ≥2.4 to ≥3.7 $\log_{10}$ within 30 min at 60°C. This is in line with published data[32] demonstrating that cell culture-derived virus stocks were already inactivated within 60 min below the limit of detection. As the current data from the pasteurization of HEV in HSA vary, and some viruses remain very stable when pasteurized in the presence of sucrose,[51] the VRF of 4.7 $\log_{10}$ observed in this study is surprisingly high as HEV was also treated in a sucrose-stabilized solution. Pasteurization is therefore an effective step in reducing HEV infectivity under the conditions used in the current study. As the stabilizer composition used during the pasteurization of plasma-derived products (to protect the heat-sensitive plasma proteins from denaturation) has an effect on the heat inactivation capacity of this manufacturing step for certain viruses,[51] it is advisable to assess the HEV inactivation capacity of other pasteurization processes in product-specific virus validation studies wherever stabilizer composition is considerably different from the composition in the VWF/FVIII intermediate.

Commonly, samples from the validation of virus inactivation procedures are assessed in cell culture systems to quantify the reduction of virus titer. Despite the fact that some HEV studies are performed employing cell culture systems, currently, a suitable and sensitive cell culture system is not available; published data show that one cell culture infectious unit corresponds to at least approximately 1000 HEV RNA copies,[23,52] whereas the minimum infectious dose for pigs corresponds to 6.5 copies (equivalent to 24 IU HEV RNA/ml).[44] Therefore, pig infection experiments are the sole possibility to determine potential HEV inactivation and remaining virus infectivity with a high sensitivity, dynamic range, and relevance for human patients, especially because pigs exhibit many similarities with humans in anatomy, physiology, and immunology.[53] We, therefore, used this species to evaluate the impact of increasing pasteurization times on stabilized VWF/FVIII (Haemate P/Humate-P) intermediate spiked with HEV-positive liver homogenate.

As recent studies suggested that HEV particles from cell culture supernatant[36,54,55] and human serum[56] are quasienveloped by a lipid layer in contrast to nonenveloped HEV[57] derived from feces, in an initial in vitro approach, the density of HEV particles from different sources was analyzed and compared with HEV particles from human plasma to select the most appropriate HEV spike material for the study. For this approach, ultracentrifugation of HEV from porcine liver, porcine feces, and human plasma was performed in a sucrose gradient. Our results show that a distinct proportion of both liver- and plasma-derived HEV particles contains a lipid layer, which was demonstrated by a shift in particle density after treatment with the detergent NP-40. No corresponding shift was observed in feces-derived HEV material as bile acts, to some extent, as a surfactant, removing the lipid from the HEV particles. The lipid layer seems to interfere with infectivity as feces-derived HEV exhibits a higher infectivity in humanized mice compared to other sources.[58] Therefore, the intravenous application of liver-derived HEV material in our experimental pig trial best mimics the transmission routes and sources of blood-transmitted HEV.

In general, increasing pasteurization times caused a significant increase in time until virus shedding in feces and in the detection of anti-HEV (up to total absence). Infections of the animals by original injection cannot clearly be discriminated from subsequent oral infections (documented for contact animals) when animals are grouped in one stable. Therefore, a secondary (oral)

infection of inoculated but not infected animals was defined when the time gaps of onset of virus shedding were 10 days or more between individuals of one cohort. Based on this definition, the virus-spiked inoculum pasteurized for 10 h infected 2 of 12 inoculated pigs, and in all other groups, all inoculated animals are considered to be infected by the inoculum.

For the welfare of animals, that is, to minimize the number of animals used in the study, no hold control was included where spiked starting material is maintained at room temperature for 10 h. This was regarded as acceptable because the VWF/FVIII intermediate at this stage contains no immunoglobulins. This was known from analytical data and pasteurization studies with Hepatitis A virus (HAV) and parvovirus B19 (B19V), where the respective hold controls showed no titer decrease when compared to the spiked starting material directly after spiking, indicating the absence of neutralizing antibodies to these viruses (unpublished data). Therefore, the likelihood that losses in infectivity in the HEV pasteurization study could be attributed to neutralization or interference rather than to the heat treatment is low.

In conclusion, we could demonstrate effective inactivation of HEV by pasteurization of the VWF/FVIII intermediate in an aqueous, stabilized solution spiked with HEV-positive liver material, with a quasienvelope comparable to that of a plasma-derived HEV and applied intravenously.

An assessment regarding the risk for HEV transmission for the VWF/FVIII product Haemate P/Humate P is performed.[59] This risk assessment takes into account the HEV load in the starting material plasma pool for fractionation (not exceeding 1000 IU/ml[47]), the volume of plasma required to produce one vial of product (considerably less than 10 L), the virus reduction capacity of the manufacturing process (pasteurization), and further manufacturing steps contributing to the overall virus reduction capacity as demonstrated for a wide range of enveloped and nonenveloped viruses,[60] and the minimum amount of HEV required to infect a patient (4.4 $\log_{10}$ IU)[61] ensures an appropriate margin of safety regarding potential HEV transmission.

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## CONFLICT OF INTEREST

NJR and WS are employees of CSL Behring and have stock interest.

## AUTHORS' CONTRIBUTION

Conceived and designed the experiments: JS, LD, ME, MHG.

Performed the experiments and necropsy: LD, JS, CF, EL, CS, ME, AG.

Analyzed the Data: JS, LD, AF, ME, CF, AG, MHG.

Wrote the paper: LD, JS, ME, MHG, AG, WS, NJR.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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