Low hepatitis E virus RNA prevalence in a large-scale survey of United States source plasma donors

Nathan J. Roth,[1] Wolfram Schäfer,[2] Rick Alexander,[3] Kevin Elliott,[3] Wlenyeno Elliott-Browne,[4] Jonathan Knowles,[4] Jürgen J. Wenzel,[5] and Toby L. Simon[1]

BACKGROUND: Hepatitis E virus (HEV) is a small, nonenveloped, single-stranded, RNA virus of emerging concern in industrialized countries. HEV transmission through transfusion of blood components has been reported, but not via plasma-derived medicinal products (PDMPs) manufactured with virus inactivation and/or removal steps. This study aimed to determine the prevalence of HEV among US source plasma donors.

STUDY DESIGN AND METHODS: Samples were collected from US source plasma donors at centers across the United States and were initially screened for HEV RNA in 96-sample minipools using the Roche cobas HEV test on the cobas 8800 system. Assuming a sensitivity of 18.6 IU/mL, the minipool screening strategy allowed for reliable detection of individual donations with HEV RNA titers of more than 2×10^3 IU/mL. Reactive minipools were resolved to individual donations, which were further analyzed to quantify viral RNA concentration, determine HEV genotype, and immunoglobulin (Ig)G and IgM HEV antibody status.

RESULTS: A total of 128,020 samples were collected from 96 CSL Plasma centers in the United States, representing 27 states. The prevalence of HEV RNA–positive samples was 0.002% with three unique HEV-positive donors identified, all HEV Subgenotype 3a. Virus titers of HEV-positive samples were relatively low (10^3-10^4 IU HEV RNA/mL). One positive donation was HEV IgG seropositive.

CONCLUSION: Routine screening of US source plasma donations for HEV would not substantially improve the safety of most PDMPs. The low prevalence and potential viral load of HEV, together with effective virus reduction steps in manufacturing processes, results in a low residual risk and acceptable safety margins for PDMPs derived from US plasma donors.

Hepatitis E virus (HEV) is a small (27-34 nm), nonenveloped, single-stranded RNA virus. Globally, HEV is the most common cause of acute hepatitis, which is usually benign, but fulminant cases have been seen in pregnant women and patients with existing liver disease. In immunosuppressed patients, there is a risk of progression to a chronic state.[1] There is one serotype but four genotypes with varying geographic distribution and epidemiologic and clinical features.[1,2] Genotypes 1 and 2 are most commonly associated with water-borne epidemics and were estimated to account for 20 million incident infections, 3 million cases of acute disease, and 70,000 deaths worldwide in 2005.[3] Genotypes 3 and 4 occur most commonly in swine. Thus, infection in humans can result from transmission through

ABBREVIATIONS: PDMP(s) = plasma-derived medicinal product(s); SCR = signal-to-cutoff ratio; WNV = West Nile virus; ZKV = Zika virus.

From [1]CSL Behring, King of Prussia, Pennsylvania; [2]CSL Behring GmbH, Marburg, Germany; [3]CSL Plasma Laboratory, Knoxville, Tennessee; [4]CSL Plasma, Boca Raton, Florida; and the [5]Institute of Clinical Microbiology and Hygiene, University Medical Center Regensburg, Regensburg, Germany.

Address reprint requests to: Toby L. Simon, MD, Plasma Product Development and CSL Plasma, 1020 First Avenue, King of Prussia, PA 19406; e-mail: Toby.Simon@cslbehring.com.

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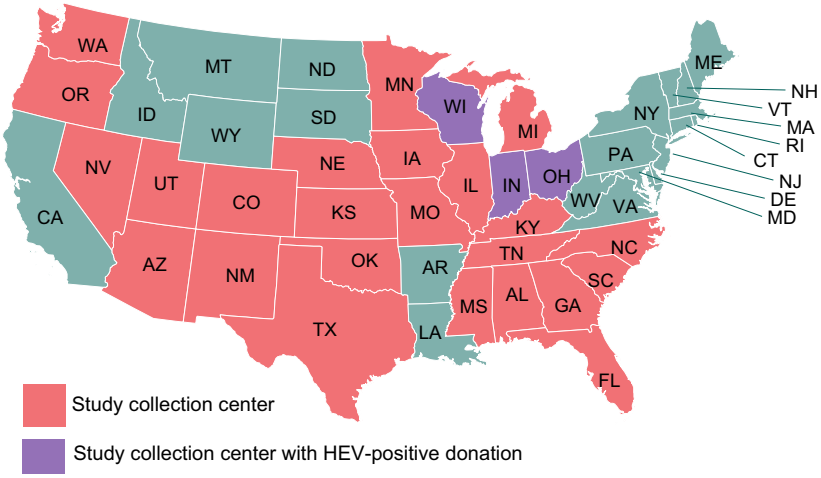

Region	Number of centers	Number of donors screened	Number HEV positive
Midwest	32	45,698	3*
Southeast	25	27,740	0
West	12	16,317	0
Northeast	0	0	0
Southwest	27	38,265	0
Total	**96**	**128,020**	**3**

*positive samples obtained from centers in Fort Wayne, IN, Racine, WI and Columbus, OH

Fig. 1. Location of study collection centers and number of donors screened by region.

food, especially raw or undercooked pork products containing liver or blood.[1]

In the United States, data from the Third National Health and Nutrition Examination Study (NHANES III), covering the period from 1988 to 1994, reported a 21% prevalence of immunoglobulin (Ig)G antibody to HEV and seven incident infections per 1000 susceptible persons per year. However, the 2009 to 2010 survey showed a seroprevalence of only 6%. Possible reasons for this difference could be assay performance, differences between an older population with infection in the remote past and younger populations, lifestyle or behavioral changes, or a change in the etiologic agent.[4] These data also suggest a lower prevalence of HEV in the United States than other parts of the world.[5]

HEV incidence and prevalence among blood donors has become a concern since the disease can be transmitted through blood transfusion. In Southeast England, Genotype 3 infections were found to be widespread in blood donors with an RNA prevalence of 1 in 2848.[6] Among German blood donors, seroprevalence of HEV was 6.8% with an annual incidence of 0.35%.[7] In the Netherlands, HEV seroprevalence has been reported as 27%,[8] and one in 762 blood donations were HEV RNA positive.[9] Seroprevalence has been reported as 10.7% in Danish blood donors.[10] In Southwest France, IgG seroprevalence was found to be 52.5% among blood donors with the endemic nature of HEV believed to be related to local dietary habits.[11] Among US American Red Cross donors low rates were found: HEV RNA prevalence of one in 9500 and anti-HEV prevalence of 7.7%.[12]

Many countries have begun HEV RNA virus screening programs for donated blood.[13] This has naturally led to the question of whether source plasma donation should also be screened, particularly since detection of HEV in manufacturing pools has been reported.[14] In the United States, source plasma donors are able to donate 400 to 800 mL of plasma a maximum of twice per week, with at least 2 days between donations.[15] Donors must be healthy; negative for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV); not involved in any high-risk behaviors; and not be showing any signs of fever.[16,17]

Dedicated virus reduction steps in manufacturing of plasma-derived medicinal products (PDMPs) are expected to be effective in mitigating the risk of virus transmission from plasma derivatives. However, it is necessary to know the anticipated virus load of the plasma pooled for manufacture to perform a risk assessment. Since the majority of the plasma used for manufacture of plasma derivatives worldwide comes from US donors, the prevalence of HEV among US-source plasma donors needs to be determined. Thus, we performed this study to evaluate the incidence of HEV in US-source plasma donors and to characterize any HEV-reactive samples identified.

MATERIALS AND METHODS

This study aimed to screen a minimum of 125,000 donors, to determine the prevalence of HEV among US source plasma donors. This target was selected, based on all existing data on HEV prevalence in the United States, to ensure a high probability of detecting one or more positives. To achieve a broad geographical distribution, samples were collected from donors at 96 Food and Drug Administration (FDA)-licensed CSL Plasma donor centers in the United States, from July 20 through August 9, 2015. The location of study collection centers is shown in Fig. 1.

The study was approved by the Copernicus Group Institutional Review Board with a waiver for consent. All samples were delinked, thereby preventing a sample being traced back to the donor. However, samples could be traced back to the collection center to identify the

geographic origins of any positives. This was accomplished with prelabeled tubes with a specific numbering sequence.

The number of prelabeled study tubes provided to each center was based on historical donor trends for each site to meet the minimum collection target of more than 125,000 unique donations from unique donors within a 2- to 3-week window. When the supply of tubes at any center was exhausted, the study ended at that center. Upon initiation of the study, the donor management system issued an electronic "flag" requesting that the donor center collect an additional 6 mL from the first donation made by the donor during the study period. During subsequent visits the electronic study "flag" disappeared from the donor management system and no prompt for an additional study-related sample was issued.

Minipools of 96 samples were created using an instrument for automated plasma sample pooling and pipetting (cobas p 680, Roche Molecular Systems), and testing was performed on a molecular testing system (cobas 8800, Roche Molecular Systems) using the cobas-HEV nucleic acid test (NAT; Roche Molecular Systems) under an investigational protocol following the manufacturer's instructions. The cobas HEV test is commercially available in countries that accept the CE mark where it has a reported limit of detection of 18.6 IU/mL (95% confidence interval [CI], 15.9-22.9 IU/mL) HEV RNA (probit analysis), following the manufacturer's instructions. Reactive minipools were resolved and tested in minipools of 12 samples and finally individual donations to identify the individual HEV-reactive samples. The cobas-HEV test claimed sensitivity of 18.6 IU/mL assures that, during minipool testing, individual positive donations with viral loads of approximately 1800, 225, and 18.6 IU/mL would reliably be detected (>95% CI) for minipools of 96, 12, and individual samples, respectively. The cobas-HEV NAT is not commercially available or approved by the FDA for use in the United States, but the test is CE marked and available for use in countries that accept the CE mark. Testing was done at CSL Plasma Laboratory.

HEV RNA–reactive samples were further analyzed to quantify the levels of HEV RNA using real-time reverse transcription polymerase chain reaction (RT-PCR) (TaqMan Fast Virus 1-Step Master Mix, ThermoFisher Scientific) in combination with published HEV-specific primers.[18] Samples were extracted using the viral nucleic acid kit (High Pure, Roche Diagnostics) and amplified on a real-time PCR instrument (Model 7500, Life Technologies). HEV RNA levels were quantified against a standard curve for the HEV-positive plasma donation, 054915007GO, calibrated against the World Health Organization HEV standard, at CSL Behring Laboratories. HEV RNA titers were verified by independent testing at the Institute of Clinical Microbiology and Hygiene, University Medical Center Regensburg.

HEV RNA–positive samples were genotyped by amplifying regions of ORF1 and ORF2 using real-time quantitative PCR and sequence determination of the resulting nucleic acid fragments, as described elsewhere.[19] Sequences were evaluated with Fasta36 against GenBank to determine the phylogenetic map for the positive samples. A maximum likelihood phylogenetic consensus tree of ORF1 sequences was constructed by using computer software (RAxML, Version 8.2.7).[20] Bootstrap values (%) were calculated from 650 bootstrap replicates.

IgG reactivity was determined using the HEV IgG enzyme-linked immunosorbent assay (ELISA; Wantai) according to the manufacturer's instructions. IgG reactivity was confirmed and IgM reactivity was determined using the HEV IgM and HEV IgG immunoblot (Mikrogen), respectively. Avidity of IgG was determined by using an HEV IgG immunoblot (recomLine, Mikrogen) and the HEV ELISA (Wantai).

Measuring avidity by immunoblot was performed with two test strips in parallel. After incubation with plasma, both test strips were washed five times with wash buffer whereas the avidity test strip was incubated with wash buffer containing 6 mol/L urea during the second washing step. Soak times were 3 minutes for the second washing step and 5 minutes for the remaining four. Avidity was quantified by determining the signal-to-cutoff ratio (SCR) of the O2CGt3 band for both the untreated and avidity reagent–treated replica blots.

Measuring avidity by ELISA was performed as previously described[21] by following a protocol published by Bendall and colleagues.[22] Briefly, plasma was tested in duplicates, whereby one duplicate was tested according to the manufacturer's instructions. The other duplicate was tested by using wash buffer containing 5 mol/L urea for the first two washing steps after serum incubation. Soak times were 5 minutes for the first two washing steps and 30 seconds for the following wash steps. Avidity was calculated as $100\% \times SCR_{treated}/SCR_{untreated}$ for both methods. Serologic tests for other virologic markers and bacterial markers were performed to clarify the relationship between two HEV RNA–reactive samples from the same plasma collection center and with identical nucleic acid sequences (see Appendix S1 and Table S1, available as supporting information in the online version of this paper).

RESULTS

Sample collection, HEV screening, and sample resolution

Samples were collected across 96 FDA-licensed CSL Plasma US centers over the period of July 20 through August 9, 2015, representing 27 states and more than 90% of CSL Plasma centers. The number of donors from each region is shown in Fig. 1.

Primary screening of samples was typically performed in minipools of 96 samples. In a small percentage of tests, smaller sample pools or individual donations were screened. A total of 128,026 donor samples underwent primary screening with 128,021 producing a final valid result. The five results that were invalid were not considered for further analysis. Of the 1352 valid primary pool tests (96-sample minipools), five returned a reactive result. Four of the five reactive primary pools were confirmed as positive during 12-sample minipool resolution testing, with a single-donor sample from each primary pool confirmed as reactive when resolved to the individual sample testing level (four of 128,021 [0.003%]). To verify that a false positive had been obtained in the fifth reactive 96-donor pool, all eight 12-donor minipools were further resolved to the single donation level and retested; all samples returned a negative result. The frequency of false-reactive primary pools was, therefore, one in 1352 (0.07%).

Of the four positive confirmed donations, one originated from Fort Wayne, Indiana; one from Racine, Wisconsin; and two from a single center in Columbus, Ohio. The unexpected yield of two positive donations from the same center prompted an investigation to ensure that the samples were drawn from unique donors. The delinked study design prevented direct confirmation; however, an extensive evaluation of viral load, genotyping, and serologic profile of the two plasma samples led to the conclusion that the two samples represented a single individual donor. The investigation and results are described in full in Appendix S1.

TABLE 1. RNA titers of HEV-reactive donations determined by RT-qPCR

Sample number	RNA titer ± SD (log IU/mL)
0065 HEV 1068	3.8 ± 0.1
0089 HEV 0499	3.0 ± 0.1
0409 HEV 0331	3.4 ± 0.1

RT-qPCR = real-time quantitative PCR.

Determination of HEV RNA titers of reactive samples

RNA titers of the three individual positive samples are shown in Table 1. Comparable results were obtained by independent testing at the Institute of Clinical Microbiology and Hygiene at the University Medical Center Regensburg (data not shown).

HEV genotyping of reactive samples

All reactive samples were identified as HEV Subgenotype 3a, based on nucleic acid fragments of ORF1 and ORF2, and were more than 87% identical (Fig. 2).

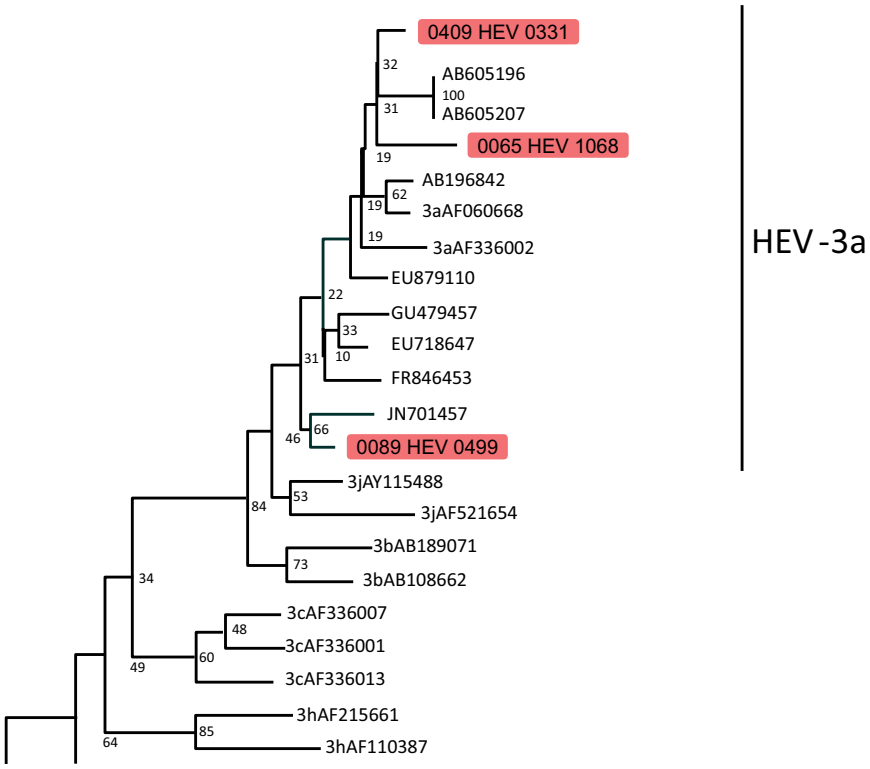

Fig. 2. Maximum likelihood phylogenetic consensus tree of ORF1 sequences showing the genotype of HEV-reactive samples. ORF1 sequences from all HEV-reactive samples cluster with HEV Genotype 3, Subgenotype 3a. Numbers at the nodes indicate bootstrap values (%) calculated from 650 bootstrap replicates. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2. Serology of HEV-reactive donations

Sample number	IgG ELISA (IU/mL)	IgG immunoblot	IgM immunoblot	IgG avidity (%)
0065 HEV 1068	0.6	Positive	Negative	84.0 (immunoblot), 80.4 (ELISA)
0089 HEV 0499	Negative	Negative	Negative	NA
0409 HEV 0331	Negative	Negative	Negative	NA

NA = not applicable.

HEV serologic characterization of reactive samples

One of the three HEV-reactive samples (0065 HEV 1068) was IgG positive on HEV IgG ELISA and IgG reactivity was confirmed by IgG immunoblot. Avidity testing of the IgG-positive sample showed a high avidity by immunoblot (84%) and by ELISA (80.4%). All three samples were IgM negative on immunoblot. These results are consistent with a pattern of reinfection for 0065 HEV 1068[23] and new infection for the other two HEV-reactive samples. Serology results for the three HEV-reactive samples is shown in Table 2.

DISCUSSION

Our data provide the first large-scale survey of the prevalence of HEV infection, as defined by HEV RNA, in US source plasma donors. We identified three unique HEV-positive donors out of approximately 128,000 donations, resulting in a frequency of around one in 42,000 donations (0.002%) being HEV positive. This frequency is lower than that reported for US blood donors (one in 9500)[12] but critical differences in HEV RNA screening strategies exist between the two studies. Stramer and colleagues[12] screened individual donations, thereby identifying positive donations with HEV viral loads as low as 10 to 20 IU/mL.

Our HEV screening paradigm was based on a 96-donation minipool strategy designed to reliably identify donations with HEV viral loads of approximately 2000 IU HEV RNA/mL or higher. This minipool screening strategy is consistent with routine NAT methods currently used to screen source plasma for HIV, HBV, and HCV. The HEV RNA titers for the three positive donations were relatively low and just sufficient to exceed the threshold level of the minipool screening strategy and reliably be detected. Additional donations with lower HEV RNA titers may have been detected, if a 12-unit minipool or single-unit donation screening strategy had been utilized. Therefore, it is possible that the HEV prevalence identified in this study is an underestimation of the true figure, as additional HEV-reactive donations may have been identified if single unit donations were screened. However, the difference is likely to be so small as to have little, if any, bearing on the conclusions made here.

During the study, two HEV-reactive donations from the same collection center, which mapped identically on phylogenetic analysis and had similar RNA titers, were identified. Extensive serologic profiling indicated that the samples came from the same donor. This may have occurred due to a laboratory or procedural error and delinking of the samples prevented them from being traced back to the donor, unlike in routine operations. Based on an audit of the study procedures, this was considered to be an isolated or extremely rare event, not believed to impact on the conclusions of the study or its scientific integrity.

The safety of PDMPs relies on the complementary approach of 1) donor selection, 2) testing of plasma donations and plasma pools for the presence of certain viruses, and 3) effective virus inactivation and removal steps within the manufacturing processes, with a high capacity to clear a broad variety of viruses. Of these complementary safety measures, arguably the most important step in assuring the pathogen safety of PDMPs from emerging viruses are manufacturing processes with robust and high virus reduction capacity. During the early phases of the emergence of a virus into the donor population, suitable virus screening tests may not be available or approved by regulatory authorities. Additionally, donor selection may be only partly effective. Source plasma is only collected from qualified healthy donors with low-risk behavior, from geographical regions with acceptable epidemiologic risk for HIV, HBV, and HCV; however, certain viruses such as Zika virus (ZKV), West Nile virus (WNV), or HEV, may result in mild or asymptomatic infections in blood and plasma donors. Donors with mild symptomatic infections would be deferred, but asymptomatic donors with no risk factors would remain eligible to donate. Nonetheless, recent history has shown that for certain emerging viruses, such as WNV, and presumably ZKV, effective virus reduction steps in combination with even partially effective donor screening measures is sufficiently robust to assure the safety of PDMPs even in the absence of NAT for screening of donations or plasma pools.[24] In contrast, nonvirally inactivated blood components are susceptible to transmission of WNV and ZKV in the absence of NAT within screening measures.

The likelihood (or margin of safety) that the final PDMP is essentially "sterile" from infectious viruses can be calculated according to European regulatory guidance and depends on a variety of factors including epidemiology of the virus in the donor population, donor frequency,

virus life cycle, virus titer in blood, product yield, starting pool size, and virus reduction capacity of the process for the virus in question. In addition, since not all virions may be packaged correctly or efficiently produce an infection in vivo, the clinical experience on the minimum infectious dose needed to produce an infection, that is, from blood component lookback studies, can also be considered in determining the margin of safety.[25] Based on the clinical experience in humans from unscreened blood components, a minimum infectious dose of 2×10^4 IU HEV RNA can be assumed.[26]

The low prevalence of HEV in the source plasma donor population suggests that, on average, not more than one HEV viremic donor's donations would be expected to enter a manufacturing pool. Although the units identified in this survey had moderate titers, a highly viremic unit with titers of approximately 10^6 IU/mL[9] could potentially enter a manufacturing pool of several thousand liters at a low frequency, which after dilution in the starting pool would result in a maximum HEV RNA titer of approximately 10^3 IU/mL, as reported by Baylis and coworkers.[14]

In the case of B19V, individual viremic units can harbor titers as high as 10^{14} IU DNA/mL,[27] which would result in maximum pool titers in the order of 10^{10} IU/mL B19V DNA in the absence of NAT screening. However, safety margins in the order of 5 to 6 log are achieved for B19V by performing minipool NAT screening, excluding highly viremic B19V units from entering the plasma pool, and the establishment of a plasma manufacturing pool limit of not more than 10^4 IU B19V DNA/mL.[28] In contrast, for HEV the relatively low maximum virus titer in individual units and manufacturing pools, in the absence of routine NAT, substantially limits the benefit that would be attainable (approx. 1-2 log) utilizing a NAT screening strategy consistent with the established commercial NAT platforms employed for testing high volumes of source plasma.

Multiple studies have confirmed that existing virus inactivation and removal steps considered to be effective (providing in the order of >4-log reduction) against relatively resistent small nonenveloped viruses are also effective for HEV.[29-31] In addition, manufacturing purification steps also have the potential to significantly contribute to reduction of HEV (approx. 1-3 log) through partitioning mechanisms.[29-31] Thus, for the vast majority of plasma-derived products, the routine implementation of HEV NAT of US source plasma would not contribute substantially to the safety of the final product. Only in a few certain processes, where insufficient HEV reduction capacity may exist, would donation and/or donor screening provide a significant contribution to the safety of the final product.

In conclusion, in this first, large-scale survey of HEV among US source plasma donors, HEV prevalence was found to be very low. One donor had serology markers and HEV IgG avidity consistent with a reinfection. The low HEV RNA prevalence provides reassuring epidemiologic data regarding the low likelihood of a maximally viremic HEV unit entering a plasma manufacturing pool. Most PDMP manufacturing processes include virus reduction steps capable of removing or inactivating HEV thereby resulting in a low residual risk and acceptable safety margins for the final products. In select processes, where HEV virus reduction may be limited, NAT screening of donations and/or manufacturing pool testing could be considered to provide additional safety margins in the order of 1 to 2 log. The results of this study are consistent with the conclusions of the European Medicines Agency reflection paper on the viral safety of PDMPs with respect to HEV and do not support a benefit from routine screening for HEV of all US source plasma donations or plasma pools intended for manufacturing into PDMPs.

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CONFLICT OF INTEREST

TLS, WS, JK, RA, KE, WEB, and NJR are employees of CSL Behring or CSL Plasma. JJW is an employee at the Institute of Clinical Microbiology and Hygiene, University Medical Center Regensburg, which received funding from CSL Behring to perform part of the studies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Appendix S1. Investigation into the origin of two HEV-reactive samples

Table S1. Serological profile of two HEV-reactive samples from the same center with identical nucleic acid sequences