The risk of transfusion-transmitted hepatitis E virus (HEV) infections by contaminated blood products remains unknown. In the present study, we evaluated and compared different nucleic acid amplification technique (NAT) methods for the detection of HEV in blood components. Minipools of a total of 16,125 individual blood donors were screened for the presence of HEV RNA using the highly sensitive RealStar HEV RT-PCR kit, revealing a minimum detection limit of 4.66 IU/ml. Thirteen donors were HEV RNA positive (0.08%), and of these donors, only three already showed reactive IgM antibody titers. The detected HEV strains all belonged to genotype 3 and were most closely related to German HEV strains from wild boars and pigs as well as from human hepatitis E cases. Furthermore, HEV RNA and HEV-specific IgM and IgG titers were determined in 136 blood donors with elevated alanine aminotransferase (ALT) levels and in 200 donors without pathological findings. HEV RNA was not detectable, but 8.08% (elevated ALT) and 0.5% (nonelevated ALT) of donors showed reactive HEV IgM titers. The overall seroprevalence rate of HEV IgG amounted to 5.94% (elevated ALT, 5.88%; nonelevated ALT, 6.0%). The clinical relevance of transfusion-associated hepatitis E infection still requires further investigation. However, in connection with raising concerns regarding blood safety, our NAT method provides a sensitive possibility for HEV testing.

In transfusion medicine, the hazards based on blood-borne viruses are separated in Germany into the categories major (obligatory testing, human immunodeficiency virus, and hepatitis B and C virus) and minor (facultative testing, e.g., parvovirus B19, hepatitis A virus, cytomegalovirus, human T cell leukemia virus [HTLV], and West Nile virus). However, the continual emergence of new infective agents introduces procedural questions about the safety of blood products. In this context, hepatitis E virus (HEV) is a potential new candidate pathogen because HEV infections are increasingly recognized as an emerging disease in industrialized countries (1, 7). HEV is a single-stranded RNA virus classified in the family Hepeviridae. It is presently grouped into four major genotypes (genotypes 1 to 4), which differ in their worldwide distribution (1). Genotypes 1 and 2 are restricted to humans and are hyperendemic in developing countries where HEV transmission by the fecal-oral route has been described as a major problem and where major waterborne outbreaks have occurred (22, 33, 40). In industrialized countries, HEV infection is considered a nonendemic travel-associated disease with a sporadic incidence (33). However, non-travel-associated infections caused by genotype 3 (Europe, United States, Japan, New Zealand, and Argentina) and genotype 4 (Japan and China) are increasingly observed (28, 41). Genotypes 3 and 4 have been isolated from humans as well as from other mammalian species (e.g., pigs, wild boars, and deer) (28), which are genetically similar and, in some cases, indistinguishable from each other (13, 23). Several findings suggest a zoonotic or food-borne transmission route by close environmental contact with farm animals or meat products or by consumption of raw meat (36, 48). Furthermore, a high prevalence of HEV-specific antibodies has been demonstrated in humans through contact with pigs (12, 28, 30, 33, 38). The transmission of HEV via transfusion has also been described in Japan, France, and the United Kingdom (3, 9, 14, 34, 35).

There have been several European studies on the HEV seroprevalence, ranging from 2.6 to 20.6% (1, 5, 10, 12, 16, 27, 32, 47). HEV infections present asymptptomatically or symptomatically with clinical features similar to those of hepatitis A. Hepatitis E is mostly self-limiting, with the potential to progress into a fulminating or fatal disease (7, 47). In 2001, HEV infection became a reportable disease in Germany, with 17 to 73 infections per annum, showing an increasing trend since 2002 (46). The fatality rate ranges from 0.2 to 4% in the general population (47) and considerably increases to 8 to 20% in pregnant women (6, 12). Chronic infections have recently been reported in transplant patients (26, 29).

In the present study, we evaluated a routine in-process screening procedure for the detection of HEV RNA in order to increase blood safety and investigated the prevalence of anti-HEV antibodies and HEV RNA in German blood donors. HEV strains detected in the blood donations were genetically analyzed to assess their possible origin and clinical potential.

**MATERIALS AND METHODS**

**Blood donors.** From July to September 2011, a total of 41,325 allogeneic blood donations from 16,125 individual German blood donors (geographic origin, North Rhine-Westphalia, Lower Saxony, Hesse; male, 57.5%; median age, 33 ± 13 years; range, 18 to 72 years; female, 42.5%; median age, 32 ± 13 years; range, 18 to 70 years) were routinely screened for HEV RNA (RealStar HEV RT-PCR kit; Altona Diagnostic Technologies [ADT], Hamburg, Germany) by the Universitätsklinik Blutspenderdiagnostik OWL. Master pools of 48 donations were set up by using a combination of 200 µl plasma (EDTA-anticoagulated); reactive pools were
followed by 45 cycles of denaturation at 95°C for 15 s, annealing at
sequence. PCR conditions were as follows: reverse transcription at
lambda gene was added to the reaction mixture as an exogenous IC

<table>
<thead>
<tr>
<th>RNA concn (U/ml)</th>
<th>recomWell IGM result (U/ml)*</th>
<th>recomWell IGG result (U/ml)**</th>
<th>MP Biomedicals IgM 3.0 result</th>
<th>MP Biomedicals IgG result</th>
<th>GLDH concn (U/l)</th>
<th>ALT concn (U/l)</th>
<th>AST concn (U/l)</th>
<th>Total bilirubin (mg/dl)</th>
<th>Day of confirmation of soroncovirase (ng/l)</th>
<th>Genotype (GenBank accession no.)</th>
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<tr>
<td>1 (3995_1)</td>
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<td>2.63E+4</td>
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<td>Negative (3.45)</td>
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<td>2.31E+4</td>
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<td>Negative (3.42)</td>
<td>Negative</td>
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<td>Negative (6.04)</td>
<td>Negative</td>
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<td>Positive</td>
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<td>Negative (61.05)</td>
<td>Positive</td>
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<td>Negative (2.85)</td>
<td>Negative</td>
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<td>41.0 0.23</td>
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<td>Negative (9.19)</td>
<td>Negative</td>
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<td>Positive (24.42)</td>
<td>Positive (23.69)</td>
<td>Positive</td>
<td>2.4 18.0</td>
<td>30.0 0.30</td>
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a NRW, North Rhine-Westfalia; HE, Hesse; LS, Lower Saxony.
b Negative, <20 U/ml; borderline, <20 to <524 U/ml; positive, >24 U/ml.

A previously described protocol was compared with a modified reverse transcription (RT)-PCR assay. For the original protocol, the 20-μl reaction mixture contained 10 μl of 2× Quantitect probe RT-PCR kit master mix (Qiagen, Hilden, Germany), primers and probes as described by Jothikumar et al. (25). This original protocol was compared with a modified reverse transcription PCR (RT)-PCR assay. For the original protocol, the 20-μl reaction mixture contained 10 μl of 2× Quantitect probe RT-PCR kit master mix (Qiagen, Hilden, Germany), primers and probes as described by Jothikumar et al. (25). The original protocol was compared with a modified reverse transcription PCR (RT)-PCR assay.
plasma samples using the respective enzymatic assays (Abbott Diagnostics Europe, Wiesbaden, Germany) on the Architect system (Abbott Diagnostics Europe).

**Statistical analysis.** All values are given as mean values ± standard deviations (SD). Mean values and SD were calculated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

**Nucleotide sequence accession numbers.** The nucleotide sequences obtained in this study are available in GenBank under the accession numbers listed in Table 1.

**RESULTS**

**Analytical sensitivity and specificity and comparison of NAT assays.** In order to evaluate the analytical sensitivity and precision of the commercially available assay applied for HEV screening in blood donors, a 2-fold dilution series of HEV RNA-positive plasma was used. The 95% detection limit was calculated to 4.66 IU/ml (confidence interval, 3.6 to 7.5 IU/ml). The reproducibility of the assay was demonstrated by analyzing the intra-assay and interassay variation for the cycle threshold (C_T) values. The intra-assay variability was calculated from eight replicates, with C_T of 34.26 ± 0.43 for the HEV target and C_T of 26.27 ± 0.24 for the internal control target. The interassay variability was determined from three independent PCR runs with eight replicates per run, with C_T of 32.85 ± 1.19 for the HEV target (25 IU/ml) and C_T of 27.89 ± 2.56 for the internal control.

Additionally, the analytical sensitivity of the RealStar HEV RT-PCR kit (ADT) was compared with two different in-house assays: a protocol originally published by Jothikumar et al. (25) and an in-house modification of this protocol including an internal control. In order to mimic routine pool screening procedure with different pool sizes, plasma samples of three different HEV RNA-positive donors were diluted with negative human plasma (Table 2). The detection frequencies and 95% detection limits of the three different NAT methods were different. The RealStar HEV RT-PCR kit, which was used for initial routine screening, showed the highest detection frequency with a 95% detection limit of 7.3 IU/ml (range, 5.2 to 15.4 IU/ml), followed by the two other methods. Modification of the original protocol by Jothikumar et al. (25) demonstrated only a marginally higher analytical detection limit of 49.2 IU/ml (range, 38.3 to 74.2 IU/ml) compared to the previously published protocol (95% limit of detection [LOD], 64.0 IU/ml [range, 48.5 to 101.5 IU/ml]).

**Screening of blood donors.** A total of 13 of 16,125 individual blood donors were HEV RNA positive (0.08%) (Table 1). RNA positivity was confirmed by analysis of a second sample aliquot. Of these 13 donors, 10 were positive only for NAT. One donor had a positive IgM titer, whereas IgG remained negative. A second donor also showed a positive IgM titer, and the concentration of IgG antibodies was determined with borderline values. One donor showed positive IgM and IgG titers. Confirmation of HEV infection was performed by determination of serostatus in subsequent samples (range, 21 to 125 days) (Table 1) showing complete seroconversion with IgM-negative and IgG-positive status for all donors. HEV RNA-positive donations were immediately rejected for transfusion.

**Prevalence of HEV RNA and antibodies against HEV in donors with elevated ALT levels.** Plasma samples of 136 donors (male, 81.0%; median age, 29 ± 10 years; range, 18 to 57 years; female, 19.0%; median age, 34 ± 13 years; range, 18 to 60 years) showing elevated ALT levels exceeding 100 U/liter (male, 188.2 ± 415.6 U/liter; range, 101 to 4,142 U/liter; female, 141.4 ± 51.7 U/liter; range, 103 to 362 U/liter) were screened for the presence of HEV RNA and HEV IgM and HEV IgG antibodies. Hepatitis A, B, and C infection was excluded. Among these 136 donors, measurement of total bilirubin showed elevated levels exceeding 1 mg/dl only in four samples (range, 1.10 to 7.85 mg/dl), and all other samples showed no elevated values (male, 0.29 ± 0.15 mg/dl; female, 0.21 ± 0.11 mg/dl). Eight plasma samples (5.88%) were evaluated as reactive for the presence of HEV IgG antibodies. Furthermore, samples of 11 donors were reactive for HEV-specific IgM antibodies (8.08%; positive, 7; borderline, 4). None of the tested plasma samples were reactive for IgG and IgM antibodies at the same sampling time point, and HEV RNA was not detectable in any sample.

**Prevalence of antibodies against HEV in donors with non-elevated ALT levels.** Plasma samples of 200 blood donors were additionally screened for the presence of HEV IgM and HEV IgG antibodies to analyze the prevalence in an unselected cohort. Of these 200 blood donors (male, 61.5%; median age, 35 ± 12 years; range, 19 to 64 years; female, 38.5%; median age, 42 ± 12 years; range, 18 to 63 years), one serum sample showed a borderline reactivity for HEV IgM (0.5%) and 12 serum samples were reactive for HEV IgG (6.0%; borderline, 1; positive, 11). Again, none of the tested sera were reactive for IgG and IgM antibodies at the same sampling time point, and HEV RNA was not detectable in any sample. Serum levels of ALT were below 100 U/liter (male, 28.38 ± 12.15 U/liter; range, 7 to 68 U/liter; female, 18.84 ± 7.88 U/liter; range, 7 to 59 U/liter).

**Sequence analysis of detected HEV genotypes.** The hepatitis E virus-specific sequences obtained from 13 donors were applied to a similarity search using the BLASTn search facility and the GenBank nr/nt database. Maximum nucleotide sequence identities between 93% and 99% were obtained with HEV strains from humans, pigs, and wild boars, all of them originating from Germany or The Netherlands. A phylogenetic tree was constructed on the basis of the 242-bp ORF1 fragment of the donor sequences.
# HEV in German Blood Donors

## DISCUSSION

Transfusion-transmitted hepatitis E infections by contaminated blood products had already been reported in Europe, Japan, and the United Kingdom (1, 9, 14, 34, 35). However, the occurrence of autochthonous and asymptomatic infection in blood donors raised the question of blood safety regarding the transmission of HEV (1). The severity and possible clinical courses (asymptomatic, mild hepatitis, and acute liver failure) in recipients are apparently variable, depending on predisposition or immune status, and the vast majority of HEV infections most likely result in an asymptomatic course (5).

A widespread distribution of hepatitis E virus was recently observed in plasma fractionation pools and plasma donations from Sweden and Germany, whereas plasma fractionation pools from the Middle East, as well as 51,075 individual donations from the United States, showed no contamination with HEV RNA (2, 4). Approximately 10% of plasma pools were positive for HEV RNA (sources, North America, Europe, and Southeast Asia) (4), and the rate of individual HEV RNA-positive donors varies from 1.7986 (Sweden, 0.012%) to 1.4525 (Germany, 0.022%) (2) to 1.7040 (United Kingdom, 0.014%) (24). Other studies from Asia revealed hepatitis viremia of 0.07% (China) (21) and 0.3% (Japan) (18) among blood donors and donors with an elevated ALT level of 1.1% (42). In the present study, we even observed a considerably higher rate of 1:1,240 HEV RNA-positive donors (0.08%). On the one hand, possible explanations are the effects of the particular donor population, especially within the context of the zoonotic potential and transmission of HEV. On the other hand, this might be due to the fact that this screening method showed a 95% LOD of 4.66 IU/ml, which is decreased by a factor of 50 compared to that of the assay used by Baylis and coworkers (250 IU/ml) (2). Observational viral loads in this study vary from 18.6 to 26.4 E+4 IU/ml, showing a range similar to those recently reported for German or Swedish donations (1.6E+3 to 4.8E+4 IU/ml) (2) or Japanese blood donors (79 to 3.1E+7 copies/ml) (42). Although highly positive viral plasma loads were observed, only three donors were positive for anti-HEV IgM antibodies and merely one of the identified HEV RNA-positive donors showed slightly elevated ALT values of 103 U/liter. Furthermore, none of the donors with elevated ALT levels were positive for HEV RNA. These results are in concordance with data observed by Baylis et al. (2), where 3 of 12 viremic donors showed elevated ALT levels.

High anti-HEV IgG seroprevalence rates have already been demonstrated among blood donors in developed countries, with considerable differences: Germany, 14.2 to 15.5% (1, 28); Switzerland, 4.1 to 5.4% (27); Denmark, 20.6% (12); Italy, 2.6% (47); Spain, 7.3% (10); France, 3.2 to 16.6% (8, 32); England, 10 to 15.8% (5, 16); Japan, 3.4% (unselected donors) (44) and 3.2 to 6.6% (preselected study population of donors with elevated ALT levels) (18, 42); China, 32.6% (21); and the United States, 18.3% (37). The seroprevalence rates observed in this study (donors with nonelevated ALT, 6%; donors with elevated ALT, 5.88%) are more comparable to rates which have been observed for Switzerland and Japan than to those observed for Germany so far. The seroprevalence rates of anti-HEV IgG have been measured less frequently, but studies in Chinese blood donors revealed a seroprevalence rate of 0.94 to 1.8% (11, 21), comparable to 0.5 to 1.6% in Japan (18, 20, 42) and the 0.5% observed in this study. We also observed a considerably higher seroprevalence rate of 8.08% in donors with elevated ALT levels, according to data obtained by Fukuda et al. (3.3%) (18). However, comparison is rather difficult due to demographic differences in the study population and differences in the sensitivity of the commercially available assays used (27).

Sequence similarity searches indicated that all HEV sequences detected in the donor samples are closely related to various genotype 3 strains, thus confirming the importance of this genotype for autochthonous infections in Germany. Interestingly, the most closely related strains originated exclusively from Germany or The
Netherlands, which may indicate a geographically confined circulation of endemic HEV strains within Germany and neighboring countries. A more detailed analysis of the sequences showed that most of the donor-derived sequences were closely related to HEV sequences from pigs, thus suggesting zoonotic transmission from pigs to humans as a major mode of infection. It has been shown that the anti-HEV IgG seroprevalence almost doubles in individuals with frequent contact to swine and other animals, e.g., slaughterers, veterinarians, and farmers (12, 28). Two of the strains showed very high percentages of sequence identity to an HEV strain detected in a porcine liver sold at a market in Germany (45), supporting the hypothesis of food-borne HEV infections. However, as pigs and pig products are the subject of extensive trade between countries worldwide, the distinct geographical origin of the pig HEV strains often remains unclear. It is therefore of special interest that the largest evolutionary cluster, containing nine sequences of the donors, also includes the wild boar strain WbGER17 (Fig. 1, Wb-DE-2006, indicated by an asterisk). This strain was originally detected in 25.9% of wild boar livers originating from a distinct region in Brandenburg, Germany, in 2006 (43). It could be speculated that these wild boars serve as a local reservoir for HEV, from which the virus could be transmitted directly or indirectly via infection in pigs to susceptible humans. Further analysis of samples from wild boars, pigs, and humans, including more-detailed geographical data, are necessary in order to understand the epidemiology of HEV infections. Most importantly, many of the strains detected in the donor samples showed up to 98% nucleotide sequence identity with the corresponding region of strains derived from human clinical hepatitis E cases. This finding may indicate that the detected strains are capable of inducing disease under certain conditions.

Our results further assume that the transmission of hepatitis E virus via transfusion is likely to be not uncommon, suggesting many subclinical infections (2, 24) with diagnostic failure. Particularly in cases of suspected posttransfusion hepatitis, the possibility of hepatitis E virus as the causative agent has to be kept in mind, followed by utilization of appropriate diagnostic efforts. So far, little is known about transfusion-associated HEV infection, but the observed severe courses of HEV infection in patients with preexisting liver disease (15), pregnant women (6, 12), transplant patients (26, 29), and immunocompromised patients (19), which is a major group of transfusion recipients, raised the question of a requirement for HEV NAT testing of blood products, at least for transfusion recipients belonging to one of those risk groups. We agreed with Baylis et al. that determination of ALT values in blood/plasma (2) and exclusive screening for the presence of HEV-specific IgM and IgG antibodies are not reliable approaches for exclusion of HEV viremic donors, although a nationwide study in Japan (42) revealed a considerably higher detection rate of HEV RNA (1.1%), HEV-specific IgM (14.0%), and HEV-specific IgG (3.2%) in donors with elevated ALT levels. Obviously, the diagnostic gap between the presence of high viral loads and serological confirmation, as well as the occurrence of apparently common asymptomatic HEV infections in blood donors, demands for NAT screening methods to effectively improve blood safety regarding the transmission of HEV. In the present study, we demonstrated an effective and sensitive semiautomated screening method during routine analysis of blood donors for the presence of HEV. This method represents a sensitive, reliable, and cost-effective method for the screening of HEV RNA equivalent to routine screening currently performed for hepatitis A, B, and C, HIV, and parvovirus B19 in Germany.

In summary, the extent of transmission and the clinical relevance of transfusion-associated hepatitis E infection still require further investigation. We are currently investigating the role of hepatitis E virus in heart transplant patients. In connection with rising concerns regarding blood components containing HEV RNA, this method provides a sensitive option for additional viral testing.

ACKNOWLEDGMENTS
We thank Sally Baylis, Paul-Ehrlich Institute, Germany, for the provision of HEV-positive material. Furthermore, we thank Sarah Kirkby for her linguistic advice.

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